KEY LEADERS’ OPINION
ON UROGENITAL CANCER:
FRONTIER AND PROGRESS

Honorary Editors: Felix K. H. Chun
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Key Leaders’ Opinion on Urogenital Cancer: Frontier and Progress
(FIRST EDITION)

HONORARY EDITORS

Felix K. H. Chun
Department of Urology, University Hospital Frankfurt a.M., Germany

Jyotsna Batra
School of Biomedical Sciences, Faculty of Health, Institute of Health and Biomedical Innovation, Australian Prostate Cancer Research Centre- Queensland (APCRC-Q), Translational Research Institute, Queensland University of Technology, Brisbane, Australia

EDITORS

Xinghuan Wang
Department of Urology, Zhongnan Hospital of Wuhan University, Wuhan, China; Center for Evidence-Based and Translational Medicine of Wuhan University, Wuhan, China

Guillaume Ploussard
Department of Urology, Saint Jean Languedoc Hospital, Toulouse, France; Division of Uro-Oncology, Institut Universitaire du Cancer Toulouse, Toulouse, France

Emmanuel S. Antonarakis
Departments of Oncology and Urology, Johns Hopkins University, USA

ASSOCIATE EDITORS

Peng Zhang
Department of Urology, Zhongnan Hospital of Wuhan University, Wuhan, China

Matteo Santoni
Oncology Unit, Macerata Hospital, Macerata, Italy

Gopal N. Gupta
Department of Urology, Loyola University Medical Center, Maywood, IL, USA

AUTHORS

Mohamed E. Abazeed
Department of Translational Hematology and Oncology Research, Department of Radiation Oncology, Taussig Cancer Center, Cleveland Clinic, Cleveland, OH, USA

Piyush K. Agarwal
Urologic Oncology Branch, National Cancer Institute, National Institute of Health, Bethesda, MD, USA

Emmanuel S. Antonarakis
Johns Hopkins Sidney Kimmel Comprehensive Cancer Center, Baltimore, MD, USA

Vanessa S. Arciero
Biological Sciences, Sunnybrook Research Institute, University of Toronto, Toronto, ON, Canada

Giorgio Astara
Department of Medical Oncology, University of Cagliari, University Hospital ‘Duilio Casula’ S.S. 554, Monserrato, Italy

Francesco Atzori
Department of Medical Oncology, University of Cagliari, University Hospital ‘Duilio Casula’ S.S. 554, Monserrato, Italy

David J. Barakat
Department of Oncology, Johns Hopkins University, Baltimore, Maryland, USA

Jyotsna Batra
School of Biomedical Sciences, Faculty of Health, Institute of Health and Biomedical Innovation, Australian Prostate Cancer Research Centre-Queensland (APCRC-Q), Translational Research Institute, Queensland University of Technology, Brisbane, Australia

Nicola Battelli
Oncology Unit, Macerata Hospital, Macerata, Italy
Robert H. Blackwell
Department of Urology, Loyola University Medical Center, Maywood, IL, USA

Alexandra M. Blee
Department of Biochemistry and Molecular Biology, Mayo Graduate School, Mayo Clinic College of Medicine, Mayo Clinic, Rochester, MN, USA

Orlando Burkhardt
Department of Urology, Cantonal Hospital Winterthur, Winterthur, Switzerland

Michele Caraglia
Department of Biochemistry, Biophysics and General Pathology, University of Campania “L. Vanvitelli”, Via L. De Crecchio, Naples, Italy

Joan Carles
Vall d'Hebron Institute of Oncology, Vall d’Hebron University Hospital, Universitat Autònoma de Barcelona, Barcelona, Spain

Liang Cheng
Department of Urology, Indiana University School of Medicine, Indianapolis, Indiana, USA; Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, Indiana, USA

Artem Cherkasov
Vancouver Prostate Centre and the Department of Urologic Sciences, University of British Columbia, Vancouver, Canada

Juan J. Chipollini
Department of Genitourinary Oncology, Moffitt Cancer Center, Tampa, FL, USA

Ananya Choudhury
Division of Cancer Sciences, The University of Manchester, Manchester Academic Health Science Centre, The Christie NHS Foundation Trust, Manchester, UK

Felix K. H. Chun
Department of Urology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

Chiara Ciccarese
Medical Oncology, University-Hospital of Verona, Verona, Italy

Alessandro Conti
Department of Urology, Bressanone/Brixen Hospital, Bressanone, Italy

Michael E. Cox
Vancouver Prostate Centre and the Department of Urologic Sciences, University of British Columbia, Vancouver, Canada

Daniel J. Culkin
University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA

Laura Demurtas
Department of Medical Oncology, University of Cagliari, University Hospital ‘Duilio Casula’ S.S. 554, Monserrato, Italy

Michael J. Donovan
Department of Pathology, Mt. Sinai School of Medicine, New York City, NY, USA

Therina du Toit
Department of Biochemistry, Stellenbosch University, Stellenbosch, South Africa

Ahmed Eldefrawy
University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA

Ihsan Y. El-Sayed
INSERM U955, Equipe 7, Creteil, France; INSERM U908, CPAC, Cell Plasticity and Cancer, Univ. Lille, Villeneuve d’Asq, France

Urban Emmenegger
Biological Sciences, Sunnybrook Research Institute, Odette Cancer Centre, Sunnybrook Health Sciences Centre, Institute of Medical Science, University of Toronto, Toronto, ON, Canada

Carrie A. Franzen
Department of Urology, Cardinal Bernardin Cancer Center,
Loyola University Medical Center, Maywood, IL, USA

Alan D. Friedman
Department of Oncology, Johns Hopkins University, Baltimore, Maryland, USA

Valentina Gallà
Unit of Biostatistics and Clinical Trials, Istituto Scienti co Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy

Anna Grimaldi
Department of Biochemistry, Biophysics and General Pathology, University of Campania “L. Vanvitelli”, Via L. De Crecchio, Naples, Italy

Carsten Gruellich
Translational Uro-oncology, Department of Medical Oncology, National Center for Tumor Diseases, Heidelberg University Hospital, Heidelberg, Germany

Gopal N. Gupta
Department of Urology, Cardinal Bernardin Cancer Center, Loyola University Medical Center, Maywood, IL, USA

Giorgia Gurioli
Biosciences Laboratory, Istituto Scienti co Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy

Jessica C. Hassel
Section of Dermato-oncology, Department of Dermatology and National Center for Tumor Diseases, National Center for Tumor Diseases, Heidelberg University Hospital, Heidelberg, Germany

James J. Hsieh
Genitourinary Oncology Service, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, USA

Michael Hsing
Vancouver Prostate Centre and the Department of Urologic Sciences, University of British Columbia, Vancouver, Canada

Haojie Huang
Department of Biochemistry and Molecular Biology, Mayo Graduate School, Mayo Clinic College of Medicine, Mayo Clinic, Rochester, MN, USA

Nuzhat Husain
Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, India

Byung Joon Hwang
Department of Molecular Bioscience, College of Biomedical Science, Kangwon National University, Chuncheon, Kangwon-do, Republic of Korea

Roberto Iacovelli
Medical Oncology, University-Hospital of Verona, Verona, Italy

Ken Inoki
Life Sciences Institute, University of Michigan, Ann Arbor, MI, USA; Department of Molecular and Integrative Physiology, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI, USA

Edith Jones
Life Sciences Institute, University of Michigan, Ann Arbor, MI, USA; Department of Molecular and Integrative Physiology, University of Michigan Medical School, Ann Arbor, MI, USA

Rattiyaporn Kanlaya
Medical Proteomics Unit, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Center for Research in Complex Systems Science, Mahidol University, Bangkok, Thailand

Yun Kee
Division of Biomedical Convergence, College of Biomedical Science, Kangwon National University, Chuncheon, Kangwon-do, Republic of Korea

Michael R. King
Meinig School of Biomedical Engineering, Cornell University, Ithaca, New York, USA

Igor Latorzeff
Department of Radiation Oncology, Clinique Pasteur,
Toulouse, France

Yi-Fen Lee
Department of Urology, University of Rochester Medical Center, Rochester, NY, USA

Yu-Ru Liu
Department of Urology, University of Rochester Medical Center, Rochester, NY, USA

Cristian Lolli
Department of Medical Oncology, Istituto Scienti co Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy

Angela Lombardi
Department of Biochemistry, Biophysics and General Pathology, University of Campania “L. Vanvitelli”, Via L. De Crecchio, Naples, Italy

Antonio Lopez-Beltran
Department of Pathology and Surgery, Faculty of Medicine, Cordoba, Spain; Department of Pathology, Champalimaud Clinical Center, Lisbon, Portugal

Ratha Mahendran
Department of Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

Brandon J. Manley
Urology Service, Department of Surgery, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, USA

Jocelyn R. Marshall
Meinig School of Biomedical Engineering, Cornell University, Ithaca, New York, USA

Filippo Martignano
Biosciences Laboratory, Istituto Scienti co Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy

Mónica Martinez-Fernández
Molecular Oncology Unit, CIEMAT, Madrid, Spain; Research Institute i+12, University Hospital 12 de Octubre, Madrid, Spain; Centro de Investigación Biomedica en Red de Cáncer (CIBERONC), Spain

Francesco Massari
Division of Oncology, S. Orsola-Malpighi Hospital, Bologna, Italy

Farhana Matin
School of Biomedical Sciences, Faculty of Health, Institute of Health and Biomedical Innovation, Australian Prostate Cancer Research Centre- Queensland (APCRC-Q), Translational Research Institute, Queensland University of Technology, Brisbane, Australia

Federica Matteucci
Nuclear Medicine Unit, Istituto Romagnolo per lo Studio e la Cura dei Tumori (IRST), Meldola (Fc), Italy

Erica J. McDonald
Biological Sciences, Sunnybrook Research Institute, University of Toronto, Toronto, ON, Canada

Alessia Mennitto
Medical Oncology Department, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

Christian Menzer
Section of Dermato-oncology, Department of Dermatology and National Center for Tumor Diseases, National Center for Tumor Diseases, Heidelberg University Hospital, Heidelberg, Germany

Omar Y. Mian
Department of Translational Hematology and Oncology Research, Department of Radiation Oncology, Taussig Cancer Center, Cleveland Clinic, Cleveland, OH, USA

M. Francesca Monn
Department of Urology, Indiana University School of Medicine, Indianapolis, Indiana, USA

Rodolfo Montironi
Institute of Pathological Anatomy and Histopathology, School of Medicine, Polytechnic University of the Marche Region, United Hospitals, Ancona, Italy

Rafael Morales-Barrera
Vall d’Hebron Institute of Oncology, Vall d’Hebron
University Hospital, Universitat Autònoma de Barcelona, Barcelona, Spain

Azfar Neyaz
Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, India

Vincent C. O. Njar
Department of Pharmacology, Center for Biomolecular Therapeutics, Marlene Stewart Greenebaum Comprehensive Cancer Center, University of Maryland School of Medicine, Baltimore, MD, USA

Jon K. Obst
Department of Genome Sciences Centre, BC Cancer Research Centre, BC V5Z 1L3, Canada

Giovanni Paganelli
Nuclear Medicine Unit, Istituto Romagnolo per lo Studio e la Cura dei Tumori (IRST), Meldola (Fc), Italy

Isabella Panfoli
University of Genova, School of Medical and Pharmaceutical Sciences, DIFAR-Biochemistry Lab., Viale Benedetto XV, Genova, Italy

Jesús M. Paramio
Molecular Oncology Unit, CIEMAT, Madrid, Spain; Research Institute i+12, University Hospital 12 de Octubre, Madrid, Spain; Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Spain

Filipe Pinto
Instituto de Investigação e Inovação em Saúde (i3S), University of Porto, Rua Alfredo Allen, Porto, Portugal; Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal

Guillaume Ploussard
Department of Urology, Saint Jean Languedoc Hospital, Toulouse, France; Division of Uro-Oncology, Institut Universitaire du Cancer Toulouse, Toulouse, France

Julio M. Pow-Sang
Department of Genitourinary Oncology, Moffitt Cancer Center, Tampa, FL, USA

Giuseppe Procopio
Medical Oncology Department, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

Marco Puzzoni
Department of Medical Oncology, University of Cagliari, University Hospital ‘Duilio Casula’ S.S. 554, Monserrato, Italy

Fabricio Racca
Vall d’Hebron Institute of Oncology, Vall d’Hebron University Hospital, Universitat Autònoma de Barcelona, Barcelona, Spain

Mohammad Ramadan
University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA

Marco Randazzo
Department of Urology, Cantonal Hospital Winterthur, Winterthur, Switzerland

Giorgia Ravaglia
Biosciences Laboratory, Istituto Scienti co Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy

Rui Manuel Reis
ICVS-Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, Campus Gualtar, Braga, Portugal; ICVS/3B’s-PT Government Associate Laboratory, Braga/Guimarães, Portugal; Molecular Oncology Research Center, Barretos Cancer Hospital, Barretos, SP, Brazil

Paul S. Rennie
Vancouver Prostate Centre and the Department of Urologic Sciences, University of British Columbia, Vancouver, Canada

Mani Roshan-Moniri
Vancouver Prostate Centre and the Department of Urologic Sciences, University of British Columbia, Vancouver, Canada

Marianne D. Sadar
Department of Genome Sciences Centre, BC Cancer Research Centre, BC V5Z 1L3, Canada
Amboise Salin
Department of Urology, Saint Jean Languedoc Hospital, Toulouse, France

Samanta Salvi
Biosciences Laboratory, Istituto Scienti co Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy

Matteo Santoni
Oncology Unit, Macerata Hospital, Macerata, Italy

Mario Scartozzi
Department of Medical Oncology, University of Cagliari, University Hospital ’Duilio Casula’ S.S. 554, Monserrato, Italy

Cristina Segovia
Molecular Oncology Unit, CIEMAT, Madrid, Spain; Research Institute i+12, University Hospital 12 de Octubre, Madrid, Spain; Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Spain

Sunao Shoji
Department of Urology, Tokai University Hachioji Hospital, Hachioji, Tokyo, Japan

Mohammad R. Siddiqui
Urologic Oncology Branch, National Cancer Institute, National Institute of Health, Bethesda, MD, USA

Susan F. Slovin
Genitourinary Oncology Service, Sidney Kimmel Center for Prostate and Urologic Cancers, Memorial Sloan Kettering Cancer Center, New York, NY, USA

Kelly L. Stratton
University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA

Amanda C. Swart
Department of Biochemistry, Stellenbosch University, Stellenbosch, South Africa

Rahul D. Tendulkar
Department of Radiation Oncology, Taussig Cancer Center, Cleveland Clinic, Cleveland, OH, USA

Stéphane Terry
INSERM UMR 1186, Integrative Tumor Immunology and Genetic Oncology, Gustave Roussy, EPHE, Fac. de médecine—Univ. Paris-Sud, University Paris- Saclay, Villejuif, France

Visith Thongboonkerd
Medical Proteomics Unit, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Center for Research in Complex Systems Science, Mahidol University, Bangkok, Thailand

Jeffrey J. Tosoian
Brady Urological Institute, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Quoc-Dien Trinh
Division of Urological Surgery and Center for Surgery and Public Health, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts, USA

Francis Vacherot
INSERM U955, Equipe 7, Créteil, France

Claudio Vernieri
Medical Oncology Department, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

Malte W. Vetterlein
Department of Urology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; Division of Urological Surgery and Center for Surgery and Public Health, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts, USA

Matthew J. Watt
Department of Physiology, Metabolic Disease and Obesity Program, and Cancer Program of the Biomedical Discovery Institute, Monash University, Clayton, Victoria, Australia

Frederik Wenz
Department of Radiation Oncology, University Medical Center Mannheim, Heidelberg University, Heidelberg, Germany
Catharine M. L. West  
Division of Cancer Sciences, The University of Manchester,  
Manchester Academic Health Science Centre, The Christie  
NHS Foundation Trust, Manchester, UK

Nami O. Yamada  
Department of Anatomy, Graduate School of Medicine, 
Gifu University, Gifu, Japan

Pina Ziranu  
Department of Medical Oncology, University of Cagliari,  
University Hospital ‘Duilio Casula’ S.S. 554, Monserrato, Italy

Corresponding Editor  
Mingzhen Gao, AME Publishing Company

Executive Typesetting Editor  
Xiaoting Xu, AME Publishing Company

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Will scholarly journals perish? This is a question that has puzzled me for years.

The introduction of online journals has resulted in the inevitable recession of print journals. The uprise of the open access journals has been changing the structure of scholarly journals ceaselessly. What keeps turning over in my mind is the open access of clinical trials data. What would the bigger picture be if open access to clinical trials data became the mainstream?

It is interesting that with the primary bottleneck being the availability of open data, the Big-data Clinical Trial (BCT) seems to stay where it was in spite of the increasingly popularity of “Big Data” among scientists. It has due to the fact that the fact that without open data, a statistical analysis is restricted to a particular area (or several areas). Even with big enough data, the study can only be termed as “research with big data sets” rather than “big data research”, which are totally different concepts. Big Data is constituted by a plurality of dimensions. On one hand, for an individual (e.g., a patient), the relevant data covering his/her disease course is large enough; on the other hand, for the entire population, as more as individuals (e.g., patients) are expected to be included, to contain all the elements just like the “universe set” in set theory; by doing so, scientists expect to carry out the so-called clinical studies in real-world settings.

Why do real-world-based clinical trials so appealing? It is understandable that there is a likelihood that the results and conclusions will be altered in studies targeting the same issue using the same research method with the sample size changed. Indeed, the probability of such a likelihood is quite high. In many top journals, it is a common phenomenon that some authors tend to validate the results of one study in another population using the same research method. However, if the results are “validated” in one population, it only means that they are “repeatable”. Will the results also be repeatable in the second, third, and populations beyond? If the attempts are not extensively iterated as they should be, this “validation” is, in a sense, equivalent to self-deception.

When clinical research data is open accessed, we can easily integrate data from multiple centers for statistical analysis and meanwhile “validate” the results in multiple populations. If this is the case, then another question arises: can everyone easily publish his/her results/papers in high-profile journals such as the New England Journal of Medicine? My answer is NO.

When the open access to clinical research data becomes mainstream, it will be easy to have access to a constantly updated database on the Internet. Simply by clicking on a button, we could obtain the statistical results of the most current data. Another click would display the validation results based on a specific population. The database would be updated at regular intervals (e.g., 1 month or 1 day), and the statistical results would likely also be changed accordingly. At that time, the question may change to “would any researchers publish their findings in a journal?” Well, even if someone is still keen to write such articles, journals may be reluctant to publish them because of the indefiniteness of the findings with the risk of being overturned at anytime.

Eventually here it comes the serious question: will scholarly journals perish? My answer is still NO. In what way then can scholarly journals continue to be relevant?

During my Business Administration course, my teacher distributed to us an article from the Case Study column of the Harvard Business Review. In this highly respected journal, articles in this column often present one case first, followed by the comments from two experts. These comments could either support or oppose each other. My teacher asked us to study the case, read through the comments and then form our own point of view on the case. He encouraged us to interpret the case from different perspectives independently, which I found to be a fairly practical exercise.

Thinking of this course brought up a possible answer to me. When the open access to clinical research data becomes mainstream, the entire publishing industry, especially the publication of “scholarly journals”, would eventually experience revolutionary change. It may no longer focus on rigid and cold outcomes but would surely shift focus towards the reflection on problems, the update of insights, and the integration of science and arts.

AME Medical Review Series is a product born of this new mentality. As an attempt, we decided to invite international experts to provide their views on a specific topic to share their insights with more clinicians with the aim that this will ultimately benefit more patients. The first chosen topic for the series is the currently controversial one: conventional surgery versus
stereotactic body radiotherapy for the early stage lung cancer. As the first book to the series, we hope it will give you a glance of the coming changes.

The book series will be written by a group of individual experts who are willing to contribute medical reviews and comments for those readers who are specially interested in clinical research and medical reviews. While the book in your hand may be about a difficult subject, we do hope we have presented it in an accessible manner. We would be overjoyed if it can, in any way, bring you thought and inspiration.

Stephen D. Wang
Founder and CEO,
AME Publishing Company
The current textbook of “Key Leaders Opinion on Urogenital Cancer: Frontier and Progress” represents a timely, complete and thus important contribution to the molecular understanding of urological malignancies in our modern times.

The book is divided into seven sections: progress in cancer genomics, molecular biology and pathology of cancer, metabolism and malignant properties, urine and exosomes, precision medicine in cancer, targeting cancer and molecular evaluation of cancer prognosis. Each section gives a comprehensive and insightful review on urogenital cancers, such as prostate cancer, bladder cancer, renal cell carcinoma, etc.

The book precisely displays the requirements of up-to-date uro-oncology, beyond clinical but genomic information enabling individual precision medicine for our patients—a “must read” for students, clinicians and scientists.

Felix K. H. Chun, MD, MA, FEBU
Professor and Chairman,
Department of Urology,
University Hospital Frankfurt a.M.,
Germany
Even though the etiology of cancer is complicated by several risk factors contributing to cancer genesis, the detection as well as treatment options for urogenital cancers (including prostate, bladder, and cervical cancers, and renal cell carcinoma) have been greatly modified in recent years with a number of nonsurgical interventions now generally available. Notwithstanding the great conceptual breakthroughs in our understanding about the nature as well causes of cancer, modifications of existing urologic techniques and discovery of novel powerful molecular therapeutic targets have contributed to additional improvement in patient outcome. Environmental factors such as smoking, drinking, food intake, viral (or other) infections, chronic inflammation and genetics are considered to be contributors in tumor progression. Many critical aspects of our understanding of these contributors that underlies the urogenital cancers has been described in this useful volume, split into seven key sections converging the unified research in the last few years.

Genetic and epigenetic factors contribute a significant fraction, producing inherited predispositions to the development of various types of urogenital cancers. In the early days, a failure to appreciate the hereditary aspects of urogenital cancer has delayed our knowledge and prevented obvious therapeutic advances in this area. Nonetheless, high-throughput gene expression analysis and next-generation sequencing (NGS) have been at the forefront of dissecting the genetic component, which has allowed the molecular analysis of the entire human genome in a matter of hours. These high throughput techniques have undoubtedly revolutionized our understanding of the disease and hold great promise for improving diagnostic and prognostic accuracy and may be now termed as Clinomics. To this effect, the first chapter (Section I) of the book by Wenz et al. summarizes a genomic classifier based on expression of predefined biomarkers to identify prostate cancer patients who may benefit from aggressive therapy in order to “hit early and hit hard”. Similarly, genomic scores could help us shortlisting patients who don’t need any aggressive treatment and are great candidates for active surveillance (Ploussard et al.). Nonetheless, these studies and also a study summarised by Tosian et al. reminded us about the tumor multiclonality and tumor heterogeneity and questioning if the genomic classifiers are not yet ready for the clinic. In an effort to provide solutions Trapani et al. in this section suggested that circulating tumor DNA analysis may allow a more comprehensive assessment of the molecular heterogeneity of the patient’s prostate cancer, which also can lead to a personalized and combinatorial treatment with targeted therapies. Segovia et al. have very well reviewed the complexity of EZH2 as treatment target covering its mutations, different roles and acquired resistance in bladder cancers. Last but not least, Manley et al. in this section has covered the novel genomic studies of renal tumors with sarcomatoid variant histology. These results have demonstrated that progressive dedifferentiation is the source of the sarcomatoid elements in renal carcinomas. Overall, these genomic studies are paving pathways towards clinical translation. More thorough studies of medical histories, family backgrounds, tumor heterogeneity and environmental exposure are now being carefully compiled and reported to enhance our comprehension of genetics of urogenital cancer.

Amalgamation of molecular biology with disease pathology has already started to bear its fruits. Consequently, firmly establishing molecular pathology in oncology practices. Development of high throughput genomic, proteomic and epigenome technologies have gradually extended the molecular diagnostic armamentarium of urogenital cancers helping early cancer detection and tumors subclassification. To this effect, in Section II, Matin et al. summarized a miRNA panel for the diagnosis of aggressive prostate cancer. Intriguingly, the elementary research has identified several molecular mechanisms communal to multiple cancers, while others are very subjective and uniquely confined to only specific cancer. For example, epithelial mesenchymal transition (EMT) is one of the key steps for fibrogenesis and cancer metastasis and several studies including the one described by Kanlaya et al. in Section II of this book has tried to understand the molecular mechanisms underlying EMT and its regulation. In the process, oncometabolite fumarate has been discovered as a potent agent inducing epigenetic regulation of EMT in kidney cancers. Similarly, rapamycin complex 1 (mTORC1) pathway has been identified as a common pathway in several cancers controlling anabolic and catabolic processes with appropriate checkpoints and balances to maintain cellular homeostasis. Jones et al. summarises a novel molecular mechanism by which oncogenic MiT/TFE transcription factors support cell growth/proliferation of cancer cells through their transcriptional regulation of the upstream of mTORC1 activator, RagD. On the other hand, pathways such as Androgen receptor (AR) pathways are not confined but specific to prostate cancer genesis. Based on this, inhibiting the production of androgens by castration or their effects by using anti-AR agents are employed as a treatment of advanced prostate cancer. El-Sayed et al. commented on elevated fibroblast growth factor (FGF) and downstream mitogen-activated protein kinase (MAPK) pathway activity as the main cellular and molecular determinants driving underlying escape of AR-directed therapy, which is also connected to EMT. Recently efforts have been
made to understand the molecular mechanisms behind rare and more aggressive form of urogenital cancers. For example, Pinto and Monn et al. have molecularly dissected the role of N-myc and their disruption by aurora kinase A inhibitors as a potential therapeutic target for treatment of small cell prostate cancer. Monn et al. and Husain et al. have commented on the emerging molecular pathways involved in a rare form, microcystic variant urothelial carcinoma. These studies undoubtedly suggest that molecular pathology has evolved into a novel focus of clinical pathology. A combination of traditional pathology and molecular pathology is bound to give rich dividend in term of guiding tumor therapy explicitly discussed in Section VII of the current book.

Cancer metabolism is an emerging hallmark of cancer, capable of segregating urogenital cancer patients into a distinctive molecular classes with variable clinical outcome. The alterations in intracellular and extracellular metabolites that can accompany cancer-associated metabolic reprogramming have profound effects on oncogene expression, cellular differentiation, and the tumor microenvironment. Protein catabolic pathways via macro autophagy is considered a critical metabolic rewiring in cancer cells. In the section III, Barakat et al. have commented on the role of autophagy in the PTEN-loss driven mouse prostate cancer in vivo model. Similarly, Watt et al. and Blee et al. have commented on the role of altered lipid metabolism mostly via secretion of adipose tissue derived proteins called “adipokines”, focussing on the effects of CCR3/CCL7-mediated cell migration. McDonald et al. summarized a study which followed bioinformatics analysis to shortlisted key regulators of prostate cancer cell metabolism, and identified peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC1α) as a key transcriptional network regulator with a role in prostate cancer metastasis suppression. In addition to metabolism of macromolecules, drug metabolism pathways are attracting special attention to improve patient outcome in drug resistant tumors. Obst et al. have covered the metabolism of abiraterone, describing six previously unknown metabolites and their effect on both androgen metabolism and tumor progression in hope to improve the treatment for castration resistant prostate cancer (CRPC).

The need for a predictor of malignancy is universally recognized. While some of the previous sections of this book has covered the use of genomics, gene expression including that of miRNA and epigenetics signatures to be at the forefront of clinical translation of biomarker discovery for early diagnosis and prognosis of urogenital cancers, Section IV amess the role of cancer-derived exosomes in tumor progression and metastasis, including that in intracellular communications (Panfoli et al.) using mediators in the forms of protein, DNA and assorted RNA molecules. Once referred to as a “rubbish bag” to wrap up and dump out waste, the term “exosome” in also gaining a newfound glory as potential novel easily accessible urine based biomarker of cancer diagnosis and prognosis (covered by Liu et al.). As of this year a urine-based, non-invasive test has become commercially available for prostate cancer to improve discrimination between indolent and aggressive disease. On the other hand, Yamada et al. have brought back the value in understanding the role of content of exosomes and their potential clinical usage. Following which Vetterlein et al. summarized a study published in JAMA suggesting the pre-biopsy use of an exosome-derived gene expression signature to avoid unnecessary invasive procedures. The study described by Donovan et al. in this section goes beyond exosomes describing the use of liquid biopsy which relies on the isolation of cellular components found in post-digital rectal exam of total urine samples of prostate cancer patients. Despite the challenges, such as streamlining the collection process, identification of a stable urine normalization control, exosomes provide a novel platform for liquid biopsy for disease diagnosis and following disease progression and recurrence. Exosomes also hold exquisite promise in the delivery of therapeutics given their low immunogenicity, the environmental protection provided by their lipid bilayer membranes, and potential for targeting to cell types of interest. Blackwell et al. have summarised their efficiency in delivering siRNAs and chemotherapeutic agents into cancer cells.

Personalized medicine is the new Buzz word in the cancer world. As per Wikipedia personalized medicine, precision medicine, or theranostics is a medical model that separates people into different groups—with medical decisions, practices, interventions and/or products being tailored to the individual patient based on their predicted response or risk of disease. The section V thus provides the clinical nutshell of the above described sections mostly incorporating genetic stratification such as polygenic risk scores (Randazzo et al.) and/or mutation profiles (Ciccarese et al.), gene signature (Lombardi et al.—for response to radiotherapy) and MRI guided biopsy (Shoji et al.) to eventually direct patient’s therapeutic regime for prostate cancer. Similarly, Roshan-Moniri et al. suggested an ERG directed therapy based on ERG and other oncogenic ETS family members expression profile as alternative or complimentary agents for the current chemotherapeutics to treat therapy resistant prostate cancer. Mennitto et al. focussed their comments on Urachal cancer, a rare and extremely aggressive
malignancy, suggesting its shared genomic alterations with colorectal carcinoma based on a case study with the presence of EGFR amplification. Matteucci et al. explored the role of the prostate-specific membrane antigen as both a diagnostic and therapeutic agent in renal cell carcinoma based on its expression dysregulation. Although these studies are highly encouraging, the results should be interpreted with pinch of salt for the validity of these signatures for the multiethnic populations.

The next phase of personalized medicine is to develop molecularly targeted therapy with minimal side-effects, which could not always be effective as hitting the target does not always mean the tumor will respond to the drug due to genomic complexity and/or tumor heterogeneity and/or tumor microenvironment. For the same reasons, the response to treatment may be temporary. The design of molecular inhibitors is inspired by the expression, function and structural determinants of the molecules within the tumor milieu. Following these classical primes and virtual drug design/screening has led to the development of small molecules that can inhibit the interaction between SPOP and its interacting partners in kidney cancers as summarized by Hwang et al. in Section VI of this book. Santoni et al. and Chipollini et al. pointed that differences in responsiveness to adjuvant and/or salvage therapy in multiple clinical trial can be streamlined if the molecular and biological features can be examined to select the patients. Similarly, Stratton et al. summarizes that a group of penile cancer patients with high PD-L1 expression in HPV negative tumors may be susceptible to novel checkpoint inhibiting therapies, also supporting this dual pathway to malignant transformation. Notwithstanding, molecular inhibitors have witnessed success as adjuvant therapy in recent years at least in experimental models. In addition, understanding the molecular mechanism of action of a drug can help in drug repositioning. Testifying the same, Nijar et al. summarized a study where clinically approved molecule/drug inhibitors of CYP17 were found to antagonize the androgen receptor and thus rationalize the clinical efficacies of “dual CYP17 inhibitors/AR antagonist” in the clinic in men with CRPC. During the last decades different immunotherapies, targeting at enabling the immune system towards recognizing cancer antigens and eliminate the tumor cells, have been trialed with some documented successes. Development of suitable in vivo models with intact immune system is a requisite to interrogate the tumor microenvironment and signaling pathways in response to single targeting agents given alone or in combination with immunotherapy agents such as checkpoint inhibitors. Slovin et al. and Ziranu et al. described the successful use of such models for assessing effectiveness of the combinatorial immunotherapy against infiltrating Myeloid-derived suppressor cells for CRPC. Menzer et al. have extended their comments on using combination therapy with ipilimumab and nivolumab for kidney cancer. Three studies in this section have covered the persistent toxicity due to Bacillus Calmette-Guerin immunotherapy for urothelial carcinoma and strategies to minimize the side effects- all pointing towards in-depth molecular characterization of the selective response.

A large volume of work presented in this book has been concerted to prostate cancer, a disease of major concern across developed countries with 1.1 million cases being diagnosed per year. Although early detection of urogenital cancers is key to better prognosis by starting early therapy; prostate cancer falls in a unique spectrum due to the availability of Prostate Specific Antigen (PSA) as a non-invasive biomarker for disease diagnosis. Since the discovery of PSA almost 40 year ago, the number of clinically reported prostate cancer cases have increased exponentially. PSA test has been recently criticized for not being able to distinguish indolent disease from aggressive prostate cancer leading to over-diagnosis and over-treatment. Given the perils of a prostate biopsy including infection, cost and diagnosis of low-risk, indolent prostate cancer, hunt for a highly specific prognostic biomarkers continues in the 21st century and is well documented in the last section of our book covering the use of gene expression profiles (Choudhury et al.) to genomic profiling including mutilations in AR genes during (CTC) (Martignano et al.), splicing of Uridine diphosphate glucuronosyltransferase 2B type 28 (Toit et al.) and circulating tumor cells (Marshall et al.). Interestingly, no differences were observed between robotic assisted laparoscopic radical prostatectomy on intraoperative levels of prostate cancer CTCs contemplating the hypothesis that the introduction of CTCs during surgery may promote cancer progression. Mian et al. summarizes the molecular markers for basal and luminal subtypes of prostate cancer and these molecular signatures could be predictive classifier in identifying the subgroup who might benefit from androgen deprivation therapy. Based on these studies, it is well anticipated that not just single but integrative molecular and classical pathology biomarkers will play an increasingly important role in risk stratification for clinical decision making not just in prostate cancer but other urogenital cancers.

It is an exciting time of translation from bench to bedside in cancer personalized medicine and molecular pathology have the potential to be the guiding hand in determining optimal treatment regimen of targeted therapies for patients with advanced diseases. Through this book, we intended to cover the recent developments in urogenital cancers at the research
and at the clinical trial fronts bringing our readers’ up to speed. We hope that the material and overview of the current state of research covered in this book will provide sufficient intellectual stimulations for our readers to venture new ideas into their research and clinical practices.

Jyotsna Batra, PhD
School of Biomedical Sciences, Faculty of Health, Institute of Health and Biomedical Innovation, Australian Prostate Cancer Research Centre- Queensland (APCRC-Q), Translational Research Institute, Queensland University of Technology, Brisbane, Australia
(Email: jyotsna.batra@qut.edu.au)
Urological and male genital cancers account for a considerable proportion of all human solid neoplasms and is leading to increased morbidity and mortality globally. The last decade witnessed dramatically boosted researches on urogenital cancers, some of which have profoundly impacted the diagnostic or therapeutic practice of the diseases and subsequently, sprung up intense discussions.

Since the year of 2016, the editorial board of *Translational Cancer Research* (Print ISSN: 2218-676X, Online ISSN: 2219-6803) has invited scientists across the world to write editorials and perspectives on pivotal articles that were published in some notable top journals such as *Nature, Cancer Cell, European Urology*. In this way, frontiers and progresses of urogenital cancer science were delivered to the readers, while key opinions from academic leaders were shared among the scholars worldwide.

The information explosion era has been challenging traditional reading pattern. Readers are usually too busy to screen out superior papers from numerous journals, and they do expect to acquire the viewpoints from academic elites on associated issues. Therefore, the present book aims at collecting key leaders’ opinions on the latest research articles of urogenital cancers and rendering them to the readers in a friendly and readable manner. Moreover, we are delighted to see the diversity of the authors: they come from medical or academical centers of the five continents, including Johns Hopkins University School of Medicine, Mayo Clinic, Cleveland Clinic and National Cancer Institute in the USA, Heidelberg University in Germany, University of Manchester in the UK, European Institute of Oncology in Italy, Australian Prostate Cancer Research Center in Australia, Gifu University in Japan, University of Porto in Brazil, Stellenbosch University in South Africa, etc.

Content comes first! The diversity of the authors ensures the diversity of the content. The book is characterized with a variety of topics on molecular biology, detection, treatment and prognosis of the diseases, containing discussions on popular fields like precision medicine and immunotherapy, which reveals the painstaking efforts of the authors and editors.

We used to wonder that what we can do in the times of translational medicine and precision medicine. Now what we are doing on this book is making a humble contribution to the challenging era. A single spark kindles a prairie fire, and we are looking forward to enjoying the next season to this first series of *Key Leaders’ Opinion on Urogenital Cancer: Frontier and Progress*!
The genomic revolution in oncology has dramatically altered our molecular understandings and clinical management of urological malignancies over the past several years. For example, in the case of prostate cancer, there are now at least two molecular classifications of the disease that have clinical relevance for therapeutic strategies. The first example, defined by prostate cancers harboring germline or somatic mutations in homologous-recombination DNA repair genes (e.g., BRCA2, ATM) appears to be associated with sensitivity to poly (ADP-ribose) polymerase inhibitors such as olaparib. The second example, defined by tumors that are deficient in DNA mismatch-repair function giving rise to microsatellite instability, is linked with sensitivity to a different class of drugs: PD-1 inhibitors such as pembrolizumab. Similar examples of genomically-targeted therapeutics are beginning to emerge for other urological cancers including bladder cancers and renal cell cancers. Moreover, as genomic and biomarker technologies become better and cheaper, it is becoming increasingly possible to use multiple body compartments (tissue, blood, urine) to assess for multiple analytes (tumor DNA/RNA, circulating tumor cells, circulating nucleic acids, exosomal contents) in an effort to achieve the dream of precision oncology as it relates to the management of genitourinary malignancies. All of a sudden, it seems that the future that we have all been waiting for has arrived.

This book has gathered together a group of elite experts in the fields of molecular genetics, basic biology, cancer metabolism, biomarker development, drug development and clinical experts in the science and therapy of urological malignancies with a focus on prostate cancer, urothelial cancer and renal cell cancer. Over a series of chapters linked by common themes, this book represents the state-of-the-art knowledge spanning basic science, translational biology and clinical management of urogenital cancers. The target audience for this book will include a broad range of individuals, from postdoctoral research students, to medical students, to translational scientists, to clinicians involved in the field of genitourinary cancers. The AME Publishing Company should be congratulated for compiling such a great collection of works representing the state-of-the-art in our current understanding of urogenital oncology. I am confident that the readership will enjoy this book very much.

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Footnote

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Progress in cancer genomics and molecular biology has led to straightforward diagnostic and clinical improvements in the context of genitourinary tumors. In a period of time of approximately 10 years these changes have opened the way to the route towards precision medicine in this field, with extremely enthusiastic consequences for patients’ prognosis and quality of life. The advances in understanding the genetic and epigenetic alterations that characterize renal, urothelial and prostate cancers have shed light on the molecular heterogeneity of these tumors. This parallels with the growing evidence on the role of metabolic alterations in the mechanisms of tumor initiation, metastatization and primary or acquired drug resistance.

The improvement on the biological basis of these diseases have provided new opportunities to enhance the diagnostic approach to urogenital cancers. In a long-term perspective, the introduction of non-invasive tests in different biological fluids, from blood to urine and saliva, will give an essential contribution to supervise real-time tumor dynamics in both early and advanced stages. Among emerging tests, the study of extracellular vesicles and exosomes has reported promising results and constitutes an evolving source of biomarkers for early detecting cancer and monitoring tumor response to therapies.

In the era of molecularly targeted approaches, manipulating tumor microenvironment is emerging as a key strategy in cancer research. Agents directed towards hypoxia-related or energy/nutrient sensing targets have been demonstrating their efficacy in a series of tumors and will probably enrich the future therapeutic armamentarium of genitourinary tumors. The possibility of handling tumor microenvironments requires also a deep comprehension of the activities of immune cells within this context. Although we have learned progressively that immune editing represents a crucial step in the early stages of tumor development, the progresses reported in the last 15 years have led to the development of novel immunotherapeutic agents that have been imposing as the new standard of care in a variety of tumors, including renal and urothelial cancers. The identification of the best therapeutic setting (adjuvant, neoadjuvant or advanced) and the molecular rational of sequencing or combining immunotherapies with targeted agents, chemotherapy or local interventions is a hot topic in oncology and will constitute the focus of the majority of future clinical trials in urogenital tumors.

This book has been assembled to provide the state of the art about current and future diagnostic and therapeutic scenarios in urogenital tumors and represents an outstanding piece of work, being composed of articles by key opinion leaders in this field.
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Biochemical recurrence after radical prostatectomy for prostate cancer can be seen in 30–50% of the cases depending on stage, PSA and Gleason score. About 50% of the recurrences become apparent within 2 years after surgery with local failure being the predominant pattern. It is therefore obvious that early post-operative adjuvant radiotherapy (ART) or PSA-triggered salvage radiotherapy (SRT) are frequently considered in these patients and improve outcome. Open questions regarding the optimal mode of treatment at biochemical recurrence especially the combination of radiotherapy with antihormonal therapy and/or other systemic agents are currently investigated. In addition current studies demonstrated the value of dose-escalated SRT especially in patients with R+ disease (1).

The current paper by Freedland et al. (2) helps to shed some light on the challenge to identify patients who may benefit from more aggressive therapy in order to “hit early and hit hard”. In a retrospective analysis, tumor material from a cohort of 170 men receiving SRT (median 66.6 Gy) after radical prostatectomy was analysed. Twenty patients (12%) developed metastases after a median follow-up of 5.7 years. The genomic classifier (GC) based on expression of 22 predefined biomarkers (affymetrix oligonucleotide microarray) predicted the risk of metastases better than established scores [CAPRA-S (3), Briganti et al. (4)] and identified a group of patients with a high risk of metastases (33.1%). On univariate analysis Gleason score 7 and higher, extraprostatic extension and pre-SRT PSA as well as GC significantly predicted post-SRT-metastases. GC remained an independent predictor after adjusting for clinical variables.

This study adds to our knowledge and represents an important milestone in personalizing treatment in order to deliver more aggressive treatment for selected patients. However, there are several open questions which need to be addressed in future analyses.

- What would have been the role of early adjuvant RT in these high risk patients as identified by the GC? Wouldn’t it be better to treat these patients with upfront radiotherapy in order to stop metastases at the source? The time delay between surgery and the initiation of SRT (median 12.4 mon) allows selected tumor cells to leave the prostatic fossa and either migrate to lymph nodes or beyond. Factors favouring early adjuvant RT were present in a high percentage of the patients (extraprostatic extension 52.7%, seminal vesicle invasion 26.6%, positive surgical margin 80.6%). So why not treat early as long as the tumor is restricted to the prostatic fossa and can be cured by RT alone?

- Focussing the genetic analyses only on tumor tissue without having normal tissue sensitivity in mind may not allow to properly increase the aggressiveness of the treatment. The therapeutic index has to be kept in mind. i.e., increasing the radiation dose or administering combined modality treatment in a sensitive subgroup of patients may induce unacceptable toxicity;
Clinomics, which is defined as the total clinical information about the patient, is heavily underutilized in investigations like this. We are well aware, that especially regarding radiation response and toxicity life style factors like smoking habits, body mass index, use of herbal additives etc. significantly influence the outcome. Like epigenetic regulation and post-translational modifications, clinical factors (the “clinome”) may influence the microenvironment. Because the mechanisms of radiation response in tissues are predominantly mediated via reactive oxygen species (ROS) e.g., smoking can lower the chance of overall survival by up to 20% (5,6) and significantly increase the risk of side effects (7).

Future studies should not put an isolated focus on genetic predictors but investigate the complete picture including the genome, the epigenome, the transcriptome, the proteome, the metabolome and last but not least the clinome.

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Footnote

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References

Bladder cancer (BC) as epigenome disease

Cancer arises as a consequence of accumulating genomic alterations, which affect primarily oncogenes and tumor suppressor genes (TSGs). This condition creates a particular landmark that can be exploited in therapy. Not surprisingly, many pharma companies launched programs aimed to inhibit the gain of function activities caused by oncogene mutation and/or amplification, and many of these inhibitors are currently in the clinics. The situation is not so simple in the case of TSGs, where a common loss of function precludes, in most cases, their actual use as therapeutic targets.

BC is a clinical challenge due to its incidence, prevalence and mortality rates (1). Moreover it also represents an economic problem as, due to its high rate of recurrence, a regular surveillance by cystoscopy and urine cytology is required (2). BCs are also characterized by the frequent alterations in genes governing chromatin organization and histone modifications, as shown by whole exome sequencing studies, leading to particular changes in the epigenome reflected in altered expression of multiple genes (3). Chromatin regulatory elements, which are epigenetic molecules that regulate flexible processes based on little post-transcriptional modifications such as acetylation, methylation or ubiquitination to express or repress genes, appeared therefore highly altered in BC and, within these alterations, at least 89% are histone remodelers and 64% nucleosome positioning genes (4). The role of epigenetics is surprisingly wide and involves not only chromatin remodelers, but also changes in DNA methylation, expression of miRNAs, lncRNAs, etc. Therefore, epigenetic factors are becoming attractive targets to develop new treatments for patients. Regarding chromatin remodelers in BCs, it is worth considering the two main pathological entities of these diseases: the non-muscle invasive BC (NMIBC), and muscle invasive BC (MIBC). While this pathological classification is now under question due to the discovery of intrinsic subtypes (5), through whole transcriptome studies, similar to other solid tumors, it also has huge relevance as it currently defines the possible therapeutic options. The NMIBC are treated by transurethral resections, in some cases followed by intravesical therapy, whereas the MIBC are regularly treated by cystectomy followed by chemotherapy. Although NMIBCs have a more favorable prognosis, they frequently recur and acquire MIBC features. Therefore, it is necessary to identify and characterize precise biomarkers of recurrence and progression for early diagnosis and follow-up, which may become possible therapeutic targets and be a non-surgical treatment option, improving patient survival and prevention of tumor recurrence. The aberrant epigenetic landscape is a hallmark of human cancer (6) and characterizes BC as an epigenome disease. The identified epigenetic alterations suggest new possibilities for the treatment of different bladder tumors, making them good candidates for epigenetic therapy.
**Therapy for synthetic lethality in suppressor genes**

Synthetic lethality occurs when the expression of two genes (or their mutated counterparts) promotes cell death. Usually these two genes are not co-expressed, are mutually exclusive, and conforming a lethal interaction. These lethal interactions are based either on genetic mutations or the introduction of molecules with a known cytotoxic effect in cancer cells or patients. The acquisition of mutations confers tumor cell advantages in proliferation, survival and even drug resistance, attributing them a pattern of differential gene regulation to normal cells. This hallmark can convert tumor cells into therapeutic targets, identifying those alterations that can be targeted to induce specifically synthetic lethality (7). Therefore, the use of synthetic lethality strategies would bring us closer to more targeted treatments for different patients (5). Nowadays, progress is being made in synthetic lethality of genes such as RB1, TP53, BRCA1, RAS and C-Myc (8).

There are different experimental methodologies to identify the inactivation of genes that can show a lethal phenotype under a given genotype. Currently, in some cancers with TSGs mutants, large libraries of small hairpin RNA (shRNA) (9), small interfering RNA (siRNA) (10), and RNA guides for gene editing using CRISPR/Cas9 or TALENs (11) are commonly used. The main difference between these techniques is the inactivation efficiency: while CRISPR engineering allows for complete silencing of genes, the use of shRNA or siRNA induces a downregulation of the genes that is temporary and with greater variability between recognizable sequences. These small RNAs can recognize other target sites, known as off-targets, which distort the interpretation of data, creating false positive genes for synthetic lethality (9). Regarding CRISPR/Cas9 approaches, they may lead to side off effects due to the guides selected. However, CRISPR/Cas9 can be so selective and efficient that the inactivation of an essential gene is lethal per se, and therefore does not allow to see the phenotype that can be observed with the use of shRNA. In addition, most of the drugs in development are competitive inhibitors, so that partial inhibition promoted by shRNA allows for the reproduction of the pharmacological effect.

In BC, substantial evidence that the epigenome shows profound alterations is reflected in a frequent silencing in some well-known TSGs such as histone demethylase KDM6A, which is mutated in 24% of BC cases, or ARID1A, which is part of the SWI/SNF complex of nucleosomes and altered in 25% of BC cases (6). Mutations in these tumor suppressors, often deep deletions or putative truncating mutation (4), may help identify patients susceptible to synthetic lethality, one of the most promising recent approaches in epigenetic therapy. In different tumor subtypes in which there is already a silenced gene, an interesting possibility could be to take advantage of this silencing to perform the inhibition of an antagonist of KDM6A and ARID1A tumor suppressors, like EZH2, to promote synthetic lethality. EZH2, a histone methyltransferase that regulates gene silencing through the H3K27me3 mark, is part of the polycomb repressive complex 2 (PRC2) together with other factors such as EED or SUZ12. Upregulated EZH2 can inactivate the transcription of many other suppressor genes, thus having an oncogenic functionality. Interestingly, EZH2 is involved in the processes of recurrence and progression in NMIBC (12). Furthermore, different studies have found that the suppressor genes KDM6A and ARID1A antagonistically regulate the expression of genes involved in cell proliferation and survival, such as PIK3IP1, which negatively regulates the PI3K-AKT pathway, and IGFBP3, which is involved in anti-proliferative signals, appears frequently silenced in BC, and is activated by inhibiting EZH2 (10,11).

Therefore, synthetic lethality emerges as a novel approach in targeted therapy for BC, as well as many other cancers, as it reduces the secondary effects of chemotherapies, tackles drug resistance, and improves knowledge of signaling pathways to define more accurately the different tumor subtypes. Moreover, synthetic lethality provides an additional way for the individualized treatment of patients (9).

**The complexity of EZH2 as treatment target: mutations, different roles and acquired resistance**

Among the most altered chromatin remodelers altered in BC, inactivating mutations in the histone H3 lysine 27 (H3K27) demethylase KDM6A (also known as UTX) were most common and enriched in NMIBCs (32–43%) (1,3), whereas inactivating mutations in the SET family histone H3 lysine 4 (H3K4) methyltransferase MLL2 were more common in MIBCs (19%), and mutations in KDM6A and MLL2 were mutually exclusive (1). This fact is not well understood at present and would require in depth chromatin immunoprecipitation/sequencing (ChIP-seq)
future studies.

*KDM6A* gene is a histone H3 trimethyl (H3K27me3) demethylase located on chromosome Xp11.2. This gene plays specific functions creating a transcription-permissive chromatin structure through its demethylase activity, and displays multiple alterations in a wide range of human tumors, in most cases leading to loss of function situation (*Figure 1A,B*). This has led to the assumption that KDM6A represents a bona fide TSG in the BC context. The KDM6A function opposes to that of EZH2, which is the catalytic subunit of PRC2, frequently overexpressed in multiple malignancies (prostate, breast, bladder, endometrial, melanoma, etc.) showing a positive correlation with high grades and worse prognosis (1,12,13). This frequent overexpression has also promoted the increased interest in developing EZH2 inhibitors still presently in clinical trials (1,13,14).

In addition, the functional antagonism between KDM6A
and EZH2 has been recently exploited to find possible new avenues in BC therapy. In a recent elegant study, Ler et al. have confirmed that KDM6A is frequently lost in MIBC and NMIBC accounting for the enrichment in PRC2-regulated signaling (11). Remarkably through various experimental approaches, they also showed that loss of KDM6A confers specific vulnerabilities to EZH2 inhibition. In particular, EZH2 inhibition delays tumor onset and induces tumor regression of KDM6A-null cells and patient-derived xenografts models. This work represents an excellent example of how TSG loss vulnerabilities can be exploited in the context of cancer therapy. Of note, similar vulnerability to EZH2 inhibitors has been previously reported for ovarian tumors bearing mutations in ARID1A gene (10). ARID1A encodes a component of the SWI/SNF chromatin-remodeling complex, and also shows a high mutation rates in multiple cancer types, thus playing various TSG functions. Similarly, loss of ARID1B in ARID1A-deficient backgrounds destabilizes SWI/SNF and impairs proliferation in cancer cells (15). The SWI/SNF complex is composed by the combinatorial assembly of around 15 subunits and contributes to DNA repair and to transcriptional regulation in a lineage-specific manner. Since genetic approaches in various organisms, including fly, showed opposite roles for SWI/SNF and Polycomb mutations, the possible susceptibility of tumors bearing ARID1A, PBRM1 and SMARCA4 alterations to EZH2 inhibition has also been tested (7). Importantly SWI/SNF-mutant cancer cells are primarily dependent on a non-catalytic role of EZH2, being only partially dependent on EZH2 histone methyltransferase activity (7).

This is also relevant in the context of BC. Indeed SWI/SNF-complex subunits are also frequently altered in BC patients (Figure 2) (4) with various mutual exclusivity/co-occurrence situations. According to this, EZH2 is an attractive target for BC management. However, further elucidation and research on this aspect is extremely needed. On one hand, among the various EZH2 inhibitors under study some are catalytic inhibitors with pleiotropic actions due to their action as SAM-hydrolase or SAM-competitive inhibiting compounds (16), whereas others may affect the stability of PRC2 complexes. Accordingly, the complete determination of the dependence on EZH2 catalytic activity in the various mutated genes is required. On the other hand, the specificity of these inhibitors over EZH2 or EZH1 is strictly required. Indeed, these two proteins may play overlapping but also different functions in normal organism development and in cancer (17). In addition, it is worth to mention that several studies have demonstrated that specific EZH2 inhibitors seem to be particularly effective in impairing cell growth in a mutant EZH2 background, showing a low effectiveness in those cases with wild type EZH2 (16,18). It is also important to note here that effects of EZH2 catalytic inhibitors are really time-dependent, requiring a long-term treatment due to the slow kinetics of H3K27me3 turnover, thus highlighting the requirement for deep pharmacokinetic and pharmacodynamics in addition to toxicity studies.

Figure 2 Alterations in chromatin remodeler genes reported in bladder cancer in TCGA database (retrieved in cBioPortal) and their percentages. Filled red rectangle represents amplification, filled blue rectangle represents deep deletion, pink rectangle represents mRNA upregulation, blue rectangle represents mRNA downregulation, filled black squares represent truncating mutation (putative driver), filled grey squares represent truncating mutation (putative passenger), filled orange squares represent inframe mutation (putative passenger), filled green squares represent missense mutation (putative passenger).
Another aspect to consider is the existence of EZH2 mutations. While these have been considered rare in BC, they are frequent in other tumor types, such as various hematological tumors. Whereas in general the mutations lead to a gain-of-function of its enzymatic activity (19), in some other the mutations account for a loss of function, thus suggesting a possible TSG functions of EZH2 (20). In fact, inactivating and loss-of-function mutations in EZH2 have been reported in myelodysplastic syndrome (MDS), myeloproliferative neoplasms (MPN), MDS-MPN overlap disorders, and T-cell acute lymphoblastic leukemia (T-ALL) (1). Interestingly, in T-ALL for instance, Ntziachristos et al. have demonstrated an interplay between an oncogenic role of NOTCH1 and tumor suppressor role for PRC2, opening a new therapeutic possibility by combining inhibitors of H3K27 demethylases and targeted anti-NOTCH1 therapies (21). These apparent discrepancies could be ascribed to different functions of EZH2 in several tissue types, implying that EZH2 role is not simply promote either stemness or differentiation per se, and leading to the idea that targeting its expression can have consequences highly cell type specific. Also in this regard, EZH2 gain-of-function, produced either by mutations or through inactivating mutations affecting others chromatin regulators antagonizing EZH2 activity, such as KDM6A mutations, can account for methylation of non-histone substrates, presenting a PRC2-independent function (22), such as those transcriptional activations mediated by AR in prostate cancer, by NF-kB or NOTCH1 in breast cancer, or by ER and WNT signaling transcription factors (1). The fact that loss of function in PRC2 genes or its substrate H3K27 is associated with oncogenesis highlights the need of being really cautious related to the use of EZH2 inhibitors in the clinic, since the possible long-term therapies needed would result in increased incidence of undesired secondary effects, including hematological malignancy development.

Considering the need of the long-term cancer treatment, the possible acquired resistance becomes a main problem. The resistance can be acquired by amplification or secondary mutations of the drug targets, or through activation of bypass signaling pathways (14). Also other epigenetic factors, such as EHMT2, have been shown to be able of compensating the loss of EZH2. Accordingly, careful studies aimed to understand the resistance mechanisms are needed. In this sense, using EZH2-mutated lymphoma cells, Gibaja et al. developed models of resistance to EZH2 inhibitor E11 by a prolonged exposure to the drug (14). Their results also supported a cooperation model between EZH2 WNT and Y641N mutants, highlighting the fact that only targeting EZH2 WT treatment could not be effective. Kim et al. also reported two novel secondary EZH2 mutations after a long inhibitory exposition in a cell line model, able of conferring resistance (1). These results implicate that new treatments should target both possibilities, EZH2 WNT or mutants, and more ideally also to EZH1, since it might also contribute to resistance. Finally, loss of PRC2 subunits has been reported to amplify Ras-driven transcription in different tumors, showing also a high correlation with resistance to EZH2 inhibition (1). Therefore, all these results point towards the essential development of new drugs or combined therapies, including different EZH2 inhibitors, in order to prevent, or bypass, the possible resistance mechanisms and achieve a more long-term effectiveness.

In conclusion, although the ongoing research provides new therapeutic options for the management of BC cancer patients based on specific mutations, and targeting epigenetic remodelers, a better understanding of their mechanistic roles is strictly required.

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**References**


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Introduction

Over the past decade alone, advances in medical and scientific technology have exponentially increased the volume of data available to clinicians. At the forefront of this movement is next-generation sequencing (NGS), which has allowed for molecular analysis of the entire human genome in a matter of hours (1). The application of NGS to prostate cancer (PCa) has undoubtedly revolutionized our understanding of the disease and holds great promise for improving diagnostic and prognostic accuracy (2). Indeed, previous authors have described genetic changes associated with unique molecular subtypes of PCa and have demonstrated that underlying genetic signatures better predicted clinical outcomes compared to traditional factors such as tumor stage, PSA level, and Gleason score (3,4).

It seems only logical that more in-depth characterization of a given cancer would lead to more accurate prediction of its clinical behavior. Nonetheless, the initial surge of data afforded by these technologies likely preceded our understanding of the disease and holds great promise for improving diagnostic and prognostic accuracy (2). Indeed, previous authors have described genetic changes associated with unique molecular subtypes of PCa and have demonstrated that underlying genetic signatures better predicted clinical outcomes compared to traditional factors such as tumor stage, PSA level, and Gleason score (3,4).

Intra- and inter-tumoral genomic heterogeneity in PCa

In their recent article (11), Wei and colleagues have made substantial progress toward better understanding these questions. Using radical prostatectomy specimens from four men who presented with NCCN high-risk (n=3) or intermediate-risk (n=1) localized PCa, the authors performed genomic and transcriptomic analysis specifically aimed at determining the extent of intratumoral (i.e., different regions within a single tumor focus) and intertumoral (i.e., different tumor foci within a single prostate) heterogeneity. In each radical prostatectomy specimen, three independent tissue cores were obtained from the index lesion (determined by size) and an additional core biopsy was obtained from each noncontiguous tumor focus. DNA and RNA were then extracted and analyzed using whole-exome sequencing, single-nucleotide
polymorphism (SNP) array analysis, and RNA sequencing.

Their findings, in summary, demonstrated considerable intratumoral and intertumoral heterogeneity. In one representative case (CAP-003), the mutation profile of one sampled region within the index lesion (Td1b) differed substantially from the other two sampled regions (Td1a and Td1c)—a most straightforward example of intratumoral heterogeneity. Moreover, the four non-index lesions varied considerably in profile—one highly similar to Td1b, another highly similar to Td1a and Td1c, and the final two lesions distinct from the others. Notably, the majority of DNA-level genomic heterogeneity was conserved at the RNA level, along with additional variability detected on RNA sequencing analysis.

The authors next explored the practical implications of their findings in the context of a recently proposed molecular taxonomy for PCa (3), whereby individual tumor foci are classified into one of seven molecular subgroups based on gene fusion status (ERG, ETV1, ETV4 or FLI1 fusions) or somatic mutations (in SPOP, FOXA1 or IDH1). They found that the majority (>60%) of foci could not be classified under any of the proposed subgroups. Analysis of 60 additional patients from four independent studies (12-15) found a similarly low rate of mutually-exclusive concordant classification across tumor foci (28%). As these findings would suggest that the specific tumor focus and region sampled differentially impact risk classification, the authors next quantitated the gene expression signatures of popular tissue-based prognostic tests. While at least two cores from each patient had similar scores for each signature, on the whole there was substantial variability in score range and direction based on the specific tissue sample analyzed. These findings appear to confirm that prognostic information obtained from tissue-based genomic testing varies substantially according to the region and lesion sampled. From this observation, the authors concluded that information from a single biopsy is not sufficient for guiding treatment decisions.

**Clinical utility of genomic classifiers in PCa**

Although not the first study to demonstrate genetic variability within and between PCa foci (16-18), this study took perhaps the most exhaustive approach to the question—analyzing between five and seven tissue cores from each prostate gland and considering both DNA variability and subsequent RNA expression. Adding these findings to the evolving context of tumor multiclonality and variable biological aggressiveness, it is reasonable to acknowledge that tissue sampling remains a crucial limitation to our ability to accurately predict the clinical behavior of an individual's PCa.

Do these findings suggest that genomic classifiers should not be used clinically? In a word—no. Even considering their limitations, several studies have demonstrated the ability of these tools to provide incremental prognostic data beyond that of existing clinical modalities (19-21). Furthermore, the future of precision oncology is almost certainly based in understanding the genetic and molecular foundations of individual cancers. Nonetheless, these studies are a sobering reminder that integrated genomic approaches remain in their infancy.

Regardless of sampling limitations, the greatest hindrance to widely using genomic data remains our very limited understanding of their specific clinical implications. The reality is that the clinical outcomes associated with even the most well-established genomic alterations are not clearly defined or sufficiently validated (21). As others have proposed (22), research efforts should shift from simply grouping molecular signatures into “high” or “low”-risk to more specifically outlining the functional consequences of specific genetic and molecular features on disease course and responsiveness to therapy (23). Innovative studies linking progressive or treatment-resistant metastases back to a multifocal primary lesion will be crucial in establishing these genotype-to-phenotype relationships (23,24). This is a daunting undertaking, no doubt, but will only grow more feasible as emerging tools such as NGS kits for formalin-fixed paraffin-embedded (FFPE) tissue become commonplace and their costs decline.

Until existing limitations are overcome, the information provided by genomic classifiers should be applied with requisite consideration. The probability that a given tissue sample does not capture the most lethal clone, practically-speaking, simply increases the false-negative rate of genomic testing (in this case, due to sampling error rather than the test itself). As such, findings may be best interpreted like any test of limited sensitivity—a negative result (low-risk classification) does not reliably rule out the presence of high-risk disease (25) and therefore should not be used as the sole basis for deferring aggressive treatment. On the contrary, a positive result (high-risk classification) appears to reliably indicate the presence of high-risk disease (4,19,20) and may be reasonably considered to encourage treatment in those otherwise appropriate for it. Certainly, acknowledging the limitations of these tools will remain an
essential part of physician-patient counseling.

Conclusions

Due to high prevalence and variable clinical behavior of PCa, the ability to accurately predict the clinical course of an individual patient’s disease is critical. The sophistication of prognostic assessment has increased remarkably in recent years, yielding a body of data so complex that cancer bioinformaticians are specially trained to synthesize and interpret it. Unfortunately, these technologies remain limited by tissue sampling in the same manner as age-old traditional approaches such as pathologic grading and staging. There is hope that any number of innovations will forestall the limitations of sampling. Nonetheless, the impact of specific genomic findings on clinical outcomes is poorly defined and requires great attention in the coming years.

Ultimately, genomic classifiers represent a clinical tool more powerful (and more complicated) than their predecessors. Like anything of great power—if used properly, this technology has the potential to improve substantially on the status quo. If used carelessly, it could have a detrimental effect. The common saying “proceed with caution” may be extreme for these purposes, but the prevailing message stands. It is critical that we utilize these tools with care—mindful of their strengths, shortcomings, and potential influence on the increasingly complex decision-making process.

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References


Genomic scores are independent of disease volume in men with favorable risk prostate cancer—implications for choosing men for active surveillance

Guillaume Ploussard\textsuperscript{1,2}, Ambroise Salin\textsuperscript{1}, Igor Latorzeff\textsuperscript{3}

\textsuperscript{1}Department of Urology, Saint Jean Languedoc Hospital, Toulouse, France; \textsuperscript{2}Division of Uro-Oncology, Institut Universitaire du Cancer Toulouse, Toulouse, France; \textsuperscript{3}Department of Radiation Oncology, Clinique Pasteur, Toulouse, France

Correspondence to: Guillaume Ploussard. Department of Urology, Saint Jean Languedoc Hospital, 20 route de Revel, Toulouse 31400, France.
Email: g.ploussard@gmail.com.

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For low-risk prostate cancer, active surveillance (AS) has been increasingly proposed as the preferential initial management strategy. AS entails a strategy by which selected men are managed expectantly with the intention to apply potentially curative treatment in case of progression signs (1). Progression mainly occurs during the first 2 years with differed treatment rates ranging from 20% to 40% among prospective series (1,2). This “rapid” progression could be explained by a not ideal initial selection rather than a real pathological progression of truly very low risk prostate cancer. Thus, for treatment decisions and inclusion of patients in AS protocols, clinicians have to deal with this clinically meaningful risk of reclassification (3-5).

Unfortunately, a consensus about the most relevant definition of low risk cancer remains elusive for men who are amenable to AS. Biopsy criteria such as the number of positive cores, tumor length (total or at any core), or percentage of cancer involvement at any core are predictive factors of tumor volume in radical prostatectomy specimens or biochemical failure after radical treatment, and, to date, are yet the main criteria used in AS protocols. Thus, published AS series used different inclusion criteria largely based on centre experiences and preferences with no hard data (1,2). However, the definition of low tumour volume/involvement strongly varies among AS program, with a relatively comparable reclassification risk whatever the retained pathologic criterion used.

How to explain this difficulty to accurately identify the truly insignificant prostate cancer? The two main explanations are probably the difficulty to precisely identify the disease molecular behavior by standard pathological tools on the one hand, and the imperfection for targeting the most aggressive part of the tumour by our standard random biopsy scheme on the other hand. However, hopefully, the future AS studies should better identify the subgroup of low risk prostate cancer men, by assessing more accurate molecular prognostic markers and imaging-based diagnostic strategies. Nevertheless, daily practice-changing studies are still awaited.

One example is the urine prostate cancer gene 3 prognostic marker that has been correlated to disease volume in low risk cancers and has been suggested to better characterize the potential aggressive behaviour of supposed low-risk prostate cancers (6,7). Unfortunately, these promising results were not significantly correlated with the reclassification risk in AS cohorts (8). Indeed, the use of a single molecular marker is probably doomed to failure. That’s why genomic tests using a panel of several genes are considered as hopeful candidates.

In the present series, from a large series of AS patients whom positive cores were tested for genomic scores, the authors have assessed the correlation between tumour...
volume on biopsies and genomic scores (9). We congratulate the authors for their findings that tended to demonstrate that genomic scores (17-gene panel, OncotypeDx™) could be of great interest at initial AS selection. Such a molecular-based prognostic assessment was not correlated with biopsy tumour volume (that was the main objective of this study) and thus, could offer an independent predictive value in addition to usual selection criteria. The genomic prostate score reclassified low risk to intermediate risk cancer in 7.2% of cases, and very low risk to low risk cancer in 6.3% of cases. These reclassification rates were in line with previous published findings confirming the reproducibility and the homogeneity of this test. Another important finding was that the genomic score confirmed the weak aggressiveness of prostate cancer in a large proportion of cases, with 43% of low risk cancers that were reclassified as very low risk tumours by the genomic prostate score. This score could be used as a reassurance tool for patients and physicians, aiming at lightening subsequent monitoring and improving AS compliance rates.

This study demonstrates that the genomic prostate score helps in reclassifying at inclusion a not negligible proportion of patients and that the prognostic information it gives are independent to those provided by tumour volume and involvement on biopsies. Nevertheless, before a wide acceptance of genomic scores in AS protocols, several limitations have to be highlighted. The main endpoint used in this study was the likelihood of favourable pathology that is a probability calculated from the initial iterations of the OncotypeDx™ test results. And this is surely not the best end point to address conclusion in men eligible for AS. This probability has been evaluated in cohorts of patients receiving radical treatments, and to the best of our knowledge, has never been correlated with outcomes in men managed by AS. The genomic tests correlate with pathologic features in radical prostatectomy specimens, biochemical failure, metastatic disease, and mortality after radical treatment. However, only extrapolations can be considered when using this test in an AS cohort. The authors assessed the reclassification rate based on this genomic testing, whereas the optimal reclassification rate should have been reported using biopsy control findings. We cannot state that genomic testing in low risk prostate cancers patients is a relevant surrogate for confirmatory biopsies or for differed treatment rates in AS programmes. The short follow-up of the present series is one explanation as well as the low number of patients undergoing differed radical prostatectomy. Indeed, the analysis of the radical prostatectomy specimens would have been interesting to confirm the correlations between disease volume on biopsies, genomic score, and pathologically confirmed tumour volume and aggressiveness in prostate specimens.

Thus, given that the main endpoint by definition depends on the genomic score testing, we cannot conclude on the inferiority of detailed biopsy characteristics, compared with genomic score, for the AS eligibility. Both information (biologic potential of the tumour measuring by genomic test, and extent of the disease assessed by biopsy features) are surely complementary for predicting reclassification rates and oncologic outcomes during conservative management.

Another pitfall of this genomic profile strategy is that gene analysis is only performed in a random part of the cancer (10). Indeed, the biopsy core number and location were not controlled and varied according to the physician. Moreover, as no data on pre-biopsy magnetic resonance imaging (MRI) and targeting was reported, we can imagine that only systematic random biopsies were performed. In that setting, we can easily believe that the genomic profile of the tumour may provide a not negligible prognostic value in addition to the Gleason score, by catching aggressive and “not visible” component of the disease, and then, by reclassifying cancers for which the random biopsies missed the most aggressive focus. The value of this genomic score remains doubtful when targeted cores can diagnose the true pathological grading of the disease.

The genomic testing is surely one of the main hopes in a near future for improving risk and prognosis assessment in prostate cancer field. Nevertheless, only long-term prospective studies comparing different inclusion criteria (imaging-based, molecular-based, volume-based) could answer the question of the ideal candidate for conservative management and definitely close the debate. This is also worthy to note, that, although the development of strict criteria based on predefined cut-offs of different variables would facilitate their use in the clinical practice, their lack of flexibility might eventually limit the number of patients potentially eligible for AS, thus exposing them to a non-negligible risk of overtreatment.

Until now, no specific molecular test, genomic score, or MRI-targeted biopsy software has definitively hit the mark. And whatever the prognostic tool used, we know that there is no such thing as zero risk. The reclassification risk will remain present justifying the monitoring strategy. By then, from our point, disease volume on biopsies should not be abandoned and still provides relevant prognostic features for decision on AS candidacy.
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References


Prostate cancer is a global health problem. Approximately 1.1 million cases are diagnosed per year, making this malignancy the second most common cancer in men worldwide and the most common cancer in men in more developed regions (1). Actually, treatment choice for castration-naïve as for metastatic castration-resistant prostate cancer (mCRPC) patients is based on clinical features of disease (2-4). The standard treatment for metastatic castration naïve prostate cancer is androgen deprivation therapy (ADT). ADT can be achieved by orchiectomy, gonadotrophin releasing hormone (GnRH) agonists, or GnRH antagonists. Through constant stimulation of the receptor, GnRH agonists lead to a down-regulation (2-4). In patients with castration-naïve metastatic prostate cancer, the upfront addition of docetaxel to ADT should be discussed with patients who are fit for chemotherapy (2-4). Progression on ADT generally occurs and optimal sequencing of endocrine agents, abiraterone acetate and enzalutamide, should be defined. A milestone for primary and acquired androgen resistance was described in 2014 by Antonarakis et al. (5). The splicing androgen receptor (AR) variant 7 (AR-V7) in circulating tumor cells
were shown to be predictive factor for resistance to next-generation AR axis-targeting agents. In the current issue, Lallous et al. utilized circulating cell-free DNA (cfDNA) sequencing technology to examine the AR gene for the presence of mutations in CRPC patients. By modifying their sequencing and data analysis approaches, they identified four additional single AR mutations and five mutation combinations associated with CRPC. Importantly, they conducted experimental functionalization of all the AR mutations identified by the current and previous cfDNA sequencing to reveal novel gain-of-function scenarios. Finally, they evaluated the effect of a novel class of AR inhibitors targeting the binding function 3 (BF3) site on the activity of CRPC-associated AR mutants (6). We endorse the conclusions of their work demonstrating the feasibility of a prognostic and/or diagnostic platform combining the direct identification of AR mutants from patients' serum, and the functional characterization of these mutants in order to provide personalized recommendations regarding the best future therapy. The detection of cf-DNA provides new opportunities for management of prostate cancer patients adding a new useful tool for diagnosis, staging and prognosis. It offers a new type of very specific biomarker that allow to identify the mutations accumulated from each tumor and to monitor the tumor burden and the response to treatment using a minimally invasive blood analyses. cfDNA analysis may allow a more comprehensive assessment of the molecular heterogeneity of the patient's prostate cancer, which also can lead to a more personalized and combinatorial treatment with targeted therapies. Mechanisms of endocrine resistance are driven by upregulation of specific pathways that can be targeted by new agents. A most unique advantage of circulating tumor DNA analysis is that it enables to follow tumor molecular evolution in time. cfDNA can be investigated repeatedly and non-invasively at different time-points through therapy. As an example, real-time monitoring of AR mutants in cfDNA could be used to design dynamic therapeutic schedules of new generation anti androgen receptor agents. A correlation of treatment response and presence of specific somatic genomic changes associated with target drugs has been observed in a longitudinal monitoring of patients participating in a phase I clinical trial of several tumors including PC (7). Providing clinicians with comprehensive catalogs of the key genomic changes in prostate cancer and disease segmentation of prostate cancer subtypes progressive to endocrine therapy will support advances in developing more effective ways to diagnose, treat and prevent cancer. The failure to deliver personalized medicine is often associated with the lack of highly bioactive and specific drugs. Experimental functionalization of AR mutants may help to dissect the intra-tumor heterogeneity and to design new agents that will overcome endocrine resistance. Liquid biopsy may overcome limitations of tumor biopsies (logistical and operational challenges, quality of tissue samples and sequencing technologies). Liquid biopsy may increase accrual in precision medicine trials. As stated in the recent “Consensus on precision medicine for metastatic cancers” (8) in individuals, the level of evidence that a genomic alteration is involved in cancer progression can vary from ‘biological interpretation without supporting data’ (level IV) to ‘evidence from clinical trials’ (level I). A most concerning term in this era is “targetable” genomic aberration, because this refers to a hypothesis, not a fact. Nonetheless, unchecked adherence to a belief in the concept of “targetability” or “druggability” of genomic aberrations with available targeted therapy, and even a “signal” of benefit from early clinical development, may reinforce a biological premise to pre-select patients based on the assay result. If a new drug has strong biological rationale and demonstrates a “signal” of activity in phase I or II studies, we still expect that results from a randomized clinical trial must demonstrate efficacy (clinical validity) before accepting that treatment as a potential standard. Functional characterization of AR mutants in order to provide personalized recommendations regarding the best future therapy is an example on how to provide druggability to a genomic tool. Another major challenge in optimizing precision medicine trials design is the appropriate use of relevant biomarkers to the molecularly targeted agents and, in case of combination of targeted agents, the setup of an appropriate treatment algorithms. There are, however, several questions to be answered. One crucial factor in evaluating cf-DNA is the standardization of assays and the definition of the optimal sampling specimen (serum or plasma) to obtain data more consistent and comparative between different laboratories. Despite these technical limitations, “liquid biopsy” may provide a unique opportunity in the field of clinical cancer research and have been already embedded in the design of several clinical trials. Another opportunity to explore the role of cf-DNA is to study the “tumor dormancy” phenomenon, very important in prostate cancer patients in order to stratify risk of relapse. A better stratification of the risk may allow an escalation of treatment. More importantly, the process of identifying specific DNA mutations for each
patient’s cancer is a laborious process that is currently too time-intensive and costly for more widespread use. Future development will have to provide a cost effective analysis mainly identifying the genes known to be recurrently mutated in each tumor. Therefore, developing standardized methodologies for cf-DNA analyses and validation in large prospective clinical studies is mandatory to implement the ‘liquid biopsy’ approach in the clinical management of prostate cancer patients.

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References

Introduction

The estimated new kidney cancer cases diagnosed each year in the United States and in the world are ~63,000 and ~300,000, respectively (1,2). Renal cell carcinoma (RCC) represents over 90% of kidney cancer and consists of a group of malignancies arising from the renal epithelium and exhibiting distinct histopathological features (3-6). The 2004 WHO classification listed 12 different subtypes of RCC (4). With a better understanding of the molecular pathogenesis of RCC, the 2013 International Society of Urological Pathology (ISUP) consensus conference added several new entities (6). Major RCC subtypes are clear cell RCC (ccRCC) (~75%), papillary RCC (pRCC) (~15%), chromophobe RCC (chRCC) (~5%), and unclassified RCC (uRCC) (4-6%) (6,7). Large-scale genomics of major RCC subtypes led by The Cancer Genome Atlas (TCGA) have been reported, which delineate the genomic landscapes of ccRCC (KIRC), pRCC (KIRP), and chRCC (KICH) (8-11). Furthermore, subsequent studies have begun to elucidate the prognostic and predictive values of prevalent mutations in ccRCC, which is likely to impact clinical management of kidney cancer patients in the near future (12-18).

Sarcomatoid components can be detected in various epithelial malignancies, featuring morphological characteristics typical of a sarcoma and implicating an underlying epithelial-mesenchymal transition (EMT) (19,20). Sarcomatoid components can arise in all subtypes of RCC (21) but with higher incidences in ccRCC and chRCC (22,23). Immunohistochemical and genetic studies indicated that sarcomatoid RCC (sRCC) does not develop de novo but results from transformation/differentiation/dedifferentiation of pre-existing RCC (21,24,25). Hence sRCC does not represent a distinct subtype and is classified according underlying histology; when no epithelial component is present, these tumors are categorized as uRCC. In general, sRCC is associated with an aggressive clinical course and portends a poor therapeutic outcome (22,26-28). Furthermore, increasing percentages of sarcomatoid component within individual RCCs are associated with worsening outcome and carry prognostic values (26,29,30). Accordingly, a better understanding of underlying molecular pathology is of paramount significance.

Genomics of sRCC

Several of the previously reported studies that have examined the genomic aberrations present in ccRCC and chRCC have included patients with sarcomatoid histology (8,10,31). However, interpreting differences in the molecular biology of patients with sRCC in these...
recently reported on their dedicated studies of sRCC tumors. One of these tumors had a mutation in the RAS family member H-RAS and another in TP53. The study included both tumors with sccRCC and tumors with sarcomatoid elements occurring in conjunction with varying histologies (e.g., papillary, unclassified, collecting duct). Both of these studies provided excellent details on the molecular aberrations specific to sRCC and the results of these studies present a number of interesting observations that will surely impact future research.

A cohort of 21 tumors with sequencing results of sufficient quality was initially included in the Bi et al. study. Each tumor had matched normal, carcinomatus, and sarcomatoid elements (microdissected), submitted for whole-exome sequencing. The mean depth of independent reads was 135, 177, and 171 for normal, carcinomatous, and sarcomatoid elements, respectively. Two of the 21 matched samples were found to have significantly higher somatic single nucleotide variants (SSNVs) in their matched tumor elements. One of these tumors had a mutation in mutS homolog 2 (MSH2), and the other had a mutation in polymerase ε (POLE). Their mutational signatures were consistent with mismatch repair deficiency, and they were excluded from the grouped analysis of the other 19 tumors.

In the 19 matched tumors samples analyzed, Bi et al. found 41.7% (45/108) of the SSNVs were shared among the matched carcinomatous and sarcomatoid elements. Most of these shared SSNVs were within genes commonly mutated in ccRCC (e.g., VHL, PBRM1, and SETD2). Among these tumors the sarcomatoid elements were found to have a significantly higher average mutational burden (45 vs. 18 SSVN) and nearly twice the length of loss of heterozygosity (LOH) events (913 vs. 460 Mb), which was also statistically significant. They also found that the sarcomatoid elements had significantly more frequent alterations occurring in known cancer genes. The most frequently mutated among these genes in the sarcomatoid element was tumor protein p53 (TP53). They reported 6 out of 19 (31.6%) sarcomatoid elements had TP53 mutations compared to zero in the matched carcinomatous elements. Bi et al. also highlighted sarcomatoid-specific mutations in BRCA1 associated protein 1 (BAP1) in 2/19 (10.5%) and AT-rich interaction domain 1A (ARID1A) in 3/19 (15.7%) samples. They also observed that mutations in TP53, BAP1, and ARID1A were all mutually exclusive among the 19 tumors. Sarcomatoid elements were also found to have more frequent LOH events among chromosomes 1p, 9, 10, 17p, 18 and 22. Ito et al. reported a similar enrichment for such copy number events in sRCC (35). Lastly, Bi et al. presented several novel SSNVs that have not regularly been associated with RCC which were found to be more common or exclusive to the sarcomatoid elements among the 19 tumors. This included alterations in; FAT atypical cadherin 1 (FAT1), FAT2, FAT3, tumor susceptibility 101 (TSG101), ligand dependent nuclear receptor interacting factor 1 (LRIF1), required for cell differentiation 1 homolog (RQCD1), and protein tyrosine kinase 7 (PTK7).

The study published by Malouf et al. included essentially three different cohorts. The first cohort was similar to the 19-tumor cohort presented by Bi et al., and it included three tumors with paired clear cell (carcinomatous) and sarcomatoid elements after microdissection. This cohort underwent targeted sequencing of both matched elements using a custom panel of 236 frequently mutated cancer-related genes and 37 introns frequently rearranged in cancer (average exon coverage of 819x). Of note this panel did not include the genes FAT1, FAT2, FAT3, TSG101, LRIF1, RQCD1, or PTK7. Also, they did not report using matched normal tissue from any patients in their targeted
sequencing analysis, which likely limits the interpretation of copy number aberrations for these tumors. The sequencing results of this cohort stand somewhat in contrast to the results of the study above. Malouf et al. reported identical alterations in two of the three matched samples (i.e., exact same type and number of alterations in both clear cell and sarcomatoid elements). In the third sample they saw similar homozygous deletions in VHL but found multiple distinct inactivating mutations in TP53 and phosphatase and tensin homolog (PTEN) that differed between the two elements. The third case also had a unique amplification of Janus kinase 2 (JAK2) in the sarcomatoid element, which, when taken together with the TP53 and PTEN mutations, may suggest a divergent course of evolution for this tumor. In the second cohort, they analyzed 23 tumors with sRCC arising from a mixture of carcinomatous backgrounds including clear cell, unclassified, collecting duct, papillary, and mucinous tubular and spindle cell carcinoma. Most of these tumors were primary kidney specimens (88.5%), except for three which were from metastatic sites (peritoneal nodule, lymph node, and liver). In this cohort, they found TP53 to be the most frequently altered gene (11/23, 42.3%). They also reported a relatively high number of cyclin-dependent kinase inhibitor 2A (CDKN2A; 7/23, 26.9%) and neurofibromin 2 (NF2; 5/23, 19.2%) alterations among these tumors. In their third cohort, the investigators employed whole-exome sequencing on four tumors with sccRCC, not microdissected. They reported a lower overall median mutation rate in these four cases (37.5 mutations) compared to the median rate in TCGA (49 mutations) for ccRCC (8). In two of these four cases they went on to test multiple regions from the primary tumors (4 regions in one and two in the other) to evaluate intratumoral heterogeneity using Sanger sequencing for VHL and TP53 genes only. For these two cases they report no finding of intratumor heterogeneity in regards to these two genes.

Integrating the results of these studies helps us answer several questions about the molecular framework of sRCC. First the truncal events and shared genomic aberrations between both the carcinomatous elements and sarcomatoid element seen in both studies confirm that sRCC arises from RCC. Next, the notion that the sarcomatoid element represents a dedifferentiated progression of RCC is supported by the increased overall mutational burden and copy number aberrations seen in the sarcomatoid elements compared to the carcinomatous elements from Bi et al. The increase in aberrations of known cancer genes (TP53, NF2, CDKN2A) also supports the sentiment that the sarcomatoid elements are driving pathogenesis in these tumors. Oda et al. published a study in 1995 reporting a mutation rate of 78.6% (11 of 14) for TP53 in the sarcomatoid elements of sRCC tumors using polymerase chain reaction (32). The carcinomatous elements, or background histology, for this cohort included both mixed and granular subtypes, somewhat limiting the application of these results. While Bi et al. clearly show an enrichment of TP53 aberrations (31.6%) in the sarcomatoid elements among primary ccRCC tumors, caution must be used when interpreting the even more enriched results (42.3%) from the 26 sRCC tumors reported in the Malouf et al. study. The latter study included diverse primary RCC histologies and also included metastatic tumors, which previously have been shown to be enriched for TP53 aberrations irrespective of sarcomatoid features (36). Similarly the finding of increased NF2 mutations occurring in sRCC may also be limited due to the diverse background of primary RCC histologies in this cohort. Our understanding of the molecular composition and the clinical implications of uRCC are both poorly defined and poorly understood. As a significant number of the NF2 and TP53 aberrations occurred in these unclassified tumors, attributing the results to sRCC may be problematic. Another interesting finding is the identification of the two tumors from Bi et al. with mutational signatures consistent with mismatch repair deficiency. Tumors such as these, and maybe even sarcomatoid variants in general, may derive significant benefit from immune checkpoint blockade in the treatment of metastatic disease (37,38). A summary of some of the differences among the tumor cohorts analyzed in these studies can be found in Table 1.

Both of these studies are novel in their attempt to better understand this very clinically relevant and aggressive disease. However, sRCC is a relatively rare entity, and both studies have small cohorts, which may hinder their generalizability. The rarity of this disease also exposes both studies to significant selection bias. This may include selection of tumors for analysis with the most tissue available (large tumors), those with the most aggressive course (likely to have been sequenced), and likely other confounding variables. The use of different sequencing platforms and the mix of histologies in the Malouf et al. study make pooling and comparing of the results difficult. While the genomic underpinnings of sRCC in approximately 1/3 of patients may be explained by the results of these studies (i.e., TP53, NF2, CDKN2A aberrations), there is still no clear molecular
Table 1 Summary of tumor cohorts

<table>
<thead>
<tr>
<th>Study</th>
<th>Pathology</th>
<th>Specimen site</th>
<th>Sequencing/median coverage</th>
<th>Results/comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oda et al. [1995]</td>
<td>sRCC (sccRCC = 7, mixed = 5, granular = 2)</td>
<td>Primary</td>
<td>PCR w/IHC</td>
<td>TP53 mutations in 11 of 14 tumors sarcomatoid elements. Only two of these tumors had TP53 mutations in both carcinomatous and sarcomatoid elements.</td>
</tr>
<tr>
<td>Bi et al. [2016]</td>
<td>scRCC</td>
<td>Primary</td>
<td>WES/normal 135x, carcinomatous 177x, sarcomatoid 171x</td>
<td>Shared mutations between elements point to common origin. High overall rate of mutations, including mutations in known cancer genes (TP53, ARID1A, CDKN2A).</td>
</tr>
<tr>
<td>Malouf et al. [2016]</td>
<td>scRCC</td>
<td>Primary</td>
<td>Targeted (255 genes)/~700x†</td>
<td>2 of 3 tumors with high fidelity of aberrations between elements. Third tumor with mutational profile consistent with divergent evolution of elements.</td>
</tr>
<tr>
<td></td>
<td>primary = 20, peritoneal nodule = 1, lymph node = 1, liver = 1</td>
<td></td>
<td></td>
<td>Enrichment for TP53, NF2, CDKN2A aberrations in sRCC tumors.</td>
</tr>
<tr>
<td></td>
<td>ccRCC</td>
<td>Primary</td>
<td>Targeted (255 genes)/~700x†</td>
<td>Comparative group.</td>
</tr>
<tr>
<td></td>
<td>sccRCC</td>
<td>Primary</td>
<td>WES/normal 71x‡, sccRCC 138x‡; sanger for two tumors (four regions in one, two regions in the other)</td>
<td></td>
</tr>
</tbody>
</table>

*, core cohort of tumors referenced in the respective study; †, mean coverage. sRCC, sarcomatoid renal cell carcinoma; scRCC, sarcomatoid clear cell renal cell carcinoma; PCR, polymerase chain reaction; IHC, immunohistochemistry; WES, whole-exome sequencing; TCGA-KIRC, The Cancer Genome Atlas Kidney Renal Clear Cell Carcinoma.

explanation of sRCC development in the majority of cases.

Conclusions

Both Bi et al. and Malouf et al. have conducted and published novel genomic studies of renal tumors with sarcomatoid variant histology. The results have definitively demonstrated that progressive dedifferentiation is the source of the sarcomatoid elements in RCC. They have also identified key genomic aberrations (e.g., TP53, CDKN2A, copy number changes) present in sRCC that may explain its aggressive clinical course and may become potential targets for therapy. We hope future research efforts build upon this work to pursue better treatment and management strategies for patients with this disease.

Acknowledgements

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Footnote

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Key Leaders’ Opinion on Urogenital Cancer: Frontier and Progress

References


Epithelial mesenchymal transition (EMT) is a naturally occurred transdifferentiation of epithelial cells that encompasses their plasticity and involves conversion from epithelial to mesenchymal phenotypes (1-3). EMT is a common program in embryonic development, organ fibrogenesis and cancer metastasis. The cells undergone EMT have been evidenced with cancer cell stemness and resistance to chemotherapy (4). Several recent studies on EMT, particularly in cancer entity, have moved forward to better understand molecular mechanisms underlying EMT and its regulation. Since EMT can be modulated by several factors at multiple steps, integrative information of EMT regulation at transcription, post-transcription, post-translation and epigenetic levels is thus required for the new approach on EMT-related cancer therapy (5). Beyond transcriptional control, DNA methylation, histone modification and microRNA (miRNA) have been recognized as epigenetic modifications that can regulate EMT-related genes (5). One of the driving forces for epigenetic modifications in cancer-related EMT is the adaptive mechanism of the cancer cells to alter their metabolism to survive under energy deprivation stage during tumorigenesis (6). Additionally, dysregulated metabolism can be affected by genetic mutations of metabolic enzymes, e.g., succinate dehydrogenase (SDH), fumarate hydratase (FH) and isocitrate dehydrogenase (IDH), that subsequently cause alterations in cellular metabolites and finally initiate cancer development and progression (7). The small metabolites with the potential to trigger cancers are termed “oncometabolites” (8,9). One of the well-known oncometabolites related to cancer progression is fumarate, which has been evidenced to be associated with the development of hereditary leiomyomatosis and renal cell cancer (HLRCC) (8).

HLRCC is an autosomal dominant hereditary cancer syndrome caused by germline mutation of the gene encoding FH, a tricarboxylic acid cycle (TCA) enzyme, which catalyzes hydration of fumarate to malate (10). The consequences of FH inactivation in kidney cancer cells include TCA cycle imbalance, fumarate accumulation, impaired oxidative phosphorylation, and metabolic reprogramming to aerobic glycolysis (also known as Warburg effect), which in turn promote tumor growth (10). Furthermore, stabilization of hypoxia-inducible factor 1 (HIF1), inactivation of AMP-activated protein kinase (AMPK), and dysregulation of Keap1-Nrf2 antioxidant system have been reported in FH-deficient kidney cancers (11-13). Although adaptive responses to fumarate accumulation are partially known, there is still a huge gap in understanding epigenetic regulation of tumorigenesis in HLRCC.

The most recent study by Sciacovelli et al. (14), has established a connection between an oncometabolite (fumarate) and epigenetic regulation of miR-200 family.
that plays important roles in EMT program of HLRCC. The authors primarily characterized the EMT phenotypes by proteome and mRNA profilings of Fb1−/− (murine) and UOK262 (human) FH-deficient cells (note that the latter was derived from HLRCC patient). They demonstrated that a mesenchymal marker, vimentin, was the most increased protein, while a counterpart epithelial marker, E-cadherin, was significantly decreased in the FH-deficient cells, suggesting the acquired mesenchymal signature in these cell lines concomitant with the increased cell migratory activity. Interestingly, epithelial features were regained and cell migratory activity was decreased when the FH-deficient cells were reintroduced with full-length FH. In addition, several EMT transcription factors, including SNAI1, SNAI2, TWIST1, ZEB1 and ZEB2, were elevated in the FH-deficient cells (14). It is known that the loss of FH in renal cancer can result in accumulation of fumarate and inhibition of HIF prolyl hydroxylase, thereby stabilizing HIF1 (11). Moreover, silencing of HIF1α in UOK262 significantly reduces invasiveness of the cancer cells (12). Unexpectedly, Sciacovelli et al. (14) found that silencing of HIF1β could not suppress EMT phenotypes, indicating that the involvement of HIF1 in cancer development/progression is (iso)form-specific.

Numerous miRNAs are supposed to be involved in EMT by epigenetic modulation of their mRNA targets especially through DNA methylation at the CpG promoter island or histone acetylation. Among these, ZEB-miR-200 axis has gained wide attention on its role for regulation of transcription factors in cancer EMT (15). miR-200 family is a potent antimetastatic miRNA cluster that suppresses expression of EMT-transcription factors as well as tumor initiation and metastatic cascade (16). Sciacovelli et al. (14) hypothesized that epigenetic modification driven by accumulation of fumarate could affect miR200-mediated regulation of EMT-related genes in HLRCC. In concordance with elevated ZEB1 and ZEB2 expression, they also showed that miR-200 family was the most down-regulated miRNA in FH-deficient cells and FH re-expression successfully rescued the expression of miR-200ab and E-cadherin, while suppressed vimentin expression (14). As expected, quantitative PCR (qPCR) revealed hypermethylation of Cpg43 in FH-deficient cells that could be returned to unmethylated state by FH reconstitution (14).

The same group of investigators also demonstrated that suppression of miR-200 cluster was a consequence of inhibition of ten-eleven translocation (Tet)-mediated demethylation (14). Tet family of dioxygenase comprises three proteins, including Tet1, Tet2 and Tet3, which are responsible for catalyzing the conversion of 5-methylcytosine (5mc) to 5-hydroxymethylcytosine (5hmc). The product 5hmC can be used to implicate the reversible process of DNA methylation/demethylation (17). This group of investigators revealed that combined silencing of Tet2 and Tet3 (Fb1β0 + shTet2-3) resulted in decreased miR-200abc and E-cadherin expression, suggesting the role of Tet-mediated demethylation in EMT regulation (14). The same results were observed when dioxygenase was reactivated in Fb1β0 + shTet2-3 cells by using dimethyl alpha-ketoglutarate (DM-a-KG) (14). To decipher whether this molecular scenario occurred in FH-deficient cells, the authors performed chromatin immunoprecipitation (ChIP) assay in combination with chromosome conformation capture (3C) analysis to pin-point the region of histone modification, indicating the opened-closed state of chromatin structure. It was clearly shown that the region adjacent to Cpg43 and transcriptional start site of miR-200ab429 was hypermethylated in FH-deficient cells, which could be restored to basal level by reintroduction of FH (14). Accordingly, the marked decrease in Tet-catalyzed 5hmC was also observed in FH-deficient cells (14). Although they did not determine the basal levels of Tet proteins in FH-deficient cells, these findings collectively suggest the role of Tet-mediated demethylation in the production of miR-200 family in FH-deficient cells. Alternatively, they confirmed the effects of oncometabolite on EMT regulation by using monomethyl fumarate (MMF), a fumarate derivative, in wild-type FH (Fb1ββ) and HK2 cells. As expected, treatment of MMF up-regulated EMT transcription factors (i.e., succinic GSH and 2-succinic cysteine), they employed SDHb-deficient cell line and demonstrated that only succinate accumulation could raise EMT phenotypes similar to those of FH-deficient cells. However, it was questionable that whether triggering EMT in SDHb-deficient cells engaged the same molecular mechanism as revealed in FH-deficient cells, since hypermethylation of Cpg43 was not predominant. Finally, the effects of oncometabolite fumarate and epigenetic regulation of EMT were validated in tumor samples collected from HLRCC patients (only two cases included in their own study) (14) and other cancers related to FH-mutation, including papillary renal cell carcinoma and clear cell renal cell carcinoma from the
published data. By this strategy, it was convincing that the decrease of FH was correlated with down-regulation of miR-200 cluster due to hypermethylation, leading to EMT features. Last but not least, the decreased FH expression was related to the worsen prognosis of the patients (14).

In summary, the study by Sciacovelli et al. (14) provided a missing piece of the puzzle and demonstrated that oncometabolite fumarate could induce epigenetic regulation of EMT in FH-deficient kidney cancers. All these findings raise the possibility that aberrant epigenetics by inhibition of Tet-mediated demethylation may be generalized in other cancers related to miR-200 suppression or loss of FH. Considering epigenetic modifications for anticancer drug design, it is still challenging to figure out the unique set of epigenetic markers specific to EMT (e.g., histone modifications, histone marks, etc.) and chromatin modifying enzymes for the precise targeting of EMT cells. In addition to the discovered linkage, further functional analyses on transcriptome, proteome, and miRNA profiling data provided in this report and by other groups are still necessary for unlocking and conveying the comprehensive message for specific therapeutics of cancers related to FH-mutation. However, it should be noted that epigenetic regulation is not limited only to the cancer entity of EMT but also organ fibrogenesis. Several studies have shown a proof-of-concept that aberrant DNA promoter methylation causing gene silencing contributes to many fibrotic diseases, including pulmonary, liver, cardiac, and kidney fibrosis (18). For example, hypermethylation of anti-fibrotic RASAL1 promoter mediated via Tet activity has been recently demonstrated in cardiac and kidney fibrosis (19,20). Nonetheless, this molecular mechanism has been revealed only in differentiated/activated fibroblasts and coronary endothelial cells. Therefore, there is still a plenty of space for investigating the role of epigenetic regulations of EMT in fibrogenesis, including a link between Tet-mediated demethylation and expression of miR-200 cluster or other genes. The study provided by Sciacovelli et al. (14) thus sheds light onto the emerging role of epigenetic modifications of EMT process not only in cancers but also for in organ fibrogenesis and may hold a promise for the novel therapeutic strategy in cancers and also in fibrotic diseases in the future.

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Footnote

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The mechanistic target of rapamycin complex 1 (mTORC1) is a major serine/threonine kinase that stimulates cellular anabolic processes including protein and lipid synthesis while suppressing catabolic processes such as autophagy in response to growth factors and amino acids (1). Upon mTORC1 activation, it phosphorylates multiple substrates including S6 kinase (S6K), eIF4E binding protein (4EBP), and unc-51-like kinase (ULK) (2). Both S6K and 4EBP are key regulators for mRNA translation and cell cycle progression (3). In addition to mTORC1’s roles in stimulating these anabolic processes, mTORC1-dependent ULK phosphorylation inhibits its kinase activity, which is essential for autophagy induction (4). Thus, mTORC1 activation in response to growth factors and amino acids promotes key cellular anabolic processes while it suppresses major catabolic processes, to build biosynthetic molecules essential for cell growth and proliferation.

Both growth factor and amino acid signals impinge on the lysosomal membrane and coordinately stimulate the activity of mTORC1 by enhancing two distinct lysosomal small GTPases, Rheb and Rags, respectively. While the Rags recruit mTORC1 to the lysosomal membrane in response to amino acids such as leucine, arginine, and glutamine (5), Rheb, which directly interacts with mTORC1, stimulates the activity of mTORC1 on the lysosomal membrane in response to growth factors (6). Mammalian cells contain four members of Rag small GTPases (RagA, B, C, and D) and form obligate heterodimers of either RagA or RagB with either RagC or RagD (7). In the active Rag heterodimer, RagA or RagB binds to GTP while RagC or RagD binds to GDP. Upon amino acid availability, the Ragulator complex, a guanine nucleotide exchange factor (GEF) for RagA/B (8,9), stimulates RagA/B GTP loading in a manner dependent of lysosomal vATPase activity (10). Likewise the folliculin (FLCN)-FLIP complex, a GTPase activating protein (GAP) stimulates RagC/D GDP loading (11). Once the Rag heterodimer is in its active configuration, it localizes mTORC1 to the lysosomal membrane (5). On the other hand, growth factors instigate the activation of the PI3K (phosphoinositide 3-kinase)/Akt pathway leading to the activation of Rheb small GTPase, which directly stimulates mTORC1 on the lysosome (6).

It is not surprising that nutritional signals collide on the lysosomal membrane. The lysosome is responsible for breaking down macromolecular components through the process of autophagy. Autophagy allows cells to respond to stress conditions such as starvation by providing nutrients through the degradation of cellular components (12). Once nutrients become available, mTORC1 is activated, stimulating anabolism while ending the autophagy response. Thus the lysosome serves as a sensing platform where the extracellular and intracellular nutritional status is carefully monitored in order to maintain a balance between catabolic and anabolic processes.

mTORC1 is well known to inhibit the induction of...
autophagy by phosphorylation of ULK (4). In addition, mTORC1 has also been shown to negatively regulate two transcription factors, transcription factor EB (TFEB) and transcription factor E3 (TFE3) (13-15), which play a key role in inducing the expression of numerous genes encoding lysosomal hydrolases, membrane proteins, and essential proteins for autophagy. Both TFEB and TFE3 are members of the microphthalmia-associated transcription factor (MiTF) subfamily of transcription factors (16). Upon mTORC1 activation, it phosphorylates these transcription factors at key serine residues, which creates a binding site for the 14-3-3 cytosolic chaperone protein, leading to the blockade of nuclear translocation of these transcription factors (17). In contrast, under starvation conditions, mTORC1 is inactivated, thus the dephosphorylated form of TFEB and TFE3 dissociates from its interaction with the 14-3-3 protein and localizes to the nucleus. Here TFEB and TFE3 recognize the coordinated lysosomal expression and regulation (CLEAR) elements in the promoter region of genes responsible for lysosomal biogenesis and autophagy (16,18). Consequently, TFEB and TFE3 transcriptionally up-regulate the capacity of degradation machineries in cells to generate nutrients for their survival under starved conditions.

Previously Martina et al. reported that TFE3 could function as part of a feedback loop leading to mTORC1 activation by increasing expression of the FLCN and two FLCN interacting proteins FNIP and FNIP2, which form the FLCN-FNIP complex, a GAP that activates RagC/D small GTPases (19). Consistently, TFE3 overexpression stimulates Rag C/D GDP loading necessary for its activation, leading to lysosomal mTORC1 localization and its activation. The same observation was also made in a model of TFEB overexpression. Thus, continuous TFE3 and TFEB activation prepare the source and machinery for mTORC1 activation and might ensure the termination of autophagy-lysosomal-mediated catabolism once nutrients become available.

Understanding the molecular mechanisms by which TFEB/TFE3 regulate mTORC1 activity is particularly relevant as the mutations in TFEB/TFE3 genes have been found in renal cell carcinoma and amongst other cancers with high mTORC1 activity (16,17). Elucidation of the molecular mechanisms by which TFEB/TFE3 mutations lead to aberrant mTORC1 activation would provide insight into the development of possible therapeutic strategies.

In this context, the study recently published in Science by Di Malta et al. provided crucial roles of MiTF transcription factor family members, substrates of mTORC1, in the activation of mTORC1. Interestingly, the study indicates that even under amino acid sufficient conditions, the inhibition of TFEB or TFE3 leads to a decrease in cellular mTORC1 activity in a variety of mammalian cells. These results suggest that the transcription factors TFEB and TFE3 are crucial for the initiation of lysosome biogenesis and autophagy under starvation conditions and are indeed required for mTORC1 activity in response to amino acids.

Yu et al. have previously shown that upon amino acid starvation, the activity of mTORC1 is abolished as expected, however, prolonged amino acid starvation restores cellular mTORC1 activity (20). They proposed that mTORC1 is stimulated in response to the availability of newly synthesized nutrients restored by autophagy during prolonged starvation conditions, bringing to an end the autophagy response. In support of this model, genetic ablation of ATG5 or ATG7, key proteins for autophagy, inhibited the restoration of mTORC1 activity under prolonged starvation conditions (20).

The study by Di Malta et al., also observed that the restoration of mTORC1 activity in response to prolonged starvation was abolished when TFEB/TFE3 were genetically ablated, indicating that these transcription factors are crucial for mTORC1 re-activation under this condition. It could be argued that the loss of mTORC1 re-stimulation under prolonged starvation conditions is due to a decrease in the capacity of cellular autophagy-lysosome degradation system caused by the lack of TFEB/TFE3-dependent expression of lysosomal and autophagic proteins. However, the authors showed that TFEB overexpression lead to higher mTORC1 activity in cells lacking the essential autophagy genes, ATG5 or ATG7 compared to control cells. Based on these results the authors propose that the MIT-TFE transcription factors may stimulate mTORC1 activity in a manner independent of their role in the induction of autophagic machinery.

Leucine and arginine have been shown to be two amino acids particularly important for mTORC1 activation on the lysosomal membrane (21,22). The study demonstrated that the sensitivity of mTORC1 activation in response to leucine or arginine was increased in cells overexpressing TFEB, yet the complete starvation of leucine largely inhibited mTORC1 activity in these cells. These results suggested that TFEB/TFE3 overexpression might support the expression of positive regulators in amino acid sensing machinery responsible mTORC1 activation. Likely candidates include the FLCN complex and the subunits of
v-ATPase, which is a positive regulator of the Ragulator complex that functions as a GEF for the RagA/B small GTPases.

As expected, enhanced gene expression in the TFEB overexpressing cells includes previously known TFEB targets important for amino acid-induced mTORC1 activation. Interestingly, the authors also identified RAGD as a putative TFEB target gene that encompasses the CLEAR element in its promoter. Strikingly, among 20 TFEB/TFE3 putative target genes that likely involve in the regulation of mTORC1 activity, RAGD was the most decreased transcript in TFEB or TFE3 silenced cells, whereas it was the most enhanced gene in cells overexpressing TFEB. Of note, consistent with the previous report by Martina et al. (19), FLCN expression was likewise affected by TFEB expression but to a much lesser extent to that compared of RAGD. Through chromatin immunoprecipitation (CHIP) and luciferase assays, the authors confirmed that RAGD is a direct transcriptional target of TFEB, as the RAGD promoter has three CLEAR sites upstream of its transcriptional start site (Figure 1). Functional importance of the RAGD CLEAR element was confirmed by generating cells bearing a deletion of a key endogenous CLEAR site through CRISPR-CAS9-mediated genome editing (RagD<sup>promedit</sup> cells). In RagD<sup>promedit</sup> cells, amino acid-induced lysosomal mTORC1 localization and its activation were significantly decreased compared to the control cells that have the intact TFEB binding CLEAR element. These observations indicate that TFEB/TFE3-induced RAGD expression plays an important role in amino acid-induced lysosomal mTORC1 localization and its activation. Although the data indicate an important role of endogenous RagD in the activation of mTORC1, it remains unclear why transcriptional inhibition of the RAGD gene is so...

**Figure 1** Dysregulation of MiT/TFE-RagD-mTORC1-MiT/TFE feedback circuit leads to cancer development. Increased expression of MiT transcription factor family members such as TFEB, recognize the CLEAR elements in the RAGD promoter located -650, -284 and -19 base pairs upstream of its transcription start site (TSS) and enhance RAGD gene expression. Increased RagD protein in turn stimulates lysosomal mTORC1 localization and its activation leading to cell growth/proliferation and tumor development even under metabolically stress conditions.
effective for mTORC1 inhibition in the presence of RagC of which expression is more ubiquitous and has a redundant function with RagD for mTORC1 regulation in response to amino acid availability. It is possible that the Rag heterodimer containing RagD might have higher activity and/or additional roles for lysosomal mTORC1 recruitment compared to the RagC containing heterodimer.

Based on these observations made in an in vitro system, the authors also addressed whether the over expression of TFEB or TFE3 had physiological relevance in tissues particularly important in adaptation to nutrient and starvation signals. In a liver specific TFEB over expression model, mTORC1 activity was indeed enhanced under nutrient rich conditions, but was inhibited under fasting conditions. In contrast, muscle specific TFEB knockout mice showed decreased mTORC1 activity in response to a post exercise leucine oral gavage, which was used to emulate the effect of a protein meal after exercise. These results pinpoint that TFEB is necessary to mediate leucine-mediated mTORC1 stimulation in vivo. Although their in vitro studies showed that TFEB is required for mTORC1 activation in response to leucine or full amino acid stimulation, it remains elusive if lack of TFEB also blocks full amino acid-induced mTORC1 activation in muscle tissues (23). In addition, the investigation of RagD expression and its role in mTORC1 activation in response to amino acid feeding in the exercised muscles will further clarify physiological relevance of the TFEB-RagD axis in the regulation of mTORC1 activity in vivo.

The TFEB/TFE3/MITF transcription factors belong to the MiT-TFE transcription factor family, and are well known oncogenes in various human tumors including renal cell carcinoma, melanoma, sarcoma and pancreatic ductal adenocarcinoma, in which aberrant mTORC1 activity is also observed. The study demonstrated positive correlations among the expression of TFE3/MITF, RagD expression, mTORC1 activity, cancer cell proliferation, and tumor development. Consistent with the other biochemical and biological observations demonstrated in this study, renal cancer cells carrying a chromosomal translocation of the TFE3 gene, pancreatic ductal adenocarcinoma bearing high MiT/TFE genes, and melanoma cells with aberrant MITF expression all showed increased RAGD transcript accompanied with increased mTORC1 activity. Silencing either these transcription factors or RagD attenuated mTORC1 activity as well as cell proliferation in these cancer cells, implying that the MiT/TFE-RagD-mTORC1 axis plays an important role in cancer cell proliferation/survival in vitro. Importantly, xenotransplantation experiments performed using the melanoma cell line showed significant reduction of xenografted tumor development upon RAGD silencing, highlighting a critical role of the MiT-TFE-RagD axis in promoting tumor development.

The MiT/TFE transcription factors are active in their dephosphorylated form under starvation conditions when mTORC1 is inactivated. However, the study reported by Di Malta et al. proposed a model where MiT/TFE-RagD-mTORC1-MiT/TFE feedback circuit is crucial for metabolic adaptation to nutrient availability. Dysregulation of this circuit such as constitutive activation of MiT/TFE leads to aberrant RagD-mediated mTORC1 activation and promotes cancer development (Figure 1). One likely physiological role of this feedback circuit is that under metabolic stress conditions, these transcription factors stimulate RagD expression and would prepare lysosomal mTORC1 localization and its activation once nutrients are replenished through extracellular influx or de novo production by autophagy. Alternatively, the MiT/TFE-RagD axis may play an emergent and specific role in keeping a low level of mTORC1 activity, maintaining cellular translational activity for the transcripts of lysosomal and autophagy components, as the restored mTORC1 activity after prolonged starvation is required for lysosomal biogenesis (20). In this regard, it is intriguing to examine the specific role of inducible RagD in the activation of mTORC1 under metabolic stress conditions.

In conclusion, this study provided a novel molecular mechanism by which oncogenic MiT/TFE transcription factors support cell growth/proliferation through their transcriptional regulation of the upstream of mTORC1 activator, RagD. The MiT/TFE-RagD-mTORC1-MiT/TFE feedback circuit precisely controls anabolic and catabolic processes with appropriate checkpoints and balances to maintain cellular homeostasis (Figure 1). Upon MiT/TFE overexpression as that observed in a variety cancers, a loss of anabolic/catabolic homeostasis occurs, leading to increased cell growth and proliferation even under metabolically stress conditions by enhancing RagD expression, and thus increased mTORC1 activity.

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Fibroblast growth factor signaling as a bypass mechanism of the androgen receptor pathway: new perspectives for castration-resistant prostate cancer

Ihsan Y. El-Sayed¹,², Francis Vacherot¹, Stéphane Terry³

¹INSERM U955, Equipe 7, Créteil, France; ²INSERM U908, CPAC, Cell Plasticity and Cancer, Univ. Lille, Villeneuve d’Ascq, France; ³INSERM UMR 1186, Integrative Tumor Immunology and Genetic Oncology, Gustave Roussy, EPHE, Fac. de médecine—Univ. Paris-Sud, University Paris-Saclay, Villejuif, France

Correspondence to: Stéphane Terry, PhD. INSERM U1186, Gustave Roussy Cancer Campus, 39 rue Camille Desmoulins, F-94805 Villejuif, France.

Email: stephane.terry@gustaveroussy.fr.

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Till now, prostate cancer (PC) appears among the most critical public health concerns in men in developed as well as developing countries. Androgens operating through androgen receptor (AR) nourish the development and function of normal prostate and may pathologically contribute to PC in case of any deformation or deregulation (1). Based on this, inhibiting the production of androgens by castration or their effects by using anti-AR agents is employed as a treatment of advanced PC disease. However, in most instances, upon therapy, cancer will develop in a form called castrate-resistant prostate cancer (CRPC). Reactivation of AR expression and activity is often noticed in this disease stage (2,3). Treatment with more potent anti-AR agents can be useful in this setting but their efficacy can be limited in time, while high therapeutic pressure will select for aggressive and highly adaptable cancer variants leading to therapeutic impasse.

Emerging observations provide relevant clues to how PC cells adapt to their microenvironment including drug treatment. They also underline the importance of heterogeneity of the tumor hosting subclonal cancer cell populations that may take advantage of the treatment selection pressure to promote tumor evolution. In particular, the studies of Himisha Beltran and other investigators previously suggested that neuroendocrine CRPC aggressive variants (CRPC-NE) might arise from CRPC variants characterized as prostate adenocarcinomas (CRPC-Adeno), and that enables tumor adaptation in response to AR-directed therapy. Indeed, CRPC-NE tumors display neuroendocrine features and an “AR-indifferent” cell state, thus hijacking the AR pathway. In vitro, in vivo, and in situ data highlighted the cellular and molecular complexity of these events and proposed numerous ways through which a prostate carcinoma cell characterized by an epithelial phenotype, can lose its epithelial “AR-driven” state while acquiring a neuroendocrine “AR-indifferent” state in a transdifferentiation process called neuroendocrine transdifferentiation (4-6). The data also suggest a spectrum of differentiation states from AR-dependent to AR-indifferent states, which echoes other types of transdifferentiation programs including epithelial-mesenchymal transition (EMT) (7). Thus, there may be CRPC diseases on the way to AR independence with no or moderate neuroendocrine differentiation that have not fully transited towards a neuroendocrine phenotype (4,8,9). The relevance of such CRPC remains an enigma, as well as the mechanisms driving their progression in the context of therapy resistance.

In Cancer Cell, Bluemn et al. (10) present an advance in our understanding of molecular mechanisms underlying
AR-independence.

In their work, the authors have investigated a small subset of CRPC characterized by AR-null/NE-null phenotype (at least null for a panel of advanced neuroendocrine markers). Importantly, such phenotype seems to be enriched in the contemporary era in which PC patients are heavily treated with AR pathway antagonists such as Enzalutamide and Abiraterone in addition to more standard castration therapies.

Since the EMT pathway came out in this analysis as highly enriched in these tumors, the double negative (AR-null/NE-null) phenotype may also highlight changes in the EMT status of CRPC escaping the AR pathway dependence. Given the small number of patient samples classified in the double negative category, it will be important to analyze additional series to further study the potential contributions of other specific EMT-related mechanisms and to deliver novel therapeutic options for this patient group. It will also be critical to the efforts to develop better biomarkers of EMT and neuroendocrine states that enable to discriminate early states from late states of differentiation. It will also be important to enlarge the number of patient samples analyzed to definitely prove that potent AR–targeted agents such as Enzalutamide and Abiraterone, rather than other therapies, promote the emergence the double negative (AR-null/NE-null) phenotype. Interestingly, through the involvement of overlapping alterations, or the expression of common driver genes, there are evidence for intimate connections among NE phenotype, EMT, epithelial plasticity and stemness properties (4,8,11-13). As discussed by Bluemn et al., it is tempting to speculate a continuum of progression from AR dependent epithelial to EMT/stemness to NE “AR independent” phenotypes, but such sequence of events is yet to be investigated. Here again, the development of more pertinent markers and tools could help statute on this aspect.

We should consider that some cancer cells are more prone than others to undergo EMT and this may favor intratumoral heterogeneity. In our previous published work, we investigated the role of CRIPTO in PC (14). CRIPTO is the founding member of the EGF-CFC (Cripto, FRL-1, Cryptic) protein superfamily. This gene is implicated in embryogenesis, oncogenesis, as well as in stemness maintenance and its expression is markedly increased in many cancer types. CRIPTO expression in these tumors was associated with poor outcomes and with EMT in in vitro models (14). In PC tumors, we demonstrated the existence of a population of CRIPTO expressing carcinoma cells exhibiting mesenchymal characteristics within the primary tumor, while other carcinoma cells expressing CRIPTO remained with more epithelial features (14). This highlights the pleiotropic nature of certain factors driving various effects in carcinoma clones and generating phenotypic diversity/intratumoral heterogeneity. The work of Bluemn and his colleagues supports the intratumoral heterogeneity notion in prostate tumors where different cellular clones with different cellular differentiation states may exist, cooperate, communicate and influence the progression of tumor. For instance, their cellular model (LNCaP double negative clones) was obtained after drastic selective pressure combining androgen deprivation and AR knock-out conditions.

Analysis of patient specimens and model systems developed by Bluemn et al. led to the identification of elevated fibroblast growth factor (FGF) and downstream mitogen-activated protein kinase (MAPK) pathway activity as the main mechanism driving the bypass of AR dependence in this setting. FGF signaling was enough to evade AR signaling pathway as they did indicate that absence or depletion of AR activity can be compensated by hyperactive FGF and MAPK pathways to maintain proliferation and survival. Meanwhile, when these two latter pathways are switched off, no growth of double negative PC cells in vitro and in vivo was observed. This finding may provide novel therapeutic opportunities. FGF/MAPK hyperactivity in certain CRPC tumors could be exploited therapeutically with fibroblast growth factor receptor (FGFR) inhibitors, and preclinical data presented by the authors are very promising in this regard.

Interestingly, FGFR1 signaling can also promote EMT program (15). Previous work also suggested the FGF/FGFR1 axis as an important element in PC initiation and progression in association with aggressiveness (15-17). In a recent study, we showed that CRIPTO overexpression mediates EMT in the CRPC model 22Rv1 cells, while this effect appeared to be promoted through parallel actions of FGFR1/MAPK and AKT signaling pathways (14). It is noteworthy that activation of FGFR1/MAPK signaling leading to EMT as a consequence of CRIPTO expression was accompanied by a marked reduction of the AR signaling (unpublished data). Interestingly, the mesenchymal-like PC cells derived in our study conserved their tumorigenic capacity in nude mice (unpublished data). These observations are in accordance with those of Bluemn et al. where elevated activity of FGF signaling is correlated with tumor progression in an AR-
indifferent manner. It is also important to consider the potential impact of interclonal cooperation and communications that might occur among the distinct clones driving intratumoral heterogeneity. In line with this hypothesis, we recently assessed the effects of the extracellular vesicles (EVs) released by mesenchymal PC cells on recipient androgen-dependent epithelial PC cells. EVs occupied wide attention among scientific community during the last years as an extracellular component impacting the intratumoral heterogeneity and interclonal communication. We showed that mesenchymal like PC derived vesicles promoted mesenchymal features in the recipient epithelial-like PC cells (18). This transformation was accompanied by reduced AR signaling and activation of TGF\(\beta\) signaling pathway. Moreover, recipient cells acquiring mesenchymal traits displayed enhanced migratory and invasive properties as well as increased resistance to the AR antagonist, enzalutamide (18). It will be interesting to see if the double negative model of PC developed in the study of Bluemn and colleagues may similarly influence the behavior and function of neighboring cells in the tumor microenvironment via secretion of vesicles or FGF species.

Overall, the study by Bluemn provides an innovative advancement of our understanding of the cellular and molecular determinants underlying escape of AR-directed therapy in CRPC. Additional studies are required to define the prevalence of these events, and their connections with cancer cell plasticity, EMT and NE phenotypes. Bluemn’s study opens new perspectives towards finding a therapeutic approach that may target and treat CRPC patients that have gained resistance to AR-directed therapy with the emergence of cancer cell clones that are null for both AR/NE features.

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None.

**Footnote**

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To date, prostate cancer (PCa) remains a medical challenge, being one of the most prevalent causes of cancer deaths in men worldwide (1). A major innovation in the management of PCa was demonstrated by the measurement of prostate specific antigen (PSA) in the serum of PCa patients in the mid-1980s; however, it is well known that measurement of PSA levels is associated with over-diagnosis and over-treatment. Although over-treatment may be reduced by improved risk stratification where very low or low risk PCa can be monitored by active surveillance and intermediate or high risk PCa can be subjected to treatment, many urologists and patients are reluctant to delay treatment due to the absence of a reliable indicator of aggressive disease and thus, there is a possibility of missing treatment of aggressive PCa patients (2,3). A single threshold PSA test is unable to distinguish between high and low risk PCa (4), and prostate biopsy is often unreliable for the prediction of cancer grade, as only a small fraction of the prostate is sampled during a biopsy for staging (5). We have recently reviewed the diagnostic and prognostic value of miRNAs, along with several detection methodologies which provides important insights into the use of miRNAs as non-invasive cancer biomarkers (9).

Knowledge about uptake, packaging and release of miRNAs is crucial to determine their regulatory functions, and double lipid membrane vesicles, called exosomes, have been found to play a crucial role in this regard (10). The usefulness of exosome miRNAs has been evaluated by several studies including a report by Li et al. showing elevated levels of exosomal miR-141 in metastatic PCa patients in comparison to benign prostatic hyperplasia (BPH) patients and healthy controls (11). Similarly, Huang et al. showed that the plasma exosomal level of miR-1290 and miR-375 was associated with poor survival of castration resistant PCa patients (12). Apart from serum and plasma, Samsonov et al. indicated upregulation of a microRNA molecular signature of aggressive prostate cancer

Farhana Matin1,2, Jyotsna Batra1,2

1School of Biomedical Sciences, Faculty of Health, Institute of Health and Biomedical Innovation, 2Australian Prostate Cancer Research Centre-Queensland (APCRC-Q), Translational Research Institute, Queensland University of Technology, Brisbane, Australia

Correspondence to: Jyotsna Batra. Translational Research Institute, 37 Kent Street, Woolloongabba, QLD-4102, Brisbane, Australia.
Email: jyotsna.batra@qut.edu.au.

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The prevailing understanding was that the genome consists of regions with little coding material of importance; however, recent advances have shown that these regions are not so barren after all. A part of the so called “non-coding” genome in fact encodes for critical gene regulators called miRNAs that are present in stable forms in the circulation and thus, can play an important role as diagnostic and prognostic biomarkers for several diseases (8). Mature miRNAs were initially detected in the cell free fractions of blood such as serum and plasma (8), and subsequently found in other body fluids and tumour tissues (9). We have recently reviewed the diagnostic and prognostic value of miRNAs, along with several detection methodologies which provides important insights into the use of miRNAs as non-invasive cancer biomarkers (9).
miR-21, miR-141 and miR-574 in urinary exosomes isolated from PCa patients and healthy controls using a lectin-based agglutination method (13). Therefore, exosomal miRNAs may be utilized as non-invasive molecular signatures specific to patients with an increased risk of developing aggressive PCa, but it is difficult to differentiate intermediate grades from aggressive forms due to the heterogeneity of PCa.

To address this issue, in a recent study published in Proceedings of the National Academy of Sciences (PNAS) (14), Alhasan and colleagues developed a high-throughput, spherical nucleic acid (SNA)- and microarray-based miRNA expression profiling platform, called the Scano-miR bioassay, to determine the exosomal miRNA expression. Authors used the Scano-miR bioassay in a discovery set of 16 serum samples from patients with varying grades of PCa, i.e., ≥8 Gleason Score for high or very high risk and Gleason Score =6 for very low or low risk PCa, and healthy individuals. Furthermore, a molecular signature score was calculated, as done by Zeng et al. previously (15), to ensure diagnostic reliability upon combining the differentially expressed miRNAs in a blind study. In this way, a molecular signature consisting of five miRNAs (miR-200c, miR-605, miR-135a*, miR-433 and miR-106a) was identified capable of differentiating indolent and aggressive forms of PCa with 89% accuracy after validation in a second cohort by quantitative real time PCR (qRT-PCR) (14). This unique molecular signature may assist in stratifying patients who may benefit from therapy from those who may only require close monitoring through active surveillance.

In the above study, the serum expression of miR-200c was highly elevated in very high risk PCa patients suggesting its role in predicting metastasis, and similar results have been obtained in studies focussing on colorectal and gastric cancer (16,17), suggesting the role of this miRNA as a general marker of metastasis. In another study, miR-200c has been found to be part of a five biomarker panel for the detection of metastatic castration resistant PCa supporting the findings of Alhasan and colleagues (18). Dysregulation of miR-106a has been reported in lung and gastric cancer (19) and has been previously linked to PCa. miR-106a was also one of the previously reported participant of several biomarker panels for high risk PCa (20), but the other miRNAs have not been previously associated with PCa.

PCa is very heterogeneous in nature where cancer specific survival rate is higher in patients with a low risk of disease progression compared to those with aggressive disease. The investigation carried out by Alhasan and colleagues lead to the discovery of a novel miRNA signature capable of differentiating indolent from aggressive forms of PCa at a higher rate than typical Gleason scoring of biopsy samples, representing a simple diagnostic tool without the need for surgical intervention. The Scano-miR bioassay does not rely on enzymatic amplification of a specific target as many of the current miRNA detection methods do, and therefore, allows multiplexing and detection of multiple miRNAs at the femtomolar levels in single samples. In addition, pathway analysis was also performed to identify targets of the miRNA panel and some of the targets were found to be known drivers of tumorigenesis.

So far, specific miRNA expression patterns have been proposed for PCa subclasses, but further studies are required and several questions need to be addressed to aid the establishment of miRNA signatures for cancer diagnosis and prognosis. Some of these necessitate an understanding of the relationship between circulating and tumour derived miRNAs, their mechanism of uptake and release, their response to inflammation and modifications, and finally their role in tumorigenesis and metastasis. Although several new techniques have improved the specificity and sensitivity of miRNA detection, the lack of referenced procedures for sample preparation, RNA extraction, endogenous control selection, sample size calculation, etc. makes it difficult to compare results between independent studies. The major challenge is to overcome these hurdles and identify reliable miRNA biomarkers for the stratification of cancer patients. The development of digital PCR would remove the dependence on a miRNA normalization control which is not yet established for qRT-PCR, where an exogenous control is used mostly. Besides, digital counting technologies such as next-generation sequencing (NGS) and the NanoString nCounter miRNA Expression Assay may be used for miRNA profiling. NGS allows the discovery of new miRNAs and confirmation of already known miRNAs as opposed to microarrays and qRT-PCR, and overcomes the limitations of background signal, microarray panel difference and cross-hybridization issues associated with the use of microarrays. The NanoString nCounter system is another new hybridization-based technology which directly detects miRNAs of interest using target specific, colour-coded probes without the need for reverse transcription or amplification by qRT-PCR (20). All these advancements in the area of biomarker research and inclusion of other criteria such patient age, PSA level, clinical tumour stage, Gleason Score, etc. for risk assessment from a PCa perspective followed by multivariate analyses, will not only help in accurate cancer detection, but will also facilitate the
development of novel strategies for cancer therapy.

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References

Prostate cancer (PCa) is the most common malignancy in men and remains a leading cause of cancer death in males worldwide (1). The mainstay for patients with advanced and metastatic PCa, including castration-resistant disease, is hormonal therapy that targets the androgen receptor (AR) (androgen deprivation therapy) (2). Recently potent AR-targeted therapies were approved for the treatment of men with castration-resistant prostate cancer (CRPC), as de case of enzalutamide and abiraterone (3,4). While the use of these agents improves the survival of individuals with CRPC, most of them eventually develop resistance to therapy with a lethal outcome (5). This phenomenon may reflect an epithelial plasticity that enables tumor adaptation in response to AR-target therapies, which is not fully understood.

It has been highlighted that androgen-deprivation therapy frequently induces the emergence of highly aggressive prostate phenotypes with neuroendocrine features, also called neuroendocrine transdifferentiation (NEtD) (6). With the introduction of this new era of potent androgen receptor-targeted agents into the clinic, there is an evolving change in the clinical landscape of advanced PCa and treatment-related neuroendocrine prostate cancer (NEPC) is becoming an even more important condition to recognize.

NEPC is a high-risk, lethal subset of disease and is distinguished from prostate adenocarcinoma by the expression of neuroendocrine markers (as synaptophysin and chromogranin) and the loss of expression of the AR and PSA (6,7). Other neuroendocrine markers have been reported, although they are not typically used in the clinical practice (8). These markers include synaptic vesicle protein 2 (9), granin-A (10) and more recently the T-Box Brachyury (11) (Figure 1). Although some of these markers are promising, more studies are necessary before they can be used in clinical practice for detecting NEPC. NEPC is often referred to as representing only 2% of all diagnosed PCa (12). However, it is believed that probably occurs far more often, since the disease is not undistinguished of metastatic CRPC, and therefore, NEPC is underdiagnosed.

In 2010, Witte's group reported the first functional study addressing the origin of PCa. The authors showed that basal cells from primary benign human prostate tissue can initiate PCa in mice through cooperative effects of AKT1 and ERG overexpression (13). Further studies from the group identified that prostate adenocarcinoma and squamous cell carcinoma can arise from a common basal cell precursor with deregulation of c-Myc expression and AKT1 (14). The proto-oncogene c-Myc is highly expressed in prostate adenocarcinomas, at variance of N-Myc that is only expressed in 5% of prostate adenocarcinomas, but is overexpressed and amplified in about 40% of NEPC (15), indicating a potential role of N-Myc as a critical oncoprotein in NEPC.

In a recent publication in Cancer Cell (16), Lee and colleagues (from Witte’s group) elegantly show the major
role of N-Myc and AKT1 in NEPC phenotype and as potential targets for therapeutic intervention (Figure 1). The authors establish a novel in vivo model that reflect the histology and molecular features of human end-stage PCa with mixed NEPC and prostate adenocarcinoma, by deregulating the expression of N-Myc and AKT1 in primary human prostate epithelial cells. This outstanding model of NEPC (M-Myc/AKT1 tumors) showed to be androgen-independent, as demonstrated by the low or absent of AR expression, a similar feature of human CRPC and NEPCs (17). The importance of N-Myc in NEPC was highlighted by the contrasting results obtained by the combination of c-Myc and AKT1, which generates prostate adenosquamous carcinomas in the same system (16). These results also demonstrate the importance of different MYC members in the susceptibility to different kinds of cancer. Importantly, this work has brought a new in vivo tool for proper studies of NEPC, that lack of suitable models with good defined genetic drivers.

NEPC usually harbor genetic abnormalities also present in prostate adenocarcinoma as ETS rearrangements and PTEN mutations (17-19). The recently identified independent prognostic biomarker in prostate adenocarcinomas, Brachyury (20), showed also to be highly associated with tumors with neuroendocrine markers, which also present ETS overexpression and loss of PTEN (11). These studies indicate that NEPC type may arise from common clonal origin. Lee and colleagues (16) successful demonstrated that the NEPC and prostate adenocarcinoma can arise from a common prostate epithelial cell with N-Myc/myrAKT1 alteration, but not from luminal epithelial cells (where the benign neuroendocrine cells are present). Future studies will be necessary to clarify the influence of N-Myc/myrAKT1 in this subpopulation of cells.

Interestingly, the authors also showed that the incidence of NEPC tumors increases compared with adenocarcinoma when the N-Myc/myrAKT1 clones are propagated in castrate conditions (16), indicating that prostate adenocarcinomas undergo NEtD under selective pressure of androgen deprivation (Figure 1). Moreover, tumors with N-Myc overexpression presented stem cell-like properties that could act as repopulation clones of NEPC and consequently therapy resistance (16). These novel evidences are in agreement with a previous study where it was shown a correlation between

Figure 1 Overexpression of N-Myc and AKT1 in human prostate epithelial cells give rise to prostate adenocarcinoma and neuroendocrine prostate cancer. Prostate adenocarcinoma under selective pressure, as androgen deprivation, undergoes neuroendocrine transdifferentiation and present increased levels of N-Myc/AKT1. Destabilization of N-Myc/AKT1 through AURKA inhibition (CD532 inhibitor) and/or other key players in neuroendocrine prostate cancer development could be a novel therapeutic strategy in this deadly disease. AURKA, aurora A kinase; AR, androgen receptor.
acquisition of stem cell properties in PCa, NEtD and resistance to conventional chemotherapy (11) (Figure 1). It was been reported that patients with prostate adenocarcinoma that develop NEPC present amplification of both N-Myc and aurora A kinase (AURKA) (18). Based in these findings, Lee and colleagues (16), have shown that inhibition of AURKA and N-Myc (by positive feedback) by using the CD532 inhibitor, reduce tumor growth in vivo (Figure 1).

Concluding, the work published by Lee et al. in Cancer Cell (16) have provided novel and important data about the etiology and molecular basis of this aggressive subset of PCa, that could be further explored to increase our ability to diagnose NEPC at an earlier stage and may guide future pre-clinical studies for the treatment of patients with NEPC.

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Emerging molecular pathways and targets in neuroendocrine prostate cancer

M. Francesca Monn1, Rodolfo Montironi2, Antonio Lopez-Beltran3,4, Liang Cheng1,5

1Department of Urology, Indiana University School of Medicine, Indianapolis, Indiana, USA; 2Institute of Pathological Anatomy and Histopathology, School of Medicine, Polytechnic University of the Marche Region, United Hospitals, Ancona, Italy; 3Department of Pathology and Surgery, Faculty of Medicine, Cordoba, Spain; 4Department of Pathology, Champalimaud Clinical Center, Lisbon, Portugal; 5Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, Indiana, USA

Correspondence to: Liang Cheng, MD. Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, 350 West 11th Street, Indianapolis, IN 46202, USA. Email: liang_cheng@yahoo.com.

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Abstract: Small cell prostate cancer remains a poorly understood, aggressive form of prostate cancer that develops either de novo or in the setting of castrate resistant adenocarcinoma of the prostate. Frequently these tumors are advanced and are challenging to treat using current modalities. Research into molecular abnormalities that drive the development and propagation of small cell prostate cancer is ongoing and has the potential to revolutionize the management of these patients. Current research into the role of N-myc (NMYC) is altering our understanding of this aggressive disease. A recent study demonstrated that NMYC over expression can lead to development of invasive and androgen castrate resistant small cell prostate cancer. Furthermore, this research went on to show that NMYC over expression can lead to transformation of prostate adenocarcinoma into small cell prostate cancer. Finally, recent research has explored the role of aurora kinase A (AURKA) inhibitors in disrupting the NMYC pathway and providing a potential therapeutic target for treatment of small cell prostate cancer. As research continues to advance our understanding of the molecular underpinnings of small cell prostate cancer, we will be able to develop novel therapeutic targets and agents in order to better treat this aggressive form of prostate cancer.

Keywords: Prostate; neuroendocrine prostate cancer; molecular genetics; N-myc (NMYC); small cell prostate cancer; molecular pathway/targets; precision medicine

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Introduction

Small cell prostate cancer remains a poorly understood, aggressive form of prostate cancer that comprises less than 2% of prostate cancer diagnoses (1-6). Small cell prostate cancer remains a histological diagnosis and develops either de novo in patients with no prior history of prostate adenocarcinoma or, more commonly, in the setting of a patient with castrate resistant prostate cancer (Figure 1). The molecular underpinnings that determine which patients develop small cell prostate cancer in the de novo or castrate-resistant setting are not well defined although significant research is ongoing.

Mutations

Among the genetic mutations most commonly described in small cell prostate cancer, p53 and TMPRSS2-ERG are most common (1-6). TMPRSS2-ERG rearrangements are present in approximately half of small cell prostate...
cancer cases and are of particular value as they are not seen in other forms of small cell carcinomas (1-6). Interestingly, recent research has reported that TMPRSS2-ERG rearrangements can be associated with androgen receptor upregulation which may be associated with disease progression (7-10). Aurora kinase A (AURKA) and N-myc (NMYC) mutations are more commonly reported in small cell prostate cancer than in traditional prostatic adenocarcinoma, with a recent study reporting that 40% of small cell prostate cancer cases compared with 5% of prostatic adenocarcinoma had associated AURKA and NMYC overexpression (11). Further studies have suggested that up to 65% of small cell prostate cancer specimens and 86% of metastatic small cell prostate cancer specimens have AURKA amplification (12).

A recent article by Lee et al. provided the first preclinical study demonstrating the role that overexpression of NMYC maintains in the development of small cell prostate cancer (13). In this study the researchers examined human epithelial prostate cancer cells—a first as prior studies have almost exclusively studied xenografts. The researchers isolated benign prostate tissue from prostatectomy specimens and enforced expression of NMYC with eventual development of both adenocarcinoma and small cell prostate cancer in the previously benign prostate tissue. They further demonstrated that the tumors generated by NMYC over expression were both invasive and lacking androgen receptor expression, rendering the tumor castrate resistant (13).

**Development**

Multiple theories for the development of small cell prostate cancer exist. Many argue in favor of a divergent clonal evolution in which small cell prostate cancer develops from castrate resistant prostatic adenocarcinoma through epigenetic changes associated with cell plasticity and androgen receptor signaling (14). Alternatively, researchers have suggested that small cell prostate cancer represents dedifferentiation of adenocarcinoma, which is supported by the common finding of concomitant small cell prostate cancer with prostatic adenocarcinoma (15-17). Finally, research has suggested that the basal progenitor cells of the prostate may give rise to both traditional prostatic adenocarcinoma and the neuroendocrine cells observed in small cell prostate cancer (17-20).

In their recent study, Lee et al. report that the progenitor cells for adenocarcinoma in the prostate were able to transform into small cell prostate cancer when there was overexpression of NMYC, which supports the theory of a common basal progenitor cell (13). Furthermore, they report that increased expression of the malignant cells in the setting of castrate resistance results in further propagation of the small cell prostate cancer as opposed to adenocarcinoma (13). They propose that NMYC overexpression may enable stem cell progenitors to repopulate prostatic based tumors after treatment. Significant research is still required to fully understand the mechanisms underlying the development of small cell prostate cancer.

**Therapeutics**

While understanding the pathogenesis of small cell prostate cancer remains a critical area of research, the true benefit in determining its molecular underpinnings lies in the ability to identify molecular targets upon which medications can intervene and therapeutics can be designed. The recent study by Lee et al. is therefore a significant step forward as it demonstrated that NMYC is essential in maintaining the
tumor through the AURKA pathway. With this in mind, the rationale behind AURKA inhibitors as therapeutic targets is strengthened. Using CD532, a novel therapeutic that interferes with the AURKA-NMYC complex (21), Lee et al. were able to demonstrate a significant decrease in NMYC activity in human prostate epithelial cells (13). This provides evidence that the AURKA and NMYC pathways may be excellent targets in the treatment of patients with NMYC overexpression small cell prostate cancer.

Prior therapeutic interventions for small cell prostate cancer have been directed by treatments for small cell cancer of the lung. Traditionally this includes cisplatin and etoposide. A study evaluating the role of docetaxel failed to demonstrate a benefit beyond that provided by cisplatin and etoposide alone (22). Beltran et al. evaluated the role of danusertib, an AURKA inhibitor, in both prostate adenocarcinoma and small cell prostate cancer. They reported that there was increased efficacy in patients who had NMYC and AURKA overexpression (11). Additional research is being performed using MLN8237/alisertib, a different AURKA inhibitor that is undergoing investigation in the setting of small cell prostate cancer and small cell cancer of the lung, although preliminary results have not yet demonstrated significant benefit (23). The research by Lee et al. demonstrating a benefit when using CD532 lends further credence to continued evaluation of NMYC and AURKA as therapeutic targets in small cell prostate cancer (13).

Continued research into the pathogenesis of small cell prostate cancer remains essential in determining therapeutic options. Recognizing the role that TMPRSS2-ERG fusion rearrangements play in small cell prostate cancer, recent studies have examined whether poly (ADP ribose) polymerase 2 (PARP1) inhibitors, which interact with ERG in prostate cancer cells, may offer an additional drug target. A recent phase 2 clinical trial of olaparib reported that 33% (n=16/49) of metastatic prostate cancer patients responded to treatment in the setting of failed treatment with docetaxel, abiraterone, enzalutamide, or cabazitaxel (24). Although this was not limited to patients with small cell prostate cancer, it is reasonable to conclude that small cell prostate cancer patients would similarly benefit based on our current understanding of the pathogenesis of small cell prostate cancer.

**Conclusions**

Based on the work of Lee et al., it is increasingly evident that NMYC plays a crucial role in both the initiation and propagation of small cell prostate cancer. These findings support what has been previously suspected and proposed but never demonstrated in human epithelial prostate cancer cells. Recognizing the essential role of NMYC provides an opportunity for targeted therapeutics that may revolutionize the treatments we use in the setting of small cell prostate cancer.

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None.

**Footnote**

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

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Introduction

Micropapillary variant (MPV) urothelial carcinoma has been reported as comprising up to eight percent of contemporary urothelial carcinoma cohorts (1-4). The majority of studies have reported that MPV urothelial carcinoma portends a worse oncologic prognosis and that the tumor demonstrates more aggressive histology (3,5-8). The optimal algorithm for patients diagnosed with MPV urothelial carcinoma remains poorly defined with many researchers arguing that even in the setting of non-muscle invasive disease, these patients should be taken for early extirpative management. As MPV urothelial carcinoma remains an uncommon entity, large and multi-institutional studies have not been conducted to evaluate the efficacy of neoadjuvant or adjuvant chemotherapy. However, retrospective institutional studies have suggested that MPV demonstrates a poorer response to standard neoadjuvant chemotherapy regimens when compared with pure urothelial carcinoma (9,10). The mechanism behind this has been poorly understood. The recent article by Guo et al. begins to explore the genetic differences in MPV compared with urothelial carcinoma (11).

Immunohistochemical evaluation

Previous immunohistochemical evaluations of MPV urothelial carcinoma have been performed to determine the best markers to identify MPV in bladder cancer specimens. Figure 1 demonstrates an H & E stain of MPV urothelial carcinoma. GATA 3 (GATA binding protein 3) and uroplakin 3 have been reported as reliable markers for urothelial tumors although the sensitivity of uroplakin 3 is worse than for GATA 3. GATA 3 is a member of a family of transcription factors involved in embryogenesis and has been reported to be the most sensitive and specific for bladder cancer (12,13). Recent studies have reported that GATA 3 levels in MPV urothelial carcinoma are similar to levels in pure urothelial carcinoma (14). Interestingly, while GATA 3 levels are similar between MPV and pure urothelial carcinoma, GATA 3 levels have been reported to be significantly lower in other variants of urothelial carcinoma such as squamous differentiation variant and sarcomatoid variant (14,15). The reason for this difference is unclear but is likely more reflective of changes in the squamous differentiation variant.
An additional marker of urothelial carcinoma is p63. Wang et al. recently reported that the presence of p63 was an independent predictor of worse survival in patients with urothelial carcinoma who underwent radical cystectomy with urinary diversion (16). Despite the majority of patients with pure urothelial carcinoma displaying expression of p63, recent studies have reported decreased p63 expression in patients with MPV, with between 27% and 54% of MPV tumors staining positive for p63 (15,17).

Choi et al. previously reported that muscle-invasive urothelial carcinoma can be divided into luminal, p53-like luminal, and basal subtypes which were predictive of response to chemotherapy and overall tumor behavior (18). The pure urothelial carcinoma cases with the basal subtype had overexpression of p63 and were more aggressive at presentation. These patients were additionally more sensitive to traditional neoadjuvant chemotherapy regimens (18,19). The luminal subtype demonstrated increased PPAR-γ expression and FGFR mutations. The p53-like luminal subtype tumors shared PPAR-γ expression and FGFR mutations but were notably chemo-resistant to current neoadjuvant chemotherapy regimens (18).

In the recent study by Guo et al., it was reported that whereas in the pure urothelial carcinoma cohort 47.2% were basal subtype, 24.7% were luminal subtype, and 28.1% were p53-like luminal subtype, when examining the MPV urothelial carcinoma cohort, 23.2% were basal subtype (n=1), 51.2% were luminal subtype (n=22), and 46.5% were p53-like luminal subtype (n=20) (11). The MPV urothelial carcinoma tumors demonstrated, almost uniformly, GATA 3 and uroplakin 2. Furthermore, the tumors demonstrated increased PPAR-γ expression and downregulation of p63. When examining the response to chemotherapy among MPV tumors, 66% (n=4/6) of tumors in the luminal subtype and 45% (n=5/11) of tumors in the p53-like luminal subtype group demonstrated response to neoadjuvant chemotherapy, similar to prior studies suggesting that the p53-like luminal subtype was less likely to respond to chemotherapy.

**Genetic alterations**

Downregulation of miR-296, which is associated with upregulation of over 300 downstream genes, was found to be a driver in the expression of MPV in the recent study by Guo et al. (11). This may be a critical pathway that could be targeted to better identify patients with this uncommon variant of urothelial carcinoma. Downregulation of miR-296 has previously been reported to be associated with aggressive changes in other cancers including prostate cancer (20-23). As part of miR-296 downregulation, the RUVBL1 pathway is activated. This is known to be associated with genes that play critical roles in metastasis, cell growth, and DNA repair. Additionally, RUVBL1 acts via p53 to block p53 mediated cellular apoptosis (24).

Furthermore, as the RUVBL1 pathway has been noted to be associated with poor response to traditional chemotherapy, it may serve as the mechanism of resistance to cisplatin based regimens. Both miR-296 and the RUVBL1 pathway could be intervened upon to prevent the aggressive changes seen with MPV urothelial carcinoma.

An additional potential intervenable pathway identified by the Guo et al. study is PPAR-γ (11). The study found that the majority of MPV urothelial carcinoma tumors, regardless of p53-like subset, demonstrate upstream PPAR-γ expression. PPAR-γ has been postulated as a target for muscle invasive bladder cancer and research is ongoing into its clinical relevance as a therapeutic target (25,26). Troglitazone, a PPAR-γ agonist, induces apoptosis and autophagy in bladder cancer cells (27); although more research is needed before these agents are used in clinical practice.

**Clinical implications**

A particularly interesting finding in the Guo et al. study is the fact that when examining tumors with MPV sections and pure urothelial carcinoma sections, the molecular signatures of the urothelial carcinoma sections were similar to the MPV sections (11). This finding would imply that regardless of the percentage of MPV in a tumor specimen, the patient will likely have a more aggressive clinical
progression of disease. Previously, authors have suggested that in the setting of only small volume variant histology (<5%), a patient could potentially be treated as if their tumor were pure urothelial carcinoma; however, the current study would suggest that these patients may be more similar to the higher volume MPV patients than previously understood and may benefit from early radical cystectomy with urinary diversion until new chemotherapeutic or immunomodulating agents are identified.

The lack of responsiveness to current chemotherapy regimens and molecular alterations indicative of an aggressive tumor suggest that patients with MPV urothelial carcinoma may benefit from early extirpative management. The approach to patients with MPV urothelial carcinoma will continue to evolve as new molecular targets are identified. As previously discussed, miR-296, RUVBL1, and PPAR-γ are potential targets that could revolutionize the way MPV urothelial carcinoma is approached.

Conclusions

MPV urothelial carcinoma remains an uncommon variant of bladder cancer that can be challenging to treat. Studies such as that by Guo et al. are landmark in building an understanding of the fundamental changes that occur in the development of MPV urothelial carcinoma. With subsequent studies of the molecular underpinnings and evaluation of therapeutic targets, management of patients with MPV will be revolutionized.

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Footnote

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References


Micropapillary urothelial carcinoma: is molecular hair-splitting on target?

Nuzhat Husain, Azfar Neyaz

Urothelial carcinoma (UC) of the bladder has been well investigated in terms of pathogenesis pathways, natural history and tumor biology. Clinically relevant biomarkers including diagnostic, prognostic and predictive molecular markers have been defined in phenotype and genotype analysis beginning with the Cancer Genome atlas (TCGA) study reported in year 2014 (1). The recent 2016 WHO Classification of Tumors of the Urinary System and Male Genital Organs enumerates several histological variants such as the micropapillary, nested, microcystic, plasmacytoid, lymphoepithelioma-like, lipoid cell, clear cell, sarcomatoid and poorly differentiated types (2). Micropapillary urothelial carcinoma (MPUC), first described by Amin et al. in 1994 has generated considerable interest (3). This aggressive variant of UC has a characteristic morphology, aggressive clinical behavior, high propensity for metastasis to regional lymph nodes and distant organs resulting in shorter survival. Recent analysis of molecular, phenotype and microRNA (miRNA) profiles of this variant define unique features which may assist in early recognition and timely treatment (4).

Micropapillary urothelial carcinoma (MPUC), first described by Amin et al. in 1994 has generated considerable interest (3). This aggressive variant of UC has a characteristic morphology, aggressive clinical behavior, high propensity for metastasis to regional lymph nodes and distant organs resulting in shorter survival. Recent analysis of molecular, phenotype and microRNA (miRNA) profiles of this variant define unique features which may assist in early recognition and timely treatment (4).

Micropapillary genotype and behavior manifests even if a small amount of micropapillary histology (>10%) is present relative to conventional UC (7). Keratin profile in micropapillary carcinoma is similar to conventional UC (Table 1). They are more likely to express cancer antigen 125 (CA 125) indicating glandular differentiation (7). Micropapillary carcinoma also shows positive immunostaining for epithelial membrane antigen, CK7 and CK20, and CD15 (5,17). Metastases are common at the time of initial diagnosis (18). The main differential diagnosis is metastatic serous micropapillary ovarian carcinoma in women or mesothelioma. Fifty six percent of MPUC harbor human epidermal growth factor receptor 2 (HER2) gene amplification which is significantly associated with poor cancer-specific survival rates in patients (14).

Several gene profiling studies have reported different sub-categorization of UC. The current accepted grouping reported by Choi et al. 2014, subtypes UC by the use of molecular markers into basal and luminal types in a pattern similar to molecular subtypes of breast carcinoma. The basal subtype is characterized by high expression levels of the markers CD44, KRT5, KRT6B, KRT14. The luminal subtype is enriched for fibroblast growth factor receptor 3 (FGFR3), KRT20, HER2, FOXA1, GATA3, TRIM24, CD24, XBP1, peroxisome proliferator-activated receptor γ (PPAR γ) (19). Alterations of the Rh pathway have been noted mainly in the basal type, while the luminal subtype is characterized by FGFR3 and TSC1 mutations and copy
### Table 1: Comparison of demographic, clinical, histological, genetic and prognostic parameters in conventional vs. micropapillary UC

<table>
<thead>
<tr>
<th>Features</th>
<th>Micropapillary UC</th>
<th>Conventional UC</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevalence estimates</td>
<td>0.7–2.2% of urothelial cancers</td>
<td>&gt;90% of bladder cancers</td>
<td>Amin et al. (3); Lopez-Beltran et al. (2)</td>
</tr>
<tr>
<td>Age (mean)</td>
<td>70.6±9.3</td>
<td>69.6±10.9</td>
<td>Guo et al. (4)</td>
</tr>
<tr>
<td>Gender</td>
<td>5.1:1</td>
<td>3.05:1</td>
<td>Guo et al. (4)</td>
</tr>
<tr>
<td>Multifocality</td>
<td>58%</td>
<td>38%</td>
<td>Fairey et al. (8)</td>
</tr>
<tr>
<td>Carcinoma in situ</td>
<td>67%</td>
<td>62%</td>
<td>Fairey et al. (8)</td>
</tr>
<tr>
<td>pT stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT1</td>
<td>12%</td>
<td>34%</td>
<td>Wang et al. (9)</td>
</tr>
<tr>
<td>pT2</td>
<td>21%</td>
<td>31%</td>
<td></td>
</tr>
<tr>
<td>pT3/4</td>
<td>66%</td>
<td>35%</td>
<td></td>
</tr>
<tr>
<td>Extravesical disease</td>
<td>66%</td>
<td>35%</td>
<td>Wang et al. (9)</td>
</tr>
<tr>
<td>Progression of NMIUC to MIUC</td>
<td>67%</td>
<td>&lt;5%</td>
<td>Kamat et al. (10)</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low grade</td>
<td>3%</td>
<td>17%</td>
<td>Fairey et al. (8)</td>
</tr>
<tr>
<td>High grade</td>
<td>97%</td>
<td>83%</td>
<td></td>
</tr>
<tr>
<td>Lymph node involvement</td>
<td>50%</td>
<td>10%</td>
<td>Wang et al. (9)</td>
</tr>
<tr>
<td>Lymphovascular invasion</td>
<td>73%</td>
<td>24%</td>
<td>Wang et al. (9)</td>
</tr>
<tr>
<td>Molecular subtype</td>
<td>Luminal (98%)</td>
<td>Luminal (53%); basal (47%)</td>
<td>Guo et al. (4)</td>
</tr>
<tr>
<td>mRNA signature</td>
<td>GATA 3, S100P, uroplakins, ERBB2, CD24, FOXA1, XBP1, thrombomodulin, MUC1, CA125</td>
<td>KRT20, GATA3, uroplakins, ERBB2, ERBB3; activated wild-type p53 gene, expression signature; CD44, CDH3, KRT5, KRT6, KRT14</td>
<td>Lopez-Beltran et al. (2); Guo et al. (4); Solomon et al. (11)</td>
</tr>
<tr>
<td>IHC profile</td>
<td>PPAR γ, GATA3 and uroplakin 2, CK7, CK 20, p63, HMWCK</td>
<td>PPAR γ, GATA3 and uroplakin 2; CK20, CK5/6, p63</td>
<td>Paner et al. (12).</td>
</tr>
<tr>
<td>Genetic profile (hierarchical cluster analysis: upregulated genes)</td>
<td>RBM38, MRPL4, ERF, C20orf96, NRR4A1, EFNB1, TRIM29, KRT5, LY6D, IRF2</td>
<td>KCNF1, TRPV6, IGDCC3, SLC30A2, PROM1, MUC1, GDPD3, ARRB1, CLDN3, MESP1</td>
<td>Guo et al. (4)</td>
</tr>
<tr>
<td>TERT gene</td>
<td>100%</td>
<td>66% muscle invasive; 74% non-muscle invasive</td>
<td>Nguyen et al. (13)</td>
</tr>
<tr>
<td>HER2 over-expression</td>
<td>56%</td>
<td>36%</td>
<td>Behzatoğlua et al. (14)</td>
</tr>
<tr>
<td>miRNA signature</td>
<td>Downregulation of miR-296</td>
<td>miR-31 and 64 miR-149 (tumour progression)</td>
<td>Guo et al. (4); Izquierdo et al. (15)</td>
</tr>
<tr>
<td>Therapy</td>
<td>Intravesical therapy is ineffective; p53 variant is more resistant to chemotherapy; early radical cystectomy is the treatment of choice for NMIUC and MIUC</td>
<td>Intravesical therapy with BCG for NMIC; neoadjuvant methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) or platinum based chemotherapy followed by cystectomy for MIUC</td>
<td>Knollman et al. (16); Guo et al. (4)</td>
</tr>
<tr>
<td>Median survival (months)</td>
<td>35.4</td>
<td>20.8</td>
<td>Guo et al. (4)</td>
</tr>
<tr>
<td>10-year CSS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unmatched</td>
<td>31%</td>
<td>53% (P=0.001)</td>
<td>Wang et al. (9)</td>
</tr>
<tr>
<td>Stage-matched</td>
<td>31%</td>
<td>40% (P=0.41)</td>
<td></td>
</tr>
</tbody>
</table>

UC, urothelial carcinoma; NMIUC, non-muscle invasive urothelial cancers; MIUC, muscle-invasive urothelial carcinoma; BCG, Bacillus Calmette-Guérin.
number changes (20). The luminal subtype is associated with better prognosis compared to the basal subtype; however a more aggressive p53-like subset exists within the luminal signature and shows overexpression of p53 (19). MPUC variant shows 98% luminal type molecular profile (4). This variant is consistently positive for expression of markers of terminal luminal differentiation such as GATA3 and uroplakin 2, as well as PPARγ (4).

Preliminary subtypes of UCs were first identified in TCGA study (19) in unsupervised clustering by non-negative matrix factorization of mutations and focal somatic copy number alterations (SCNAs) which identified three groups: group A, highly enriched in focal SCNAs in several genes, as well as mutations in MLL2. Group B, the “papillary CDKN2A-deficient FGFR3 mutant,” enriched in papillary histology with loss of CDKN2A, and 1 or more alterations in FGFR3 and group C, “p53/cell-cycle mutant,” which has p53 mutations in nearly all samples, and enrichment for Rb1 mutations, and amplifications of E2F3 and CCNE1 (20). Clusters I and II both express high HER2 levels and have an elevated estrogen receptor beta signaling signature, suggesting a relationship to HER2-positive breast cancers as well as those of luminal A breast cancer and has high expression of GATA3, FOXA1 and uroplakins. Cluster II differs from cluster I in the absence of papillary morphology or FGFR3 events. In contrast, cluster III (“basal/squamous-like”) is similar in some respects to both basal-like breast cancer which express high levels of keratins 5, 6 and 14 (19,21). These groups are clinically relevant with basal bladder cancers carrying the poorest prognosis and shortest disease-specific survival (19,20). Pathway analysis has led to identification of Stat-3, nuclear factor-κB, HIF-1, and p63 as probable transcriptional drivers of basal gene expression (19) and correspondingly, PPAR-γ and estrogen receptor as drivers of the luminal gene expression pattern.

Low-grade non-muscle invasive urothelial cancers (NMIUC) form approximately 70% of UC. They have a good survival, however they have a tendency to recur and hence require regular monitoring and follow-ups. On the other hand, high-grade muscle-invasive urothelial carcinoma (MIUC) progress rapidly to become metastatic and carry high mortality (19). In terms of invasive and non-invasive UC two divergent pathways of tumorigenesis in bladder cancer are either FGFR3 mutation based or carry p53 mutation. The key genes involved in the FGFR3 pathway are RAS, STAT1, PIK3 and Cyclin D1. These are associated with low grade lesions which carry a low risk of invasion, present at a lower stage have low risk of recurrence and overall carry a good prognosis. The p53 mutation pathway involves Rh gene, p21, hax, bcl2 and TSP1 and is seen in high grade UC and carries a high risk of invasion, tumors present at high stage, recur and progress early in the disease and carry an unfavorable prognosis. NMIUC tumors frequently exhibit FGFR3 and PIK3-kinase catalytic subunit A (PIK3CA) mutations, few chromosomal changes, and low mitotic rate and Ki67 activity. Low-grade non-invasive papillary carcinoma is often multifocal and tends to recur following resection, but rarely progresses to invasive disease. In contrast, micropapillary variant presents with muscle invasive disease in 95% cases (Table 1). Genetic pathways which form targets for therapy are also activated including the mitogen activated protein kinase (MAPK) pathway, phosphoinositide 3-kinase (PI3K) pathway, which affects downstream protein kinase B (AKT) and mammalian target of rapamycin (mTOR) pathways are activated in low grade UC. Upstream of the RAS protein is FGFR3, a tyrosine kinase receptor. FGFR3 or HRAS mutations are present in almost 82% of non-muscle invasive bladder cancer. The more aggressive muscle-invasive tumors carry p53 mutations, and show a high proliferative activity as well as signs of genomic instability. Rb1 deletions and low expression of CDKN2A (p16) forms a parallel pathway in p53 mutated cases. p53 and CDKN1A gene generate p21 protein, a cyclin-dependent kinase inhibitor. A molecular signature combining multiple genes including FGFR3, PIK3CA, KRAS, HRAS, NNRA, p53, Rb1, CDKN2A and TSC1 correlates well with histologic categories, and can accurately predict whether a tumor fits into noninvasive low-grade papillary or high-grade in situ and invasive groups (22).

The difference and similarities in genotype and phenotype of high grade muscle invasive MPUC and UC have recently been detailed in an interesting study based at the MD Anderson Institute (4). The study compares muscle invasive micropapillary UC, conventional UC and areas of both types of tumors from same patients in two independent publicly available cohorts of conventional UC and micropapillary UC. Two distinct clusters obtained in hierarchical clustering include: cluster A containing UC exclusively and cluster B with mostly micropapillary tumors. MPUC is enriched with expression signatures involved in multiple important oncogenic pathways converging on transformation (mechanisms of cancer, mechanisms of glioma/glioblastoma, RhoA, and p53), cell cycle regulation (cyclins, G1/S checkpoint), DNA damage repair (BRCA1), and signal transduction (ephrin signaling).
It is interesting to note that a micropapillary expression signature is also present in the conventional components of the tumors that contained foci of micropapillary carcinoma (4). TERT promoter mutations are present in MPUC, UC with micropapillary areas and conventional UC. Mutations have been identified at positions-124 (C228T) (85%) and -146 (C250T) (12%) upstream of the TERT ATG start site. Concordant mutations have been identified in heterogeneous tumors with MPC and non-MPC areas as well as corresponding conventional UC (13). In view of the similarity in gene signatures within heterogeneous tumors it appears that there is a common oncogenesis origin of UC and its variant histology in individual cases. HER2 protein overexpression or gene amplification has been shown in urothelial bladder cancer. This could be helpful when using targeted anti-HER2 therapy on these tumors. Fifty six percent of MPUC showed HER2 overexpression (3+ staining) while conventional UC show HER2 overexpression in 36% cases and 50% in in situ carcinoma. All low grade noninvasive tumors have been reported to be HER2 negative (14).

Studies of miRNAs in bladder cancer indicate that their specific species can be associated with bladder cancer behavior and chemosensitivity. Downregulation of miR-296 has been reported in many human cancers. It occurs in later phases of carcinogenesis and is associated with the progression to aggressive disease (23). A conclusive observation in the study by Guo et al. [2016] is the confirmed downregulation of miR-296 in MPUC and the over expression of RUVBL1 (4). MiRNA-296-5p modulation was been shown to be associated with altered viability of cell lines exposed to cisplatin. This explains the chemoresistance encountered in MPUC (24). Similarly, activation of RUVBL1 is associated with clinically aggressive disease (23). The RUVBL1 molecule belongs to the family of AAA+ adenine triphosphatases which are scaffolding proteins for chromatin-remodeling complexes and control diverse functions including DNA damage repair, proliferation, and invasion (23).

Outcomes of radical cystectomy for patients with MIUC are similar to those with UC when controlling for other clinical and pathologic factors (8). Conventional prognostic parameters include pathologic TNM stage, multifocality and presence of concurrent carcinoma in situ, lymphovascular invasion, histologic grade and adjuvant chemotherapy. Survival analysis using micropapillary gene expression signature with hierarchical clustering shows aggressive behavior is associated with micropapillary tumors as compared to conventional UC (Table 1). The so-called superficial micropapillary carcinoma, which is a high grade MPUC in stage T1, should be offered aggressive therapy instead of intravesical immunotherapy to improve long-term survival (25). The classical morphology and molecular events allow early detection of even a 10% surface micropapillary component and hence MPUC can be detected at an early stage. Prognosis is also related to the proportion and location of the micropapillary component, with higher risk in cases with extensive micropapillary component (7). The p53-like type forms a bad prognosis group with response rates of 45% as compared to 66% in the luminal group, but the difference is statistically insignificant. A small percent of cases with a micropapillary signature exist within a genomically unstable group that overlaps with the luminal and p53-like categories (4). Bacillus Calmette-Guérin (BCG) treatment does not appear to be effective in non-muscle invasive MPUC which progresses in 67% of patients despite intravesical therapy as compared to a progression rate of less than 5% in non-muscle invasive conventional UC (10). Radical cystectomy is hence recommended by some urologic oncologists for even superficial MPUC while others have supported neoadjuvant chemotherapy followed by early cystectomy (6). However concern has been raised related to a potential poor response to cisplatin based neoadjuvant chemotherapy in MPUC (12), a fact explained at MiRNA level by the upregulation of RUVBL1 (4). The so-called p53-ness in MPUC has also been associated with chemoresistance to cisplatin-based neoadjuvant chemotherapy (19).

In view of the key molecular pathways activated in MPUC and UC, potential therapeutic targets and drug interventions include HER2, epidermal growth factor receptor, fibroblast growth factor receptor, programmed cell death ligand 1 (PDL1) and programmed cell death protein 1 (PD1), vascular endothelial growth factor receptor (VEGFR) and vascular endothelial growth factor (VEGF). The receptor tyrosine kinase (RTK)/RAS pathway involved in cell cycle signalling is altered in 44% of tumours and tyrosine kinase inhibitors may form a treatment modality (1,22). Genes involved in regulating chromatin, the structure of DNA and proteins that makes up chromosomes, are frequently mutated and represent novel targets for bladder cancer (1,22). It seems hair splitting of UC into variants with specific molecular signatures will help define targets for therapy. It is an exciting time of translation from bench to bedside in cancer therapeutics and molecular pathologists have the potential to be the guiding hand in
determining optimal treatment regimen for patients.

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None.

**Footnote**

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

**References**


Protein catabolic pathways are of critical importance to cellular physiology. The breakdown of proteins and organelles serves not only as a form of protein and organelle quality control, but also to generate amino acids and free fatty acids for reuse by the cell. Macroautophagy (hereafter referred to as autophagy) is a major pathway of protein degradation in eukaryotic cells that has been preserved through evolution. There has been much interest in the role of autophagy and cancer because mutations in autophagy genes have been observed in human cancers, autophagic activity is altered in cancer cells, and suppression of autophagy could be utilized as a cancer therapy (1,2). A recent report by the DiPaola group (3) demonstrated for the first time that autophagy is essential for the development of prostate cancer driven by PTEN-loss in a mouse model.

**Autophagy: a core degradation pathway**

Autophagy or “self-eating” is the process, by which long lived cytoplasmic proteins, receptors and organelles are loaded into autophagosomes (double-membrane vesicles), which subsequently fuse with lysosomes for degradation of their cargo by lysosomal enzyme activity. The formation of the autophagosome is an incredibly complex process that has been reviewed in detail by others (4). In brief, formation of the autophagosome occurs in three steps: initiation, nucleation, and expansion/closure (Figure 1). In response to nutrient deprivation, a well-established trigger for autophagy, the Beclin-1 and ULK1 protein kinase complexes are activated and recruited to the endoplasmic reticulum (ER). The Beclin-1 complex is composed of VPS34, a type-III phosphatidylinositol-3 kinase (PI3K), Beclin-1, and ATG14L (4). The ULK complex phosphorylates Beclin-1 to promote translocation of the complex to the ER (5). Once activated, the Beclin-1 complex enriches localized sites on the ER membrane with phosphatidylinositol-3 phosphate (PI3P) via VPS34 activity. These PI3P-enriched domains are critical for nucleation of the isolation membrane or phagophore, a pre-autophagosome structure. The PI3P-enriched phagophore serves as a docking site for WIPI2 (6). The expansion of the autophagosome from the isolation membrane requires the ATG16L1 protein complex by promoting LC3 family protein lipidation and incorporation into the isolation membrane/autophagosome (7). The ATG16L1 complex is recruited to the isolation membrane by interaction with WIPI2, where it promotes LC3 family protein conjugation to ATG3, which promotes phosphatidylethanolamine (PE) lipidation of LC3 (7). The lipidated LC3 proteins are incorporated into the emerging isolation membrane/autophagosome to favor its expansion and closure (8,9). LC3 proteins are also critical for cargo specific loading of autophagosomes via interaction with p62- and NBR1-bound to targets (e.g., mitochondria or protein aggregates) destined for autophagic catabolism (10,11).

**Autophagy and cancer**

The relationship between autophagy and cancer is complex. It has been widely observed that deficiencies in core autophagy genes (i.e., genes essential to the autophagy conjugation system) lead to increases in cytoplasmic
Subsequently, oxidative DNA damage and DNA double strand breaks are observed in cells lacking core autophagy genes. However, the relationship between autophagy deficiency and cancer is not linear. Where monoallelic loss or down-regulation of core autophagy genes can promote the development of cancer, complete deletion of these same genes can inhibit cancer growth and progression (1,13). It was recently shown that homozygous deletion of ATG7, a regulator of LC3-I conjugation and ATG16L1 complex formation, suppresses melanoma growth by generating high levels of DNA damage and by activating senescence in cancer cells (14). Thus, it could be hypothesized that increased DNA damage as a result of a mild or intermediate defect in autophagy could promote tumorigenesis and drive cancer progression by increasing mutation frequency, whereas complete inhibition of autophagy disrupts organelle and protein quality control mechanisms and generates irreparable DNA damage to trigger senescence and suppress cancer. Direct evidence for the role of monoallelic loss of ATG5 in promoting melanoma progression in human cancer was recently demonstrated (1). This study showed that monoallelic loss of ATG5 in melanoma patient samples was associated with metastatic disease and predicted worsened overall patient survival. The authors also showed that ATG5 haploinsufficiency increased tumor burden and metastasis in a mouse melanoma model driven by PTEN-loss and activated BRAF and promoted resistance to BRAF chemical inhibitors. However, complete ATG5 knockout in this model of melanoma increased sensitivity to BRAF inhibitors and ameliorated tumor burden. Complete loss of ATG7 was also found to delay tumor onset in this same model of melanoma and in a BRAF-driven model of lung cancer (1,14,15). A recent study analyzing single nucleotide polymorphisms (SNP) from 458 patients with localized prostate cancer found that there was an association with biochemical recurrence (BCR) and ATG16L1 SNPs (16). Patients with at least one ATG16L1 rs78835907 A allele showed a 22% reduction in the risk of BCR with an associated increase in ATG16L1 gene expression. Overall, these studies suggest that upregulation of autophagy genes suppresses cancer progression, haploinsufficiency of core autophagy genes promotes cancer progression, and homozygous loss suppresses tumor growth and promotes susceptibility to targeted therapy (Figure 2).

**Role of autophagy in PTEN-null prostate cancer**

Santanam and colleagues found that loss of ATG7, a core autophagy gene, suppresses prostate tumorigenesis in a PTEN-loss driven mouse prostate cancer model (3). The authors used a novel transgenic mouse with tamoxifen-
inducible Cre under control of the Nkx3.1 promoter for inducible expression of Cre in prostate epithelial cells. Prostate specific knockout of PTEN and ATG7 was achieved by administration of tamoxifen to transgenic mice with floxed PTEN and ATG7 alleles. This mouse model of prostate cancer is a significant improvement upon the prior Probasin-Cre mouse because it circumvents the requirement for Cre to be inherited from the male and gives control over PTEN deletion via Cre induction with tamoxifen. As expected, mice with double knockout showed a deficiency in autophagy within their prostate tissue compared to mice harboring PTEN knockout alone as evidenced by increased cytoplasmic volume and accumulation of p62. Concurrent loss of prostastic ATG7 and PTEN resulted in decreased prostate epithelial cell proliferation and apoptotic cell death. Similar to mouse lung and skin cancer models, suppression of autophagy delayed prostate tumor growth and promoted an ER stress response (17). Lastly, the authors castrated control and ATG7 conditional knockout mice and evaluated prostate tumor growth. The ATG7 conditional knockout prostate tumors, also lacking PTEN, showed a greater decrease in prostate volume in response to castration. As the PTEN loss driven prostate cancer model is thought to be castrate-resistant (18,19) it may have been additionally informative to determine whether ATG7 deletion increased the apoptotic response to surgical castration, as these mice showed an increase in ER-stress signaling within their prostate tissue and surgical castration increases apoptosis in prostate cells in this model (18).

### Figure 2

Autophagy’s non-linear relationship to cancer. Heterozygous loss of core autophagy genes leads to a decrease in cell protein and organelle quality control mechanisms and, subsequently, increases DNA damage; complete disruption of autophagy by deletion of core autophagy genes represses cancer growth by activation of senescence pathways and can promote susceptibility to targeted therapies.

### Future directions

This study adds to our understanding of the role of autophagy in the PTEN-loss driven mouse prostate cancer model, although several questions still remain. It is unclear whether autophagy is changed in the prostate in mice with prostatic PTEN KO alone relative to benign prostate tissue. PTEN has previously been shown to activate autophagy, so it is possible that loss of PTEN in the prostate may down-regulate autophagy to drive cancer development (20). Further, it is not currently known whether heterozygous loss of ATG7 would promote prostate cancer progression similar to the effect of monoallelic loss of ATG5 in melanoma (1). As the authors suggested, it would be of interest to test whether suppression of autophagy augments response to chemotherapy in this model of prostate cancer. Our own group has found that suppression of autophagy by knockdown of C/EBPβ, a transcriptional regulator of multiple autophagy genes, suppresses the growth of PC-3 xenografts and increased their response to bortezomib (21). ATG7 knockout would be predicted to promote prostate cancer apoptosis in response to docetaxel, as chemical inhibition of autophagy was found to increase docetaxel sensitivity in prostate tumor xenografts (22). Others have shown that blocking autophagy with lysosomotropic agents augments killing of prostate cancer cells by AKT inhibitors (23). Another intriguing possibility is whether the loss of ATG7 increases senescence in PTEN-loss driven prostate cancer as was observed in a mouse model of melanoma (14). Several groups including our own have observed that androgen-deprivation promotes senescence in androgen sensitive prostate cancer cells (24,25). Because senescence occurs in response to loss of PTEN or loss of core autophagy genes, it is possible that ATG7 KO increases the senescence response to PTEN loss and surgical castration as an underlying mechanism for increased sensitivity to androgen deprivation in these mice (26). In conclusion, autophagy plays a critical role in the progression of PTEN-loss driven cancers. Small molecules targeting the autophagy pathway could serve as an adjunct to chemotherapy or androgen deprivation in the treatment of prostate cancer. Future studies aimed at determining the role of autophagy in prostate cancer progression and metastasis are warranted.
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Prostate cancer is the second most commonly diagnosed malignancy in men; and in spite of recent therapeutic advances, it remains the fifth leading cause of male cancer deaths worldwide (1,2). Aside from prostatectomy and radiation, used with curative intent for the treatment of localized prostate cancer, approved life-prolonging treatment options for metastasized prostate cancer are limited to androgen receptor signaling inhibitors, microtubule targeting taxane chemotherapeutics, the bone targeted radioisotope Ra223, and active immunotherapy with sipuleucel-T (2). Hence, to improve prostate cancer care it is essential to identify novel treatment targets (3).

Comprehensive genetic analyses have revealed a number of actionable molecular aberrations, including alterations of the PI3K-AKT and WNT signaling pathways, and DNA repair defects (4-6).

Although alterations of metabolic pathways have been recognized as essential aspects of cancer progression and metastasis, there is a need to better understand the molecular mechanisms behind how the metabolic landscape of cancer cells changes in coordinated manner to support tumor growth (7). In a detailed and elegant study Torrano et al. examined the peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC1α, also PPARGC1A) transcriptional network and its role in suppressing prostate cancer metastasis (8). Initially, a bioinformatics analysis was completed to identify regulators of prostate cancer metabolism responsible for disease progression. Amongst three metabolic co-regulators identified in most or all data sets studied, covering different stages of prostate cancer (i.e., PGC1α, PGC1β, HDAC1), the expression of PGC1α was the only one found to be associated with the Gleason scoring system and disease-free survival. The expression pattern of PGC1α was characteristic of a tumor suppressor.

Next, PGC1α was chosen for further analyses. While PGC1α deletion did not promote prostate cancer initiation, it was shown to be responsible for impairing prostate cancer growth and metastasis. PGC1α has been identified as a promoter of inflammation, angiogenesis and the production of reactive oxygen species; neither of these processes, however, contributed to the PGC1α-mediated anti-prostate cancer effects described by Torrano et al. In gene expression profiling and metabolomics analyses, PGC1α was found to significantly alter gene expression and metabolite levels with respect to mitochondrial catabolic programs and oxidative processes such as fatty-acid β-oxidation. Altogether, the findings suggest that in prostate cancer PGC1α may serve as a metabolic regulator balancing a catabolic, tumor suppressive state (high PGC1α expression) versus an anabolic, tumor promoting state (low PGC1α expression) (Figure 1).

Finally, Torrano et al. used promoter and gene set enrichment analyses to identify estrogen-related receptor alpha (ERRα or ESRRA) as a major transcription factor mediating the tumor suppressive activities of PGC1α. Furthermore, using two independent patient gene expression data sets they demonstrated that the PGC1α/ERRα transcriptional program is positively associated with...
time to prostate cancer recurrence.

ERRα and two additional ERR isoforms (i.e., ERRβ and ERRγ) belong to a subfamily of constitutively active, orphan nuclear receptors that share high homology with estrogen receptors, have been implicated in metabolic regulation, are involved in prostate and breast cancer biology, and often exert opposing biological effects (9). A number of natural phytoestrogens have ERR-activating properties and could serve as lead compounds for the development of potent and specific ERR agonists with potential anti-prostate cancer properties (9). Yet, are the findings by Torrano et al. compelling enough to consider PGC1α/ERRα activation as a novel treatment strategy for prostate cancer? Fittingly, the authors are cautious with the interpretation of their results.

The importance of metabolic rewiring for malignant growth in general, and the role of the PGC1 co-activator and ERR transcription factor families for oncogenic metabolic reprogramming in particular, are widely recognized (7,10). However, the consequences of PGC1/ERR signaling are highly context-dependent. Torrano et al. describe prostate cancer suppressive PGC1α/ERRα activities. Conversely, the expression of PGC1α/ERRα has been associated with tumor promoting properties in prostate and other cancers by others. As an example, Fradet et al. showed that ERRα promotes prostate cancer progression in bone (11). Although the opposing conclusions of the Torrano and Fradet studies are not easily reconciled, one notes that the anti-metastatic consequences of inducing PGC1α expression in PC-3 prostate cancer cells in the study by Torrano et al. are more pronounced regarding visceral (i.e., lung) than bone metastases (8). Could it be that the role of PGC1α/ERRα signaling is not only tumor type dependent, but also dependent on the organ site of metastasis?

Although a PGC1α/ERRα activation strategy may be particularly promising in tumors with low PGC1α and/ or ERRα activity, it is currently not known how frequently such a constellation would exist in prostate cancer. Copy number alterations or mutations of PGC1α and ERRα are rarely found in prostate adenocarcinomas, and therefore are unlikely genetic driver aberrations (Table 1) (4,5). Indeed, PGC1α deletion alone was not found to initiate prostate carcinogenesis in the study by Torrano et al. (8). On the other hand, the PGC1α transcriptional network is under the control of MYC, a master regulator of cancer cell metabolism (12). MYC and PGC1α expression are inversely related to each other (Figure 1) (13,14). Intriguingly, MYC is amplified in more than 50% of neuroendocrine prostate cancers (Table 1), which are characterized by aggressive clinical behavior, are rarely diagnosed de novo, but are increasingly recognized as a prostate cancer phenotype in patients with inherent or acquired resistance to androgen receptor signaling inhibitors (6). Furthermore, the PC-3 prostate cancer cell line, prominently used in the study by Torrano et al., harbors neuroendocrine features. Thus, MYC amplification may contribute to a PGC1α low state, potentially amenable to a PGC1α/ERRα activation strategy, and may serve as a predictive marker for such a treatment approach.

In summary, the findings by Torrano et al. identify the PGC1α/ERRα transcriptional network as one of only few well-defined molecular mechanisms of prostate cancer metastasis. Accounting for the opposing biological functions attributed to PGC1α and ERRα in different tumor models, the study by Torrano et al. serves as an invaluable starting point to obtain a more detailed picture of the complex interplay between tumor cell metabolism and prostate cancer metastasis. Only time will tell if PGC1α/ERRα modulation will become a strategy to treat prostate cancer. The latter may apply especially to neuroendocrine prostate cancer.
cancers, an area of increasing therapeutic need, due to the widespread use of second-generation androgen receptor signaling inhibitors.

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**References**


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In their recent report published in *Nature*, Li and colleagues investigate the feasibility of exploiting drug metabolism pathways to improve abiraterone treatment for castration resistant prostate cancer (CRPC). Abiraterone is a 17α-hydroxylase/17,20-lyase (CYP17A1) inhibitor, and is used in the context of androgen deprivation therapy to prevent the de novo generation of androgens by the tumor from cholesterol or adrenal precursor molecules. While abiraterone initially blocks androgen synthesis and prolongs survival, the disease will ultimately progress despite treatment (1,2).

Dr. Sharifi’s group has previously demonstrated that one of the initial metabolites of abiraterone—Δ4-abiraterone (D4A)—shows improved anti-tumor efficacy with respect to inhibiting androgen synthesis, as well as direct androgen receptor (AR) inhibition compared to the parental compound itself (3). The focus of their current report was to examine other downstream metabolites and determine whether they provide a positive or negative role in the context of disease progression. Additionally, the authors explored the approach of “fine-tuning” abiraterone metabolism in an effort to select for metabolites of interest, specifically D4A.

D4A is generated by metabolism of abiraterone by 3β-hydroxysteroid dehydrogenase (3βHSD) and, based upon its structure, is predicted to be a substrate for steroidal 5α-reductase (SRD5A). Using LC-MS/MS Li et al. confirmed when LAPC5, C4-2 and VCaP cell lines were treated with abiraterone or its metabolite D4A, that D4A was first irreversibly reduced to 3-keto-5α-abiraterone (5α-abi) or 3-keto-5β-abiraterone (5β-abi). These in turn are further metabolized by 3βHSD into their respective 3α-OH and 3β-OH congeners (4). Indeed, the authors were able to detect all six 5α- and 5β-reduced metabolites in the serum from 12 patients undergoing abiraterone treatment. 5α-reduction of steroids preserves the planar structure of biologically active androgens, while 5β-reduction renders them inactive and promotes their clearance (4-6). Therefore the authors focused primarily on characterizing the three 5α-reduced metabolites of D4A.

While D4A was able to inhibit the activities of CYP17A1, 3βHSD and SRD5A (as indicated by HPLC analysis of enzyme substrates), its 5α-reduced metabolites demonstrated a significant reduction in activity. Interestingly, while the affinity of 5α-Abi to the AR<sup>TRT77A</sup> and AR<sup>WT</sup> was comparable to D4A, the former acted as an agonist rather than exhibiting the antagonistic properties of the latter. This was confirmed by measuring mRNA transcript levels of the AR regulated gene PSA in both LNCaP (AR<sup>TRT77A</sup>) and LAPC4 (AR<sup>WT</sup>) cells. Additionally, 5α-Abi treatment significantly shortened progression-free survival of castrated hosts compared to controls bearing VCaP (AR<sup>WT</sup>) xenografts. In light of these data, the authors postulated that resistance to abiraterone could occur through selective metabolism of its more potent metabolite D4A by SRD5A to the 5α-reduced metabolites—at least...
one of which demonstrated statistically significant tumor promoting activity. To test this hypothesis, abiraterone- and D4A-resistant cell lines were generated by chronically treating VCaP and LNCaP cells with respective compounds over the course of 6 months. It was shown that the resistant cell lines displayed increased SRD5A1 mRNA, protein levels, and a compensatory increase in consumption of SRD5A1 substrates compared to a control cell line. In agreement with this hypothesis, enzalutamide-resistant VCaP and LAPC4 lines did not show any difference in SRD5A1 protein or mRNA levels over the course of their generation.

Finally, the authors asked whether the levels of the D4A metabolite could be specifically increased by co-treatment with abiraterone and the dual SRD5A inhibitor dutasteride. An ongoing phase II clinical trial (NCT01393730) exploring dutasteride treatment following abiraterone allowed the investigators to directly measure the effect SRD5A inhibition had on abiraterone metabolism. As predicted, serum D4A levels increased significantly (9.96 to 18.20 nM; P=0.002) and 5α-Abi levels decreased (25.80 to 2.94 nM; P<0.001) following the addition of dutasteride to the treatment regimen. Unfortunately, no levels of any biomarkers of AR transcriptional activity (such as PSA) were reported in these patients. Thus the implied benefit of increasing circulating D4A lacks definitive clinical evidence.

The authors concluded that sustained AR signaling through the persistent synthesis of androgens can contribute to both CRPC and abiraterone resistance via upregulation of genes involved with steroidogenesis. Specifically, this may occur through increased SRD5A activity; modulating not only dihydrotestosterone (DHT) synthesis, but also the metabolism of the potent abiraterone metabolite D4A into one with tumor-promoting attributes. Exploiting drug metabolism may prove a powerful therapeutic tool in selectively preventing unwanted metabolites from being created, while retaining only the most potent ones.

This report is the first to examine and describe six previously unknown D4A metabolites and their effect on both androgen metabolism and tumor progression. The authors of this study are also commended on their approach of using this knowledge to specifically fine-tune abiraterone metabolism; and its potential impact on advancing CRPC therapy is recognized. While undeniably valuable as a proof-of-concept study, there is however some concern that the emphasis on D4A and 5α-Abi may be overstated. The concentration ranges of D4A (up to 10 µM) which were used to complete the HPLC experiments were significantly higher than what was reported in patient’s serum (~8 nM), bringing into question the clinical relevance of some of these experiments. Specifically, while D4A is indubitably as potent as abiraterone in the context of inhibiting CYP17A1 activity in the low nM range, inhibition of 3βHSD and SRD5A1 required doses of 1 and 10 µM D4A respectively.

Similarly, the 5α-metabolites (5α-Abi and 3α-OH-5α-Abi) that the authors focused on when examining PSA and TMPRSS2 mRNA transcription levels, also employed concentrations in large excess (~3 orders of magnitude) of that measured in the clinical samples (~40 and ~6 nM respectively). Additionally, even at concentrations that were up to 2000x that of 0.5 nM DHT, the potency of 5α-Abi and 3α-OH-5α-Abi on AR transcriptional activity was only fractional to that achieved with DHT. This begs to question whether metabolism of D4A to 5α-Abi is really a major contributor to abiraterone resistance compared to elimination of abiraterone (I.E. through 5β-reduction). Clearance of abiraterone and its non-functional metabolites would allow the restoration of synthesis of androgens from precursor steroids, and may play a more relevant role in clinical abiraterone resistance. This interpretation is supported by the fact that the patient data presented in this report show 5β-Abi and its 3α-OH and 3β-OH congeners are the more prevalent metabolites following abiraterone and D4A metabolism.

The importance of understanding abiraterone metabolism should not be understated, and the data presented here provide a solid foundation for examining this phenomenon. Li et al. describe the generation of novel metabolites and offer a validated proof-of-concept for driving selective metabolite production in the clinical setting. Further study is warranted to better combat abiraterone resistance through studying mechanisms of its metabolism. This work will hopefully shed light not only on the functionality of specific metabolites, but also on preventing their inactivation and clearance.

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Fat talks: a new role for adipose tissue in promoting prostate cancer in obesity

Matthew J. Watt, Renea A. Taylor

Department of Physiology, Metabolic Disease and Obesity Program, and Cancer Program of the Biomedical Discovery Institute, Monash University, Clayton, Victoria, Australia

Correspondence to: Matthew J. Watt, PhD; Renea A. Taylor, PhD. Department of Physiology, Monash University, Clayton, Victoria, 3800, Australia. Email: matthew.watt@monash.edu; renea.taylor@monash.edu.

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Why is it that obese men have more aggressive prostate cancer and die faster from their cancer compared with lean men? This question has remained largely unanswered, but a recent study by Laurent and colleagues (1) has provided one possible explanation: chemokine signals from prostate-associated fat cells directly communicate with cancer cells to promote local dissemination.

Obesity is a major health issue with approximately two in every three men in Westernized countries now classified as overweight or obese and obesity prevalence is increasing in all global regions (2). Obesity is a major risk factor for life-threatening diseases including cardiovascular disease, type 2 diabetes and many cancers, accounting for approximately one-third of cancer related deaths in 2012 (3). With respect to prostate cancer, obesity is not associated with evidence of increased incidence of disease but, importantly, is a significant risk factor for more aggressive prostate cancer with increased diagnosis of advanced, high-grade prostate cancer, increased biochemical recurrence after primary treatment, and increased prostate cancer-specific mortality in obese compared with lean men (4,5).

Intervention studies in mice have generally supported the view that obesity increases the risk of more aggressive disease. Feeding mice a high-fat diet recapitulates many of the metabolic and endocrine abnormalities of human obesity such as insulin resistance and hyperinsulinemia, dyslipidemia and low-grade inflammation. When mice are fed such high-fat diets for prolonged periods, there is clear evidence of increased tumour mass of xenografted human prostate cancer cells (6). Likewise, obesity accelerates progression in transgenic mice that are predisposed to prostate cancer (6).

Despite the well-documented association between these diseases, the biological mechanisms linking obesity and aggressive prostate cancer remain unresolved. Based on the understanding of tumorigenesis in other solid cancers (7), and a limited number of mechanistic murine studies (6), it has been proposed that both systemic and/or adipose tissue-derived factors promote prostate cancer progression. Systemic promoters are produced as a consequence of obesity co-morbidities and include altered circulating lipids resulting from dyslipidemia, insulin resistance and mild hyperinsulinemia resulting from the development of pre-diabetes, and alterations in several endocrine cascades including the growth hormone/insulin-like growth factor-1 axis, renin-angiotensin system and steroid hormones (Figure 1). Dramatic changes in adipose tissue morphology and function occur in obesity (8), and altered lipid metabolism, secretion of adipose tissue derived proteins called “adipokines”, and the development of subclinical inflammation secondary to immune cell infiltration into adipose tissue have been postulated to drive cancer pathogenesis.

The prostate is covered anteriorly by the periprostatic adipose tissue (PPAT), which is prominently positioned to
participate in bidirectional paracrine communication with prostatic cells (Figure 1). In this way, the aforementioned adipose-derived factors are postulated to perfuse the prostate gland to impact the tumour microenvironment and promote tumour growth, local invasion such as extracapsular extension into the PPAT, and possibly distant metastases. The evidence that PPAT secretes pro-tumourigenic factors is underscored by studies in which cell culture medium enriched with PPAT secretions increased tumourigenesis in prostate cancer cells (9). Moreover, the PPAT secretions from obese men were more pathogenic to cultured cells than secretions from lean men (10), providing a plausible link between obesity and aggressive prostate cancer. In this regard, prospective diagnostic studies show that increasing thickness of the PPAT is associated with high-risk disease (11). Molecules implicated in driving this association are limited to IL-6 (12), matrix metalloproteinases 2 and 9 (9) and the fatty acid composition within adipocytes (10), although notably, none of these factors have been shown to be as causative.

In the most notable recent advance in the field, Laurant et al. (1) have employed an array of eloquent experiments to unravel a previously unidentified chemokine axis controlling prostate cancer migration. The focus on chemokine signaling was well justified based on the documented role of chemokines and their receptors in prostate cancer and other cancers (13), their known production and secretion from adipose tissue, which increases in obesity (14,15), and their ability to induce chemotaxis and cell migration. The authors identified the chemokine, C-X-C motif chemokine ligand 7 (CCL7), as a factor secreted from immortalized murine adipocytes that promoted the migration of prostate cancer cells in a manner dependent on the receptor CC chemokine receptor 3 (CCR3), thereby establishing the CCL7/CCR3 axis. CCL7 was shown to be secreted from adipocytes and not immune cells located within adipose tissue, and further studies showed that CCL7 was secreted by human PPAT, demonstrating relevance to human biology.

The authors next procured human PPAT and prostate tissue using serial punch biopsies along a continuous gradient and demonstrated progressive decreases in CCL7 expression from PPAT. This established the potential for PPAT secretion of CCL7 through the prostate capsule to signal to CCR3 expressing cancer cells, which are generally present in the peripheral zone of the prostate. The authors demonstrated potential relevance of the CCL7/CCR3 axis for prostate cancer severity by showing in two prospective cohorts that CCR3 is expressed in prostate cancer and that its expression was positively correlated with the occurrence of aggressive prostate cancer, including Gleason grade, T stage, lymphatic

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**Figure 1** Factors secreted by distant and local adipose tissue influence prostate cancer progression. Left: schematic depicting the secretion of endocrine factors from visceral and subcutaneous adipose tissue depots that are postulated to increase tumourigenesis; center: the prominent periprostatic adipose tissue (PPAT) and possibly adipose tissue located near the peripheral zone secretes proinflammatory cytokines/chemokines (CCL7/CCR3 axis), proteins and lipids to influence cancer aggressiveness and potentially promote extracapsular extension; right: haematoxylin and eosin stained human prostate tissue showing adipose tissue adjacent to prostate cancer, separated by a fibromuscular pseudocapsule; adipocytes are rarely found in intra-prostatic tissue. Scale bar =200 µm.
emboli, surgical failure and biochemical recurrence.

In a final proof-of-concept study, the investigators used short hairpin RNA technology to partially knockdown CCR3 in immortalized murine prostate cancer cells (TRAMP-C2). In contrast to the parental cells, CCR3 knockdown cells were refractory to migration upon the addition of adipose tissue secreted factors to the cell culture medium. When these same cells were transplanted into mice, the tumour mass was reduced in the CCR3 knockdown cells compared with the parental TRAMP-C2 cells. Interestingly, the parental tumor cells growing adjacent to adipose tissue induced a reactive stromal phenotype where adipocytes disappear, fibroblast-like cells accumulate and a desmoplastic stroma ensues, indicating that bi-directional ‘cross-talk’ alters the adipose tissue phenotype and promotes the tumor’s proliferative and invasive capacities.

The authors then provided the link between obesity and cancer aggressiveness by showing that CCL7 secretion was upregulated in obesity, that tumour growth of CCR3 deficient prostate cancer cells was completely attenuated in obese mice and that extraprostatic extension and local dissemination were increased in obese patients. Extraprostatic extension to PPAT is clinically significant as these so-called ‘cancer-associated adipocytes’ exhibit more aggressive behaviour characterized by increased proliferative and invasive capacities and, at a clinical level, this switch from a prostate-confined tumour to a locally disseminated cancer is also viewed as a crucial step in the progression of the disease (16).

The current study unravels a new pathway with therapeutic potential. Whilst the authors suggest that blocking CCR3 offers a new strategy to treat advanced prostate cancer, the chemokine-induced migration leading to extracapsular extension, and possibly metastasis, would have already occurred at this stage of disease progression. Hence, a more rational strategy might be to antagonize CCR3 in a preventative setting, when the disease is organ-confined. In addition, while the authors speculate that this treatment would be efficacious in obesity, it is likely to benefit all men in whom the CCL7/CCR3 axis and interactions with PPAT are present. CCR3 antagonists are currently being developed for other diseases, such as asthma, but their application to prostate cancer would be completely novel.

This work also raises broader issues with respect to understanding the anatomical location of adipose tissues and possible paracrine/endocrine communication with the prostate gland. An extensive pathological examination of prostatic specimens showed that unlike breast, the presence of intra-prostatic adipocytes is extremely rare (17) (Figure 1). The most prominent adipose tissue mass is the PPAT, which is located at the anterior surface of the prostate covering the central zone. There are also small depots of adipose tissue that lie along the periphery of the posterior surface of the prostate, in close proximity to the capsule of the peripheral zone where tumours are most commonly located. Laurent and colleagues suggest that factors from the PPAT can diffuse through the prostatic stroma to reach cancer cells (1); however, the evidence demonstrating direct portal circulation or even perfusion from the PPAT to prostate remains unclear. Others have suggested that adipocytes themselves may enter the tumor microenvironment to occupy the peritumoral space through the local vasculature or systemic circulation (18), but the chemo-physical properties of adipocytes makes transvascular transport highly unlikely and this hypothesis is devoid of direct experimental support. These are critical points that require clarification in order to interpret the current findings, but also in elucidating the broader relationship between local adipocytes, PPAT and prostate cancer cells.

In closing, the conceptual advance of this work is the demonstration that the CCL7/CCR3 axis links adiposity to cancer cell migration and predicts aggressive prostate cancer. While this provides important information on the biology of prostate cancer progression and a putative therapeutic target, we need to be cognisant that obesity is a complex disease and that a variety of factors are likely to impact on cancer pathogenesis, including both local and systemic influences (Figure 1). Future studies aimed at deciphering the complex mixture of factors derived from PPAT and their role in directing tumour progression will be required in formulating a broad spectrum of treatment modalities.

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References


Although the majority of prostate cancer (PCa) cases are now detected and treated at early stages, PCa remains the second leading cause of cancer death in American men (1). In the case of more advanced tumors, androgen deprivation can prevent cancer progression temporarily, but this inevitably leads to castration-resistant prostate cancer (CRPC). In recent years, several targeted therapies have entered the clinic that inhibit the androgen receptor (AR), a key driver of CRPC, but tumors often gain secondary resistance to these therapies (2). Additional studies that explore novel mechanisms of PCa progression could contribute to development of more effective therapies. In a paper published this year in Nature Communications, Laurent et al. identified a novel chemokine signaling pathway in PCa that links adipocyte-secreted CCL7 to PCa cell migration (3), suggesting that this signaling pathway is a potential therapeutic target.

Besides the predominant role of AR in PCa, other signaling pathways are also important for PCa progression, including chemokines such as CXCL12 (4). PCa cells express the chemokine receptor CXCR4 which is activated by CXCL12, a chemokine expressed by bone stromal cells (5). As a result, CXCR4-positive PCa cells frequently metastasize to the bone (6). Similarly, adipose tissue is recognized as an endocrine organ that can secrete a variety of factors (adipokines) and influence cell behavior (7). Not surprisingly a correlation exists between obesity and PCa aggressiveness (8-10), suggesting that secreted adipokines may influence PCa cell behavior.

Of particular interest to Laurent and colleagues was the interplay between periprostatic adipose tissue (PPAT) and PCa tumor cells, and how obesity affects this interplay. The authors first demonstrated that condition media from adipocytes can direct the migration of DU-145 and PC-3 PCa cell lines. The authors further identified that this migration is due to chemokine signaling between chemokine receptor CCR3 and ligand CCL7 secreted by mature adipocytes. Importantly, they found that CCR3 was expressed in these PCa cell lines as well as in patient tumors, and that their expression levels correlated with cancer aggressiveness. Perhaps most exciting, the authors demonstrated that the CCR3/CCL7 axis-mediated migration can be blocked by a CCR3 inhibitor or monoclonal antibodies for CCR3 and CCL7 (Figure 1).

Because obesity correlates with increased CCR3 expression that may reflect increased secretion of CCL7, Laurent et al. also assessed the effect of obesity on tumors in a murine orthotopic graft model of PCa. In this system, obese conditions led to an increase in tumor size that was dependent on CCR3 expression. Further investigation on metastases and animal survival could yield important insights into how the CCR3/CCL7 axis might affect PCa cell migration in vivo.

In their recent publication, Laurent et al. characterized a novel chemokine signaling pathway in PCa which links signaling molecules secreted by PPAT with tumor progression. The authors’ findings bring to light a few key questions about PCa progression and interplay between different components of the tumor microenvironment. Gaining a better understanding of the mechanism of increased CCR3 expression in PCa cells, the downstream
targets of active CCR3 that trigger cell migration, and the long-term effects of obesity on CCR3/CCL7-mediated cell migration are essential. In addition, further studies to uncover relationships between CCR3/CCL7 signaling in PCa and other cancer-associated signaling pathways could reveal a complex, well-orchestrated network. One prediction could be that CCR3/CCL7 signaling induces PCa cells to disseminate from the primary tumor into adjacent tissue, while another chemokine signaling pathway like CXCR4/CXCL12 may encourage already freely circulating tumor cells to metastasize to distant secondary sites. Therefore, the findings by Laurent et al. have paved the way for these future studies, as well as the development of therapeutics that may have the potential to block tumor cell migration at a very early stage.

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Footnote

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References

have more advanced and more aggressive prostate cancer at time of surgery than non-obese men after adjusting for screening PSA level and age: results from two independent nested case-control studies. Prostate Cancer Prostatic Dis 2013;16:352-6.

The term Exosome has come into use to define nanovesicles contained in multivesicular endosomes (MVE), secreted by fusion of MVE with the plasma membrane (1-3). Exosomes are secreted in vivo by almost any cell type and can be isolated from body fluids (4-6). Indeed, circulating vesicles account for both exosomes and microvesicles (MVs), which can be purified by various purification methods and fully discriminated according to their shape, size and CD-markers (7,8). Due to their protein and RNA content, once internalized, exosomes have the potential to act as “translational” organelles, altering the expression pattern of recipient cells, their growth, and fate. In fact, exosomes are involved in many of physio-pathological processes, thereby including cancer (9).

The work recently published by Franzen et al. in The Journal of Urology (10) examined the role of exosomes in intercellular communication and their potential as noninvasive cancer biomarker source to assess disease and its progression, or vehicles for therapeutic delivery in urologic cancer (11). The need for a predictor of malignancy is universally recognized. This is particularly true in the case of early tumors. There is evidence that cancer-derived exosomes contribute to tumor progression and metastasis (12). Tumor cells produce exosomes, emerging as a potential for the early detection or therapy of human cancer (13).

Human urinary exosomal proteome has been extensively studied (7). Urinary exosomes come from every cell type of the urinary tract, kidney and prostate (14-16). There is increasing interest in urinary exosomes, due to their ability to carry information specific of the tissue of origin. I am positive about the idea that exosomes, which can be easily isolated from human urine by minimally invasive techniques, can allow to detect biomarkers in patients with urogenital cancer, with a wealth of applications in therapy and diagnosis, as we have already observed (17). Bladder cancer is one example, as it represents a serious health problem, (about 8% of all human malignancies), still burdened by a high percentage of relapse (18).

However, I am sceptic to the idea that exosomes can be used in therapeutics as RNA or therapy delivery vector to target cancer cells. Surely, RNA would be protected by the membrane from degradation. Nevertheless, it seems that we still know too little about the surface proteins of exosomes. We have shown that among the exosomal surface proteins there are the respiratory chains and F1Fo-ATP synthase, conducting an oxidative phosphorylation (19,20). Before seeing the contents of a package, its envelope must be opened. Similarly, before the RNA content of an exosome complex is shed, and it can affect the cell expression pattern, its surface proteins will have interacted with the cytosol. We have reported the proteome analysis of urinary exosomes, studied by Orbitrap mass spectrometry, compared to urinoma (20). Cytoscape software analysis of the data elucidated the enriched presence in urinary exosomes of proteins clustered to aerobic metabolism. Moreover, functional experiments showed that urinary exosomes carry out oxidative phosphorylation. The same applies to exosomes derived from human umbilical cord mesenchymal cells (MSC), which are able to conduct an aerobic metabolism (19). Such emerging metabolic function...
for both human MSC and urinary exosomes should not be ignored. It appears consistent with the report that exosomes can transfer the aerobic metabolism capacity to profoundly hypo-metabolic cells in less than one hour (21). This dramatic effect overturned the fate of doomed cells long before any transcriptional event can have occurred. In case of tumor cells, this may not be desirable. For example it was found that various concentrations of exosomes purified from the supernatants of human bladder cancer T24 cell cultures induce the proliferation and decrease the rates of apoptosis (22). Having the observations been limited to late events, posing ATP availability as a prerequisite (viability assay of at 72 hr, Annexin V and transcription factors activity), an involvement of early metabolic effect cannot be excluded. In fact, it was also found that bladder cancer exosomes can cause urothelial cells to undergo epithelial-mesenchymal transition (EMT). Authors treated primary urothelial cells with the exosomes isolated mRNA and evaluated the expression of several mesenchymal genes necessarily after a 4- and 6-h time to reveal newly transcribed mRNAs (23). While it is known that the EMT induces invasive properties in epithelial tumors, very little is known about EMT-induced metabolic changes. A study on HER2-positive BT-474 breast cancer cells showed enhanced aerobic metabolism, along with the overexpression of specific glucose transporters (24).

A method to be used clinically should be simple, cost-effective and minimally invasive, which exosome collection from urine appears to be. In conclusion, I am optimistic that further studies about the potential for exosomes will help in diagnosis, treatment and prognosis assessment of urinary tract cancer. However, several unanswered questions regarding the metabolic potential of exosomes remain. In this context, improved knowledge about the metabolism of exosomes are needed.

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The first publication on exosomes dates back to the 1980's. Exosomes are membrane-derived vesicles that are specified by particle size (30–100 nm in diameter), density (1.13–1.19 g/mL), and surface markers. Once referred to as a “rubbish bag” to wrap up and dump out waste, the term “exosome” gained newfound meaning following the discovery of its biogenesis mechanism via multivesicular bodies and the joint effort of Nobel Prize winners, Südhof, Schekman and Rothman, in discovering the machinery of vesicle transport. For the past decade, the number of exosome-related publications upsurged from fewer than 20 in the year 2001 to more than 1,000 in the year 2015. The nomenclature pertaining to “exosomes” was ambiguously used in literature, that is, “extracellular vesicles (EVs)” and “exosomes” were often used interchangeably or imprecisely. The criteria for exosomes have been refined over the decades to distinguish exosomes from other EVs (e.g., ectosomes, microvesicles and apoptotic bodies, see Table 1) by expression of distinct molecular markers such as TSG101, Aliex and CD63, and nanometer size in exosomes. However, the surface markers are overlapped in EV subgroups, making it fairly difficult to set a clear boundary. In this article, we will use “exosomes”, unless a specific subpopulation of EV is mentioned.

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Safe and luxury journey of miRNA intercellularly

Most cells secrete exosomes, which act as an ‘intercellular postal service’ as the exosomes facilitate intercellular exchange of molecular information in the forms of protein, DNA and assorted RNA molecules. With the advantage of bilayer membrane, small fragments of RNA such as pre-miRNAs in the exosome are protected from ubiquitous RNase and undergo maturation by exosomal Dicer, Ago2 and TRBP (1). This renders the exosome a perfect shuttle of miRNA. Compared to healthy cells, cancer cells secrete a greater amount of exosomes that aid tumorigenesis. Locally, cancer exosomes not only advance cancer cells’ malignancy by promoting cell proliferation, migration, invasion and angiogenesis, but also cancerize the surrounding non-cancerous tissues. Distantly, cancer exosomes prepare an ideal metastatic site on preferential organs through circulation (2).

Obstacles of urinary exosome study

Among all body fluids, urinary exosomes provide a unique opportunity for studying urological diseases. In the May 2016 issue of the Journal of Urology, Drs. Gupta et al. published a review article on the subject of urinary exosomes for their roles in urological cancer malignancy (3). In this review, they summarize the bioactive oncoproteins and oncomiRNAs identified from urinary exosomes and derived from genitourinary cancers. The reported oncogenic properties of urinary exosomes include the promotion of cell migration and angiogenesis, aversion of apoptosis and impairing immune cells, and even facilitation of treatment resistance. Because of the huge volume and concentration variations of void urine among each individual, it is not easy to study urinary exosome on a fair
Identification of validated “internal” control for urine normalization is urgently needed for developing a reliable urine based bioassay. Total protein concentration, urinary creatinine levels, or particle numbers have been suggested for valuing the quantity of exosomes despite the subpopulation of exosomes that carrying different markers (4). In particular, the noticeable effect of polymeric Tamm-Horsfall protein—also known as uromodulin, and one of the most abundant proteins in urine—often diminishes the procedure’s reproducibility (5). In addition, longitudinal patients’ sample collection and verification of differences in exosome contents derived from urine as opposed to blood of the same patients will be critical information for developing the exosome-based biomarkers for monitoring tumor evolution, dynamics, and therapy response in clinical application.

**Naturally-borne nanoparticles for disease biomarkers and drug delivery**

More recently, scientists have also tapped into key exosome attributes for biomarkers and drug delivery potential. With minimally invasive clinical sampling and the improvement of the sensitivity of bioassays, exosome-based body fluid biopsy has opened a new avenue of biomarkers for diagnosis and prognosis of human disease. Several studies were conducted in finding miRNA, mRNA and protein content of exosomes as biomarkers such as periostin in bladder cancer (6) and PCA3 and TMPRSS2:ERG in prostate cancer (7). The updated results are summarized in Dr. Gutpa’s review. Exosomes are enriched in urine providing sources of biomarkers for urological diseases. Yet, the lack of tissue specific exosome markers that can distinguish prostate versus kidney versus bladder could hinder their further application. To determine cell origin and destination of circulating exosomes, researchers have focused on finding the specific surface molecule responsible for exosome binding and internalization of target cells. The discovery of glypican-1, a cancer exosome marker with 100% cancer specificity in pancreatic patients, generates excitement (8). It has not yet been verified if glypican-1 does present specifically in urological cancer exosomes.

As for exosomes in drug delivery application, due to their nanometer size, low immunogenicity, fast uptake rate and RNase-free environment, such naturally-borne nanoparticles become very attractive vehicles that can deliver therapeutic small molecules such as miRNA and peptides to a specific, affected area. For instance, miRNA
and pharmacologic agents were reported to be successfully transferred in exosomes and delivered to cells. The exosome bearing IL-12, a key cytokine to induce tumor rejection response, has been suggested as a cancer vaccine for cancer treatment.

**Something good of exosomes in urology**

The prevalent body of research into exosomes has been focused on their roles in diseases, yet their roles in normal genitourinary physiology are often overlooked. For instance, in the reproductive organs, exosomes are found to be critical for gametes maturation. The ovarian follicle derived exosomes contain miRNAs that not only reflect the aging-related quality changing of oocytes, but also function in regulating estradiol and progesterone concentration levels during oocyte maturation (9,10). Epididymis and prostate secrete epididymosomes and prostasomes, enabling sperm cells to undergo necessary biochemical, biophysical, and molecular compositional changes prior to reaching oocytes to promote fertilization ability of the sperm cells (11). Furthermore, epididymosomes can transfer proteins P25b that are necessary for the sperm-egg binding, and contain the enzymes aldose reductase and sorbitol dehydrogenase that are involved in modulating sperm motility during the epididymal transition (12).

A unifying model was proposed in which prostasomes are involved in sperm capacitation and acrosome exocytosis processes (11), thereby protecting the sperm from the female’s immune system (13) and inhibiting late capacitation event and acrosome activation until sperm cells reach the oocytecumulus complex in the oviduct (14). In the kidney, exosomes are involved in cell-to-cell communication and tissue repair. Aquaporin 2 (AQP2), which functions in water molecules transfer, is one of the main components found in collecting duct cell originated urinary exosomes, and it can be delivered into AQP2 negative cells, and increase water flow (15). The TGF-β1 containing exosomes derived from injured kidney tubular epithelial cells initiate tissue regeneration (16). It is generally believed that the majority of urinary exosomes originate from renal tubular epithelia; however, their function is hardly known. Hiemstra et al. (17) conducted an in-depth research on protein contents in urinary exosomes by mass spectrometer, and surprisingly found the enrichment of antibacterial proteins and peptides. This suggested that those exosomes function as innate immune effectors for inhibiting uropathogenic bacteria in the renal tract.

**Light behind the clouds**

Taken together, emerging evidence has shown that urinary exosomes are involved in complex physiological and pathological genitourinary processes. This developing thought has stirred growing interest in their clinical applications. However, several issues remain critical for establishment of clinical relevance in applying exosomes. First, with respect to the mixed population of urinary exosomes, most exosome isolation methods collect particles that have the same density but not precisely separate sub-populations by their biological origin. This collection process could lead to data misinterpretation. Discovery of tissue and disease specific exosome markers would be highly significant. Second, the large variation in urine volume and density among individuals present another issue. As one example, the quantification of exosome concentration would be compromised. Identification of a stable urine normalization molecule would be critical. Lastly, the clinical studies that collect patients’ samples longitudinally with longer patient follow-up on clinical outcome are lacking. Despite these challenges, exosomes provide a novel platform for liquid biopsy for disease diagnosis, prognosis and therapy. Compared to blood borne exosome application in genitourinary diseases, urinary exosomes have the advantage of a less invasive sampling method and closer proximity to targeted secreting cells. Notwithstanding, the ability of fast uptake rate and site specific targeting makes exosomes a perfect drug delivery vehicle intravesically for bladder cancer therapy.

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Extracellular vesicles in cancer: current status and challenges

Nami O. Yamada

Department of Anatomy, Graduate School of Medicine, Gifu University, Gifu, Japan

Correspondence to: Nami O. Yamada, DVM, PhD. Department of Anatomy, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu-city, Gifu 501-1194, Japan. Email: nyamada@gifu-u.ac.jp.

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Small membranous vesicles released from the cell surface, which can be frequently observed by electron microscopy, were considered as just an artifact for a long time (Figure 1). The hypothesis that these vesicles, now called extracellular vesicles (EVs), are not mere artifacts but important and primitive cell-cell communication tools, was proposed for the first time in 1984 (1). An increasing number of studies have demonstrated that EVs contain a variety of biomolecules such as proteins, mRNAs and microRNAs, and that their profiles reflect the state of their donor cells. These cargos can be maintained in a remarkably stable state within biofluids including plasma, urine, saliva, breast milk, and culture media because EVs are composed of a lipid bilayer. Through the horizontal transfer of the cargos, EVs modulate various biological processes in both physiological and pathological conditions. In particular, cancer cells actively secrete and utilize EVs to educate stromal cells in the tumor microenvironment and to arrange the metastatic niche at distant sites for their prosperity. Understanding these roles of EVs has given novel insights into cancer research and encouraged further studies on EVs as potential non-invasive biomarkers and therapeutic targets for cancer. “Liquid biopsy” targeting circulating EVs is now a subject of great interest in cancer diagnosis.

With regard to liquid biopsy, urine can be collected easily, non-invasively and in large volumes compared with the other body fluids. However, EVs isolated from blood have been the focus of EV research so far and studies on urinary EVs have not flourished yet. The recent review article published in European Urology by Junker and colleagues intelligibly organizes the eligible articles regarding urinary EVs in genitourinary tumors (2). To date, 16 studies have examined urinary EVs as biomarkers for bladder, kidney, and prostate cancer and the authors of these studies suggest the great potential of urinary EVs as novel non-invasive biomarkers for these malignancies. However, they also raise several issues in this developing research field.

First, they refer to the current situation that there has been no standardized method for the isolation, confirmation, and quantification of EVs yet. The golden standard method for the isolation of EVs is ultracentrifugation or ultracentrifugation plus filtration, both of which provide fairly pure EVs. In addition, other isolation methods such as sucrose gradient density ultracentrifugation, magnetic beads coated with an EV-specific antibody, and commercially available extraction kits. Methods for the confirmation and quantification of EVs are also varied among studies, for instance, using NanoSight (Malvern Instruments, Malvern, UK) to estimate the size and number of EVs, electron microscopy to confirm their morphological characteristics, and western blot to detect specific markers of EVs (CD9, CD63, and CD81). Furthermore, one of the greatest problems is that a common and reliable internal control for EV-content is not available between independent studies. Therefore, different studies may draw different results even when they examine the same malignancy. Although a consensus has not yet been achieved, the International Society for Extracellular Vesicles (ISEV) is now in a series of enthusiastic discussions regarding the standardization of methods for the isolation and analysis of EVs (3,4).

Second, EVs from different biofluids of the same patient may contain different biomolecules. It is reasonable that the intercellular communication via EVs at one site should
be different from that at another site. In their review, the authors refer to a study by Armstrong et al. that compared microRNA profiles of tumor tissue, plasma EVs, urinary EVs, and WBCs from patients with bladder cancer (5), and encourage large-scale profiling studies on EV-contents across biospecimens to discover true and reliable biomarkers. The NanoString nCounter Vantage assay, which Armstrong et al. introduced in their study, seems a fine tool to accelerate these profiling studies.

Third, there is great interest as to how exactly EV-contents are sorted into EVs; are EVs randomly selected packages of molecules or specific molecular groups with the same ultimate goal to manipulate the recipient cells? Accumulating studies on the function of EVs and EV-contents have partially deciphered the contributing factors or unique packaging of specific molecules within EV-contents to the phenotypic alteration of the recipient cells (6,7). However, until now, the majority of EV-research is aimed at biomarker discovery. To use EV-contents as biomarkers, we first need to elucidate their function in each fluid and validate the precise mechanisms by which the cargos are sorted into EVs and released into the biological environment.

The EVs and their contents are now the hot topic in cancer research. Liquid biopsy for EVs is a promising non-invasive method for cancer diagnosis and monitoring in the near future. I hope that the increasing number of studies on EVs will provide us with exciting knowledge and renewed focus to fight cancer.

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Exosomes: an evolving source of urinary biomarkers and an up-and-coming therapeutic delivery vehicle

Robert H. Blackwell¹, Carrie A. Franzen¹², Gopal N. Gupta¹²

¹Department of Urology, ²Cardinal Bernardin Cancer Center, Loyola University Medical Center, Maywood, IL, USA

Correspondence to: Gopal N. Gupta. Department of Urology, Loyola University Medical Center, 2160 S. First Avenue, Fahey Center, Room 247, Maywood, IL 60153, USA. Email: gogupta@lumc.edu.

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Background

Exosomes are a subset of small, extracellular vesicles secreted by all cell types, and can be isolated for virtually all bodily fluids that have been investigated. The importance of exosomal signaling in normal physiology and cancer is clear, with the transfer of host-cell cytoplasmic RNA, intracellular and membrane-bound proteins being well-described.

Locally, cancer-derived exosomes are able to be internalized and even alter recipient-cell expression and behavior (1). Epithelial-to-mesenchymal transition, a process associated with tumor progression and malignant transformation, has been observed with the application of cancer-derived exosomes on normal cells in several malignancies, with documented stimulation of angiogenesis, as well as enhanced cell migration and invasion (2,3). Distantly, the excretion of cancer-exosomes systemically has been shown to be an organotropic determinant of metastases for specific malignancies via the delivery of specific integrins expressed by tumor-derived exosomes (4).

With the role in signaling exosomes demonstrate in malignancy, they represent a potential, largely-untapped, reservoir for biomarker identification and delivery of therapeutics.

Obstacles of urinary exosome study

We agree with Drs. Lee and Liu on their commentary regarding obstacles to the study of urinary tract exosomes (5). With multiple exosomal sources (including renal, urothelial and prostatic), there is a definite need for characterization of exosomal markers to specifically delineate the source. We have found that by performing a bladder barbotage (a standard procedure in the evaluation for bladder cancer in which at the time of cystoscopy saline is irrigated vigorously in the bladder and the fluid sample taken, typically for cytologic analysis) exosomal yields are higher than in the voided urine, and that barbotage samples had increased expression of mesenchymal markers than in the voided urine (2). In these cases where any residual urine had been emptied from the bladder, the saline barbotage sample should provide a more homogenous sample of urothelial exosomes from the bladder, with less contamination from upper tract (renal) and prostatic sources.

While this is a promising first step in using exosomes for the detection and study of bladder cancer, improvement in specific exosome markers will allow the voided urine sample, an entirely non-invasive test, to become more practical in the evaluation of the mixed population of urinary exosomes.

Use of exosomes in diagnostics

At present there are only two commercially-available exosome-based diagnostic tests on the market, for lung and prostate cancer. Despite the limitation noted above, for prostate cancer a non-invasive test using a patient’s voided urine sample to assess exosomes has been developed based
on a proprietary, three-gene signature for the detection of high-grade prostate cancer. In the recently published validation study, McKiernan et al. demonstrate that the use of this test, compared to standard of care, demonstrated improved prediction of clinically significant prostate cancer on biopsy, as opposed to less aggressive disease or a benign biopsy (6).

**Use of exosomes for delivery of therapeutics**

Exosomes hold exquisite promise in the delivery of therapeutics given their low immunogenicity, the environmental protection provided by their lipid bilayer membranes, and potential for targeting to cell types of interest.

Bladder cancer is unique given its precedent for direct delivery of therapeutic agents intravesically in the treatment of cancer (e.g., mitomycin C, Bacillus Calmette-Guérin vaccine). In *vitro*, we have demonstrated that following co-incubation with non-cancer-derived exosomes, bladder cancer cells internalize exosomes at a 50-fold higher rate than normal uroepithelial cells (7). We were then able to load exosomes with siRNA directed toward the gene Polo-like kinase-1 (PLK-1, a key regulator of mitotic progression) via electroporation (confirmed via Amnis ImageStreamX), and subsequently treat bladder cancer cells lines with PLK-1-loaded exosomes. With this technique, we were able to demonstrate not only knockdown of PLK-1 gene expression (via qRT-PCR), but also induction of apoptosis and necrosis of bladder cancer cells compared to those treated with negative control siRNA (7).

A recent study lead by Dr. Kim demonstrated the use of exosomes in the delivery of a chemotherapeutic agent (8). In this study, paclitaxel was loaded into exosomes via sonication and, in *vitro*, loaded-exosomes were shown to have uptake into cancer cells with demonstration of increased cytotoxicity compared to standard chemotherapeutic administration. In an *in vivo* mouse model for pulmonary metastasis, intranasal administration of loaded-exosomes demonstrated not only co-localization of exosomes with cancer cells on confocal microscopy, but also greater inhibition of metastasis growth compared to negative controls or chemotherapeutic administration.

While the above studies use a direct delivery of loaded-exosomes to the tumor cells, promising work in the systemic administration of targeted exosomes has also begun. In the *in vivo* mouse study by Dr. Alvarez-Erviti *et al.*, it was convincingly shown that by producing self-derived exosomes engineered to express a modified exosomal membrane protein (Lamp2b) fused to a cell specific peptide (in this case rabies viral glycoprotein (RVG), a neuron-specific peptide), that exosomes could be injected systemically and not only specifically target neuronal structures, but in doing so also cross the blood-brain barrier (9). When these exosomes were loaded with siRNA to BACE1 (a therapeutic target of Alzheimer’s disease), there was significantly decreased mRNA and protein expression in neural tissue compared to controls.

These results should address Dr. Panfoli’s concern regarding the ability of exosomes to not only deliver an RNA (or chemotherapeutic) payload, but to also affect target-cell expression (10).

**Conclusions**

Urinary exosomes represent the forefront of innovation and discovery, and we anticipate great strides to be made in the near future in biomarker discovery and therapeutic advancement. As of this year a urine-based, non-invasive test has become commercially available for highly prevalent prostate cancer to improve discrimination between high- and low-risk disease. The ability to determine exosomal biomarkers to similarly detect urothelial or renal cell carcinoma, or benign but progressive renal conditions, may provide opportunities to spare patients from invasive procedures, or to improve clinical risk stratification of disease. Further, the promise of packaging biologically active molecules or chemotherapeutics for targeted delivery is an exciting prospect in the treatment of benign and malignant disease.

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The role of the liquid biopsy as a clinical tool for early prediction in prostate cancer

Michael J. Donovan

Department of Pathology, Mt. Sinai School of Medicine, New York City, NY 10029-6574, USA

Correspondence to: Michael J. Donovan PhD, MD. Department of Pathology, Mt. Sinai School of Medicine, 1468 Madison Avenue, New York City, NY 10029, USA. Email: michael.donovan@mssm.edu.

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The evolution of the liquid biopsy from a novel biomarker discovery platform to a clinical (molecular diagnostic) assay represents a true inflection point in the practice of medical (oncologic) pathology. Initially associated with the quantitation of breast cancer circulating tumor cells (CTCs) found in blood as a measure of tumor burden, the field quickly has expanded to include the isolation and capture of cell free/tumor DNA, exosomal RNA species and peptide-protein analytes in all body fluids including CSF and urine (1-4). The improvements in specimen handling, isolation techniques and the robust identification of low abundance nucleic acids have continued to advance the field; however, challenges persist as investigators attempt to understand the importance of rare variants in a complex setting of tumoral heterogeneity, drug resistance pathways and host-immune response. Recent success including the development of the first Food and Drug Administration (FDA) approved blood-based (liquid biopsy) companion diagnostic for the drug Tarceva (erlotinib) in patients with non-small cell lung cancer have further realized the potential (5). A simple, non-invasive, liquid biopsy approach for men with a suspicion of prostate cancer that offers insight into early detection of clinically significant disease while not over-diagnosing low-risk prostate cancer would have a critical impact on reducing the number of prostate needle biopsies and most importantly limiting over-treatment (6,7).

The current study by Van Neste et al. 2016 in the journal European Urology is an example of such a liquid biopsy assay, which relies on the isolation of cellular components found in post-digital rectal exam (DRE) total urine samples from men presenting to a urologist for either an initial or repeat biopsy. The primary objective of this study was to validate the performance of a previously reported gene signature when combined with clinical variables would accurately predict high-grade (Gleason score 7) prostate cancer from GS6 and benign disease on prostate biopsy. There are currently two post-DRE urine assays commercially available including the United States FDA approved PCA3 test (Progensa; Hologic) which detects PCA3 mRNA transcripts normalized with KLK3 (PSA) mRNA from sloughed epithelial cells and a second urine test that combines total serum PSA, the PCA3 assay described above and the TMPRSS2:ERG fusion transcript known as the Mi-Prostate Score (MiPS) from the University of Michigan (8,9). The Progensa (PCA3) assay was originally FDA approved for men who had a prior negative biopsy but has shown efficacy in both the initial and repeat biopsy setting while the MiPS is currently used for both types of patients. These three assays require an ‘attentive’ DRE before urine collection and expedited specimen handling in a special transport tube and are able to predict a patients risk for having both any prostate cancer and intermediate/high-grade GS7 disease. Furthermore, both the MiPS and the current Van Neste assay incorporate clinical variables directly into the test results to achieve optimal predictive accuracy.

Of note, a urine-based exosome-derived gene expression (mRNA) test which includes PCA3 combined with total ERG (V-ets erythroblastosis virus E26 oncogene homologs)
normalized with SAM pointed domain-containing Ets transcription factor (SPDEF) was recently validated to predict GS7 disease at initial biopsy for men with equivocal PSA from 2–10 ng/mL (10,11). In distinct contrast to the previously described urine tests, this assay does not require a DRE, and there is no need for expedited transport or special handling. As with any new assay, in addition to accuracy, the ability to easily introduce into clinical practice will be a significant factor towards adoption. Furthermore, the exosome assay assesses total ERG, which also includes the fusion transcript addressing some of the recent reports that total ERG RNA levels are associated with clinical characteristics of higher risk prostate cancer (12,13).

Van Neste et al. used training and test cohorts (n=519 and n=386, respectively) which included men scheduled for either an initial or repeat biopsy based on an elevated PSA ≥3, abnormal DRE, or family history of prostate cancer. All urine samples were collected after a standard DRE, subsequently transferred to a specialized carrier tube, shipped at room temperature and then stored at −80 prior to analysis. Some important clinical characteristics are noted in the training and test cohorts, including fairly high median PSA values (16 vs. 12 ng/mL), high percentage of men with abnormal DRE’s (38% vs. 31%) and a high percentage of ≥GS7 prostate cancer (51% vs. 50%). Also noteworthy is the prior biopsy rate of 21 vs. 11%. In addition, the total combined cohort was predominantly (>95%) white. A qPCR amplification kit was utilized for RNA isolation with a one-step RT-qPCR and the KLK3 PSA gene used as a normalizer. Standard statistical analyses were employed including AUC of the ROC.

The authors in the current study compared a series of novel genes initially using a fixed sensitivity of 90% with pre-determined cut-offs and identified that the homeobox C6 (HOXC6), and distal-less homeobox 1 (DLX1) had the best combined AUC of 0.76 for predicting high grade disease. The gene combination was subsequently validated with an AUC of 0.73. They then introduced a series of clinical variables into the primary gene expression model to assess performance. Two models were created, ± DRE as an additional clinical risk factor that included both HOXC6, DLX1, combined with the clinical variables: PSAD, previous negative biopsy, total serum PSA, family history and age. With or without DRE risk factors, the AUC in validation ranged from 0.86–0.90. There are a few additional points worth noting. The authors observed that a model developed with only traditional clinical risk factors in the test cohort produced an AUC of 0.87 (by report mainly driven by PSAD) and that the addition of the two genes would increase the AUC to 0.90. Although the difference is statistically significant (P=0.018) it is not certain whether this will be clinically relevant.

In addition, when the final test model which included the gene signature was applied to men with a total serum PSA <10 ng/mL, the true ‘gray zone’ population where a biopsy decision is most challenging, the models AUC with or without DRE risk factors ranged from 0.78 to 0.86, respectively. Noteworthy is that the ‘gray zone’ population was limited to 264 men from the test cohort of which 86% had no or low grade G6 prostate cancer. The number of men who had a prior negative biopsy in this group was also not reported. The Prostate Cancer Prevention Trial risk calculator (PCPTrc) 2.0 (which includes percent free-PSA) was used as the main benchmark for all models performance (14). In the validation/test cohort, the PCPTrc v2.0 yielded an AUC 0.77 and when PCA3 was included, the AUC increased to 0.80; however, in the gray zone population the PCPTrc AUC was 0.66 and with PCA3 increased to 0.72.

It is widely accepted that integration of composite tools to define patient risk are important elements of personalized medicine. The more quantitative the outcome, the more precise and useful they become. Given the hazards of a prostate biopsy including infection, cost and diagnosis of low-risk, indolent prostate cancer, it is imperative that the clinician be well informed on the specifics surrounding the development of new assays prior to incorporation. This includes parameters of trial design, target population, accuracy metrics and ability to implement in clinical practice.

The current study was not designed to evaluate the PSA 2–10 ng/mL gray zone population presenting for their initial biopsy and although sub-group performance was quite good, the evaluable patient cohort is small and additional features including prior negative biopsy status would be helpful to understand performance. Additionally, as prostate cancer risk models move towards the prediction of clinically significant disease, it will become increasingly important to discriminate GS7 prostate cancer based on the International Society of Urological Pathology (ISUP) categorization of 3+4 vs. 4+3 as improved classifiers for evaluating significant disease (15). As part of this effort, investigators will need to provide false negative assessment of the clinical significant Gleason 4+3 population.

As demonstrated in the published literature, the performance of the urine-based gene expression only models to predict high-grade prostate cancer, including the Van
Neste, are all quite comparable with AUCs that range from 0.68–0.73. Given the impact of clinical variables alone on performance, especially as observed in the current study, one possibility is to retain gene risk models as independent patient-specific phenotypes and have the treating physician use this information in conjunction with on-line clinical nomograms such as the PCPTrc 2.0 to facilitate more informed decision-making. Furthermore, an additional challenge is the requirement of a DRE prior to urine collection and the need for special specimen handling. These aspects may negatively affect general implementation in a busy clinical practice setting.

In closing, liquid biopsy assays, especially those derived from urine and blood, will no doubt advance and become fully integrated into the precise ‘diagnostic-prognostic’ pathology tool kit. The appropriate assessment of these tests will continue to require diligence, along with extended validation and clinical utility studies to expand our understanding of their performance in sub-groups and ultimate impact on health outcomes. For patients and their treating physician, the ability to utilize a waste product to predict pathologic outcomes is an important milestone for the early detection (and future management) in the field of prostate cancer.

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References

Over the past five years, prostate-specific antigen (PSA)-
based screening programs have come under scrutiny given 
concerns about the accuracy of PSA and its downstream 
effects on diagnosis and treatment.

Specifically, PSA is not cancer-specific and thus, several 
benign conditions are associated with elevated serum PSA 
levels. Even though several adjuncts to conventional PSA 
have been proposed (e.g., PSA density/velocity, PSA doubling 
time, percentage of free PSA, and several isoforms), there is 
no optimal threshold value to distinguish between prostate 
cancer and benign conditions (1). Furthermore, evidence is 
scarce showing that those PSA ‘modifiers’ provide additional 
accuracy relative to serum PSA alone (2).

Moreover, the extensive use of PSA screening has led
to a significant increase of diagnostic prostate biopsies and higher detection rates of clinically insignificant tumors, which will likely remain indolent over time (3). Hence, influential public health guideline panels, such as the United States Preventive Services Task Force (USPSTF), have recommended against PSA-based screening in all men (4) to minimize the risks of overtreatment of low-grade tumors, as well as associated health care costs and psychological burden to the patient (5,6). Nevertheless, those recommendations have potentially significant consequences for patients harboring intermediate- to high-risk disease, as diagnoses might be delayed up to a certain point where potentially curative treatment is no longer possible. Given the established survival benefit of surgery or radiation therapy in patients with high-grade prostate cancer [Gleason scores (GS) ≥7 or locally advanced clinical stages] (7,8), the ideal prostate cancer early detection tool would be able to (I) identify patients with high-grade tumors to initiate diagnostic and treatment pathways and (II) avoid unnecessary biopsies and overtreatment in men with low-grade or without malignant cancer.

The perfect biomarker for general use in prostate cancer management needs to meet certain strict criteria. In addition to the required high sensitivity and specificity, the ability to differentiate benign from malignant, as well as indolent from aggressive tumors, the ideal marker has to be an inexpensive, easily accessible, and ideally non-invasive test. The concept of urinary prostate cancer biomarkers is not novel. To date, two urinary markers have been identified and adopted into clinical prediction tools to improve prostate cancer diagnosis and risk assessment.

Prostate cancer antigen 3 (PCA3) was initially described in 1999 as a prostate specific messenger ribonucleic acid (mRNA), which was overexpressed up to 66-fold in more than 95% of prostate cancers (9). In several follow-up studies, PCA3 demonstrated superior predictive abilities compared to serum PSA. In 2006, the PCA3 assay was translated into the commercially available Progensa™ PCA3 test (10). PCA3 was included into a predictive nomogram (11), which was externally validated in 2010 (12). Importantly, de la Taille et al. showed that PCA3 was superior in predicting initial biopsy outcome, compared to total PSA, PSA density, and %free PSA (13). Thus, in 2012, the US Food and Drug Administration (FDA) approved the test as a decision tool for the repeat biopsy setting, given that the likelihood of harboring prostate cancer increases, as the PCA3 score is higher (14,15). Specifically, in a cohort of 127 patients with a suspicious digital rectal exam (DRE), and/or persistently elevated PSA levels, and previous suspicious histology on the initial biopsy, Uprich et al. confirmed that PCA3 was the best predictor of prostate cancer at first repeat biopsy, compared to total PSA alone (16). Nevertheless, its role to distinguish indolent from aggressive tumors remains equivocal. In a retrospective study of 305 patients who underwent radical prostatectomy, the PCA3-score was not an independent predictor of extraprostatic extension, seminal vesicle invasion or high-grade disease (GS ≥7) (17).

Second, another important group of genes that are differentially expressed was identified in 2005: the ETS family (v-ets erythroblastosis virus E26 oncogene; ERG and ETS variant gene 1; ETV1). Tomlins et al. showed that these genes were overexpressed in approximately 57% of prostate cancer cases (18), and that this overexpression was most likely driven by an androgen-regulated fusion with the Transmembrane Protease, Serine 2 (TMPRSS2) (18). Following this, TMPRSS2-ERG fusion transcripts were shown to be detectable in urine samples (19). A meta-analysis of 61 studies evaluating men with fusion-positive prostate cancers did not find TMPRSS2-ERG to be a strong predictive marker of disease outcome after radical prostatectomy, as the fusion status was not associated with risk of GS ≥7 vs. GS ≤6 or GS =7 vs. GS ≤6 (20).

These two urine-based prostate cancer early detection biomarkers—PCA3 and TMPRSS2-ERG—along with serum PSA were subsequently combined into another urine test, the Mi-Prostate Score (MiPS) (21,22). MiPS + PSA outperformed both PCA3 + PSA and PSA alone for prediction of high-grade prostate cancer defined as GS ≥7 (22). Of note, both Progensa™ and MiPS require pre-collection DRE, which might be perceived as an invasive intervention and thus, do not meet the stringent definition of a ‘perfect’ detection tool.

Against this backdrop, a study by McKiernan et al. in JAMA Oncology found promising results for a novel urine-based gene expression assay to predict high-grade prostate cancer at initial biopsy (23). The authors used an exosome-derived gene expression signature, which included PCA3 and ERG RNA. While the underlying genes are not novel per se, McKiernan et al. were the first to isolate exosomal RNA without previous prostate examination, derive a molecular prostate cancer signature, and prospectively validate the predictive accuracy of this diagnostic tool.
Exosomes are miniscule tissue-derived vesicles, which can be secreted by different cell types, including tumor cells, and carry proteins and RNAs that are representative of their tissue origin (24). In this study, 255 patients with serum PSA levels of 2–10 ng/mL were examined to assess the prognostic accuracy of the ExoDx Prostate IntelliScore urine exosome assay. The derived score was then validated in an intended-use population of 519 patients from 22 facilities in the United States. Patients were considered eligible if they had no history of prostate cancer or biopsy, were 50 years or older, and referred for initial prostate biopsy due to a suspicious DRE finding and/or serum PSA levels from 2–10 ng/mL. When estimating the area under receiver operating characteristic curve (AUC) for discrimination of GS ≥7 vs. GS <7 or benign disease, the novel urine exosome gene expression assay in combination with standard of care (PSA, age, race, and family history of prostate cancer) was superior to standard of care alone (AUC: 0.73 vs. 0.63; P<0.001) (23). Similar results were found when the target population was extended to include patients with a serum PSA level of 10–20 ng/mL.

This new tool relies on previously established genomic markers, but is solely first-catch urine-based. While the MiPS does incorporate previously established genes and serum PSA, the ExoDx Prostate IntelliScore urine exosome assay is different as it does not require a DRE (23).

Notably, the predictive accuracy of this novel exosome-derived test is no better than MiPS, which was previously introduced in 2015 (22) and relies on TMPRSS2-ERG, PCA3, as well as clinical variables included in the Prostate Cancer Prevention Trial risk calculator (PSA, family history, outcome of DRE, and prior biopsy) (25). While the AUC for MiPS was 0.779 for predicting high-grade cancer at biopsy in the validation cohort (22), McKiernan et al. reported an AUC of 0.73 in the external validation of 519 patients to discriminate between GS ≥7 vs. GS <7 or benign disease (23). However, it is convincing that the novel test can be conducted by any health professional without precise knowledge of the performance of an adequate DRE and not only by urologists or physicians. Also, patients could be spared another DRE, which may raise compliance and eventually facilitate the clinical workflow, indeed.

Despite these advantages, patients were considered eligible for the novel exosome-derived test if they presented with an elevated serum PSA ranging from 2–10 ng/mL (2–20 ng/mL in subanalyses) and/or a suspicious DRE. Whether a patient with suspicious DRE should undergo this test is a complex manner. Given that a substantial proportion of prostate cancers detected by DRE at PSA levels ≤4 ng/mL are associated with clinically highly aggressive tumors (26), it is debatable if a patient who presents with a suspicious DRE should undergo this test, as it is unlikely to change clinical decision-making.

Novel tumor targets are anxiously needed, and the combination of biomarker templates seems to be a promising approach to improve the prediction of prostate cancer and prostate cancer aggressiveness at biopsy. However, adequate internal and external validation of these markers are necessary. Specifically, prospective validation in randomly invited population-based cohorts is the gold standard to test the predictive accuracy of those novel markers. As such, the Stockholm 3 study group recently validated a new predefined model in a screening cohort of 113,082 men to identify high-risk prostate cancer (GS ≥7) with better accuracy than PSA alone (27). The model included a combination of several plasma protein biomarkers and performed significantly better than PSA alone (27). Nevertheless, regarding exosomes, further research is eagerly awaited. If researchers are able to gain higher yields of exosomes from urine samples, this may help in finding new bladder, prostate, or renal cancer-specific miRNA and mRNA biomarkers.

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Urachal carcinoma is a rare and extremely aggressive malignancy deriving from an embryological remnant of the urogenital sinus and allantois. It represents <1% of all bladder cancers, with a prevalence of approximately 0.2% and a higher incidence in males than in females (1,2). About 90% of urachal cancers are adenocarcinomas and half of them share histological and molecular features with colorectal cancer (CRC); indeed, they have a common embryological origin from the cloaca (2).

The 5-year survival rate is less than 50%, with a median survival for locally advanced or metastatic disease ranging between 12 and 24 months (3,4). This poor prognosis can be attributed to the following factors: (I) the tumour originates in the anterior portion of the bladder, thus causing delayed symptoms presentation and compromising an early diagnosis; (II) the molecular pathogenesis of the tumor, as well as its sensitivity to specific chemotherapy treatments or molecular targeted therapies, is largely unknown, and no treatment standardization actually exists. This latter aspect is common to all rare cancers, in which collecting sufficient biological material to perform in vitro and in vivo biological analyses, and enrolling a sufficiently high number of patients in prospective randomized trials, is a challenge for both scientists and clinicians.

Due to the lack of published randomized trials, there are no reference guidelines for the treatment of urachal cancer. In the case of localized disease, surgically removing the tumour is the only strategy that can guarantee cancer cure in a long-term perspective. The standard surgical approach consists in performing partial cystectomy, bilateral pelvic lymphadenectomy and umbilicus plus umbilical ligament resection (1,3). Local recurrence rate within the first two years after resection is reported to be of 15% to 41%, with the pelvis, bladder, and the surgical incision or abdominal wall being the most frequent sites of relapse. The most common sites of metastatic spread are the liver, lymph nodes, lungs and bones (particularly the spine) (1,3). Risk factors predicting early tumour relapse are: positive surgical margins, lymph node involvement, high tumour grade and advanced TNM stage. Patients at high risk of local or distant relapse could be potentially treated with adjuvant local or systemic treatments, similarly to what is currently done in the case of CRC or genitourinary tumours arising in the pelvis. However, urachal carcinoma tends to be relatively resistant to radiotherapy (2), while the role of neoadjuvant and adjuvant treatments is still unclear.

While localized disease can give rise to metastatic spread after surgical removal, approximately 30% of patients present with metastatic disease at diagnosis. In this setting, no standard-of-care therapeutic options exist. In different published patient case series, single agent or combination chemotherapy has demonstrated antitumor activity and clinical benefit. The most commonly used chemotherapeutic agents are cisplatin and 5-fluorouracil (4,5), while targeted therapies, including gefitinib, sunitinib and cetuximab have recently demonstrated clinical activity in some patients (6,7). Due to the paucity of published studies and the lack of randomized trials, defining the best therapeutic strategy for individual patients with advanced urachal carcinoma is usually left to the discretion of the treating physician.
The result of this common practice is a high treatment heterogeneity and arbitrariness, which results in poor treatment optimization and poorly interpretable results emerging from single, small published results.

The article by Collazo-Lorduy et al. reports the case of a young male patient with urachal carcinoma metastatic to the lung, who was successfully treated with cetuximab as a third-line therapy. After cystectomy and two subsequent lines of systemic chemotherapy with gemcitabine-FLP (5-fluouracil, leucovorin and cisplatin), discontinued because of unacceptable toxicity, and doublet carboplatin-paclitaxel chemotherapy, precociously stopped because of progressive disease, targeted genome sequencing performed on the primary tumour revealed the presence of EGFR amplification, which was subsequently confirmed by fluorescent in situ hybridization (FISH). Moreover, no KRAS gene mutations were detected. The patient was therefore treated with cetuximab monotherapy, and reported a radiological partial response (25% decrease of tumor diameters on computed tomography scans) lasting for about 8 months. Whole-genome sequencing was then performed to better characterize the genetic landscape of the primary tumour. However, no alterations linked with tumour sensitivity/resistance to cetuximab other than EGFR amplification were found. Then the authors investigated the prevalence of EGFR alterations in nine additional patients, but no EGFR mutations or amplifications were found. On the other hand, they found alterations in genes that are often involved in CRC cancerogenesis, and converge on activating the MAPK pathway, such as KRAS, NRAS and MAP2K1 activating mutations (8).

Similar data have emerged from a recent molecular analysis published by Módos et al., who also found BRAF mutations occurring with a similar frequency as in CRC (9). It is currently unknown if urachal carcinomas with different molecular profiles result in different biological and clinical behavior. However, based on the accumulating experience in other cancer types, specific gene mutations could have a prognostic (such as the case of BRAF mutations in CRC) or predictive (such as EGFR mutations in lung adenocarcinomas treated with EGFR inhibitors or RAS-mutated CRCs) value (10-12). Understanding the molecular mechanisms driving urachal cancer growth, as well as the genetic alterations conferring sensitivity or resistance to specific therapies, might guide treatment personalized.

Since published data suggest that the molecular landscape of urachal carcinoma could be similar to that of CRC, molecularly targeted treatment could parallel the recent successes obtained in CRC therapy. For this reason, and due to the limited therapeutic options available for urachal carcinoma, routine genomic assessment for actionable mutations may provide useful information to guide treatment personalization. For example, the absence of KRAS, NRAS and BRAF mutations could predict sensitivity to the EGFR inhibitors cetuximab and panitumumab, while BRAF mutations could predict tumor sensitivity to combinations of BRAF inhibitors (e.g., vemurafenib or dabrafenib) with EGFR or MEK 1/2 (e.g., trametinib) inhibitors (13-16).

Despite these promises, the following critical aspects need to be discussed.

Firstly, because of the low incidence of urachal carcinoma, all patients with this form of cancer should be sent to reference centers with the aim of collecting tumour tissue samples to comprehensively investigate the mutational landscape and molecular pathogenesis of this cancer type. Indeed, one crucial aim is to provide the most exhaustive view as possible of occurring genetic alterations and their frequency, so to understand which alterations are worth being routinely assessed and therapeutically targeted.

Secondly, the correct timing for tumour genetic assessment and targeted therapy administration needs to be established. In commonly occurring cancers, such as CRC or lung cancer, randomized trials have been performed to clarify the clinical efficacy of molecular targeted therapies before, after or concomitant with first-, second- or third-line chemotherapy treatments. Results emerged from the studies are not universal, and depend on both tumor site and tumor biology. For example, combination of standard cytotoxic chemotherapy with anti-EGFR monoclonal antibodies has emerged as the most effective first-line treatment in advanced CRC with wild-type KRAS/NRAS/BRAF. On the other hand, combining EGFR-mutated small tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib with first line chemotherapy in lung adenocarcinoma has not proven to be more effective than single TKI or chemotherapy treatment (17-21). Due its rarity and the lack of established chemotherapy treatments, it will be impossible to replicate such big studies in urachal carcinoma. For this reason, there will be poor space for rationally combining biological therapies with cytotoxic chemotherapy, and molecular-targeted treatment options will probably consist in single- or combination biological treatments targeting molecular alterations that likely drive cancer growth. One different scenario could emerge in the case that the mutational landscape of urachal carcinoma will be found to significantly
overlap with that of CRC. In this case, there is a hope to translate results deriving from big studies in advanced CRC directly to the treatment of urachal carcinoma, including possible combinations of chemotherapeutic treatments with molecular targeted therapies. However, the fact that sunitinib and gefitinib have shown activity in urachal but not CRC suggests that the genetic landscape, molecular pathogenesis and sensitivity to treatments by these tumors is not completely overlapping (6,7,22,23).

Lastly, the increasing necessity to extend genetic profiling to individualize patient care collides with the high costs of diagnostic tests and currently available molecular targeted therapies. However, this problem is common to all cancer types in this historical period. Once the biology of urachal carcinoma, as well as its disease-relevant and “druggable” targets, will be identified, treatment personalization will allow to restrict genetic/molecular profiling studies and costly treatments to patients more likely to specific patients, while sparing useless analyses and treatments to the remaining patients.

Despite the lack of prospective studies and treatment standardization in patients with advanced urachal carcinoma, the availability of compounds targeting crucial biological pathways, such as EGFR and VEGFR inhibitors, has recently expanded the potential therapeutic armamentarium against this type of cancer. This fact, combined with the availability of sensitive and potent molecular biology techniques that are able to reveal drivers of cancer growth, will likely improve patient outcomes compared to historical data. In the perspective of treatment personalization, it is mandatory to define a clear picture of occurring molecular alterations, so to make specific genetic and molecular tests widely available to patients, and to approve the use of compounds targeting the most frequently deregulated pathways.

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References

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The prostate-specific membrane antigen (PSMA) is a membrane glycoprotein (type II-carboxypeptidase) encoded by the FOLH1 gene, which is hyper-expressed in prostate cancer (PCa). 68Ga-PSMA PET/CT is a new diagnostic tool for the localization of PCa foci in patients with biochemical recurrence. Its use in pre-surgical staging as well as in early treatment evaluation is also under investigation (1). Moreover, the possibility of labeling PSMA antagonists with radionuclides emitting α and/or β-particles is becoming an interesting therapeutic application for the same molecule (2). However, PSMA, despite its name, is not really specific for PCa as it is also expressed by endothelial cells in the neovascular tissue of many solid tumors including kidney cancer. It has also been reported that the expression of PSMA in normal renal parenchyma can be detected within the brush borders and apical cytoplasm in a subset of proximal tubules (3,4).

Recently, Spatz et al. (5) evaluated PSMA immunohistochemical expression in neovascularized tissue in a cohort of 257 patients with renal cell carcinoma (RCC), mostly clear cell RCC (ccRCC) and, to a lesser extent, papillary RCC (pRCC) and chromophobe RCC (chRCC). The authors correlated PSMA expression with clinical-pathological parameters related to 68Ga-PSMA PET/CT. They also investigated the possible prognostic role of FOLH1 (folate hydrolysis 1) gene encoding for PSMA in patients with ccRCC and pRCC. Results of immunohistochemical analysis revealed that PSMA hyperexpression was only present in the endothelium of neovascular tissue in RCC samples. In particular, 82.5% of ccRCC and 71.4% of chRCC samples expressed PSMA glycoprotein, whereas only 13.6% of pRCC showed PSMA staining.

For the first time, Spatz et al. highlighted a significant correlation between increasing levels of PSMA expression and overall survival among patients with ccRCC. The association between PSMA expression and overall survival also maintained its significance (HR 2.02; 95% CI: 1.08–3.79) after correlation with key clinical features such as tumor grade, primary tumor stage and metastases. These results were largely supported by the data analysis of RNA expression from the Cancer Genome Atlas (TCGA). The authors reported a significant correlation between the expression of FOLH1 mRNA and survival in both univariate and multivariate analysis in pRCC patients, whereas it was only significant in univariate analysis in ccRCC patients. These results allow us to hypothesize an important role of PSMA in the management of RCC patients.

Recently, a potential role of 68Ga-PSMA PET/CT was reported in the preoperative evaluation of patients with RCC (6,7). The main advantage of 68Ga-PSMA PET/CT compared to conventional methods, in particular CT, lies in its ability to detect small lymph node lesions that do not exceed CT volumetric limit. In addition, the possibility of performing a whole body scan enables distant metastases to be detected.

PSMA could also be used to evaluate response to therapy...
in RCC. At present, inhibition of angiogenesis represents a new treatment strategy for RCC.

It is estimated that more than 300 compounds are currently being investigated for their potential anti-angiogenic effect, and a large series of inhibitors of angiogenesis have shown great potential in (pre)clinical studies for the treatment of many tumors.

Sorafenib and sunitinib, two tyrosine kinase inhibitors of the VEGF and PDGF receptor, were recently registered for the treatment of metastatic RCC.

68Ga-PSMA PET/CT could be a triage test for antiangiogenic therapy and for subsequent evaluation of the response, monitoring the variations of “PSMA expression” as a surrogate of neo-angiogenesis in vivo. To date, only one study performed in eight RCC patients revealed a potential role of 68Ga-PSMA PET/CT in the evaluation of early response to systemic therapy compared to MRI and CT (8).

According to Spatz et al., PSMA may represent a molecule to be used as both a diagnostic and therapeutic agent in RCC. The latter application could be very important if we consider that RCC remains largely incurable, despite the increasing number of currently available drugs.

Like PCa, the possibility of labeling PSMA antagonists with α and/or β particles (9,10) could also pave the way for an effective radionuclide therapy in patients with RCC.

Although there is still a long way to go, in our opinion we are heading in the right direction. However, large-scale prospective studies are warranted.

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Footnote

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References


Prostate cancer (PCa) is marked by a broad heterogeneous spectrum of clinical behavior, ranging from indolent subclinical forms up to aggressive metastatic and rapidly lethal tumors. This complex landscape of PCa behavior denotes the extreme genomic heterogeneity of this tumor type (1). Interestingly, the genomic heterogeneity of PCa is observed not only between the primary (localized) tumor and the advanced (metastatic) tumor samples, but also within each of the two disease stages. Certainly, PCa molecular characterization could provide an important impact in defining the patients’ prognosis and guiding therapeutic decisions. In this light, a pivotal contribution in understanding localized PCa molecular taxonomy comes from the whole exome sequencing molecular analysis of more than 300 primary PCa performed by the Cancer Genome Atlas (TCGA) study group (2). This analysis confirmed the androgenic dependence of primary PCa, the significant incidence (about a quarter) of activating mutations of the phosphoinositide 3-kinase (PI3K)/Akt/mTOR and MAPK signaling pathways, and the possibility to classify the vast majority of PCa tumors into seven subtypes defined by specific gene fusions (ERG, ETV1/4, and FLI1) or recurrent mutations in specific genes (SPOP, FOXA1, and IDH1) (2).

Treatment plans personalization based on genomic classification although promising is still highly unripe. In addition, a further complication of this scenario lies in the vast inter- and intra-tumor genetic heterogeneity. In particular, when considering a radical prostatectomy specimen, multiple different intraprostatic neoplastic foci can significantly differ in their genomic profile and therefore in biological aggressiveness (3,4). The highly genomic heterogeneity of multifocal PCa tumors, the possible co-existence of PCa foci of independent clonal origin, and the subsequent diverse tumor evolution of each PCa lesion greatly complicate the management of localized PCa. Therefore, PCa risk stratification and the consequent treatment algorithm cannot rely exclusively on limited sampling of the prostate.

Wei et al. (5) presented the results of a comprehensive genomic analysis using whole-exome sequencing, single-nucleotide polymorphism arrays, and RNA sequencing performed on multiple non-microscopic and noncontiguous PCa foci in radical prostatectomy specimens derived from four patients with clinically localized National Comprehensive Cancer Network intermediate- or high-risk PCa who did not receive neoadjuvant therapy. DNA and RNA were extracted from three independent tissue cores of the index lesion (based on size) and from one core obtained from all additional spatially distinct tumor foci. The aim of this analysis was to create a genomic fingerprint for each PCa lesion within each prostate gland in order to delineate genomic heterogeneity within the index PCa lesion (intratumoral heterogeneity) and between the different
PCa foci (intertumoral heterogeneity). According to the results of previous studies, this analysis confirms the significant intratumoral and intertumoral heterogeneity in somatic DNA alterations between different tumor foci within the prostate gland, thus emphasizing the need and the complexity to identify that specific aggressive focus responsible of tumor recurrence and/or metastatic spread.

In addition, the important contribution of Wei and colleagues depends particularly on the demonstration that:

(I) The majority of DNA-derived genomic heterogeneity is conserved at the RNA level, and the combined assessment of both DNA variants and RNA expression has been shown to be more powerful at differentiating subgroups of PCa than either alone. Additional variability in gene expression and gene fusions has been identified when analyzing RNA;

(II) The bulk of PCa foci analyzed could not be classified as belonging to any of the seven subgroups of the TCGA molecular taxonomic system (2). Accordingly, same results derive from an extended analysis that includes additional 163 tumor foci from 60 men from four public studies; The lack of a correlation with the TCGA taxonomy underlines the importance of further deepening the current knowledge on PCa molecular characterization, stresses the limitations of the classification tools available, and supports the need of novel more reproducible molecular clustering;

(III) Considerable intratumoral and intertumoral heterogeneity has been also observed between the scores of different commercially available genetic prognosticators, which quantify for each PCa focus the expression of gene signatures able to stratify indolent versus aggressive tumors: the Decipher (a 22 genes set that estimates the probability of metastatic disease), Prolaris (31 cell-cycle progression genes indicating PCa aggressiveness), and Oncotype DX (12 genes predicting PCa recurrence after surgery). Prospective, systematic analyses of large cohorts of PCa specimens are required to verify if taking into account the range rather than the absolute value of these scores, the average score from two or more intraprostatic PCa foci, cooperativity between scores from different assays on the same tissues, and inclusion of DNA-based data may improve the performance of current prognostic risk tools;

(IV) The androgen receptor (AR) activity, assessed for each PCa lesion by measuring the expression of a select set of 20 AR target genes, is remarkably diverse both within and among PCa specimens. AR activity does not correlate with any other scores or with the prostate region from which the cores were obtained, but correlates with Gleason score. Albeit with the limitation of the specific AR-dependent gene set analyzed, the heterogeneity of AR activity raises the question of a possible molecular selection for guiding the androgen deprivation adjuvant therapy indication.

Beyond the fundamental contribution that this study brings in the perspective of a molecular stratification of PCa, several limitations should be taken into account:

(I) The small sample size of only four PCa patients analyzed. Although this study has extracted the highest number of samples from each prostate gland—between five and seven from each prostate—with each sample highly representative of neoplastic tissue, a larger cohort is strongly suggested to validate and better delineate the hypothesis of multifocal PCa genomic heterogeneity (6);

(II) The clinically restricted PCa patients (at high-risk of relapse) selected for molecular characterization. PCa molecular profiling should help clinicians in the management of the most critical clinical situations where treatment decisions are not unequivocally accepted.

It means, for instance, in low-risk apparently indolent PCa where active surveillance represents a possible therapeutic option to consider together with active locoregional treatments. Do we think that mapping genetically biopsy specimens may contribute substantially in treatment decisions? The identification of a PCa focus (although not in the index lesion) with aggressive molecular properties could prompt the clinician to an active treatment? Similarly, the genomic characterization of an intermediate or high risk PCa will give substantially information about the selection of patients who will benefit most from adjuvant hormonal therapy (taking into account the heterogeneity of AR activity)?

Moreover, considering that single tumor-biopsy specimen reveals a minority of genetic aberrations that are present in an entire tumor, the problem of adequate sampling of the prostate remains.

In addition, this study lacks a correlation between the genomic profile of the PCa primary tumor and that of
cancer cells responsible for relapse/progression/metastasis. It would be very fascinating to being able to identify the aggressive PCa subclone both in the primary tumor and in the metastatic sites, or—even more interesting—in an earlier disease stage through DNA analysis of circulating tumor cells, and to characterize the genomic differences at different disease stages. An indirect comparison of the key aberrations observed in localized versus advanced PCa revealed that: (I) metastatic castration resistant PCa (mCRPC) has a higher mutational load (more copy-number alterations and mutations); (II) AR signaling, TP53, and PI3K pathway are more commonly mutated in mCRPC compared to primary PCa; (III) no genes are selectively mutated in primary PCa (2,7).

Can we assume in the future to draw an integrated prognostic model that includes biochemical data [prostate-specific antigen (PSA) levels], radiological findings (staging, disease extension), histological features (Gleason Score), clinical parameters and molecular characteristics of the tumor? Such a model should be seen as a dynamic system that periodically guides clinicians to the best therapeutic strategy during the course of the patients’ clinical history. Tumor progression is a multistep process that reflects the progressive accumulation of genetic mutations, which confer a selective advantage to cancer cells proliferation. Therefore, the best prognostic model should provide an inherent dynamism and reproducibility at different stages of PCa disease.

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References


Introduction

Population-based screening for prostate cancer (PCa) using prostate-specific antigen (PSA) has shown to reduce the cancer-specific mortality but was associated with a high rate of overdiagnosis (1). The reason for this is the high prevalence of PCa (2). Thus, 27 men had to be diagnosed with PCa in order to prevent 1 PCa-specific death in the cited mass screening trial. It is important to recognize, that the mentioned screening study was a population-based trial (= every eligible participant providing consent was tested). There was no risk-stratification intended prior to PSA-testing. A PSA cut-off was the only trigger for biopsy irrespective of risk factors. In addition, conditions increasing the PSA-value such as benign prostate enlargement were not considered in the study protocol. The value of PSA screening is higher among individuals defined by particular characteristics, such as family history of PCa, ethnicity, increasing age, or genetic factors. Therefore, a pre-diagnostic information on the future risk for aggressive PCa might be of important clinical value in order to stratify individuals at risk. In an attempt to categorize men according to their future risk profile, efforts have been made including baseline PSA-values at younger age (3), family history (4) and single nucleotide polymorphism in the kallikrein 6 region (since PSA is a member of the kallikrein-family) (5).

Recently, a new study was published in the British Medical Journal. The authors aimed to identify men who might be at risk for PCa development and therefore would be candidates for a more focused (and earlier) PCa screening. More than 200,000 single nucleotide polymorphism (SNP) were analyzed in a development set by performing a stepwise regression framework in order to calculate the individual genetic risk. This yielded 54 SNP that were incorporated in a risk model. Clinical data was obtained from 31,747 men of the international “Prostate Cancer Association Group to Investigate Cancer Associated Alterations in the Genome” (PRACTICAL), which is a collaborative group of researchers investigating the inherited risk of PCa. Aggressive PCa was defined as Gleason Score \( \geq 7 \), cT3/cT4 disease, PSA \( \geq 10 \) ng/mL or cN1/cM1 disease. Finally, the model was tested in a validation set of 6,411 men from the ProtecT-Study (6) [1,583 men with an PCa, 632 with aggressive PCa, 220 with very aggressive PCa all diagnosed by transrectal ultrasound biopsy (TRUS) and 4,828 controls]. ProtecT assigned 1,643 men with localized PCa to active monitoring, surgery or radiation therapy. The authors conclude that the “polygenic hazard scores can be used for personalized genetic risk estimates that can predict for age at onset for aggressive PCa”. Any effort to minimize overdiagnosis should be welcomed with open arms. However, a few questions remain:

Is the polygenic hazard scores safe? Does it reduce the unnecessary biopsy? Does it help preventing overdiagnosis? And finally: Does the polygenic hazard scores reduce PCa-specific mortality? Any combination variants might be used...
for risk prediction in order to improve screening for lethal
disease. However, although there is a plethora of research
on SNP’s for aggressive cancer (7-10), the puzzle for genetic
variants is still not gaining shape for clinical purposes.

Conclusions

The variable clinical course of aggressive PCa makes the
risk prediction difficult. “Proceed with caution!” (11) or
in other words “publish these data with care” is one of the
dictums standing for the current scientific situation. It is
debatable, whether adding more SNPs will help preventing
unnecessary biopsies, overdiagnosis or even death from PCa
in the future.

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In the era of personalized medicine, there is significant emphasis on the development of companion diagnostics and/or molecular signatures to guide therapeutic decisions (1). For example, two recurrence risk signatures (Oncotype Dx® and Mammaprint) are commonly used to guide chemotherapy in women with node-negative breast cancer (2-5). An evolution of Oncotype® (that is a centralized method of determination of the gene signature in breast cancer) is EndoPredict. The latter is a not centralized method of genotyping of the breast cancer that associates a signature of genes to the clinical staging of the patients and that was recently demonstrated to give higher performance if compared with the other well established genotyping system Oncotype. In fact, the test can predict whether breast cancer will spread in women with estrogen receptor positive, HER2 negative (ER+/HER2−) disease (6). Women with ER+/HER2− breast cancer are given endocrine therapy after surgery to treat their cancer. They receive also chemotherapy if there is a reasonable risk of the cancer spreading to other organs (referred to as secondary or metastatic breast cancer). This is because if the cancer spreads to certain organs, it may not be possible to treat it and the cancer can become incurable. EndoPredict analyses the activity of eight different genes within a tumor sample, and uses this information alongside the patient’s tumor size and nodal status to give an ‘EPclin’ score estimating their risk of developing advanced breast cancer. The EPclin score is then used to categorize patients into low or high risk groups, with a cut-off point of 10% risk over 10 years. When Oncotype DX was used to identify the third of lymph node-negative patients with the lowest risk of secondary disease, 7% went on to develop secondary breast cancer after 10 years. Of the third of patients identified as having the lowest risk according to the EPclin score, only one of 227 (0.5%) patients developed secondary disease. By identifying a large group of patients with a very good prognosis, EPclin could offer to clinicians and particularly to patients a reliable reassurance that chemotherapy can be avoided. In the field of the prediction of response to anti-tumor agents, K-ras and N-Ras mutations has been shown to be predictive of panitumimab and cetuximab nonbenefit in colorectal cancer (7,8). Furthermore, epidermal growth factor receptor (EGFR) mutations have been shown to predict benefit from tyrosine kinase inhibitors (TKI) and more recently, ALK gene rearrangement has shown to be predictive for crizotinib benefit in non-small cell lung cancer (NSCLC) (9-11). Moreover, fibroblast growth factor receptor (FGFR) mutations and/or amplification are emerging as new predictor markers of response to agents raised against FGFR in a variety of tumors, e.g., adeno- and squamous cell carcinoma of the lung, glioblastoma multiforme and bladder cancer. FGFR3 mutations are reported in up to 50% of cancers of all stages from the lower and upper urinary tract with p.S249C being the most common mutation, found in 61% of cases. Mutation is inversely correlated with tumor stage and grade, and
mutated tumors are associated with a favourable clinical outcome. The mutation analysis potentially allows a further stratification of patients, and should be additional evaluated in larger cohorts of invasive tumors. Bearing in mind that recurrence-free survival is an indicator for disease severity and risk of progression, FGFR3 mutations in squamous differentiated bladder tumors may indicate potential for FGFR inhibitor treatment in these tumors (12). On the other hand, clinical decision making in radiation oncology is still mainly based only on clinicopathologic features. Therefore, there is a great need to develop molecular diagnostics to more efficiently use radiotherapy (RT). Effective predictive biomarkers are a central requirement for the development of personalized treatment in clinical oncology. Unlike prognostic biomarkers, which predict clinical outcome independently from treatment, predictive biomarkers are treatment specific and thus are critical for therapeutic decision making (13). For example, several targeted drugs are now routinely offered to patients whose tumors harbour a specific marker for benefit or nonbenefit [i.e., HER-2/neu expression and trastuzumab benefit (14), K-ras mutation, and panitumumab nonbenefit] (7). In contrast, radiation therapy is still recommended on the basis of standard clinicopathologic features, which generally address tumor burden/aggressiveness and serve as prognostic biomarkers of outcome rather than a specific marker for RT therapeutic benefit. It is estimated that approximately a third of patients with localized or locally advanced prostate cancer undergo external beam RT with curative intent (15). The use of RT in combination with androgen-deprivation prolongs survival (16), and has contributed to the increase in 5-year survival rate from 30% in the 1970s to 80% in 2009 (17). Late toxicity following irradiation for prostate cancer includes damage to the bladder, bowel and erectile function. The median rates of late gastrointestinal (GI) and genitourinary (GU) toxicity are reported to be 15% and 17%, respectively (18). Studies are attempting to identify the genetic variants that increase an individual's risk of radiation toxicity (19,20). Moreover, a low number of studies have made efforts to identify biological features of prostate cancer tissues and immunologic circulating biomarkers able to identify patients responsive to RT. In this light, Nardone et al. (21) have recently found that tumor infiltration by different lymphocyte subsets predicts the outcome of patients with prostate cancer showing only local relapse after primary surgery and subsequently receiving RT. They establish a premise for a possible immunological therapy associated with RT for selected patients. In fact, the chemotherapy/RT could induce DNA double-strand breaks that, in turn, produce mutations and neo-antigens generation, promote immunological danger signals and reduce tumor infiltrating immunosuppressive cell populations, such as inhibitory myeloid cells. All these events are necessary to trigger antigen-specific CTLs. The critical role of the tumor immunologic microenvironment in conditioning both tumor development and survival offers the rationale to design new immunotherapeutic strategies for patients with prostate cancer associated to radiation treatment. On the light of the genetic scores of prostate cancer, in a matched retrospective analysis reported in *The Lancet Oncology*, Zhao *et al.* (22) identified and validated a 24-gene predictor of response to postoperative RT in prostate cancer. In the training cohort (n=196 from one study) and pooled validation cohort (n=330 from four remaining studies), patients who had post-operative RT were matched with patients who did not receive RT based upon clinical and pathological parameters including Gleason score, PSA level, surgical margin status, extracapsular extension, seminal vesicle invasion, lymph node invasion, and androgen-deprivation therapy. In the training cohort, a 24-gene Post-Operative Radiation Therapy Outcomes Score (PORTOS) was used to predict response to post-operative RT. The 24-gene set included 6 genes related only to DNA-damage response, 4 genes related to both DNA-damage and radiation response, and several genes involved in immune response (including *IL1B*, *IL7R*, *PTPN22*, and *HCLS1*). The primary endpoint was development of distant metastasis. They used high-throughput gene expression and clinical data to develop and validate 24-gene expression signature that predicts response to post-prostatectomy RT (PORTOS) in matched training and validation cohorts of patients with prostate cancer. They show that, in patients receiving RT, patients with high levels of 24-gene expression had a lower incidence of distant metastasis than in patients with low scores. In the same study, the new signature score PORTOS was compared to the predictive value of the already standardized methods of prediction Decipher, CAPRA-S, and microarray version of the cell cycle progression (CCP) signature. Decipher is a genomic test, which evaluates the activity of genes in the tumor that are shown to be involved in the development and progression of prostate cancer. In details, Decipher measures the expression levels of 22 RNA biomarkers involved in multiple biological pathways across the genome that is associated with aggressive prostate cancer. CAPRA-S is a straightforward instrument for
facilitating disease risk classification. A CAPRA score is valid across multiple treatment approaches and predicts an individual’s likelihood of metastasis, cancer-specific mortality, and overall mortality. The score is calculated using points assigned to: age at diagnosis, PSA at diagnosis, Gleason score of the biopsy, clinical stage and percent of biopsy cores involved with cancer (again clinical and pathological features of the tumor). Genes whose expression is regulated as a function of CCP were originally identified as having RNA expression levels that oscillated as cells progressed through various stages of the cell cycle. Since the expression levels of CCP genes probably reflect fundamental aspects of tumor biology, 31 CCP genes were selected and tested for their ability to predict disease outcome using a predefined score based on their expression levels. Using the median score as the cutoff point, the interactions between the Decipher, mCCP, and CAPRA-S prognostic models with RT were not significant (interaction between RT and score, Decipher P interaction =0.99, mCCP P interaction =0.34, CAPRA-S P interaction =0.34). In details, patients with high Decipher, mCCP, or CAPRA-S scores do worse than do those with a low score regardless of treatment, and patients treated with RT have improved outcomes regardless of risk score. In conclusion in this interesting report, in comparison to PORTOS, the widely used genomic and clinical risk tools Decipher, mCCP, and CAPRA-S did not predict response to post-operative RT. However, a combination of Decipher and PORTOS could allow for selection of patients who need post-operative RT (using PORTOS), and help decide whether to irradiate in the adjuvant or salvage setting (using Decipher). These evaluations were not conducted in routine clinical settings. No evidence was identified to address the question of clinical utility. Future research should focus on evaluating clinical validity more extensively and robustly in the general clinical populations, and on comparing PORTOS panel directly with the existing standard care and diagnostic standards. In addition, emphasis should be given to the finding of new circulating genetic biomarkers that can be easily assessed through not invasive procedures in prostate cancer patients. In these lights, circulating micro-RNAs and circulating tumor DNA (ctDNA) were recently investigated. The following circulating miRNAs were found to be associated to the development and progression of prostate cancer. miR-21 expression increases together with clinical parameters (such as Gleason score or lymph node metastases) and is correlated with castration resistance and metastatic disease. MiR-21 and miR-18 are also useful as biomarkers in prediction of progression of prostate cancer. Another oncogenic miRNA overexpressed in prostate cancer and positively correlated with poor overall and PSA recurrence free survival, is miR-4534. It is hypermethylated in normal cells and tissues compared to those of prostate cancer and exert its oncogenic effects partly by downregulating the tumor suppressor PTEN gene. Its overexpression induces pro-cancerous characteristics in non-cancer cell line whereas its knockdown impair cell proliferation, migration/invasion and induce G0/G1 cell cycle arrest and apoptosis in prostate cancer. MiR-32 is highly expressed in castration resistant prostate cancer (CRPC) samples compared to benign prostatic hyperplasia samples. The reduction of miR-145 expression in prostate cancer was correlated with higher Gleason scores, advanced clinical stage, larger tumor diameter and higher PSA and follow-up PSA levels. miRNAs are important modulators of gene expression. They are frequently altered in prostate cancer and as such offer the potential to be used as biomarkers or novel therapeutic targets. However, none of them are still validated for clinical use. Regarding the cell-free ctDNA and circulating tumor cells (CTCs) are plasma sources of tumor DNA that have been investigated for non-invasive detection and monitoring of patient tumors but have not been analyzed or directly compared across multiple tumor types. ctDNA liquid biopsy allows to understand specifically what kind of molecular changes are happening in the tumor in real time, which is a very big step beyond where CTCs are today in clinical terms. Perhaps the most promising applications of CTCs and ctDNA are molecular analyses that can inform the rational selection of appropriate therapies for patients. In example, in the treatment of a patient with prostate cancer, alterations in the androgen receptor variant 7 mutation, detected in CTCs, predicts the lack of response to abiraterone or enzalutamide and can be useful to provide the most immediately actionable information regarding the choice between AR-targeted therapies or non-AR-targeted therapies such as cytotoxic chemotherapy (for a summary of the scores available for the prediction of response in prostate cancer see Figure 1) (23). The use of this information in real time can guide the clinicians in the choice of the best-personalized therapy in the case of androgen depletion therapy. It has also to be considered that PORTOS diagnostic system can open a new scenario of investigations coming back to the bench in order to study if any of the evaluated genes can be efficiently assessed directly in the blood of the patients and if some mutations can be revealed that correlate to the response to
post-operative RT, for which the information is still limited. Another chance given by the authors of the manuscript by Zhao et al. is to integrate PORTOS score with other circulating miRNAs and/or ctDNA and/or CTCs in order to increase the prognostic accuracy in the same set of patients.

In conclusion, the manuscript by Zhao et al. disclosed the possibility to study genetic scores of the prostate cancer that correlates to the response to post-operative RT independently from the conventional clinical and pathological features of the disease and strongly encourages additional studies on new intratumor or circulating biomarkers in this subset of patients.

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References


Clinically significant prostate cancers have been rendered detectable with the advent of multi-parametric magnetic resonance imaging (mpMRI) (1). Diagnosis and spatial localization of these lesions is important in their management and/or active surveillance (2). In-bore MRI-guided biopsy (MRGB) can be performed with mpMRI localization (3), although the procedure can be difficult and time-consuming, and is not considered routine for several reasons. First, the biopsy takes at least 30 minutes, a long time for patients to lie prone. Second, MRI-safe biopsy devices are very expensive.

In this issue, Venderink et al. show the usefulness of the Prostate Imaging and Reporting and Data System (PI-RADS) for the classification of lesions, including significant cancers, although PI-RADS had changed from version 1 to version 2 during the study period (3). They did show that the combined use of PI-RADS and prostate-specific antigen (PSA) levels made it possible to avoid unnecessary biopsies (3). These findings would contribute to detect the significant cancer with minimum time of prostate biopsy.

A limitation of the study was that systematic biopsy was not performed in the patients, and >70% of the patients did not have follow-up histology, PSA levels, or mpMRI examinations (3). In a previous study, the detection rate of higher grade cancers [Gleason score (GS) ≥7] with systematic biopsy, excluding ROIs designating known suspicious lesions on mpMRI, was 11%, and the authors cautioned against using mpMRI alone for risk stratification because of this (4). In another study, GS concordance rates between targeted prostate biopsies and radical prostatectomy specimens were: 63%; systematic: 54%; and combined targeted + systematic: 75%; they concluded that the combined approach best predicts the highest tumor grade (5). Based on these results, follow-up information, and the comparison of the pathological findings between biopsy results and whole-gland specimens (a surrogate for systematic biopsy) would be required to evaluate the usefulness of MRGB for the detection of significant cancers.

Recently, MRI-transrectal ultrasound (TRUS) fusion image-guided prostate biopsy has become more widespread due to its ability to detect significant cancers (6). In this method, systematic biopsy is generally performed in addition to targeted biopsy (6). In the present economic situation, the MRI-TRUS fusion image-guided biopsy is more common than MRGB. MR images are the result of just as much software manipulation. Also, a fused image contains the MR image, plus an ultrasound image. Although one could question the accuracy of their superimposition, each image is the product of a different modality and is subject to that modality’s inaccuracies. Multi-parametric ultrasound (mpUS), which includes grayscale, Doppler, dynamic contrast-enhanced, and elastographic imaging, has been widely used to guide prostate cancer biopsies. Using mpUS, the cancer detection rate was improved over that with grayscale only (7,8). Beyond the reproducibility of mpUS, real-time image-guided biopsies are generally easier...
to perform, and may become the major biopsy procedure. Accurate localization, measurement, and Gleason scoring of significant cancers with imaging would enable tailored treatment of localized prostate cancer from active surveillance to radical treatment.

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Prostate cancer (PCa), the most common, non-cutaneous male malignancy (1), is primarily driven by androgen signaling. Thus, clinical management of the disseminated disease is dominated by ever-improving androgen receptor (AR) pathway inhibitors (ARPIs) that have contributed to 30–40% decline in disease-specific mortality observed over the last two decades (2). Nonetheless nearly all ARPI therapy patients eventually develop resistance to these agents (2,3), calling for the development of novel targeted therapeutics for additional molecular lesions in PCa.

Although confounded by disease heterogeneity, PCas harbor a number of specific genomic alterations linked to the disease occurrence, progression, and outcome (1,3,4). Some of the most prevalent lesions correlated with metastatic castration-resistant PCa (mCRPC) include: AR overexpression and mutations, loss/mutations of key tumor suppressors [including TP53, RB and phosphatase tensin homologue (PTEN) among others], and prominent gene fusions that direct aberrant expression of members of the E26 transformation-specific (ETS) family, such as TMPRSS2-ERG (5,6). These fusion events have been observed in approximately half of PCas, and represent the most common PCa-associated abnormality documented to date (7).

ERG is one of several members of the ETS transcription factor family known to have oncogenic potential (8,9) and the fusion of androgen-responsive elements of TMPRSS2 with open reading frame sequences of ERG (TMMPRSS2-ERG) directs aberrant AR-driven ERG expression (1). It is broadly accepted that TMPRSS2-ERG rearrangements represent early events in PCa initiation and are strongly associated with higher Gleason score, aggressive disease, and poor prognosis due to activation of aberrant ERG-driven transcriptional programs that promote migration, invasion and epithelial-mesenchymal transition (10). Importantly, ERG expression has been shown to persist during disease progression and to regulate taxane sensitivity in PCa. Thus, it is expected that therapeutic targeting of ERG could have immense clinical significance (11).

While TMPRSS2-ERG diagnostic and prognostic methods undergo very active development, there are yet no approved ERG-directed therapeutics. The absence of agents targeting any ETS factors makes developing therapeutics targeting these major oncoproteins a critical step towards new therapeutics for PCa and other ETS factor-driven malignancies. With urine tests available to detect the TMPRSS2-ERG fusion event (12,13), the development of ERG-targeted drugs would offer a specific ‘precision medicine’ approach for PCa patients. Here we discuss the recent report by Wang et al. “Development of Peptidomimetic Inhibitors of the ERG Gene Fusion Product in Prostate Cancer” (14), and that effort to develop such needed ERG-targeted therapy.

Using a phage display random peptide library screen, Wang et al. identified ERG inhibitory peptides (EIP) that bound directly to the DNA binding (ETS)-
domain of ERG and disrupt ERG-ETS domain/DNA interactions. Mutagenesis of the ETS domain and peptides demonstrated reciprocal requirements for the selective affinity. Furthermore, cell permeable peptides prepared via conjugation of HIV-TAT sequence to EIPs retained ERG-ETS affinity, exhibited nuclear co-localization with ERG, and blocked invasive properties of ERG-expressing PCa models. While retro-inverso (RI) EIP versions exhibited no significant effect on angiogenesis in several models, they promoted ERG degradation, decreased ERG target gene expression, offered improved stability when delivered via intraperitoneal administration, and demonstrated inhibition of tumor growth and metastasis.

The results of this study represent a significant step towards the development of an ERG-targeted therapeutic and bolster the ever-increasing recognition of the importance of persistent ERG expression in TMPRSS2-ERG PCas. The lack of overt murine toxicity of the developed candidates is encouraging; however, there is a need to characterize the affinity of these peptides with other ETS family members since these genes are involved in maintenance and oncogenesis in several tissue types and target selective peptidomimetic agents would undoubtedly be of value. The details of molecular interactions between the developed EIPs and ERG-ETS target as well as the specifics of the competition with DNA binding remain to be described. For the latter, as for any mutational efficacy study, the lack of observed activity in the binding assay needs to be considered with respect to differential domain folding, or indirect allosteric changes to the protein structure. Finally, ERG is a key regulator of fate determination and differentiation of several tissues, including chondrogenesis (15), hematopoiesis (10) and, as tested by Wang et al., endothelial development. It is perplexing that a potent ERG antagonist would not exhibit an impact on the array of angiogenic assays performed.

As has been previously reviewed (16,17), peptidomimetics have several advantages and disadvantages in their use as therapeutic agents. While complexity of peptide-based therapeutics affords their high target affinity and specificity, as well as generally low side effect and toxicity, the important issues of tissue accumulation of the corresponding drug candidates, their metabolic stability and solubility, membrane permeability and delivery obstacles, along with rapid clearance and high cost of development, represent well-known drawbacks for their clinical development (16). With that being said, it is important to stipulate that the use of peptides as ERG-directed therapeutic agents represents an exciting avenue for PCa treatment and that result by Wang et al. provide an important stepping stone for overcoming limitations associated with the use of peptides as therapeutic agents.

On another hand, it should also be noted that there are concurrent efforts to develop small molecule ERG antagonists as a more clinically viable alternative for peptide-based agents. The first reported small molecule ERG inhibitor YK-4-279 was initially discovered as an antagonist for FLI1 protein, a close homologue of ERG and a known oncogene implicated in Ewing’s sarcoma (18-21). The pre-clinical development of its derivative TK216 is currently in phase 1 trial (ClinicalTrials.gov Identifier: NCT02657005) (22) that is expected to significantly impact the future development of ETS targeted therapies.

Other recent efforts to directly target ERG protein with small molecules include rational computer-aided discovery of a compound VPC-18005. It has been shown that this compound directly binds the ERG-ETS domain and suppresses ERG transcriptional activity at low micromolar concentrations, while it is also capable of suppressing metastatic potential of ERG-expressing PCa cells (23). Other small molecules include ERGi-USU, a small molecule that can selectively suppress growth of ERG-expressing cancer cells (24), and heterocyclic dithiophene diamidines that target the ETS consensus DNA motif to block ERG-DNA interactions (25).

To conclude, it is important to outline, that therapeutic targeting of ERG, as well as other oncogenic ETS family members represents a promising avenue for the development of novel precision oncology strategies. It is anticipated that an entirely novel class of ERG inhibitors (whether peptide- or small molecule-based) are urgently needed and can be used as alternative or complimentary agents for the current ARPIs and chemotherapeutics to treat PCa even in its most deadly resistant and metastatic form.

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**Footnote**

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References


Renal cell carcinoma (RCC) arise from the tubules of the nephron and account for over 90% of kidney cancers. The most common RCC subtypes are clear cell (cc)RCC (70–75% of cases), papillary (p)RCC (10–15% of cases), and chromophobe (ch)RCC (5% of cases). Other less frequent subtypes include oncocytomas and carcinosarcoma of the collecting duct (1). Patients with stage I or II (localized) RCC have a >70% 5-year survival rate following radical or partial nephrectomy, however prognosis is poor in patients with stage III (regional spread) or IV metastatic (m)RCC. mRCC is resistant to chemo- and radio-therapies and virtually incurable (1).

Genome-wide association studies (GWAS) have identified components of the VHL-HIF pathway as major drivers of ccRCC pathogenesis. Under normoxic conditions, hydroxylation of HIF-1α at two conserved proline residues facilitates VHL binding and subsequent cullin-RING ligase (CRL)-type E3 ligase complex-mediated degradation by the ubiquitin-mediated proteasomal pathway. However, under hypoxia, HIF-1α degradation is blocked by the failure of non-hydroxylated HIF-1α to bind VHL, and thus interact with the CRL-type E3 ligase complex. HIF-1α then forms a transcriptional activator complex with HIF-1β, promoting target genes expression relevant to angiogenesis, glycolysis, cell proliferation, invasion and metastasis (2). Loss-of-

Small molecule inhibition of speckle-type POZ protein-substrate interactions for the treatment of renal cell carcinoma

Byung Joon Hwang¹, Yun Kee²

¹Department of Molecular Bioscience, ²Division of Biomedical Convergence, College of Biomedical Science, Kangwon National University, Chuncheon, Kangwon-do, Republic of Korea

Correspondence to: Byung Joon Hwang, PhD. Department of Molecular Bioscience, College of Biomedical Science, Kangwon National University, Chuncheon, Kangwon-do, 24341, Republic of Korea. Email: bjhwang@kangwon.ac.kr.

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Abstract: Renal cell carcinoma (RCC) is the most common type of kidney cancer and is highly resistant to therapy, clear cell (cc)RCC accounts for 70–75% of cases. Current treatment options include high-dose interleukin-2 (IL-2), and inhibitors of mTOR and HIF-1 downstream signaling. Recently, speckle-type POZ protein (SPOP) has emerged as a promising therapeutic candidate for ccRCC treatment. SPOP is a subunit of the cullin-RING ligase (CRL)-type E3 ligase complex that plays important roles in regulating cell death and proliferation. In 99% of ccRCC tumors, SPOP is overexpressed and mislocalized to the cytoplasm where it acts to lower levels of tumour suppressor genes such as PTEN and DUSP7 by targeting them for ubiquitin-mediated proteasomal degradation. Guo et al. have reported the identification of small-molecule inhibitors that block SPOP-substrate interactions, preventing SPOP-mediated ubiquitination and degradation of PTEN and DUSP7, and suppressing the growth of ccRCC cancer cells in vitro and tumor growth in vivo. These data suggest that therapeutic targeting of SPOP may provide new opportunities for the treatment of patients with ccRCC.

Keywords: Speckle-type POZ protein (SPOP); renal cell carcinoma (RCC); small-molecule inhibitor; E3 ubiquitin ligase; targeted protein degradation

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Renal cell carcinoma (RCC)

RCC arise from the tubules of the nephron and account for over 90% of kidney cancers. The most common RCC subtypes are clear cell (cc)RCC (70–75% of cases), papillary (p)RCC (10–15% of cases), and chromophobe (ch)RCC (5% of cases). Other less frequent subtypes include oncocytomas and carcinomas of the collecting duct (1). Patients with stage I or II (localized) RCC have a >70% 5-year survival rate following radical or partial nephrectomy, however prognosis is poor in patients with stage III (regional spread) or IV metastatic (m)RCC. mRCC is resistant to chemotherapy and radio-therapies and virtually incurable (1).
function VHL mutations are common in ccRCC and lead to hypoxia-independent stabilization of HIF-1α and enhanced expression of downstream target genes including vascular endothelial growth factor (VEGF) A, glucose transporter (GLUT) 1, epidermal growth factor receptor (EGFR), and platelet-derived growth factor (PDGF) B (1-4).

Epigenetic regulators are the second most commonly mutated genes in ccRCC, these include PBRM1 (SW1/SNF chromatin remodeling gene), SETD2 (histone H3K36 methyltransferase), and BAP1 (BRCA1-interacting deubiquitinase). It is not clear how mutations in these epigenetic regulators drive the development of ccRCC, but breakdown of genomic stability appears to be involved in the process (1).

The PI3K-AKT-mTOR signaling pathway is a key regulator of cell growth and proliferation and PI3K associated genes are commonly overexpressed or mutated in many types of cancer, including ccRCC. The tumor suppressor, PTEN, acts as a negative upstream regulator of PI3K, and PTEN deficiency and activation of AKT are associated with poor cancer prognosis. The mTOR pathway acts as an upstream translational activator of HIF-1α (1).

Several drugs have been approved for treating patients with ccRCC, including high-dose interleukin-2 (IL-2), anti-programmed cell death protein 1 (PD1) antibody (nivolumab), mTOR inhibitors (everolimus, temsirolimus), and small molecule/antibody inhibitors of the VEGF and PDGF β pathways (axitinib, bevacizumab, cabozantinib, lenvatinib, pazopanib, sunitinib and sorafenib). Although drugs targeting mTOR and VEGF/PDGF β pathways show better therapeutic responses than conventional IL-2 therapy, it remains important to continue improving patient outcomes by developing new combinations of existing treatments and identifying new targets for drug development.

**Targeted protein degradation**

The selective promotion of specific protein degradation using degron technology may have therapeutic potential (5,6). In this methodology, target proteins are linked to a destabilization domain (DD) and subsequent exposure to small molecules or light promotes the degradation of the target-DD fusion proteins by the ubiquitin-mediated proteasome pathway (7-12). Although degron technology has many diverse chemical biology applications, the requirement to molecularly engineer target proteins has so far limited the development of therapeutic reagents.

To circumvent the limitations of degron, several groups have developed small molecules, peptides, and proteins that promote the selective degradation of target proteins without prior molecular engineering. Small molecules such as protein-targeting chimeric molecule 1 and phthalimide-conjugated compounds simultaneously bind to both E3 ligase and a target protein, inducing target polyubiquitylation and subsequent degradation by the 26S proteasome (13,14). Similarly, a synthetic death associated protein kinase (DAPK)-binding peptide containing a chaperone-mediated autophagy-targeting motif was shown to promote lysosomal DAPK degradation (15). E3 ligases can also be engineered to selectively bind target proteins, resulting in specific target degradation by the ubiquitin-proteasome pathway (16,17). Synthetic E3 ligases have been developed by the fusion of a target protein-specific nanobody to a truncated form of E3 ligase, in which the substrate-recognition domain was deleted. In the case of the Ab-speckle-type POZ protein (SPOP) synthetic ligase, target proteins were depleted in the cell nucleus, but not in the cytoplasm, and protein degradation occurred more efficiently than treating cells with the corresponding siRNA (17).

Several groups have developed small molecules that prevent interactions between E3 ligase and its substrate, inhibiting protein degradation. The interaction between p53 tumor suppressor and MDM2 E3 ligase for example, has been inhibited by synthetic small molecules (chalcone derivatives and some polycyclic compounds), chlorofusin (a fungal metabolite), and by synthetic peptides (18). Similarly, the small molecule inhibitor MLN4924 prevents the ubiquitin-like polypeptide NEDD8 from activating E3 CRLs (19). CRL-dependent protein degradation is selectively inhibited by MLN4924, leading to the death of human tumor cells (20).

Virtual drug design/screen identified a small molecule that inhibits the interaction between Skp2, a substrate-binding subunit of SCF E3 ligase, and p27, a tumor suppressor that acts as an inhibitor of cell cycle dependent kinase (CDK) (21). Since Skp2 is overexpressed in several cancers, particularly those with poor prognosis and highly metastatic, Skp2 inhibitors could restrict cancer stemness and potentiate sensitivity to chemotherapeutic agents (6).

**SPOP and RCC**

Ubiquitylation of a specific protein is achieved by the sequential activity of three different classes of enzymes
(22,23). Firstly, ubiquitin-activating enzyme (E1) activates the C-terminal carboxyl group of Ub to form a thioester linkage with the active-site cysteine of E1. Next, ubiquitin-conjugating enzyme (E2) transfers the Ub molecule from E1 to its own active-site cysteine. Finally, ubiquitin-ligating enzyme (E3) binds to both E2 and the target protein, bringing the target close to the E2 enzyme. Thus, E3 helps E2 transfer Ub from its charged cysteine to the lysine amino group of the target protein. Subsequently, proteins tagged with polyubiquitin chains are degraded by the proteasome (24,25).

In humans, there are two E1 genes, about 40 E2s, and more than 600 E3s (26,27). Thus, the fate of most intracellular proteins is determined by temporal and spatial expression patterns, intracellular localization, and substrate specificities of E3 ligases (26,28). There are around 30 HECT domain E3 ligases, in which active-site cysteine forms an Ub-thioester intermediate during the transfer of Ub from E2 to substrate (29,30). The vast majority of E3 ligases belong to the group of RING and RING-related E3s that serve as a scaffold to bring E2 and substrate together, enabling the direct transfer of Ub from E2 to substrate (22,28). CRLs form a prominent subclass of RING-type E3 ligases, they consist of cullin (CUL) isoforms, RING-BOX (RBX)-containing proteins, and various adaptor-substrate recognition proteins that bind to a variety of substrates for ubiquitylation (31,32). NEDD8-activating enzyme (NAE) inhibitor studies suggest that at least 20% of all proteasome-mediated protein degradation is CRL-dependent (20).

Structural analysis indicates that all CRLs share a common elongated molecular architecture: CUL is bound to RBX at the C-terminal domain, and to an adaptor protein at the N-terminal domain. Interaction with target proteins usually occurs via a separate substrate-recognition protein that binds to the adaptor protein, the exception being CUL3, where both substrate-recognition and adaptor proteins are merged in a single “broad complex-tramtrack-bric-a-brac” (BTB)-domain-containing polypeptide (33-35).

SPOP is a subunit of the CRL-type E3 ligase complex, containing domains for substrate-recognition (MATH) and CUL3-binding (BTB-3-box) in single polypeptide (34). In normal cells, SPOP is localized in the nucleus through a nuclear localization signal at its C-terminus (36). The BTB domain is involved in homo- or hetrodimerization with other BTB-containing proteins, which is necessary for its E3 ligase activity (17,34,37). The C. elegans SPOP ortholog, MEL-26, degrades MEI-1 to promote the meiotic-to-mitotic transition (38), and in Drosophila, SPOP degrades Cubitus interruptus (Gli transcription factor) and Puckered (JNK phosphatase) to regulate Hedgehog (Hh) and tumor necrosis factor (TNF) pathways (39,40). In addition to its conserved role in Hh and TNF pathways, human SPOP plays important roles in cell death and proliferation, as well as epigenetic regulation by degrading several proteins including death-domain-associated protein (Daxx) (41), the Polycomb group protein BMI1 (42), the variant histone MACROH2A (42), the proto-oncogene DEK (43), tripartite motif-containing (TRIM) 24 (44), and Nuclear receptor coactivator (NCOA) 3 (45). Wild-type SPOP also appears to enhance homology-directed DNA repair (HDR) presumably by degrading an unidentified substrate (46). Thus, when SPOP is mutated, non-homologous end joining predominates, resulting in more genomic errors and rearrangements (47).

SPOP is mutated in 8% to 14% of prostate and endometrial cancers; in prostate cancer, the mutations are confined to the substrate-binding MATH domain, suggesting that the mutations affect its interaction with substrates (48,49). An ubiquitylome analysis measuring the interactions between the mutated SPOP proteins and their interacting proteins showed that SPOP mutations found in prostate cancer caused a dominant negative effect (repressing the function of the wild-type SPOP), and did not result in a gain-of-function effect (increasing the binding affinity for the same substrates), nor a neomorphic effect (binding to new substrates) (37). Since SPOP homodimer, not monomer, ubiquitylated substrates (17,34), SPOP mutations that form heterozygous dimers with the wild-type allele are able to decrease the ubiquitylation and subsequent degradation of substrates in a dominant-negative manner.

Tissue microarray screening has shown that SPOP is overexpressed, without mutation, in kidney cancer (85%, 17/20), uterus/endometrial cancer (71%, 10/14), and testis/germ cell cancer (90%, 18/20) (40). In corresponding normal tissues, SPOP was expressed very weakly or not at all. When expression levels were measured in different types of RCC, most ccRCC cells overexpressed SPOP protein (179 positive and 1 negative), but penetrance was low in other types of RCC cells (40), suggesting that overexpressed SPOP could drive the pathogenic development of ccRCC. In normal kidney cells and non-ccRCC cell lines, SPOP is predominantly localized within the nucleus, however in ccRCC, SPOP accumulates predominantly in the cytosol (50). Cytoplasmic SPOP appears to promote tumorigenesis by degrading DAXX, Gli2, and other targets including the...
tumor suppressors, PTEN and DUSP7 (an ERK-specific MAPK phosphatase that acts as a negative regulator of the ERK pathway) (50). Under hypoxic conditions, HIF proteins directly activate SPOP transcription and SPOP protein accumulates in the cytosol (50). Since hypoxic stress and HIFs play important roles in a wide range of tumors, the mechanisms permitting targeted overexpression and mislocalization of SPOP in particular cell types, such as RCC, endometrial and germ cell tumor cells should be the focus of future research.

The work of Guo et al. is based on a virtual drug design/screening and has led to the development of small molecules that inhibit the interaction between SPOP E3 ligase and tumor suppressors, including PTEN and DUSP7 (51). Using a computational screen that combined pharmacophore modeling, molecular docking, and chemical scaffold diversification, 109 small-molecule candidates predicted to inhibit the interaction between SPOP and a peptide containing a SPOP binding consensus (SBC) were identified. From these candidates, the small molecules 6a (initial-hit compound, $K_D$: 62 µM) and 6b (lead compound after synthetic optimization, $K_D$: 35 µM) were confirmed to physically interact with SPOP and PTEN in vitro and inhibit the proliferation of the A498 ccRCC cell line.

Several assays were performed to assess the suitability of compound 6b as a potential therapeutic reagent. Circular dichroism (CD) spectra analysis suggests that 6b does not dramatically perturb the structure of SPOP, and HPLC analysis has confirmed its purity. Dynamic light scattering showed that 6b is highly soluble in cellular assay media, does not aggregate to form particles, is not itself fluorescent, has excellent cellular permeability, and accumulates rapidly inside cells. Compound 6b directly binds to SPOP in vitro, as indicated by surface plasmon resonance and NMR techniques, and in vivo cellular thermal shift assay. Binding to SPOP inhibits not only ubiquitylation and subsequent degradation of the tumor suppressors, PTEN and DUSP7, but also the proliferation of ccRCC cell lines and primary human ccRCC cells. Evidence supporting the specific targeting of SPOP in ccRCC by 6b is provided by experiments using ccRCC lines treated with shRNA to reduce SPOP expression; in these modified cells 6b does not inhibit cellular proliferation. Finally, 6b inhibits the growth of A498 tumor cell xenografts in nude mice, without histological changes in multiple organs except kidney. Combined with the very low level of toxicity to mice (toxicity was not observed by daily treatment of 120 mg/kg for 6 days), 6b appears to be a promising therapeutic reagent for treating patients with ccRCC (51).

Ubiquitin-mediated protein degradation and modification pathways play major regulatory roles in maintaining genome integrity, gene expression, and various cellular processes including cell cycle, death, differentiation, proliferation, and signaling (27,28). E3 ligases are implicated in a number of disease pathologies, making them attractive therapeutic targets (52-55). Ubiquitin-mediated pathways are regulated by the selective binding of over 600 E3 ligases to a variety of intracellular proteins. Of over 600 E3 ligases in the human genome, physiological roles are understood for only a small number. Thus, as shown in the case of SPOP, systematic efforts are necessary to identify E3 ligase target proteins, determine their expression patterns in normal and pathological cells and tissues, and correlate their distribution with that of target proteins, providing opportunities to develop new targeted therapeutics.

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Footnote

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5. Crunkhorn S. Anticancer drugs: Selectively targeting


We read with great interest the paper entitled “Adjuvant Treatment for High-Risk Clear Cell Renal Cancer: Updated Results of a High-Risk Subset of the ASSURE Randomized Trial” by Haas et al. (1). Last year, the same group published the results of a randomized phase III trial ASSURE comparing 1-year treatment with sorafenib (400 mg twice daily), sunitinib (50 mg/day for 4 weeks of every 6 weeks), or placebo as adjuvant therapies for patients with completely resected renal cell carcinoma (RCC), without reporting significant improvements of the disease-free survival (DFS) in the study arms (2). More recently, Ravaud and his group firstly showed the results of a 750-patient randomized study, S-TRAC (3), (sunitinib 50 mg daily with 4/2 schedule vs. placebo in clear cell RCC predominant pT3-4 or node-positive disease). The authors showed an advantage in terms of DFS of 1.2 years (6.8 vs. 5.6 years) in this population of patients with high-risk of recurrence treated with sunitinib as adjuvant therapy compared to placebo, without mature results on overall survival (OS) at time of data cut-off (3).

Based on these new evidences, Haas et al. performed an updated analysis (1) of data from the Assure trial (2) focusing on the high-risk population. This subgroup was composed by patients with pT3 and higher stage (with tumor growing into a major vein, such as the renal vein or the vena cava, or into tissue around the kidney, but not invading the adrenal gland or overcoming beyond Gerota’s fascia, Figure 1) or node-positivity. Differently from S-TRAC study (3), they did not find a significant benefit in terms of DFS (5-year rates were 47.7%, 49.9%, and 50.0% for sunitinib, sorafenib and placebo, respectively) or OS (5-year rates: 75.2%, 80.2% and 76.5%) by treating patients with anti-vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitors (TKIs) compared to placebo (1).

So how can we explain the different results obtained by these two studies? Should we treat or not patients with high-risk of recurrent RCC after nephrectomy with sunitinib or do we need more data to better identify the subpopulation of patients who will certainly benefit from this approach? Firstly we can start analysing this questions underling the different population enrolled in these two studies. Indeed, the ASSURE trial included also patients with non-clear cell histology, who represented more than 20% of the whole study accrual (1) and were excluded from the S-TRAC trial (3). Moreover, risk assessment was calculated following AJCC indications (1), while in the S-TRAC study it was used the UISS system (3). In addition, only 67.7% of patients started at full sunitinib dose in the study by Haas et al. (1) and it was followed to reduce the dose till a minimum of 25 mg, while Ravaud et al. (3) included only patients treated at the beginning with 50 mg of sunitinib, with a maximum reduction allowed till 37.5 mg.

The study by Haas et al. also investigated the impact of receiving higher or reduced doses of sunitinib or sorafenib...
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in terms of patients’ outcome. They reported that starting dose as well as dose reductions were associated with a DFS that didn’t differ from that registered by patients treated with higher doses. These data are consistent with those published by Iacovelli et al. (4), who revealed that toxicity-related dose reductions in 591 patients treated with first-line sunitinib or pazopanib were correlated with longer OS and with a better outcome with second-line treatments (4).

Another very interesting topic is represented by the responsiveness to target agents or immunotherapies of patients with recurrent disease following adjuvant therapy. What we still don’t know is the complex series of changes caused in the tumor microenvironment by adjuvant therapy, which is aimed to prolong the time from nephrectomy to tumor recurrence. In this view, it is important to consider the results published by our group in 2014 focused on the biological features of patients with metastatic RCC relapsed >5 years from nephrectomy (5-7). We showed that this group of patients with a long DFS presented a different pattern of metastatic spread, involving unusual site of metastases, such as stomach and glands (5,6), and were particularly responsive to first-line sorafenib, sunitinib or pazopanib without significant differences (5,6). In late-relapsing patients, inflammation resulted highly prognostic, with patients with higher neutrophil to lymphocyte ratio (NLR) associated with shorter PFS and OS compared to patients with lower NLR (7). Taken together, these data underline the particular biological features that characterize patients with prolonged DFS, thus suggesting that patients with increased DFS due to adjuvant therapy should be carefully studied in order to optimize the diagnosis and to select to most potentially effective strategies at tumor recurrence. At this regard, a phase II study (NCT01649180, NEXT, PrE0801) was planned to assess the efficacy of anti-VEGFR TKI axitinib at recurrence after adjuvant therapy in RCC.

Based on the results obtained by Nivolumab in patients with metastatic RCC (8,9), the use of immune checkpoint inhibitors in the adjuvant setting should be carefully evaluated. A reason to be potentially optimistic is the ability of anti-PD-1 and anti-PD-L1 inhibitors to shape memory phenotype CD8 T cell subsets (10). At present, four different phase III studies are investigating the efficacy and tolerability of Pembrolizumab (NCT03142334, KEYNOTE-564), Atezolizumab (NCT03024996, IMmotion010), Durvalumab alone or with anti-CTLA-4 tremelimumab (NCT03288532) and the combination of Nivolumab and Ipilimumab (NCT03138512, CheckMate 914) as adjuvant therapy in patients with RCC. These studies are actively enrolling at this time and the results are awaited in the next 5 years. An evolution of PD-1/PD-L1 approach is under evaluation in the URroRCC study (NCT02429440), a phase I/II trial that will test the efficacy and safety of intradermal application of adjuvant peptide vaccine (developed by using tumor associated peptides) in combination with either granulocyte macrophage colony stimulating factor (GM-CSF) or Montanide ISA-51 in patients with clear cell and not-clear cell RCC histology.

In conclusion, the results of this sub-analysis of ASSURE trial focused on high-risk RCC patients underline the necessity of more selective criteria in the adjuvant setting, not only based on tumor staging but also on tumor biological and molecular features. This is absolutely required in order to carry the adjuvant approach for RCC patients into the era of personalized and precision medicine.

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Footnote

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Lu et al. (1) introduce a novel chimeric model of prostate cancer to interrogate the tumor microenvironment and signaling pathways in response to single targeting agents given alone or in combination with checkpoint inhibitors. This provides new preclinical data that reinforces clinical observations that combinatorial approaches for treating metastatic castration-resistant prostate cancer (mCRPC) may be more beneficial when compared with monotherapies (Figure 1). It also provides a rationale for the combination of biologic receptor and pathway targeting agents with the family of different checkpoint inhibitors. While the first demonstration of survival benefit by an immunotherapy for a solid tumor was in patients with minimally symptomatic or asymptomatic CRPC using an autologous dendritic cell product, sipuleucel-T (Provenge®) (3), immunotherapeutic approaches with single agent peptide or DNA vaccines using novel viral platforms, CAR T cells, and checkpoint inhibitors have all shown limited or minimal impact on the disease.

To date, it remains unclear as to the rationale for the suboptimal responses to checkpoint inhibitors in prostate cancer. Studies have suggested prostate cancer is not a hypermutated disease (4) compared with other genitourinary malignancies such as bladder and renal cancers, however, others have postulated that the absence of or lack of expression of PD1, PD-L1, or polymorphisms in molecules such as CTLA-4 may have some indistinct role. Despite these negative results, there are prostate cancer patients who have had dramatic, durable responses following treatment with ipilimumab alone or in combination with radiation therapy (5-7). Graff et al. (8) reported results from a pilot trial of prostate cancer patients with late disease and significant tumor burden demonstrating several dramatic responses when pembrolizumab was administered post enzalutamide failure, suggesting that the preclinical observations of enzalutamide (9) as an immune modulator may in fact be contributing to the response. The variations in responses to checkpoint inhibitors have now shown that not all cancers respond equally to the same checkpoint inhibitor and that a particular cancer may have unique responsiveness to a specific checkpoint drug.

In the “Letter” published by Lu et al. (1), the authors present data to confirm their hypothesis that a combination of immune checkpoint agents together with a targeted agent could affect myeloid-derived suppressor cells (MDSCs) in the setting of preserving normal T cell function. MDSCs contribute to an immune suppressive environment and have been implicated in cancer progression. However, how MDSCs respond to treatment and their role in providing a mechanism by the tumor microenvironment can be positively or negative influenced have not been completely studied. Clinically, MDSCs have been studied as potential biomarkers to assess response to treatment as well as disease progression. Preliminary retrospective data presented by Autio et al. (10) used a novel platform for a biomarker based assay in whole blood that enumerated MDSC from 36 patients with either metastatic castration sensitive prostate cancer (CSPC) and mCRPC. The results did not confirm any impact of chemotherapy on MDSCs nor were...
they significantly higher in those with visceral metastases, though a trend existed (20.3 vs. 24.0, P=0.076). There was a trend for higher MDSC values in patients with visceral metastases, which historically are associated with worse prognoses.

Lu et al. (1) tested the impact of novel immune oncology and biologic agents using a chimeric mouse model of mCRPC that could exhibit autochthonous tumor evolution. A novel non-germline mCRPC model in a C57BL/6 background was used via a JH61 and JH58 mouse embryonic stem cell clone. These were derived from several genotypes including the PB-Cre<sup>Pten<sup>+/−</sup></sup> smad4<sup>−/−</sup> mTmG<sup>+/−</sup> LSL-LUC<sup>L1+</sup> genotypes. These animals developed metastases to lymph nodes and micrometastases in lungs and could provide an in vivo window into mechanism and response to therapies. As such, a panel of checkpoint inhibitors that have been shown to be safe and have a clinical signal in selected patients in early phase trials but did not impact on overall survival in phase III trials, have some measure of preclinical activity, and/or induce immunomodulation were studied. Among the drugs studied were dasatinib (Sprycel®, a synthetic small molecule-inhibitor of SRC-family protein-tyrosine kinases), cabozantinib [Cometriq<sup>®</sup>, a small-molecule inhibitor of tyrosine kinases, including MET, VEGF receptors (VEGFRs), and AXL], BEZ235 [a phosphoinositide 3-kinase (PI3k)/mTOR dual inhibitor], along with anti-CTLA4 (Yervoy<sup>®</sup>) and anti-PD1 antibodies. These mCRPC-bearing chimeric mice received either checkpoint inhibitors alone or in combination with these drugs. As expected, the respected target agent monotherapies as well as the immune agents had minimal impact on the prostate tumor mass but the combination of cabozantinib and immune agent or BEZ plus immune agent showed potent synergistic efficacy both against the primary and metastatic lesions. Marked reduction of disease burden in addition to reduced proliferation and apoptosis were seen histologically. Dasatinib showed minimal activity when given in combination with a checkpoint drug. However, there was some impact on the disease as determined by a significant reduction of tumor-infiltrating lymphocytes (TILs) T cells suggesting impact on the tumor microenvironment. Depending on the murine model used, there was significant impact on the tumor microenvironment as assessed by a variety of signaling assays including phospho-receptor tyrosine kinase as well as cytokine assays. MDSCs showed a significantly higher sensitivity to cabozantinib and BEZ but not to dasatinib. In addition, when MDSCs were isolated from CRPC tumors treated with cytokines that were downregulated as a result of pretreatment of the tumor with cabozantinib or BEZ, significant upregulation of Arg1, Cybb, Ncf1 and Ncf4 were observed. The authors concluded that prostate cancer cells were capable of driving immunosuppression-related gene expression in MDSCs via the secretion of multiple cytokines. This was extrapolated further to suggest that there was paracrine signaling that was impaired by using cabozantinib or BEZ treatment.

These observations provide a real-time window into the interrogation of novel agents and their combinations with immune oncology drugs and provide further insight into the tumor microenvironment, the immune mechanisms at work, and the signaling pathways that are affected by drugs given singly or in combination with these immune agents. It may also explain mechanistically the lack of responsiveness.

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**Figure 1** The interactions of combination immunotherapy, checkpoint and signaling pathways and the tumor environment (TM). AR, androgen receptor; ACT, adoptive cell transfer. Reproduced from Bryant et al. (2).
to these single agents in prostate cancer. However, it does not completely explain why the majority of patients using single agent therapy fail and why individual patients may have durable responses. Nevertheless, it is a novel foothold by which the biology of these drugs may or may not show impact on the tumor microenvironment. Caution should be exercised that while preclinical models using novel drugs have often been successful in reducing or eliminating tumor burden, their use clinically may not similarly translate to comparable findings.

Despite these caveats, there are multiple studies that support the use of combinatorial approaches in prostate cancer many of which have been based on sound preclinical work. Ardiani et al. (9) studied the combination of drugs targeting the PI3k/Akt pathway and the androgen-receptor (AR) axis. They studied the combination of AZD5363, an adenosine triphosphate-competitive pan-Akt inhibitor and enzalutamide (9), an AR targeted drug was given at time of castration similarly and resulted in significant regression of tumors. The combination of AZD5363 and enzalutamide significantly delayed the development of resistance to enzalutamide in preclinical models via synergistic increases in cell cycle arrest as well as apoptosis. The authors support the idea that greater efficacy may result with earlier combination treatment.

In the TRAMP-C2 model, in vitro treatment with enzalutamide resulted in the up-regulation of MHC-I and Fas (11). Treatment with enzalutamide also induced a modest up-regulation of tumor antigens and cell-surface molecules in AR-expressing LNCaP human prostate carcinomas. Of note, enzalutamide or the AR-directed adrenal agent abiraterone when given in vitro, mediated major changes in several apoptotic genes in LNCaP cells. NAIP, a member of a family of inhibitors of apoptosis proteins that inhibit cell death via the inhibition of activated caspases was markedly down-regulated in LNCaP cells treated in vitro with enzalutamide (14-fold) or abiraterone (5-fold) (11). This family of inhibitory proteins has been shown to be overexpressed in a variety of malignancies and may contribute to the resistance of apoptosis, drug resistance, and tumor progression.

Other combinatorial approaches have evaluated sorafenib, a tyrosine kinase inhibitor along with enzalutamide in a CRPC model (12), enzalutamide combined with sorafenib decreased cell proliferation and induced apoptosis in the prostate cancer line, LNCaP. Tumor growth was suppressed in castrate-resistant LNCaP xenografts with the combination of these agents compared with each alone. While AR was down-regulated per Western blot, the ERK pathway was inhibited. Marques et al. (13), also using twelve human prostate cancer cells lines to study whether the combination of hormonal therapy with AZD5363 and AZD8186 could upregulate AR-target genes. The combination with hormonal therapy improved the efficacy and resulted in durable remissions. These data suggested that the combination resulted in upregulation of AR-target genes upon PI3k/Akt inhibition could result in efficacy via some form compensatory crosstalk between the AR and PI3K/Akt pathways. Similar observations have been reported by Toren et al. (14) who also supported the premise of crosstalk between the PI3k/Akt/mTOR and RAF/MEK/ERK signaling pathways. Castration sensitive, castration resistant, and enzalutamide-resistant prostate cancer cell lines were treated with AZD5363, an Akt inhibitor and PD0325901, a MEK inhibitor, either alone or in combination. The authors confirmed that the co-targeting of these pathways showed that Akt inhibition induced apoptosis and inhibited cell growth in PTEN null cell lines; that MEK inhibition had a greater effect on the 22RV1 cells compared with AR-expressing LNCaP, or enzalutamide resistant cells. But there was synergy using Akt and MEK blockade in some of the cell lines but this was inconsistent among the cell lines studied.

These studies all serve to highlight the potential for combinatorial approaches for the treatment of prostate cancer. The chimeric models introduced by Lu et al. (1) provide a means to better explore in vivo the effects of combination drug and immune therapies. However, despite the usefulness of this approach, the overall heterogeneity of prostate cancer continues to limit the rapidity by which preclinical success can translate into clinical implementation.

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**Footnote**

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Androgen receptor antagonism and impact on inhibitors of androgen synthesis in prostate cancer therapy

Vincent C. O. Njar

1Department of Pharmacology, 2Center for Biomolecular Therapeutics, 3Marlene Stewart Greenebaum Comprehensive Cancer Center, University of Maryland School of Medicine, Baltimore, MD, USA

Correspondence to: Vincent C. O. Njar, PhD. Department of Pharmacology, University of Maryland School of Medicine, 685 West Baltimore Street, Baltimore, MD 21201-1559, USA. Email: vnjar@som.umaryland.edu.

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In a recent article by Norris and colleagues, published in high-impact J Clin Invest (1), the authors investigated the potential role of androgen receptor (AR) antagonism and the efficacy of cytochrome P450 17A1 (hereafter called CYP17) inhibitors in prostate cancer models. The manuscript begins with a clear and thorough description of experimental/clinical candidate small molecules and clinically approved drugs designed as inhibitors of CYP17 (inhibitors of androgen synthesis) (2,3) or antiandrogens, agents that competitively antagonize the AR (4-6) (Figure 1), with the goal of treating castration resistant prostate cancer (CRPC). Of the promising CYP17 inhibitors, a clinical candidate, seviteronel (sev; VT-464) (Figure 1), shown to be a ‘C17,20-lyase selective inhibitor’, was also reported to have direct effects on AR function, although the molecular basis for this activity was not investigated (7).

The focus of this current report by the group led by Dr. McDonnell was to evaluate the potential AR antagonism activity of clinically relevant CYP17 inhibitors. In addition to the importance of the secondary activity to the efficacy of these inhibitors in cellular and animal models of prostate cancer, the author document that the ability of abiraterone (abi) and sev to inhibit CYP17 is dispensable for their efficacies against enzalutamide (enz)-resistant AR-F876L xenografts. Based on biochemical data and Phase I clinical experience with sev, the authors evaluated the effects of sev and other well-known CYP17 inhibitors (Figure 1) on AR activity (1).

Using a whole-cell radioligand-binding assay, the investigators showed that unlike orteronel (ort), the other CYP17 inhibitors effectively displaced [3H]-R8118 (a potent AR antagonist) from the AR, albeit with varied potencies, some of which were comparable to the efficacies of the benchmark FDA-approved antiandrogens, hydroxyflutamide, bicalutamide (bic) and enz (Figure 1). In complementary AR transcriptional assays, the most potent AR antagonists, sev, galeterone (gal) and abi, which were further investigated, and were shown to effectively inhibit AR transactivation. These three inhibitors were also found to be equally efficacious progesterone receptor antagonists, but they did not impact glucocorticoid or mineralocorticoid receptor functions. These CYP17 inhibitors were also shown to be pure AR antagonists as they did not exhibit significant AR agonistic activities (no impact on reporter gene activity). Additional studies revealed that sev was as effective as enz in preventing androgen-mediated target gene expression, while gal and abi were less effective.

Using two complementary assays, the investigators showed that the CYP17 inhibitors did not deplete AR protein levels in LNCaP cells. However, because gal has been shown to induce degradation of AR in several in vitro and in vivo prostate cancer models by several independent groups (8-14), the results presented here should be treated with caution. Using LNCaP and VCAP prostate cancer
cells, Norris et al. showed that the CYP17 inhibitors blocked androgen-mediated growth of AR-expressing prostate cancer cell lines, and also showed that the AR-negative DU-145 cell line was less responsive. We note that the authors fail to cite previous reports showing the effects of gal on the proliferation and inhibition of DU-145 and PC-3 cells and tumors (9,15).

It was shown that conformational change in the AR induced by the CYP17 inhibitors closely resembles the unliganded AR (apo-AR), suggesting that these compounds are mechanistically distinct from other classes of AR antagonists and would have clinical utility in the contexts in which existing antagonists have proven to be ineffective. The unique nature of these CYP17 inhibitors was further demonstrated using studies that validated their ability to prevent AR translocation to the nucleus and attenuating their interaction with target gene enhancers. Additional studies clearly demonstrated that the CYP17 inhibitors function as antagonist in AR-overexpressing CRPC models and that the inhibitors effectively antagonize the transcriptional activity of several clinically relevant AR mutants expressed in CRPC samples. Notably, the CYP17 inhibitor, ort, that lacked AR antagonistic activity, failed to inhibit the transcriptional activity of antiandrogen-resistant AR variants, even at concentrations up to 100 µM.

Studies using LNCaP cells engineered to overexpress AR-F876L (model of enz-resistant CRPC), showed that in contrast to the finding that enz was as effective as testosterone at activating AR target gene transcription, abi, gal and sev were ineffective. Additional studies in this model revealed that the CYP17 inhibitors did not stimulate AR-F876L nuclear accumulation and they also inhibited testosterone-induced recruitment of AR-F876L with AREs in the KLK and NKX3.1 genes. As expected, bic and enz treatment increased the proliferation of AR-overexpressing (LNCaP-AR) and enz-resistant (LNCaP-F876L) prostate cancer cells; however, the CYP17 inhibitors were without effects in either cell line. Collectively, these results led the authors to conclude that CYP17 inhibitors may have therapeutic utility in the management of CRPC patients who fail enz therapy due to selection for the AR-F876L mutation.

Finally, encouraged by these in vitro promising results and because of the importance of validation of efficacy using in vivo antitumor efficacy of lead drug candidates, in view of translation into the clinic, the authors assessed the efficacies of two CYP17 inhibitors (abi and sev) compared to enz using well-established hormone-sensitive and enz-resistant CRPC tumor xenograft models. Following the establishment of the effective dose of sev (100 mg/kg, twice daily) using the hormone-sensitive LNCaP xenograft models, the impact of sev and abi on the growth of enz-resistant LNCaP-F876L xenografts was determined. This later study was designed to isolate the CYP17-inhibitory activity from the AR antagonistic activity. Their data clearly showed that enz (30 mg/kg, once daily) had no effect on LNCaP-F876L tumor growth, and, neither did testosterone. In contrast, both abi and sev (each at 100 mg/kg, twice daily), significantly inhibited tumor growth (P<0.0001), regardless of testosterone administration. Importantly, the efficacy of sev was superior to that of abi, despite its lower molar dose,

![Figure 1 Structures of CYP17 inhibitors and antiandrogens.](image-url)
since the molecular weight of sev is 1.14-fold that of abi. In addition, analysis of plasma at termination of the study showed that the drug exposure was similar to that observed in patients. Together, the authors rightly concluded that the in vitro and importantly the in vivo antitumor efficacy data clearly support the role of AR antagonism in the mechanisms of action of the CYP17 inhibitors, sev, abi and gal, although gal was not assessed for its effects on tumor growth inhibition.

This is the first comprehensive head-to-head study of experimental/clinical candidate small molecules and clinically approved drugs that were designed as inhibitors of CYP17, with the strategy to validate the ability of some of these CYP17 inhibitors to also antagonize the AR. The authors are commended for their rigorous and comprehensive studies and report. The studies have validated previous studies by other research groups (2,12,16) and provides strong evidence to rationalize the clinical efficacies of “dual CYP17 inhibitors/AR antagonist” in the clinic in men with CRPC.

Although this report serves as a valuable proof-of-concept for the potential impact of ‘designed CYP17 inhibitors’ in the treatment of CRPC, additional studies would further enhance its potential impact. First, in the antitumor efficacy studies, given that the molar doses of sev and abi are 7.75- and 8.86-fold, respectively, higher than that of enz, at least one acceptable higher dose of enz should have been assessed in the study. Second, given that up-regulation of AR-V7 is a major mechanism of drug (enz and abi)-resistance in CRPC therapy, sev should have been tested in a CRPC model such as CWR22Rv1 with overexpressed AR-V7. Third, because of the high effective in vivo doses of sev and abi, and also because of AR antagonist association with convulsions/seizures (due to binding and activation of the central nervous system (CNS)-based GABA<sub>a</sub> receptor) (17,18), not only plasma, but also brain concentrations should have been determined to support the safety assessments of sev and abi. Indeed, assessment of the binding affinities of these agents to the GABA<sub>a</sub> receptor would also be valuable. Overall, given the current state of drug development for effective treatment of CRPC, the work should capture the interest of the wide prostate cancer therapy audience, and it is timely.

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**Footnote**

Conflicts of Interest: VC Njar is the lead inventor of Galeterone.

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For decades, lymph node metastasis (LNM) at the time of radical prostatectomy (RP) has been considered as a poor prognostic sign. Currently, the appropriate timing for androgen deprivation therapy (ADT) remains controversial. Only one small prospective randomized study (ECOG 3886) has shown improved survival for immediate vs. delayed ADT in this select group of patients (1). However, given that most urologists will not delay hormone therapy for evidence of bulky metastatic disease as was done in that trial, the study’s findings do not apply to the contemporary management of biochemically recurrent (BCR) prostate cancer. In today’s PSA era, immediate ADT would lead to overtreatment for a significant number of patients along with its associated risks and adverse effects (2,3).

The presence of LNM, or pN+ disease, has traditionally been seen as a sign of disseminated disease with lymphadenectomy playing more of a staging rather than therapeutic role. However, emerging evidence has provided insight into this complex issue; with longitudinal data demonstrating a considerable subset of men can be free of disease at 10 years with lymphadenectomy alone (4). Patients with low Gleason score and low number of metastatic lymph nodes appear to be a favorable group for whom an extended pelvic lymph node dissection (PLND) may be beneficial (5,6). The need and timing of adjuvant treatments remains less clear with current national comprehensive guidelines labeling ADT and ADT plus pelvic radiotherapy (RT) as a category 1 and 2B, respectively (7). Only one retrospective report from the Vita-Salute San Raffaele University (Milan, Italy) demonstrated improved BCR-free and cancer specific survival for men treated with ADT plus RT vs. ADT alone after RP and PLND (8).

This same center partnered with the Mayo Clinic and Memorial Sloan-Kettering Cancer Center in the latest issue of European Urology examining long term outcomes between different management strategies for pN+ men (9). Based on practice patterns at each institution, their large cohort of 1,388 men was comprised of three arms: observation (28%), ADT (49%), and ADT + RT (23%). Of note was their median follow-up of 69 months with 368 (26%) men followed longer than 10 years. Their results showed ADT + RT was associated with better overall survival than ADT [hazard ratio (HR): 0.46, 95% CI: 0.32–0.66; P<0.01] or observation alone (HR: 0.41, 0.27–0.64; P<0.01). This benefit seemed greater for those with high-risk disease features such as high Gleason score, pathologic T3b/T4 stage, and positive surgical margin; which correspond with previously reported data that also included Milan and Mayo Clinic patients (10).

Interestingly, there were no differences in survival between ADT and observation alone; with lifelong adjuvant ADT associated with increased risk of death from other causes (HR: 3.05, 1.45–6.40; P=0.003). However, it is noteworthy that approximately 77% of patients in the ADT arm came from US centers which tracked deaths using the Social Security Death Index (as opposed to the Italian National Civil Registry) so these results may be affected by
spurious differences in coding between the two registries. Additionally, most of the ADT + RT patients (83%) came from Milan so these findings may not necessarily translate to North American cohorts with distinct lifestyle, medical, and environmental factors which can confound retrospective studies such as this one. Nevertheless, the authors are to be congratulated for providing the largest experience to date on the post-operative management of pN+ disease after RP and PLND. These findings add to the evidence for the benefit of surgical resection and local control for a complex and heterogeneous disease state in which prospective data is unlikely to be forthcoming given the downward stage migration caused by widespread PSA screening (11,12).

Selecting patients who would benefit from adjuvant treatments remains difficult. What is clearer, as data continues to show, is the dogma that pelvic LMN is invariably a ‘game-over’ for prostate cancer patients. Even those with ominous pathologic features may derive benefit from aggressive local consolidation, and referral to centers of experience with multidisciplinary management of high-risk prostate cancer should be considered. With advances in systemic therapy targeting the androgen receptor, there is also an opportunity to improve hormonal manipulation and assess oncologic benefit in both adjuvant and salvage settings. What remains to be better defined is the subset of patients who may not require or benefit from adjuvant therapies, thus also sparing them the adverse consequences of castration and radiation effects on quality of life. Additionally, there is an opportunity to evaluate novel molecular biomarkers which could allow for better risk stratification with the goals of maximizing oncologic benefit while minimizing morbidity of overtreatment.

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Is dose-dependent response to bacillus Calmette-Guérin treatment in urothelial carcinoma?

Fabricio Racca, Rafael Morales-Barrera, Joan Carles

Vall d’Hebron Institute of Oncology, Vall d’Hebron University Hospital, Universitat Autònoma de Barcelona, Barcelona, Spain

Correspondence to: Joan Carles, MD, PhD. CNS and Sarcoma Tumor Unit, Vall d’Hebron University Hospital, Vall d’Hebron Institute of Oncology (VHIO), Universitat Autònoma de Barcelona, Universitat Internacional de Catalunya, 08035 Barcelona, Spain. Email: jcarles@vhio.net.

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Abstract: Bacillus Calmette-Guérin (BCG) intravesical instillation therapy has been established as a standard of care treatment for high-risk non-muscle invasive urothelial carcinoma (NMIUC). To date, many clinical have demonstrated a direct antitumor activity, prevention of tumor recurrence, reductions in tumor progression and, consequently, tumor specific mortality and improvement in overall survival. Nevertheless, the local adverse events arising from BCG intravesical account for about one-third of treatment discontinuations. Strategies to minimize the side effects are needed.

Keywords: Non-muscle invasive (NMI); urothelial carcinoma (UC); toxicity; Bacillus Calmette-Guérin (BCG); standard doses (SD); high doses

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Bladder cancer (BC) is a major global health challenge with 430,000 new cases and nearly 165,000 deaths during 2012. In 2012, estimated BC incidence and mortality in Europe were 151,297 and 52,411 cases, respectively (1). Urothelial carcinoma (UC) is the most common histologic subtype of BC, and represents nearly 90% of all cases. Approximately 75% of patients with BC present with a disease confined to the mucosa (stage Ta, CIS) or submucosa (stage T1) (2). The high grade tumors have been recognized as an important prognostic factor with regard to the potential for disease recurrence and progression. In patients with high-risk non-muscle invasive UC (NMIUC) the transurethral resection with intravesical Bacillus Calmette-Guérin (BCG) is the standard treatment (3).

Immunotherapy with BCG results in a massive local immune response characterized by induced expression of cytokines in the urine and in bladder tissue, and by an influx of granulocytes into the bladder wall. A larger set of cytokines, including TNF-α, GM-CS, IFN-γ and several IL has been detected in the urine of patients treated with intravesical BCG. These cytokines are involved in the initiation and maintenance of inflammatory process (4). Martínez-Piñeiro et al. studied the relationship between dose reduction and efficacy of intravesical BCG, which compared the standard dose (SD) of 81 mg of BCG with a reduced dose of 27 mg for the treatment in patients with NMIUC. Reduced dose had similar results for recurrence and progression but with significantly less toxicity (5). In another study Martínez-Piñeiro et al. compared if a third of the dose of intravesical BCG is as effective as the SD (6). On the other hand, Shah et al. reported that higher dose of BCG showed a better antitumor effect in two human UC cell lines than a SD. The authors suggest that there is an optimal dose of BCG measured by a cellular response to BCG and there is a rational for perform a dose escalation...
for improve response rates (7).

The authors of this paper use two human cell lines derived from UC at different concentrations of in vitro BCG from 1:5 to 1:500 confirming the adhesion and internalization of BCG in most of the different groups of cell lines. They demonstrated that UC cells exposure to BCG generated an activation of NFκB and this activation was correlated with BCG dose. The effect of the BCG dose on UC cells and gene expression was evaluated by quantitative RT-PCR. Only in the cell line 253J there was a relationship between BCG dose and gene expression (iNOS, CD45, IL6, CXCL1, CXCL3, IL8 and CCL20) in response to BCG.

Intravesical BCG is a treatment that causes serious side effects in many patients. The largest published study with BCG including 1,316 reported 62.8% of local side effects and 30.6% had some form of systemic side effects (8).

There are several strategies to diminish the toxicity. One of them is lowering the BCG (6). This approach diminishes the frequency of side effects but certainly not the severity of adverse events, which are the reason for stopping the treatment with BCG. Another strategy is the preventive systemic administration of isoniazid (INH). EORTC study 30911 addressed this clinical question. However, INH provoked transient liver toxicity in several patients. So, the use of prophylactic INH is not recommended (9). The last approach is the preventive symptomatic treatment with anticholinergic drug as such oxybutynin. The results were disappointing as the significantly worse outcome was in the oxybutynin arm when was compared with placebo arm (10).

The next question is how we can go move forward to increase the doses of BCG in the clinical setting? The authors propose further research will be conducted in a phase I trial in population of patients refractory to SD.

In contrast to the general belief that side effects increase over time, frequency was similar in the induction treatment and the maintenance therapy. Most treatment discontinuations for severe side effects occurred in the first year, so severe adverse events can already appear at the first instillation, this observation reveals that side effects are not dependent on the number of instillations but upon the host. Recently, Serretta et al. reported that almost 60% of patients interrupted the treatment due to persistent toxicity with SD of BCG (11).

At the moment data demonstrated that none of the earlier advocated methods to prevent BCG toxicity are effective. On the other hand, severe complication will occur if the patients are treated with higher doses of BCG.

Therefore, since the study lacks for decrease the toxicity of BCG and unacceptable toxicity induced by higher doses of BCG, any dosing strategy that increases the SD of BCG will produce an absolute intolerance to intravesical BCG.

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References


Intravesical instillation of Bacillus Calmette-Guerin (BCG) is the first line adjuvant therapy to transurethral resection of bladder tumor (TURBT) for patients with intermediate- and high-risk non-muscle invasive bladder cancer (NMIBC) lesions (1). Its effectiveness, however, comes at a cost of complications, with up to 91% of patients developing some type of irritative local symptoms (1). Additionally, about 10–20% of responders and 66% of non-responders eventually progress to muscle-invasive disease, leaving them with an invasive option of cystoprostatectomy (2).

As mentioned in the current paper, researchers have been attempting to find ways to reduce morbidity associated with intravesical BCG therapy without compromising its efficacy. To that effect, trials have shown that a third of the standard dose has similar efficacy in intermediate-risk superficial tumors with improved side effect profile. However, what can improve response rates to the above therapy, especially in BCG unresponsive patients, remains unknown. This paper takes a key step in this direction by evaluating the effects of BCG dose escalation in both in vitro and in vivo settings. By using two different TCC cell lines, T24 and 253J, they have shown that BCG dose escalation from the standard cell-to-BCG ratio of 1:50 to up to 1:500 led to dose-associated response at the cellular level. This is manifested in improved BCG attachment and internalization in cells, increased activation of several signaling pathways, increased RNA levels of key immune response genes, and increased cell death. Additionally, the in vivo study involving an orthotopic murine model of bladder cancer showed improved response to escalating intravesical BCG dose.

While the reported findings are encouraging and warrant further research in BCG dose escalation, it is noteworthy that the two cell lines behaved differently at the same concentrations of BCG. For instance, CEBP intracellular signaling pathway had its peak activity at 1:200 and 1:500 for T24 cells and 253J cells, respectively. Similarly, only 4 of 7 BCG related genes were activated in T24 compared to 7 of 7 genes in 253J cell line. From these results, it is clear that there are fundamental biological differences between TCC cell lines that we do not, yet, fully understand. This also holds true for actual bladder cancers, as reflected in some lesions being more BCG susceptible than others.

In addition to the highlighted molecular differences, the in vivo data must also be cautiously evaluated. With the short follow-up after intravesical BCG instillation and no reported data on changes in mice weight after treatment, it is difficult to discern how well these mice tolerated the escalation in BCG dose. Given that a large proportion of patients receiving standard BCG dose experience significant, yet tolerable, side effects, it will be important that any escalation in BCG dose does not achieve its efficacy at the expense of increased morbidity. Hence, a fine line between efficacy and morbidity will have to be maintained.

In conclusion, we are desperate for better treatment options for patients who fail intravesical BCG therapy for bladder cancer. This could be due to the fact that our current standard dose is insufficient, as proposed by this
current paper, or that some patients have biological factors that deem them BCG unresponsive. While we wait for better treatments to be developed, it may be worthwhile to evaluate the escalation in the BCG dose in such patients. After all, the clock is ticking on them!

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**References**


**Bladder cancer**

According to Globocan 2012, bladder cancer is the eleventh most common cancer worldwide. It occurs more commonly in Europe, North America, North Africa and Western Asia (1) with a greater occurrence in males rather than females and in older people. The majority (75–85%) of bladder cancers are confined to the mucosa (Ta or carcinoma in situ) or submucosa (T1). Though the incidence rate of bladder cancer is decreasing worldwide (1), it still poses a significant problem as patients who have bladder cancer are prone to frequent recurrences. These may eventually progress to muscle invasive disease. The cause of recurrence has been attributed to either remnant tumor cells missed at surgery or precancerous lesions that later develop into cancer. Without therapy the majority (88%) of patients with bladder cancer are likely to have a recurrence (2).

The gold standard treatment for intermediate and...
high grade non-muscle invasive bladder cancer is surgical removal of the tumor followed by Mycobacterium bovis, Bacillus Calmette-Guérin (BCG) immunotherapy. It is the most successful immunotherapy in clinical practice and reduces the incidence of recurrence (2,3).

**BCG immunotherapy**

The first use of BCG immunotherapy for the therapy of bladder cancer was by Morales in 1976 (4). Morales developed this trial based on the studies of several researchers who showed in animals that BCG instilled in the bladder could induce immune activation (5,6). Since then many clinical trials have been performed. The therapy consists of weekly intravesical instillations of BCG. It is believed that the instilled BCG induces a non-specific immune response in the bladder (7) in an attempt to remove the bacteria which inadvertently removes the remnant tumor cells and/or precancerous cells that give rise to recurrent tumors. Almost every type of immune cell has been shown to play a role in the immune response to BCG therapy and this is well presented in the review by Redelman-Sidi et al. (8). However there are still a significant number of patients (30–50%) who do not respond to therapy and why this is so is not known. The dose of BCG, frequency of BCG instillations, BCG strain and patient genetics are all important variables that impact patient response to therapy.

**Frequency of BCG instillations**

The South West Oncology Group (SWOG) recommends six weekly instillations of BCG (induction phase) followed by three instillations BCG (maintenance therapy) every 3 months for 3 years (9). The latest meta analyses show that better recurrence and progression free survival are associated with maintenance therapy (10). But the long therapeutic schedule can be burdensome for patients.

There is evidence that prior BCG vaccination and the presence of pre-existing immunity results in improved recurrence free survival (11). Zlotta et al. assayed lympho-proliferation against mycobacterial antigens in patients receiving BCG immunotherapy pre therapy and weekly post BCG therapy (12). They found that subjects with reactivity to mycobacterial antigens pre BCG immunotherapy had maximal lympho-proliferation after the 3rd or 4th week of BCG instillations. For those without any reactivity pre-BCG therapy maximal proliferation was observed at 6 weeks. This was confirmed in mice where prior vaccination with BCG and confirmation of the generation of BCG T cells correlated with improved response to BCG immunotherapy (11).

The impact of the frequency of BCG dosing was evaluated by de Boer et al. who showed in mice that reducing the number of BCG instillations from 6 to 2 (13) did not reduce efficacy in terms of cytokine gene expression. Normal mice instilled with BCG at the 1st and 6th week resulted in similar cytokine gene expression as mice given a weekly dose of BCG for 6 weeks. However, they did not evaluate this therapeutic schedule in tumor bearing mice.

**BCG strains**

The original Mycobacterium bovis vaccine strain was generated by Calmette and Guérin, in the early 20th century, by maintaining the bacteria in continuous culture (230 passages) over a period of 13 years from 1908–1921. This resulted in attenuation of the strain. By 1924 BCG was distributed to several countries. But as the technical ability to lyophilize BCG and/or store samples at −80 °C were not available until the 1960s, BCG was maintained in continuous culture leading to further attenuation and differences between the strains in terms of antigenic potential (14) and loss of T cell epitopes (15). These are probably a cause of the variability of the efficacy of the vaccine strains. For bladder cancer therapy several different BCG strains have been utilized but very few comparative studies have been performed on their efficacy.

In a randomized Phase III trial conducted in Switzerland the efficacy of BCG Connaught and TICE was evaluated on 142 patients. There was a median follow-up of 25 months and the 5 years recurrence free survival was 74% for Connaught and 48% for TICE (P=0.0108) (16). The poorer performance of TICE may be attributed to its lower survival in cells (17). Another trial compared BCG Tokyo (BCG Japan) and Connaught in patients with carcinoma-in-situ (CIS), but due to the lack of the Connaught strain this trial did not achieve significance (18). The 2-year recurrence-free survival was 73.2% and 68.8%, for Tokyo and Connaught, respectively. BCG Japan has better survival in macrophages being a strain that produces methoxymycolate (14). BCG Japan, is the most attenuated BCG strain and is associated with the least complications; strongest tuberculin reactivity and the best viability after lyophilisation (11,16). Trials with other BCG strains are small and underpowered to determine efficacy differences. BCG sub-strains used
in clinical therapy include BCG Pasteur, Glaxo, Moscow, Moreau, A Frappier, S African, Copenhagen, Romanian, RIVM/1 (19). Good outcomes have been obtained with BCG Moreau (19) and BCG Pasteur Danish strain 1331 for patients with TIS (19). BCG RIVM was as effective as mitomycin C for patients with pTa, pT1 and CIS (20).

**BCG interaction with cancer cell lines**

A comparison of eight BCG sub-strains (Japan, Moreau, Russia, Connaught, Danish, Glaxo, Phipps and Tice) for their ability to kill bladder cancer cells and induce cytokine production revealed that Russia (an early strain) and Connaught (a late strain) were the most effective at killing tumor cells and inducing cytokine production (17). A comparison of Moreau, Tice and RIVM for direct anti-proliferative effects on T24 cells and indirect effects via activation of dendritic cells (DC) and peripheral blood mononuclear cell (PBMCs) showed that these three strains had similar indirect effects on the bladder cancer cells but little direct effects (21).

It is known that BCG interacts with the fibronectin receptor (α5β1 integrins) (22). Binding to α5β1 integrins triggers p21 dependant cell arrest and reduces apoptosis (23). BCG increased HMGB1 secretion (24) in bladder cancer cell lines and BCG internalization modulated cellular redox levels (25). The viability of BCG modulates the amount of reactive oxygen species generated (26,27) and this is related to BCG induced cell death.

The relevance of BCG effects on human bladder cancer cell lines is questionable because BCG therapy commences after removal of the tumor mass. Thus there should be only a few cancer cells present except in the case of TIS.

**Immune cells modulate response to BCG**

Analysis of patient tissue has shown the importance of immune cells in the bladder environment. Increased CD4+ and GATA3+ T-cells in the tumor environment was associated with increased recurrence free survival post-BCG immunotherapy (28) while Tissue associated macrophages (TAMs), T regulatory cells (Tregs) and T-bet+ T-cells are associated with poor outcomes (28,29). The importance of the Tregs was confirmed in a clinical study using anti-CTLA4 blockade which resulted in increased CD4+ICOShi IFNγ expressing T cells over Treg cells in the bladder and peripheral blood (30). But after CTLA4 blockade more of the CD4+ICOShi T cells could recognize tumor antigens (31).

**Dose of BCG**

The normal dose of BCG used, termed standard dose contains between 1×10^8–1×10^12 colony forming units (CFU) of lyophilized BCG depending on the strain used. But the viability of BCG in lyophilized preparations for different strains also varies. Some patients are unable to tolerate standard dose BCG and thus 1/3 (32) and 1/6 (33) doses of BCG have been evaluated for immunotherapy as well as 1/3 dose BCG and IFNα (34). The lowering of BCG dose reduces side-effects and adverse events. In clinical trials, standard dose and 1/3 dose BCG seem to have similar outcomes (35).

Shah et al.’s study takes a different view of the dosage of BCG rather than decreasing the dose of BCG, they considered the effect of increasing the dose of BCG (36). They report that increasing the ratio of BCG to bladder cancer cells resulted in increased adherence; NFκB function and cytokine production with a plateau around 200:1. When they examined cell death by necrosis the amount of necrosis started to decrease at higher doses. But a higher ratio of BCG to cells resulted in better outcomes in mice with bladder tumors. They evaluated three instillations of BCG given at a 3–4 days intervals. The short duration of the animal studies performed by Shah et al. means it is difficult to determine the efficacy of this strategy. The authors do acknowledge that the use of a higher dose of BCG has to be considered in the context of likelihood of adverse events. In a Phase III trial evaluating TICE some 20% of patients had to stop therapy due to side-effects (37) during the maintenance phase. In this trial patients received 2×10^5–5×10^6 CFU of TICE. Increasing the amount of BCG used in intravesical therapy will increase side-effects.

This work also disregards the impact of immune cells which are known to be major players in the modulation of the response to BCG. The dose of bacteria and timing of the exposure are known to differentially modulate immune cells. For Lactobacillus species exposure of DC to a higher dose of Lactobacilli results in tolerance rather than immune activation (38).

**Genetic control of BCG survival in macrophages and patient response to therapy**

A further complication is that genetic differences could influence response to BCG as well. Skamene et al. were the
first to show that the ability of host macrophages to respond to BCG was controlled by the BCG gene later identified as the natural resistance associated macrophage protein 1 (Nramp1) gene (39,40). Kadhim et al. demonstrated that the Nramp1 gene controlled response to BCG immunotherapy in a murine orthotopic model of bladder cancer (41) using BCG sensitive and resistant mice strains. The BCG sensitive mice respond to BCG immunotherapy. BCG survival in macrophages from BCG sensitive and BCG resistant mice is related to the production of nitric oxide (NO) (38). The BCG resistant cells produce more NO. How this is linked to the NRAMP gene is not known?

As a consequence of these results, single nucleotide polymorphisms (SNPs) in the NRAMP1 gene have been evaluated in patients with respect to the response to BCG therapy. The NRAMP1, SNP analyses have produced contradictory results (42-44) in bladder cancer patients. This is likely due to differences in genotype expression at polymorphic sites in different human populations. Polymorphisms in a variety of cytokine genes (45,46); DNA repair enzymes (47), FASL (48) oxidative stress response genes (49,50) have been evaluated as well.

A recent study has shown that besides germline mutations somatic cell differences such as E2F4 expression in tumor versus normal tissue was predictive of the response to BCG (51). Predictive signatures have been found in T1 tumors that correlated with response to BCG therapy (52).

Conclusions

Shah et al. have introduced an interesting concept that an increased BCG dose would improve response to BCG immunotherapy. However more work needs to be performed taking into account the importance of immune cells, BCG strain differences and patient genetic factors before it can be determined if this would be of clinical benefit. If a higher dose of BCG is to be evaluated it may be a better strategy to reduce the dosing schedule as suggested by de Boer et al. (13). Forty years since the introduction of BCG immunotherapy we are still trying to understand how it works. Because without understanding, it is not possible to improve or optimise this therapy. The quest is on-going to determine the optimal dose; dosing schedule; BCG strain and adjuvant therapy.

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Footnote

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Editorial on the use of immunotherapy in renal-cell carcinoma—promising results in combination therapy with ipilimumab and nivolumab

Christian Menzer¹, Carsten Gruellich², Jessica C. Hassel¹

¹Section of Dermato-oncology, Department of Dermatology and National Center for Tumor Diseases, ²Translational Uro-oncology, Department of Medical Oncology, National Center for Tumor Diseases, Heidelberg University Hospital, Heidelberg, Germany

Correspondence to: Jessica C. Hassel, MD, Head of section of Dermato-oncology. Department of Dermatology and National Center for Tumor Diseases, Heidelberg University Hospital, Im Neuenheimer Feld 460, 69120 Heidelberg, Germany. Email: Jessica.Hassel@med.uni-heidelberg.de.

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Immunotherapy in cancer

During the last decades different immunotherapies have been used in the treatment of cancer without great success. However, stimulation of the immune system seems reasonable as there are immunogenic tumors such as melanoma and renal cell carcinoma which are known to rarely spontaneously regress when the immune system of the patient regains the ability to control the cancer (1). Multiple immune escape mechanisms are described which might be targeted by immunotherapies. The aim of all approaches is to enable the immune system to again recognize cancer antigens and eliminate the tumor cells (2). In contrast to chemotherapies and targeted therapies, immunotherapies thereby have the chance to lead to durable responses.

The breakthrough of immunotherapy came with introduction of the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4)-inhibitor ipilimumab which showed revolutionary results in the treatment of metastatic melanoma as compared to standard therapies at that time (3,4). In a phase III study ipilimumab was the first systemic treatment to prolong overall survival (OS) of melanoma patients with a median OS of about 10 months, which was significantly superior to the results seen in patients treated with the peptide vaccine gp100 (4). Ipilimumab is an IgG1 monoclonal antibody directed against CTLA-4, a classical immune checkpoint. It is expressed on cytotoxic T-lymphocytes and physiologically deactivates them to prevent autoimmune activity. The blockage of the CTLA-4 receptor finally prevents this “switch-off”-mechanism and allows T-cell immune response against the neoplastic cells. Further investigation of the interaction between immune and tumor cells resulted in the development of other immune checkpoint blockers with different molecular targets such as the programmed death-1 (PD-1)-inhibitors nivolumab and pembrolizumab which are meanwhile approved by the Food and Drug Administration (FDA) for the treatment of metastatic melanoma, lung cancer (5,6), renal-cell carcinoma, and others. PD-1 is a human immunoglobulin G4 antibody which blocks the interaction between the PD-1 receptor on activated T-cells and its ligand PD-L1/PD-L2 on tumor and dendritic cells. The overall response rates in studies with PD-1-inhibitors vary between different tumors. They were reported at a range of 30–40% and thereby superior to the prior results seen with ipilimumab treatment in patients with metastatic melanoma (7,8). Finally, metastatic melanoma is the first indication for which the combination of ipilimumab and nivolumab is approved. This combination led to even higher objective response rates (ORR) of up to almost 60% and a significant advantage in progression-free and OS could
be seen compared to either agent alone (9). For metastatic melanoma, the approved doses in the combination treatment are 3 mg/kg ipilimumab and 1 mg/kg nivolumab 4 times in 3 weeks intervals followed by 3 mg/kg nivolumab every other week for up to 2 years. In the phase 1 trial this dosage of the combination revealed the highest antitumor activity at first evaluation and was hence chosen for further investigation in the CheckMate 067 phase 3 trial (10).

However, the beneficial effects of an enhanced immune activity came at the cost of, partly severe (grade 3 or 4), immune-related adverse events (irAEs), especially treatment-induced hepatitis and enterocolitis which require immunosuppressive treatment (11). Even though the nature of the side effects is similar between the different immune checkpoint blockers, frequency differs greatly. PD-1 monotherapy leads to grade 3/4 treatment-related adverse events (AEs) in 15–20% of patients, ipilimumab in 20–30%, and the combination treatment in more than half of the patients with metastatic melanoma (12,13). Interestingly, even though toxicity is increased by ipilimumab in the combination treatment, grade 3/4 AEs did not differ much between the different tested dosages of the combination treatment in patients with metastatic melanoma in the phase 1 trial with about 66% of grade 3/4 AEs with 1 mg/kg nivolumab plus 3 mg/kg ipilimumab (N1I3; cohort 8) and 69% of grade 3/4 AEs with 3 mg/kg nivolumab plus 1 mg/kg ipilimumab (N3I1; cohort 2a) (10).

Meanwhile, the combination of ipilimumab and nivolumab is or has been tested in other tumor entities such as lung cancer, head and neck-, and renal-cell carcinoma.

First approaches with immune checkpoint blocker treatment in renal cell carcinoma

Immunotherapies have been used in metastatic renal cell carcinoma similar to metastatic melanoma. Especially treatment with cytokines such as interferons and interleukin-2 has been applied with limited success. Standard first-line treatment to date is the application of vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitors such as sunitinib and pazopanib or the anti-vascular endothelial growth factor (VEGF) antibody bevacizumab in combination with interferon. Further tyrosine kinase inhibitors such as axitinib, cabozantinib, and lenvatinib are approved for second or later lines (14–16). Concerning immune checkpoint blockers, ipilimumab induced partial responses in 8% of patients in a phase II study (17). A third of patients suffered from grade 3 or 4 AEs, mainly enteritis and endocrine deficiencies with a positive correlation between irAEs and tumor response, as it had been previously reported in metastatic melanoma (18). The PD-1 inhibitor nivolumab is already approved for second-line treatment of renal cell cancer. It was investigated in a phase 3 study in which 821 patients with clear-cell renal cell cancer received therapy with either nivolumab or everolimus, a mammalian target of rapamycin (mTOR)-inhibitor widely used as a second-line agent in renal cell carcinoma (19). In this study, nivolumab showed a favorable side effect profile and improved quality of life compared to everolimus and a superior efficacy with an ORR of 25% and significauntly longer median OS (25 vs. 19.6 months, respectively). Whereas in melanoma patients PD-L1 expression of tumors was associated with better response to PD-1 inhibitors, no significant differences in response could be detected in advanced renal-cell carcinoma (19,20).

Thus, efficacy of immune checkpoint blockers in renal cell carcinoma had been demonstrated. Yet, responses to ipilimumab and nivolumab monotherapy did not reach as high results as seen for advanced melanoma with response rates of 12% and 40%, respectively (9).

The CheckMate 016 study—newest advances in renal cell carcinoma

The encouraging results for metastatic melanoma on the combination treatment of ipilimumab and nivolumab led to several similar clinical trials for other tumor entities. In patients with metastatic renal cell carcinoma a phase I study with the combination treatment of nivolumab and ipilimumab, the CheckMate 016 study, was installed and recently published (21). Five treatment arms existed, three of which consisted of the combination therapy of nivolumab and ipilimumab [nivolumab 3 mg/kg plus ipilimumab 1 mg/kg (N3I1)], nivolumab 1 mg/kg plus ipilimumab 3 mg/kg (N1I3), and nivolumab 3 mg/kg plus ipilimumab 3 mg/kg (N3I3)], and two consisted of the combination of nivolumab with a tyrosine kinase inhibitor for which results have not yet been released. Regardless of dosage, the combined treatments of nivolumab and ipilimumab were administered intravenously every 3 weeks for up to four doses (induction phase) after which the regimen was switched to nivolumab monotherapy 3 mg/kg every other week until disease progression or intolerable
toxicity. The primary objective of this study was to determine a recommended phase II dose regarding safety and tolerability. Forty-seven patients were assigned to each the N3I1 and N1I3 arm. In the N3I3 arm all 6 included patients had to be censored early because of disease progression (3 patients), treatment-related toxicity (2 patients), or withdrawal of consent (1 patient). Because of this high censoring percentage, no confirmed responses were found in this treatment arm, and efficacy hence could not to be evaluated. In both remaining treatment arms ORR was 40.4% with more complete responses (CR) in the N3I1 arm compared to the N1I3 arm (10.6% vs. 0% of patients). In the N3I1 arm 42.1% of responses were ongoing compared to 36.8% in the N1I3 arm. Median PFS was 7.7 months for the N3I1 arm and 9.4 months for the N1I3 arm, respectively. At 12 and 24 months, OS was 81% and 67% in the N3I1 arm and 85% and 70% in the N1I3 arm, respectively. Hence, preliminary data did not show leading differences in treatment efficacy.

In contrast, toxicity was lower in the N3I1 arm with only 38.3% of patients developing grade 3/4 AEs compared to 61.7% in the N1I3 arm. Colitis and hepatitis were again the most common treatment-related AEs requiring short-term systemic glucocorticoids, confirming the experiences that had been gathered in the melanoma studies. However, in the phase 1 trial in metastatic melanoma, grade 3/4 toxicity did not differ much between N1I3 and N3I1 (10). Hence, side effects of immune checkpoint blockers seem to vary in patients with different tumor entities. Another example is the higher rate of pneumonitis in patients with lung cancer (12).

Similar to the melanoma studies, the combination of ipilimumab and nivolumab showed promising efficacy with acceptable toxicity in renal cell carcinoma. The synergistic effects of PD-1- and CTLA4-inhibition again seem to lead to a more effective T-cell-mediated anti-tumor response compared to the respective monotherapies. ORR and OS were similar in both dosage groups of this phase 1 study described by Hammers et al. Yet, the safety profile with significantly less cases of grade 3 and 4 AEs favors the N3I1 dosage of the combination therapy for further clinical development (CheckMate 214; NCT02231749). Further studies investigating the combination of VEGF-targeted therapy with immune checkpoint inhibitors have already shown promising results in early phases and phase 3 data of first-line trials are expected to be presented soon (IMmotion 151, NCT02420821; Javelin renal 101, NCT02684006; Keynote-426, NCT02853331).

**Conclusions**

In summary, comparable to the results in metastatic melanoma, treatment of renal cell carcinoma with combined ipilimumab and nivolumab leads to promising responses and improved survival of patients. Side effects are well-known in the meantime and can be safely managed based on our experience in other tumor entities, such as melanoma. How the combination treatment performs first-line compared to sunitinib is under investigation in a phase 3 trial. Further ongoing strategies explore the efficacy of a combinatorial approach of VEGF-targeted and immune checkpoint blockade.

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**References**

Effective combinatorial immunotherapy for castration-resistant prostate cancer: new future chance?

Pina Ziranu, Francesco Atzori, Marco Puzzoni, Laura Demurtas, Giorgio Astara, Mario Scartozzi

Department of Medical Oncology, University of Cagliari, University Hospital ‘Duilio Casula’ S.S. 554, Monserrato, Italy

Correspondence to: Francesco Atzori, MD. Department of Medical Oncology, University of Cagliari, University Hospital ‘Duilio Casula’ S.S. 554, km. 4500, Bivio Sestu, 09042 Monserrato (CA), Italy. Email: francescoatzori74@yahoo.it.

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Prostate cancer (pCa) is the second most common non-cutaneous malignant neoplasm in men worldwide, with an estimated incidence of 1.1 million new cases per year. Furthermore, it is the fifth leading cause of cancer-related death, representing 6.6% of total male mortality (1).

New cases of pCa mostly show a localized disease at first presentation diagnosis and they are potentially curable, unfortunately relapses occur in 20% to 30% of these patients despite a curative intent therapy. Moreover, the current incidence of lethal pCa metastatic evidence at the time of diagnosis has declined to 5% approximately (2,3).

Androgen deprivation therapy (ADT) is a standard of care against pCa. This therapy reduces at first the tumor burden and/or circulating PSA to low or undetectable levels (4). However, the response duration should be variable from months to years with unavoidable disease progression in patients with metastatic disease (5). The pCa growing despite adequate ADT is defined as castration-resistant prostate cancer (CRPC) (5).

Several new agents have been developed in metastatic CRPC (mCRPC) treatment, leading to the ability of androgen receptor (AR) signaling inhibition. These strategies include those drugs interfering with androgenic stimulation as abiraterone and enzalutamide, already approved in mCRPC disease over the last decade (6-9).

Despite a survival related improvement due these second-generation AR targeted therapies, acquired or inherent resistance may occur in all patients so that metastatic pCa currently remains incurable. Failure of ADT and chemotherapies (docetaxel and cabazitaxel) is the major cause of death in patients with CRPC (10,11).

New strategies will be necessary to improve cancer management. In order to assess an adequate process of anticancer therapy, targeting immune system represent a promising option. Checkpoint blockade immunotherapies have shown exciting results in several tumor types as NSCLC, melanoma and renal-cell cancer (12).

The presence of inflammatory cells and T-cell infiltrates in pCa tissues provides the activity of a host immune-response towards this neoplasm (13,14). Potential benefit from immunotherapeutic strategies in patients with CRPC is further suggested by preclinical studies achievements in experimental pCa models and the clinical activity results of sipuleucel-T (15-17).

Despite that premises, current data demonstrates failures of various immune system targeting agents in mCRCP. Two phase III clinical trials assessing ipilimumab versus placebo after progression to docetaxel-chemotherapy and ipilimumab versus placebo in chemotherapy-naïve mCRPC setting showed no significant difference between the ipilimumab group and the placebo group in terms of overall survival (18,19).

Resistance towards immune checkpoint blockade (ICB) in pCa has to be still identified.

Myeloid-derived suppressor cells (MDSCs) play an important role in immunotherapy failure as well as pCa promotion and progression. In healthy subjects, immature myeloid cells (IMCs) generated in bone marrow differentiate into mature macrophages, granulocytes or dendritic cells. In cancer patients appears a partial blockade
in IMCs differentiation, which produce an expansion of this population. Moreover, among this pathological context there are evidences of upregulated expression of immune suppressive factors (ARG1, NOS2, NO, ROS) resulting in IMC population spreading with immune suppressive activity; these cells are known as MDSCs (20,21). A high amount of circulating MDSCs are associated with prostate-specific antigen levels and higher risk of metastasis in pCa (21). Furthermore, a better knowledge of the immune infiltrate composition and interactions between cancer and immune system would help to identify proper candidates for immunotherapy (22).

In this clinical scenario Lu and co-workers hypothesized that the combination of target therapy against mCRPC-infiltrating MDSCs with ICB agents may improve the response to immunotherapy (23).

They conducted a preclinical trial using a novel chimeric mouse model of mCRPC, engineered with signature genes mutations implicated in the genesis of human pCa. The genetically engineered mice exhibited autochthonous tumor evolution among an intact immune system (23).

Traditionally, preclinical studies have largely used xenograft models of human pCa, using cell lines of prostate tumor implanted into immune-deficient mice. However, xenograft models have several important limitations due to heterologous microenvironment and absence of endogenous immune response. Thus, the study of combination therapy using xenograft models appears to be inappropriate (24).

Lu and his colleagues employed novel non-germline mCRPC model availing mouse embryonic stem cell clones (JH61 and JH58) derived from PB-Cre+p53−/−, Pten−/−, Smad4−/−, mTmG−/−, LSL-LUC−/− (CPPSML) genotypes which exhibited age-dependent green fluorescent protein (GFP) LUC pCa growth (23).

Mice that developed GFP+ cancer cells at 3 months, with a dissemination of cancer cells also to lung and lymph nodes, underwent to androgen deprivation therapy protocol (castration followed by enzalutamide-admixed diet) in order to induce CRPC. Then, CPPSML chimaeras with MRI documented mCRCP were assigned to therapeutic trials (23).

The selected target agents were the tyrosine kinase inhibitors dasatinib (Dasa) and cabozantinib (Cabo), and the phosphoinositide 3-kinase PI3K/mTOR dual inhibitor BEX235 (BEZ). Moreover, a combination of anti-CTLA-4 and anti-PD1 was used for ICB.

CPPSML chimaeras were randomized to receive single agent or combination treatment for 4 weeks. The combination CABO + ICB and BEZ + ICB showed a synergic efficacy to bring a significant burden disease reduction. On the contrary, administration of target single agents, dual ICB cocktail or DASA + ICB had minimal impact on prostate tumor mass and metastasis reduction (23).

Furthermore, authors explored tumor micro-environment modifications using CyTOF analysis of mouse prostate tumors (23).

They demonstrated that Cabo + ICB and BEZ + ICB treatment was not associated with significant reduction of tumour-infiltrating T cells, but showed a reduction of Gr-MDSCs and an increase of CD8+/Treg ratio. Cabo or BEZ in combination with ICB mitigated the suppressive activity of intratumoral MDSCs on CD4+ and CD8+ T-cell proliferation (Table 1) (23).

Moreover, as parallel evidence, CD4+ and CD8+ T-cell proliferation was entirely blocked by Dasa. A significant reduction of tumour-infiltrating T cells was associated with Dasa treatment, related to T cell depletion into tumor microenvironment, probably due to the small impact of Dasa + ICB.

Cabo and BEZ combination treatment with ICB induced downregulation of pEGFR, pErbbB2, pErbbB3, pAxlin and pPDGFRα, and reduced phosphorylated MET and VEGFR2. Finally, these combinations affected cytokine production in primary CRPC, with CCL5, CCL12, CD40, HGF reduction and IL-1ra, CD142 and VEGF increase. These cytokines modifications, less pronounced in Dasa + ICB treatment, may influence the activity of myeloid cells and upregulate the gene expression responsible of MDSC-induced immune suppression (Arg1, Cybb, Ncf1, Ncf4) (Table 1) (23).

In conclusion, it appears reasonable believing that synergic effects of ICB and target therapies against mCRPC-infiltrating MDSCs, might be related to the selective MDSCs depletion and tumor microenvironment changes.

Lu and colleagues confirmed the immunosuppressive T cells activity caused by MDSCs into tumor microenvironment, generating resistance to ICB. Whereas treatment with targeted agents against MDSCs enforced T cells, enhancing ICB.

On top of that, this paper highlights the importance of longitudinal immune-response study approach. In fact, the dynamicity of the immune system prevents conduction of data analysis extrapolated from a specific time point. Thus, exploring the microenvironment tumor changes and MDSCs levels appear incredible interesting.

Genetically optimization of an engineered mouse model
of pCa leaded to significant advances to understand cellular pathways from cancer initiation to castration resistance, through observation of disease progression.

The development of a CPPSML chimeric mCRPC model in mice, looking for an efficient combinatorial immunotherapy, plays a promising approach in order to understand the relationship between novel therapies and microenvironment modifications. Probably, this model will provide important insights into pCa mechanisms.

Based on these preclinical trials results, future clinical studies in human mCRCP patients should explore molecular mechanisms causing immunotherapy de novo resistance in pCa, in order to achieve the best combination therapy, identifying the most effective schedule protocols.

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Penile cancer is a relatively rare malignancy with approximately 2,000 men diagnosed in the United States annually and approximately 300 deaths (1). Penile cancer stage and nodal status are strong prognostic factors with patients who have advanced disease having poor cancer specific survival (2). Risk factors for penile cancer development include poor hygiene, phimosis, smoking, lack of circumcision, increased number of sexual partners, and balanitis (3). In addition to these factors, nearly half of all penile cancers are found to be associated with human papillomavirus (HPV) infections (4). Taken together, two pathways for penile cancer development have been proposed, one resulting from HPV infection with high risk genotypes such as HPV 16 or HPV 18 and another resulting from chronic inflammation. Histologically, HPV infection has been shown to occur more frequently with warty and basaloid squamous cell carcinoma (SCC) as opposed to usual type and verrucous SCC (4). HPV-related penile cancer has also been evaluated as a prognostic marker for survival. In other HPV-associated tumors, such as oropharyngeal SCC and anal
cancer, HPV positivity was associated with improved survival (5,6). Early studies found that HPV associated penile cancers were associated with an independent and significantly better disease-specific survival (7). These studies were confirmed in a similar study of a more contemporary cohort (8).

Unlike penile cancer, nearly all invasive cervical cancers are associated with infection by oncogenic HPV (9). Without an available cure for HPV infection, preventing infection through vaccination has been a major focus of women's preventative health measures. The success of female vaccination and the resulting reduction in HPV-associated lesions led to studies of HPV vaccination in men. Early vaccination studies showed a significant reduction in HPV-associated genital lesions (10). Over time, the Centers for Disease Control and Prevention altered its stance on vaccination from an option to vaccinate to a recommendation for vaccination in all men beginning at the age of 11 with either the quadrivalent or 9-valent vaccine (11).

While the success of HPV vaccination should reduce HPV-associated penile cancer occurrences, clinicians must still manage the cancers that are found to be HPV negative. Unfortunately, these are the same cancers that have been shown to have worse survival. Surgical advances in the treatment of penile cancer have resulted in potential decreases in morbidity, but remain centered on early intervention with local resection, preservation of function when possible, and lymphadenectomy based on pathologic features and risk of metastases. For patients with locally advanced disease or metastases, obtaining long-term survival remains a challenge. Traditional chemotherapy regimens can be effective, but often there is eventually cancer progression. For some cancers, it is felt that immune system evasion may be one factor resulting in cancer progression. Recently, immune checkpoint inhibition has been proposed as a new treatment paradigm. In particular, new agents targeting programmed death ligand 1 (PD-L1) have been proposed. In cancers such as melanoma and non-small cell lung cancer, PD-L1 inhibition has been shown to be an effective treatment (12,13). Recently, the PD-L1 targeted agent atezolizumab, was found to be active in patients with metastatic urothelial carcinoma who had progressed following treatment with chemotherapy (14). This resulted in a new second line agent for bladder cancer.

Prior to evaluating the efficacy of PD-L1 targeted treatments in patients with advanced penile cancer, it is important to understand if PD-L1 expression occurs in penile SCC and if this expression may reflect the underlying aggressiveness of the tumor. An initial study was recently conducted to evaluate PD-L1 expression in a series of 37 patients with penile cancer. It was found that PD-L1 expression occurred in 62.2% of primary tumors and that expression was associated with worse survival (15). However, this study was limited to a small number of patients with relatively low incidence of HPV-associated penile cancer (15.2%). In the current study, Ottenhof et al. evaluate a larger cohort of patients with a higher proportion of patients having HPV-associated cancers (16). From 200 tumors, they found that 75% of tumors were negative for high risk HPV genotypes. Previous studies in this cohort of patients had shown that presence of high risk HPV genotypes provided a survival benefit (8). PD-L1 expression was detected in 48% of tumors. Tumors negative for high risk HPV had a significantly increased frequency of PD-L1 expression. Diffuse PD-L1 expression was associated with a significant increase in lymph node positive disease and PD-L1 was prognostic of lymph node involvement on multivariable analysis. For PD-L1 positive tumors, diffuse PD-L1 expression was associated with worse disease-specific survival. This was even more pronounced in cases without high risk HPV. In a multivariable analysis of survival, PD-L1 expression pattern was a significant predictor of survival. Once again, this was even more pronounced in tumors negative for high risk HPV.

For the past decade, the most important advances in penile cancer therapy had been the development of a vaccine that could prevent HPV-associated genital lesions and tumors. Increasingly both men and women are receiving this vaccine and this will remain a cornerstone of cancer prevention. However, there was growing evidence that tumors arising from an alternative pathway may be even more lethal than cancers that are HPV positive. A gap developed between our understanding of this more challenging prognosis and treatments that may prevent poor outcomes. The significance of this new study is that it identifies a group of patients (PD-L1 positive), occurring more frequently in HPV negative tumors that may be susceptible to novel checkpoint inhibiting therapies. New strategies for penile cancer therapy may include testing for PD-L1 expression in addition to HPV status. Before we get to this point, it will require that clinical trials evaluating the efficacy of PD-L1 treatments in penile cancer be conducted. Although tumor cell expression brings hope for activity, differing expression between tumor and immune cells may be more predictive of treatment response. Further, studies have found that some targetable genetic alterations may...
occur at a high enough frequency that additional drug options may be available. It is our hope that over time, with growing utilization of HPV vaccines, that HPV associated cancers will decline and that simultaneously, we can exploit vulnerabilities inherent to the remaining tumors using a combination of judicious surgical resection and targeted chemotherapy treatments. We await the results of clinical trials that are currently under consideration or about to open for enrollment.

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Traditional prognostic factors PSA, Gleason score and TNM staging allow patients with prostate cancer to be classified into low, intermediate and high risk groups (1). These prognostic groups are used not only to predict clinical outcome, but also to discuss appropriate treatment options with patients. Recently the Gleason score was further refined into the Gleason Group Grade to reflect the differences in clinical outcome between Gl 3+4 and Gl 4+3 (2).

The landmark ProTect study published last year (3) confirmed what clinicians had known for some time, that low and intermediate risk prostate cancer can be managed conservatively with excellent outcomes. For those who go on to have radical treatment surgery and radiotherapy are equally effective for cancer outcomes although long term side-effects are more marked with surgery. The outcomes for low and intermediate risk prostate cancer are excellent, but 10% of patients treated with state-of-the-art radiotherapy will experience biochemical relapse (4,5). For men with high-risk, locally advanced prostate cancer, 3-year recurrence rates can be in the order of 30% (5) and the 5-year failure-free survival rate for those with metastatic disease is around 30% (6). However, there is a spectrum of response even within the defined risk groups. Clearly there are limitations to the current risk classification groups, and underpinning this variation in clinical outcome is the genetic heterogeneity of prostate cancer. Analysis of 4,938,362 mutations from 7,042 cancers showed the diversity of mutational processes underlying the development of cancer. The prevalence of somatic mutations was highly variable between tumor types being high in melanoma low in acute lymphocytic leukemia and intermediate in prostate cancer (7). The level of genomic alterations was also highly variable within each tumor type.

In recent years there has been a plethora of advances in molecular technology. It is now easier and cheaper than ever to interrogate the cancer genome. Although associations have been found between individual genes as well as gene panels, there are no robustly validated genetic biomarkers which are used routinely in clinical practice (8). A number of commercially available products show potential, but there is still some way to go before there are enough data to convincingly demonstrate added value to prostate cancer patients. The commercially available RNA based signatures for prostate cancer are the 22-gene Decipher assay that is prognostic for risk of metastasis following prostatectomy; the 31-gene Prolaris test (46 genes including internal reference genes) that assesses aggressiveness; and the 17-gene Oncotype DX Prostate Score that tests the probability of metastatic disease (9).

There is now a DNA classifier to add to the validated prognostic RNA based signatures. In the November 2016 on line issue of European Urology, Lalonde et al. (10) presented work aimed at validating a prognostic DNA
genomic classifier and progressing its translation into an aid to guide treatment planning. In an earlier publication, the collaborative group derived a DNA-based 100-locus copy number alteration (CNA) genomic classifier that stratified localized prostate cancers into groups with low and high risks of recurrence (11). In the European Oncology paper, the classifier was reduced to a 31-locus test by evaluating changes in RNA levels. Loci were selected where RNA expression reflected the copy number state. Thirty-one loci were identified that involved 109 genes, and the reduced signature was validated in four retrospective cohorts totalling 563 radical prostatectomy patients. The 31-locus genomic classifier identified patients with an increased risk of biochemical relapse [hazard ratio (HR) = 2.73, P < 0.001] and risk of metastasis (HR = 7.79, P < 0.001). Combining the classifier with standard prognostic variables outperformed use of clinical models alone. A further cohort of 102 patients was used to measure and validate the 31-locus classifier using the NanoString platform, which is suitable for clinical application. The 100-locus genomic classifier was shown in an earlier publication to outperform published RNA signatures including the OncoType DX Genomic Prostate Score and Prolaris test (11).

Precision medicine initiatives are striving to optimize therapies for patient sub-groups based on genetic or molecular profiling. The current most promising prostate cancer signatures have been validated in terms of prognostication to justify their use to aid decisions of whether to treat or to intensify treatment. They have not, however, been evaluated prospectively to show they improve outcomes or can predict benefit from specific interventions. Lalonde et al. highlight this limitation and state that future prospective trials will need “to evaluate whether the genomic classifier can serve as a predictive biomarker”, i.e., show that treatment intensification improves outcomes. The use of a standardized NanoString platform will aid future prospective validation.

The design of a follow-on interventional trial requires consideration of the choice of appropriate treatment. In a low risk group it would be important to select the patients who are not suitable for active surveillance so that either surgery or radiotherapy could be discussed. Where radiotherapy is the treatment of choice decisions regarding dose escalation or de-escalation could permit tailored treatment optimizing both cancer outcome and long-term toxicity risk. Defining groups of patients who would benefit from combined androgen deprivation therapy (ADT) and radiotherapy as well as those who would benefit from longer term ADT would allow targeting of treatment that can be beneficial, but can also have significant effects on a man’s quality-of-life.

One of the most important questions in radiotherapy is when to irradiate the pelvic nodes for patient benefit, and prognostic stratification could select a group for which lymph node irradiation increases cure. For patients with high risk prostate cancer, the STAMPEDE trial has shown the benefit of early chemotherapy (12). However, there is little doubt that chemotherapy is toxic and for some patients can adversely affect their quality-of-life so any steer towards selecting patients who benefit would be welcome.

The use of genomic signatures to improve prognostication would be a game-changer, but even more exciting would be the use of genetic indicators to predict specific treatment benefit. Connectivity mapping has been used to identify link RNA signatures with novel or re-purposed drugs which may be used to enhance treatment (13). Future research could use a network of genes based on the transcriptomic signature associated with the genomic classifier, and then use connectivity mapping to identify possible FDA approved agents for re-purposing. An ultimate goal for a radiotherapy-predictive biomarker would be to stratify patients who benefit from different modes of radiotherapy: low dose-rate brachytherapy, high dose-rate brachytherapy, protons or photons. Research aimed towards the latter requires generation of cohorts reflecting the different types of radiotherapy. As almost all signature generation to date has involved surgical cohorts and given the importance of radiotherapy in the treatment of the disease, there is a clear need to collect radiotherapy cohorts.

The speed of technological development highlights the challenges faced in translating gene signatures into the clinic. Biomarker discovery is easy but it is much harder to obtain the funding for qualifying a biomarker for clinical use. Tests need to be validated analytically and clinically and then shown to have clinical utility and an ability to improve healthcare (Figure 1). It is a highly competitive field that requires multi-disciplinary expertise and multi-center collaboration. The paper by Lalonde et al. illustrate the depth and breadth of research required. The work also illustrates the potential. However, it is a competitive field and the need to show clinical utility is paramount within an increasingly crowded area.
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Emerging mutations and functional changes of androgen receptor associated with treatment resistance in prostate cancer

Filippo Martignano¹, Cristian Lolli², Giorgia Ravaglia¹, Valentina Gallà³, Giorgia Gurioli¹, Samanta Salvi¹

¹Biosciences Laboratory, ²Department of Medical Oncology, ³Unit of Biostatistics and Clinical Trials, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy

Correspondence to: Samanta Salvi. Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, 40 P. Maroncelli Street, 47014 Meldola, Italy. Email: samanta.salvi@irst.emr.it.

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Abstract: Androgen receptor (AR) signaling is deeply involved in prostate cancer (PCa) development and growth, and castration-resistant prostate cancer (CRPC) transformation. Currently, the use of anti-androgen drugs, such as abiraterone and enzalutamide, has temporary effects on CRPC patients due to several patient-related resistance mechanisms, such as the development of AR mutations. Extensive research is being conducted on AR mutations in both tissues and cell free DNA (cfDNA), in order to identify those mutations responsible for treatment resistance. A recent study identified AR mutations in cfDNA samples from CRPC patients treated with different anti-androgen drugs. In particular, these mutations occurred in exon 8, which codes for ligand binding domain (LBD), causing functional protein changes in vitro, leading to anti-androgen drugs resistance. Moreover, a novel drug tested on PCa cell line, VPC-13566, has been proposed as a potentially alternative therapeutic approach in presence of AR-LBD mutations. This evidence underlines the importance of monitoring AR mutations in cfDNA in order to obtain information about the most efficacious treatment and timely therapy switch.

Keywords: Androgen receptor (AR); prostate cancer (PCa); mutations; anti-androgen treatment; castration-resistant prostate cancer (CRPC); cell free DNA (cfDNA)

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Introduction

Androgen receptor (AR) signaling axis seems deeply involved in prostate cancer (PCa) development and growth making androgen-deprivation the first therapeutic approach. However, PCa can temporarily benefit from androgen-deprivation, progressing to a castration-resistant prostate cancer (CRPC) status after some months of treatment (1). Despite resistance to hormonal drugs, AR axis remains the favorite target for the next generation hormonal therapies, such as abiraterone and enzalutamide (2,3). Abiraterone inhibits cytochrome P450 17 a-hydrolase (CYP17A1) reducing androgen production in the adrenal glands, testicles and tumor microenvironment (4). Enzalutamide has a great affinity for AR, inhibiting its interactions with dihydrotestosterone (DHT) (5). The use of these drugs has led to an increase in the overall survival of CRPC patients: their maintained efficacy after resistance to older anti-androgen drugs such as bicalutamide and hydroxyflutamide encouraged further testing of novel anti-androgen drugs (6-10).

AR aberrations such as AR copy number variations (CNVs), alternative splice variants and AR point mutations are among the main causes of resistance to anti-androgen
treatment (3,11-13). AR mutations are directly related to protein changes, which could lead to an enhanced affinity for ligands, cofactors and DNA, resulting in increased activity (5). Mutations affecting the AR ligand binding domain (LBD) are likely to be responsible for resistance to anti-androgen drugs which impair the interaction between the AR protein and its natural ligands such as DHT (14). Such mutations produce promiscuous AR mutants able to evade anti-androgen action converting AR-antagonists into AR-agonists (15), and allowing AR to bind with alternative ligands (16).

In the past years, many studies investigated primary, biopsy and autopsy tissues from CRPC patients in order to identify AR mutations causing treatment-resistance (17,18). The analysis of serum/plasma cell free DNA (cfDNA) can overcome the limitations of tissue-based approaches, giving a real time picture of disease evolution and treatment efficacy (11-13).

A study investigated CNVs of PCa-related genes (including AR) and mutational status of AR exon 8 in plasma from CRPC patients who had progressed on enzalutamide, abiraterone or other treatments (19). They identified AR amplification and three novel AR mutations (D879E, L881I and E893K) not as yet described in literature. They confirmed other well-known AR mutations, in particular H875Y, F877L, T878A.

Unfortunately, data about AR mutational status were not available for some samples as AR sequencing was impossible to perform due to low DNA yield. Such data have been updated by Lallous et al. in their recent study featuring an improved sequencing pipeline with a whole-genome pre-amplification step of cfDNA and characterized AR mutational status of all patients recruited in previous case series. Deep sequencing was performed for AR exon 8, which codes for AR-LBD, detecting four additional novel AR mutations (H875Q, D891H, E898G, T919S). In addition, the authors performed in vitro AR functional studies evaluating the effects on AR-LBD of the mutations detected in CRCP patients and of other mutations already described in literature.

**AR mutations and treatment resistance**

The majority of documented AR mutations falls in the LBD or cofactor binding regions (20). Alterations in the LBD can interfere with the action of AR-antagonists, turning them into AR-agonists and leading to treatment resistance, as it often happens with first-generation AR-antagonists such as hydroxyflutamide and bicalutamide (21). Such mutations are also able to alter AR specificity for ligands leading to a great affinity for other hormones, such as progesterone, with a pivotal role in the development of resistance against CYP17A1 inhibitors (16). Lallous et al. sequenced AR exon 8 in order to identify mutations which alter the AR-LBD and that could be responsible for anti-androgen treatment resistance.

AR mutations in codon 878 (T878A and T878S) are among the most investigated mutations in PCa patients (22-26). Functional studies have shown that in presence of T878A and T878S mutations hydroxyflutamide acts as an AR-agonist (27-29). According to Lallous et al. also bicalutamide and high concentration enzalutamide and ARN509 exhibit an AR-agonist behavior in presence of these two mutations, with an important role also in new-generation AR-antagonist treatments. In addition, T878A and T878S could be activated by estrogens (2). T878A is frequent in abiraterone-treated CRPC patients producing a progesterone-activated AR mutant protein leading to abiraterone-resistance (16). Similarly, H875Y is associated with elevated AR promiscuity, in particular with increased AR affinity for progesterone (30,31) and also estradiol and hydroxyflutamide (32). Lallous et al.’s findings are concordant with these previous studies. They found in vitro that T878A/S and H875Y mutants convert AR-antagonists into AR-agonists, and obtain higher affinity for progesterone and estradiol binding. In fact, the authors frequently found these three mutations in cfDNA from both abiraterone- and enzalutamide-resistant patients.

L702H mutation was reported in abiraterone- and enzalutamide-resistant patients receiving glucocorticoid treatment (11,18). This agrees with Lallous et al. functional studies, showing that L702H is the only single mutant activated by hydrocortisone. The authors did not found the mutation in cfDNA, probably because none of the patients had undergone glucocorticoid-based treatment.

Another critical mutation is F877L: several studies reported its capacity of inducing resistance against new-generation antiandrogens, converting both enzalutamide and ARN-509 into AR-agonists (33-36). Lallous et al. reported a partial agonist effect of these drugs on AR- F877L in vitro, while F877L/T878A haplotype was far more sensitive to enzalutamide and ARN-509 agonist action. This finding agrees with a recent work reporting only a mild AR- F877L affinity for enzalutamide and a strong agonist activity of enzalutamide against the F877L/T878A haplotype (37). Interestingly, only one patient carried the F877L/T878A haplotype after enzalutamide treatment, which was absent.
after bicalutamide, suggesting that it could be related to the enzalutamide resistance mechanism. On the other hand, bicalutamide showed no agonistic activity on F877L or F877L/T878A in vitro.

**Novel treatment strategies**

Nowadays, direct anti-AR drugs target AR-LBD, which often acquires genetic variations as mechanism of resistance. In order to overcome treatment resistance, Lallous *et al.* highlighted the importance of developing novel therapeutic strategies with an impact on other AR domains than the LBD. The strategy proposed by the authors is to target the AR binding function-3 (BF3) pocket, i.e., a site distant from the LBD essential for AR transcriptional activity and for recruiting AR co-regulators such as FKBP52 and Bag-1 L (38,39).

VPC-13566 is a quinolone derivate with different pharmacodynamics from classical anti-androgen drugs, targeting BF3 functionality (40). According to Lallous *et al.*, VPC-13566 proved effective also in presence of mutations which confer resistance to enzalutamide and ARN-509. The authors proposed it as a promising option against AR-mutants, either alone or in combination with LBD-targeting agents.

VPC-13566 is not the only novel drug targeting a region outside the LBD. Comparison of VPC-13566 activity with other drugs under investigation would be advisable: EPI-001 and its trans isomer EPI-002 are able to bind covalently the AR N-terminus by blocking it from activating downstream signaling pathways (41,42). EPI-001 has proven effective in CRPC xenograft models, and an analogue of the EPI compounds is currently being evaluated in phase I/II trials (NCT02606123) (41). The goal of these compounds is to inhibit both ligand-dependent and -independent activation of AR (41,42). EPI-002 significantly reduced tumor growth even in presence of AR splice variants in a xenograft model (43). Unfortunately, no studies regarding EPI compounds effects and AR-mutants are available. However, thanks to the ability of EPI compounds to inhibit AR in a ligand-independent way, they are likely to maintain their effects also in presence of mutations in the LBD.

Another novel drug under trial is galeterone, a next generation CYP17 inhibitor similar to abiraterone with an additional inhibitory action against AR. It is able to compete with DHT in binding to AR LBD (42), to impair AR binding to DNA (44) and to mediate AR degradation (1). Interestingly, galeterone showed a degrading effect also against the T878A mutant (42). Thanks to its multiple actions galeterone can potentially overcome constitutively-active AR splice variants: this is currently under investigation in a phase III clinical trial (ARMOR3-SV) (6).

The next-generation AR-antagonist ARN-509 is structurally and mechanistically similar to enzalutamide (7); in fact, according to Lallous *et al.*, it suffers the negative effects of certain AR-mutations as well as enzalutamide does. Other promising novel anti-androgens, such as the CYP17 inhibitor VT-464 and the AR-antagonist ODM-201, have different biochemical structures than, respectively, abiraterone and enzalutamide (8-10). Therefore it would be interesting to investigate if AR-LBD mutations impair their activity just as it happens with abiraterone and enzalutamide.

**Conclusions**

Based on the work of Lallous *et al.*, several AR mutations in exon 8 showed a strong effect on AR protein promiscuity, causing resistance to anti-androgen drugs.

In particular, the authors highlighted that H875Y and T878A/S mutations are involved in resistance to AR-antagonists (hydroxyflutamide, bicalutamide, enzalutamide and ARN-509) and abiraterone in vitro. These data suggested that the detection of these mutations in cfDNA could lead to alternative therapeutic strategies, which target another AR domain.

In addition, F877L mutation also caused resistance to enzalutamide and ARN-509 in vitro, maintaining its sensitivity to bicalutamide. The authors hypothesized that switching back to a bicalutamide-based treatment could be an option for a carrier of this mutation.

Due to the effect of the mutations analyzed on AR-LBD, the authors also proposed the use of VPC-13566 drug, with proven efficacy also against the AR-mutants investigated in vitro. Further studies could compare the effects of VPC-13566 with those of other novel anti-androgen drugs in clinical trials.

However, in CRPC, mechanisms of resistance may be also associated with deregulation of other pathways as PTEN/PI3K/AKT or with the activation of AR-independent pathways as neuroendocrine differentiation, suggesting the importance of targeting both AR and other pathways (45-49).

The cfDNA from CRPC patients was characterized for predictive information about different treatments such as abiraterone and enzalutamide. As Lallous and coworkers...
collected plasma samples at abiraterone and other treatments progression, but not at enzalutamide progression for all patients, no data are available on the AR mutational status subsequent to enzalutamide treatment. However, the few data available on the samples of three patients collected during enzalutamide treatment showed interesting mutation status: two of them carried additional mutations, absent during previous treatments, suggesting that they could have developed after the administration of enzalutamide.

In addition to other well-known AR mutations, Lallous et al. found four new AR-LBD mutations (H875Q, D891H, E898G, T919S) in cfDNA of CRPC patients, potentially important for predicting treatment efficacy. Further studies are needed to better understand how these mutations are involved in disease evolution.

In conclusion, a biological characterization of CRPC is pivotal to better select tumor treatments, in addition to clinical poor prognostic factors, such as presence of visceral metastases, early PSA progression, early metabolic progression, or increase of inflammatory biomarkers (50-56).

On the basis of Lallous et al.’s research, the monitoring of AR mutations in cfDNA could provide additional information about timely treatment change, aiming to improve patient survival.

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**Footnote**

**Conflicts of Interest:** The authors have no conflicts of interest to declare.

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Prostate cancer is the second most common cancer in men worldwide, accounting for 15% (1.1 million) of the total new male cancer cases and 6.6% (307,000) of the total cancer deaths in men (1). In the U.S., 161,360 new cases and 26,730 deaths from prostate cancer are estimated for 2017 (2).

The management of localized prostate cancer is guided by clinical and pathologic criteria including stage, grade, and serum prostate specific antigen (PSA) levels (3). Based on these criteria, men with non-metastatic prostate cancer were stratified into three broad and clinically heterogeneous risk categories (4). Over the ensuing decades, algorithmic treatment schemas emerged from prospective clinical trials based on this clinicopathologic risk stratification system (5) and formed the current basis for management decision making (6).

Some of the earliest studies in gene expression profiling of prostate cancer demonstrated distinct taxonomies that were associated with more aggressive forms of the disease (7). However, the clinical translation of these findings has remained largely unrealized. In contrast, breast cancer taxonomies have been more effectively utilized for clinical decision making. This was largely based on the seminal work of Sørlie and Perou (8). Subsequent years saw the development of expression based biomarkers to estimate the risk of breast cancer recurrence in women with early stage disease and to select patients who may benefit from endocrine therapy or chemotherapy (9).

Molecular profiling of prostate cancer has more recently emerged as a reliable method for predictive modeling and clinical risk stratification (10). Indeed recent retrospective data suggest gene expression based classifiers may outperform traditional clinicopathologic criteria for selecting men with a diagnosis of prostate cancer for active surveillance (11) or men with adverse pathology following prostatectomy for adjuvant radiotherapy (12,13). Given the wide spectrum of prognosis and the myriad therapeutic options available to patients with prostate cancer, a significant unmet need persist for the development and analytic validation of predictive biomarkers.

**Basal and luminal subtyping in prostate cancer**

In 2009, Parker and colleagues described the PAM50 classifier in breast cancer, which separated tumors into four distinct classes: luminal A, luminal B, basal and amplified human epidermal growth factor receptor 2 (HER2) (14,15). PAM50 subsequently gained U.S. Food and Drug Administration clearance as a tool for risk stratification in breast cancer. Prostate cancer bears similarities to breast cancer in that both are driven by gonadal hormones and endocrine therapy can be highly effective in both diseases. In this context, Zhao and colleagues explored whether the basal/luminal classification might therefore also be relevant in prostate cancer (16).

In their study, Zhao et al. applied the PAM50 classifier...
across gene expression data, generated using a commercially available array-based clinical assay (GenomeDX, San Diego, CA), from 3,782 archived radical prostatectomy specimens. These specimens were derived from six institutional retrospective cohorts and one prospectively collected cohort. They excluded the HER2 subtype from their analysis, noting that HER2 is not amplified in prostate cancer as it is in breast cancer. They found that the 1,576 retrospectively analyzed prostate tumors clustered in nearly equal proportions across the three remaining subtypes: luminal A (34.3%), luminal B (28.5%) and basal (37.1%). These proportions were conserved in 2,215 expression profiles from prospectively collected prostatectomy specimens in the Genome DX Decipher GRID post prostatectomy cohort.

In their retrospective cohorts, for which follow-up data were available, the authors investigated the prognostic significance of PAM50 clustering. Patients with luminal B tumors were found to have consistently worse outcomes for all clinical endpoints examined, including biochemical recurrence-free survival (bRFS), distant metastasis-free survival (DMFS), prostate cancer-specific survival (PCSS), and overall survival (OS). This contrasts with breast cancer where basal-like expression confers a poor prognosis. The PAM50 proliferation score (a composite of proliferative gene expression in the PAM50 cluster) was highest for the luminal B subtype, in line with the relatively more aggressive clinical behavior of this subset. The luminal B subtype was similarly associated with adverse clinical and pathologic characteristics including higher PSA, Gleason score, and rates of extracapsular extension and seminal vesicle invasion. After adjusting for these clinicopathologic variables in multivariate analysis, the luminal B subtype remained independently prognostic of unfavorable bRFS, DMFS, and PCSS.

The authors performed gene set enrichment analysis (GSEA) which demonstrated the androgen receptor (AR) pathway was enriched in luminal (A and B) tumors compared to basal tumors. They found that the luminal and basal subtypes had conserved markers for both luminal and basal lineages, respectively. Specifically, the basal CD49f signature was enriched in the basal cluster, while luminal markers NKX3.1, KRT18, and AR were enriched in the luminal subtypes.

Considering the observed variation in AR signaling, the authors hypothesized that luminal tumors may exhibit increased sensitivity to androgen deprivation therapy (ADT). They explored the predictive utility of PAM50 with respect to ADT response in patients who either did or did not receive androgen deprivation in the adjuvant/salvage setting. They performed an exploratory subgroup analysis by retrospectively matching clinicopathologic variables [Gleason score, PSA, lymph node involvement (LNI), extra-capsular extension (ECE), seminal vesicle invasion (SVI), and positive surgical margin status] and radiotherapy treatment status in 315 patients treated with ADT (n=105) or not treated with ADT (n=210). For their analysis luminal A and basal subtypes were pooled and compared with the luminal B subtype.

Importantly the authors found that, with a median follow-up of 13 years, luminal B patients benefitted from postoperative ADT while luminal A and basal patients did not. In the luminal B subtype, which had the poorest prognosis, patients treated with ADT had improved DMFS (10-year metastasis rates: ADT, 33% vs. no ADT, 55%). On the other hand, non-luminal B subtypes treated with ADT had poorer DMFS compared with untreated patients (10-year metastasis rates: ADT, 37% vs. no ADT, 21%). Separating patients receiving adjuvant or salvage therapy in the matched cohort resulted in a similar trend, although no longer statistically significant, which the authors attributed to reduced numbers.

The PAM50 classifier as a predictive biomarker

In addition to its established role in breast cancer, the PAM50 classifier has been successfully applied to bladder (17) and lung (18) cancer, where basal/luminal classification again appears to confer predictive value (19). Zhao and colleagues now show that PAM50 subtyping is able to stratify patient outcomes and may have value in predicting androgen response in prostate cancer (16). There are several notable limitations to the study reported by Zhao et al., which the authors fastidiously point out in their manuscript. Most important among them is the retrospective nature of the study, rendering it impossible to completely account for confounders and selection bias.

In addition, a question arises as to why luminal B cancers would preferentially respond to ADT compared to luminal A tumors, which are similarly enriched for AR pathway activation. The authors maintain that luminal B tumors are biologically distinct from both basal and luminal A lineages with respect to proliferative index and expression of oncogenic drivers. Luminal B tumors represent a more aggressive subset, and therefore could be reasonably expected to exhibit a greater relative response to treatment.
intensification. However, the absence of any response to ADT in luminal A tumors remains incongruous with their AR activation state and represents an aspect in need of further study.

There are inherent limitations to taking a diagnostic optimized in one cancer and applying it to another. Breast and prostate cancer, while similar are not identical. A priori, it is plausible that a more tailored de novo classifier embedded in the gene expression data may more accurately model risk and predict response in prostate cancer. Moreover, owing to methodological limitations, a measure of intra-tumoral heterogeneity is absent from this analysis. Basal and luminal subtypes are likely to co-exist within the same tumor, and may arise from a common progenitor, a phenomenon that has been described in organoid models of prostate epithelial differentiation and tumorigenesis (20).

Despite these limitations, the findings reported by Zhao and colleagues are promising and prospective validation of the utility of the PAM50 classifier in identifying the subgroup who might benefit from ADT is warranted. If confirmed, the PAM50 classifier may identify patients for the appropriate application of ADT in the post-operative setting. In the wake of recent randomized trials demonstrating a cumulative benefit to the addition of ADT in the post-operative recurrence setting (21,22), clinicians find themselves in need of tools to better identify exactly which men derive a benefit from concurrent ADT and salvage radiotherapy. Similarly, the optimal timing for initiation of ADT in pathologically node positive disease remains an open question (23). Given the parallels one can draw between breast and prostate cancer, it is not surprising that a uniform predictive algorithm may apply in both diseases. Based on its utility in breast, bladder and lung cancer, it stands to reason that the PAM50 gene expression classifier has more broad applicability and may transcend both tissue of origin, and perhaps even the basal/luminal framework, as a predictive tool in prostate cancer.

Prospective trials are needed to definitively establish the utility of the PAM50 classifier in prostate cancer. An upcoming cooperative group study, NRG-GU-006, will enroll patients with a rising PSA after prostatectomy, randomizing between salvage radiotherapy alone or salvage radiotherapy concurrent with a second-generation AR antagonist (apalutamide, ARN-509) (24). Importantly, this will be the first study in localized prostate cancer to stratify patients prospectively based on a predictive biomarker, the PAM50 classifier. This innovative study design should definitively answer the question of whether the PAM50 classifier can predict both prostate cancer outcomes and response to ADT in the post-operative setting.

**Molecular profiling in prostate cancer: looking to the future**

As molecular stratification in prostate cancer comes of age, and as cost barriers associated with clinical genomics become more permissive, emerging biomarkers may increasingly rely on more comprehensive integrative analyses. The Cancer Genome Atlas (TCGA) Research Network published their landmark report on the molecular taxonomy of primary prostate cancer in 2015 (25), wherein they examined genomic alterations, gene expression, and epigenetic changes in 333 primary prostate carcinomas. They found that 75% of primary prostate cancers fell into 1 of 7 subtypes defined by specific gene fusions (ERG, ETVI/4, and FLI1) or mutations (SPOP, FOXA1, and IDH1). These subtypes demonstrated substantial heterogeneity with respect to epigenetic profiles as well as AR activity, which clearly clustered in a subtype dependent manner. For example, the IDH1 mutant subset was associated with a hyper-methylator phenotype and SPOP and FOXA1 mutant tumors had the highest levels of AR-induced transcripts. In addition, 25% of the prostate cancers they examined had “actionable” lesions in the PI3K or MAPK signaling pathways. They also found DNA repair genes inactivated in 19% of localized prostate cancers. This degree of molecular heterogeneity infers the existence of distinct taxonomies, defined by genomic alterations, transcriptional states, and epigenetic marks, conferring differential sensitivity to therapies such as ADT, chemotherapy, and radiation therapy.

Integrative molecular biomarkers will play an increasingly important role in risk stratification for clinical decision making in prostate cancer. Scenarios posing management dilemmas in contemporary multidisciplinary prostate cancer clinics include: (I) which men with favorable risk disease can be safely observed; (II) which men with unfavorable risk localized disease need treatment intensification, for example with a combination of surgery, radiation and androgen deprivation; (III) which men receiving salvage therapy will benefit from concurrent androgen deprivation and for how long; (IV) which men with low volume metastatic disease may be rendered disease free with combinations of systemic therapy and local therapy; and (V) how to best sequence available systemic therapies in men with castrate resistant metastatic prostate cancer. These scenarios are becoming
both increasingly common and more complex as clinicians attempt to incorporate novel functional imaging modalities and new therapies, including DNA damage response modulators and immunotherapy.

In conclusion, the incorporation of molecular profiling in the management of prostate cancer is entering the mainstream. As such, a working knowledge of emerging molecular diagnostics is fast becoming a pre-requisite for contemporary high-quality care of the prostate cancer patient.

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Footnote

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Perspective on the regulatory role of UGT2B28 as a conjugating enzyme in the progression of prostate cancer

Therina du Toit, Amanda C. Swart

Department of Biochemistry, Stellenbosch University, Stellenbosch 7600, South Africa

Correspondence to: Amanda C. Swart. Department of Biochemistry, Stellenbosch University, Private Bag X1, Matieiland, 7602, South Africa. Email: acswart@sun.ac.za.

Provenance: This is an invited Perspective commissioned by Section Editor Peng Zhang (Department of Urology, Zhongnan Hospital of Wuhan University, Wuhan, China).


Abstract: The maintenance of steroid homeostasis in the prostate is critical, with perturbation of steroidogenesis contributing to the modulation of active ligands in the androgen pool. In this scenario, enzymes catalysing the biosynthesis, inactivation and conjugation of steroids are the key players, regulating active ligand levels and in so doing, the activation of the androgen receptor (AR). The glucuronidation of potent ligands renders them unable to bind the AR, allowing the secretion of conjugated steroids. Uridine diphosphate glucuronosyltransferase 2B type 28 (UGT2B28), one of the UGT enzymes catalyzing the glucuronidation of androgens, has recently been given a prominent role in the regulation of prostate steroidogenesis—one which stands in contrast to the accepted dogma that lower androgen levels resulting from increased conjugation are associated with decreased prostate cancer (PCa) risk and disease progression. Increased DHT and its precursors, T and androstenediol, were reported to be associated with increased UGT2B28 tumor expression levels, linked to lower PSA levels but higher Gleason scores and increased PCa risk. In addition, the complete deletion of UGT2B28, was associated with decreased T, DHT and glucuronide derivatives when compared to patients carrying both alleles. UGT2B28 is encoded by a single gene giving rise to UGT2B28 type I which catalyses androgen glucuronidation and, due to alternative splicing, also produces two distinct transcripts, UGT2B28 type II and III. Type II with its premature stop codon, is devoid of the cofactor binding domain while type III is devoid of the substrate binding domain, both catalytically inactive, truncated proteins. Increased UGT2B28 mRNA expression was reported in primary tumours, and while variable nuclear and strong cytoplasmic staining were distinctive of tumour cells, the expression levels and compartmentalization of the specific protein isoforms remain unknown. While increased expression of type I would contribute towards lowering androgen levels, increased expression of types II and III would not. The abundance of type III transcripts in multiple tissues may provide insight into a regulatory role with truncated isoforms possibly affecting androgen levels by regulating substrate and/or co-factor availability, dimerization or the formation of protein complexes with other UGTs, while protein-protein interaction may also impact cascade signaling pathways in PCa development and disease progression.

Keywords: 11keto-testosterone; copy-number variation; dihydrotestosterone; prostate cancer prognosis; UDP-glucuronosyltransferase

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In their article “The UGT2B28 sex-steroid inactivation pathway is a regulator of steroidogenesis and modifies the risk of prostate cancer progression” published in *Eur Urol* in April 2016, Belledant *et al.* report a regulatory role to the enzyme uridine diphosphate glucuronosyltransferase 2B type 28 (UGT2B28), modulating prostate cancer (PCa) progression. This role is separate from that of UGT2B28’s function as a conjugative inactivating enzyme, with UGT2B28 influencing circulating androgen levels in PCa patients as well as clinical and pathologic factors associated with the disease (1).

Placing the work in context, we will discuss the contribution of steroidalogenic enzymes towards the production and maintenance of active androgens which interact with the androgen receptor (AR) and the crucial role that these enzymes play—not only in normal prostate homeostasis but also in PCa. We will also briefly discuss genetic variability within UGT2B enzymes, focusing on B28, after which we will review the concepts highlighted by Belledant *et al.* and provide a perspective on the role of this conjugating enzyme in PCa.

Active androgen levels are maintained by both metabolic and catabolic enzymes with 5α-reductase type 1 (SRD5A1) and type 2 (SRD5A2) catalyzing the biosynthesis of dihydrotestosterone (DHT) from testosterone (T) or alternatively from androstenedione (A4) and the 5α-androstan-3,17-dione (5αDIONE) intermediate. The interconversion of A4 and T and their 5α-reduced metabolites by the reductive or oxidative 17β-hydroxysteroid dehydrogenase (17βHSD) enzymes further adds to the complexity of steroidogenesis in the prostate. The equilibrium is however perturbed in PCa and tumour development is associated with the modulation of enzyme expression. Increased expression of the reductive enzyme 17βHSD3 (31-fold) has been reported in the prostate tumour microenvironment (2) as well as increases in 17βHSD5 (AKR1C3) expression ranging 2–5-fold, favouring T biosynthesis. In conjunction with the increased expression of the reductive enzymes, a 7-fold decrease in the expression of the oxidative enzyme 17βHSD2 which catalyzes the reverse conversion of T to A4, has been reported which again diverts the flux towards T production. In addition, while SRD5A2, which is expressed in normal prostate tissue, is decreased (2–4-fold), the expression of SRD5A1 has been shown to be increased (2-fold) in castration-resistant prostate tumours thus maintaining DHT levels in the prostate ([3] and the references therein).

Contributing to the active androgen levels in the prostate microenvironment are the inactivating and conjugating enzymes. Only androgens with a hydroxyl group at C17 or C3 are potential substrates for conjugation by UGTs and as such both T and DHT can be converted to their glucuronide derivatives, rendering them inactive to be secreted into circulation. DHT together with 5αDIONE are, however, also inactivated by 3α-hydroxysteroid dehydrogenase type 3 (AKR1C2), which catalyzes the reduction of the keto group at C3, forming 5α-androstan-3α,17β-diol (3αDIOL) and androsterone (AST), respectively, allowing the subsequent addition of the glucuronide moiety at C3. While AKR1C2 also exhibits oxidative activity, this reverse reaction is primarily catalyzed by 17βHSD6, 17βHSD10 and retinol dehydrogenase 5 expressed in the prostate (4–7). In primary PCa, AKR1C2 expression levels have been reported to be significantly decreased compared to benign tissues (7–9), which contributes to significantly higher DHT levels in primary PCa tumours (10). In malignant epithelial cells, increased expression (3-fold) of 17βHSD10, which mediates the conversion of 3αDIOL to DHT, increased DHT tumour production (11). Furthermore, androgen deprivation therapy led to a 2-fold increase in 17βHSD6 expression levels, also associated with the biochemical progression of PCa (11). It is therefore apparent that the intricate homeostasis in the prostate is modulated by the perturbed expression of the steroidalogenic enzymes catalyzing DHT production and those catalyzing the inactivation of androgens.

The expression of the UGT2B enzymes are tissue and substrate specific. UGT2B7, B15 and B17 are the three major UGT2Bs primarily responsible for androgen conjugation in humans and of these only B15 and B17 are actively expressed in the prostate (12). The UGT2B enzymes conjugate androgens in a regiospecific manner, either catalyzing the addition of the glucuronide moiety at C17 and/or at C3. UGT2B7 conjugates 3αDIOL and AST at C3 and T, DHT and 3αDIOL at C17; UGT2B15 only at C17 of T and 5α-reduced androgens, DHT and 3αDIOL; UGT2B17 at C3 and C17 of T, DHT, 3αDIOL and AST and UGT2B28 conjugates 3αDIOL at both C3 and C17 (low efficiency), as well as AST and T. Although UGT2B28 conjugates 3αDIOL, its capacity to conjugate 3αDIOL is much lower than that of B15 and B17 (12,13). In addition, UGT2B28 also conjugates estradiol (E2), etiocholanolone and 5β-androstan-3α,17β-diol. The latter is the product of androstenediol (5-diol) catalyzed by AKR1C2 and SRD5A1 (13,14). Interestingly, 5-diol was shown by Belledant *et al.* to be increased and associated with increased UGT2B28 levels in prostate tumour tissue.
In contrast to the steroidogenic enzymes catalyzing biosynthesis pathways, the genes encoding the UGT enzymes are characterized by substantial genetic variability brought about by polymorphisms, the production of alternative transcripts through alternative splicing/last exon/internal exon use and exon skipping, together with copy number variation (CNV) and alternative promoters. These factors all contribute to the complexity of the role of the UGT enzymes in steroid inactivation, impacting protein expression levels and enzymatic activities which in turn impact circulating androgen levels and PCA outcomes (15).

While comparative studies relating to circulating hormone levels are complicated by interindividual variations, biochemical studies inevitably yield tangible data regarding enzyme kinetics as, for example, in the case of the Asp85Tyr polymorphism of UGT2B15. This functional polymorphism encodes a protein with the Asp85Tyr mutation and has been shown to be expressed either homozygously for one or the other as well as heterozygously in patients with both alleles. Similar Km values for DHT and 3αDIOL are reported but the enzyme containing Tyr residue catalyzes the inactivation more efficiently with a 2-fold higher Vmax value (16,17). UGT2B15, B17 and B28 are encoded by a single gene (15), with polymorphisms in the former two genes having been shown to significantly impact circulating levels of free unconjugated T and AST (18-20), and the intra-prostatic levels of 3αDIOL-3G and 3αDIOL-17G (21). Whole gene deletions were reported for UGT2B28 in this paper and previously by Nadeau et al. (18), in which they also showed that in UGT2B17 (−/−) PCA patients circulating 3αDIOL-17G levels were significantly reduced. Although T and DHT levels were not significantly lower, AST levels were increased, indicative of increased flux in these metabolic pathways and if unconjugated, would allow the reactivation of AST and a concomitant increase in AR signaling. This study also showed that in UGT2B28 (+/−) patients, circulating androgen levels were not affected. However, patients with a single copy in conjunction with the UGT2B17 (−/−) deletion not only had significantly increased AST levels but also significantly decreased AST-G and 3αDIOL-17G levels (15,18).

Investigations into complete UGT2B28 deficiency by Belledant et al. (1) reported significantly decreased circulating T, DHT AST-G, and 3αDIOL (both C3 and C17 derivatives) in patients when compared to UGT2B28 (+/+ ) patients. In addition, their study also reported that high tumour expression levels of UGT2B28 were associated with lower protein specific antigen (PSA), smaller tumour volume, but with higher Gleason score and positive nodal status. Patients with increased nuclear and cytosolic UGT2B28 expression in tumours together with significantly increased circulating T and DHT levels suggested an association with progression to a more aggressive disease.

It should be noted that three UGT2B28 isoforms are expressed in humans—type I, II and III characterized in 2001 by Lévesque et al. (13). RT-PCR data identified the three transcripts in mammary gland tissue and in LNCaP prostate cancer cells with only type III expressed in prostate and benign prostatic hyperplasia tissue. The active UGT2B28 type I was shown to catalyze the conjugation of E2, T, AST and 3αDIOL efficiently. UGT2B28 type II contained a 308 bp deletion, amino acid residues 335 to 437 in the cofactor binding domain and contained a premature stop codon, and UGT2B28 type III lacked residues 105 to 221 in the putative substrate binding domain. Both truncated isoforms yielded a non-functional protein. Western blot analyses, using the polyclonal EL-93 anti-UGT2B17 antiserum, specific to the UGT2B enzymes, showed the three isoforms to have apparent molecular masses of 52, 35 and 42 kDa, respectively. While UGT2B protein was also shown to be present in liver preparations together with RT-PCR identifying UGT2B28 type II and III transcripts (13), the present study by Belledant et al. showed the polyclonal EL-93 antibody yielding a positive signal for all the UGT2B proteins. In contrast, the UGT2B28 antibody was shown to specifically bind UGT2B28 protein only and since it was raised against a peptide sequence spanning residues 113–124 the antibody only recognizes the type I and type II isoforms. Since only type III mRNA transcripts were shown in normal prostate (a single prostate tissue sample purchased from Clontech (13), it is therefore interesting that immunohistochemistry (IHC) analyses showed the presence of UGT2B28 in normal prostate tissue, in the nucleus of basal and some secretory cells, pointing to the presence of both the type I and II isoforms. Cancer tumour cells from UGT2B28 (+/−), on the other hand, showed strong nuclear and cytoplasmic staining (1) and while it is very possible that type III is also present, the data cannot distinguish between type I and II isoforms. Cancer tumour cells from UGT2B28 (+/+), on the other hand, showed strong nuclear and cytoplasmic staining (1) and while it is very possible that type III is also present, the data cannot distinguish between type I and II isoforms. Interestingly comparative analyses of UGT2B28 expression and gene copy number showed, in both the nuclei and cytoplasm, that expression levels were similar between +/- and +/- patients. Strong nuclear staining was associated with significantly lower PSA levels and patients presented with smaller tumours, while strong cytoplasmic staining was associated with higher Gleason scores and positive nodal status. Considering circulating androgens, significantly
increased T and DHT levels were also associated with increased nuclear UGT2B28 expression. Although these androgens also increased with increased cytosolic expression, their levels were not significant. 5-diol, the product of dehydroepiandrosterone catalyzed by AKR1C3/17βHSD3, also increased with increased nuclear (P=0.079) and cytoplasmic (P=0.026) expression of UGT2B28 (1), and would as such contribute towards T levels due to the presence of 3β-hydroxysteroid dehydrogenase type 2 catalyzing the conversion of 5-diol to T.

Analyses of circulating androgens in UGT2B28 (−/−) PCa patients showed significantly reduced T, DHT as well as downstream conjugated metabolites, AST-G and 3αDIOL-3G and 3αDIOL-17G levels in comparison to UGT2B28 (+/+ ) patients. Circulating A4 was significantly increased in the UGT2B28 (−/−) cohort (1) which would contribute to the production of DHT via the 5αDIONE pathway in prostate steroidogenesis (22). Although all downstream intermediate steroid metabolites were not reported in this study, analyses of the ratio of A4, T and DHT to their conjugated metabolites, 8.59 [UGT2B28 (+/+)] and 8.97 [UGT2B28 (−/−)] offers some perspective into the metabolic flux. However, these steroids cannot be regarded in isolation as we have also shown the hydroxylation of A4 by cytochrome P450 11β-hydroxylase leads to the production of 11β-hydroxyandrostenedione (11OHA4) in the adrenal, which would also be increased in UGT2B28 (−/−) patients with higher A4 levels, and would certainly contribute to the androgen pool via the 11OHA4-derived pathway (23,24).

The data presented is certainly open to interpretation and the manner in which the enzyme isoforms would impact PCa complex. Inactive androgens in circulation are also products of other UGT enzymes. When considering increased circulating T, DHT and 5-diol being associated with increased tumour expression of UGT2B28, it is prudent to note that androgens in the prostate and in circulation would be conjugated by UGT enzymes expressed either in the prostate itself or in other target tissue and therefore circulating levels cannot be regarded in isolation. In addition, the expression levels of UGT2B28 isoforms in the prostate and prostate tumours are unclear, since the expression can be that of type I or type II. It is furthermore possible that type III is also present. In the translation of UGT2B28 transcripts encoding type II, the deletion of exon 4 and 5 excises the UDPGA binding site and disrupts the open reading frame with the ensuing premature stop codon yielding a protein fold different to that of type I. Despite not having a transmembrane domain, the truncated type II was reported to nevertheless be present in the endoplasmic reticulum and in the perinuclear membrane as in the case of type I. Type III on the other hand contains all the relevant structural domains but not the substrate binding domain. Although the data was not shown Lévesque et al. also reported that type III was capable of homodimerization (13). It is interesting to note that in their study in which the three isoforms were expressed in HEK293 cells, Western blotting showed type I and III to be represented by single bands while only type II showed additional bands of greater apparent masses indicating possible protein aggregation, either type II with itself or with the other two isoforms. Although one would normally not expect protein aggregation under denaturing electrophoretic conditions, it is fairly common that proteins form stable aggregates as for example in the case of cytochrome b₅, which electrophoreses as dimer and tetramer aggregates even under stringent denaturing conditions (25).

Besides the IHC data not distinguishing between type I and II, it is unclear if isoform expression would be compartmentalized as in the case of UGT2B15 and 17 with the former being expressed in the luminal cells and the latter in the basal cells (26), further contributing towards the fine regulation brought about by compartmentalized conjugation and ligand availability to the AR. An interesting aspect brought to the fore by the Belledant et al. study is indeed a novel tier of regulation other than that of the inactivation of androgens. Increased T and DHT levels associated with increased UGT2B28 protein expression and more aggressive PCa could perhaps also be indicative of decreased UGT2B15 and B17 expression since androgens have been shown to negatively regulate their conjugation by these enzymes (27).

While an increase in the active isoform may contribute towards high levels of circulating conjugated androgens, the expression of the inactive forms would not contribute towards conjugated androgen levels. Linking UGT2B28 (+/+ ) to an increase in biochemical recurrence (BCR) and overexpression to increased PCa risk and potent androgens, would possibly implicate the expression of the inactive isoforms and an impaired ability to conjugate androgens. On the other hand, the isoforms may aggregate as has been shown for the UGT1 and UGT2 enzymes (28,29)—not only forming dimer-dimer complexes but also aggregates with other UGT2B enzymes thus rendering them unable to catalyze the conjugation of androgens.

Whether UGT2B28 is the active androgen-inactivating
UGT isoform under high-androgen exposure as the authors suggested is unclear as the expression of UGT2B28 in the prostate remains to be fully characterized. While type I would certainly contribute towards androgen inactivation in the prostate, the expression of the isoform types in normal and PCa tissue and the expression level of the three isoforms as well as possible compartmentalization remain unknown. Since data thus far indicate tissue-specific processing of mRNA transcripts, investigations into the regulation of post-transcriptional activities in the expression of UGT enzymes will shed light on the role of the UGT2B28 isoforms. It is possible that type III may be involved in regulating co-factor availability while type II may sequester androgens, rendering them unavailable for AR activation, depending on the level of expression. In addition, the role of UGT2B28 type I in the inactivation of estrogens rendering these steroids incapable of activating the estrogen receptor, AR or mutated AR may be one of critical importance. Furthermore, UGT2B28 has been considered only in terms of T and DHT—C_{19}, C_{21} and C_{11}-oxy steroids would certainly also impact PCa and possibly find associations with BCR. Previously published data suggests a regulatory role for UGT2B28 in terms of estrogen conjugation as AST and E2 were efficiently conjugated while more than 50% estrone (E1) remained in the free form in breast cyst fluid (13). Lévesque et al. (13) showed the presence of all three isoforms of UGT2B28 in mammary gland tissue, however, when transiently expressed, E1 was not conjugated by UGT2B28 type I—suggesting that other UGT2B enzymes are involved in the conjugation of E1. However, since the conjugation of E2 has been shown, a role for UGT2B28 in breast cancer is highlighted. Furthermore, with perturbed UGT2B28 expression and E2 conjugation decreased, promiscuous binding of E2 to the mutated AR in PCa may also occur (30).

Interestingly while the three UGT2B28 transcripts were detected in breast tissue none were detected in the ovary or placenta and thus the presence of UGT2B28 type III in the testis, prostate and BPH tissue (13) suggests a prominent role for UGT2B28 in male steroid homeostasis. It was previously reported that the C_{11}-oxy C_{19} steroids were poor substrates for conjugation and that UGT2B17 exhibits lower catalytic activity towards C_{19} steroids containing a C_{11}-hydroxy group, even though it has been shown that 5a-androstane-3a,11α/β-17β triol and T are conjugated at similar rates (15,31). We recently reported that 11OHA4 is metabolized to 11keto-dihydrotestosterone in PCa cells and have shown that it is as potent as DHT in activating the AR (32). We have since shown that 11keto-testosterone is not glucuronidated efficiently in LNCaP cells and as such would readily activate the AR as T and DHT are fully conjugated. Furthermore, we reported that the C_{11}-oxy C_{19} steroids are present at significantly higher levels than the C_{19} steroids in circulation, in PCa tissue (33) and in BPH tissue (unpublished data), drawing attention to C_{11}-oxy metabolites in PCa.

In summary, the contribution of UGT2B enzymes to active ligand availability can only be fully assessed in the context of all steroids which may contribute to PCa and its aggressive progression. This will certainly lead to a better understanding of the complex regulation by these inactivating enzymes, modulating receptor signaling, not only in terms of C_{19} steroids but also in terms of the C_{18} and C_{21} steroids as well as the C_{11}-oxy steroids. As has been demonstrated by this study, genetic variations together with tissue-specific mRNA processing and the biosynthesis of different isoforms, as well as CNVs, contribute to the complexities of the UGT enzymes and their impact on PCa development and disease progression. Both UGT2B15 and B17 have also been reported to conjugate pharmacological compounds while the contribution of UGT2B28 to drug inactivation remains unknown. UGT2B17 and B28 are also among the UGT genes of the human genome which are the most commonly deleted genes and as such may impact drug metabolism and therapeutic strategies. While the usage of an alternative promoter may modulate the expression and/or activity of UGTs, it may also contribute to variability in the glucuronidation pathway and steroid hormone levels observed in patients. In the case of UGT2B17, gene deletions and CNVs have been shown to affect steroid inactivation, altering tissue and circulating androgen levels, supporting the general hypothesis of reduced inactivation by UGT enzymes resulting in increased active ligands, risk of PCa and its recurrence (34). The current report by Belledant et al. (1) showing increased UGT2B28 expression linked with increased T, DHT and 5-diol and a more aggressive disease, together with CNV associating gene deletion with decreased active AR ligands and conjugated downstream derivatives, points to other mechanisms at play involving pathways other than steroid inactivation.

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of genetic aspects.

**Footnote**

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

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Surgical resection of tumors is a common practice in breast, lung, melanoma, and many other cancers, and is known to extend life expectancy significantly. However, recurrence and metastasis are still frequently seen post-resection. Distant metastasis occurs when cells from the primary tumor enter the bloodstream, adhere to the endothelium, infiltrate a distant site and proliferate. The number of circulating tumor cells (CTCs) in the vasculature has been shown to correlate with patient survival and prognosis (1). CTC count perioperatively has been under investigation to determine whether surgical procedures introduce additional CTCs into the bloodstream. While this postsurgical CTC increase has been observed for various cancer types, many studies have shown that CTC counts normalize and often decrease after surgery (2). Still, the long-term effects on progression and survival of surgical release of CTCs have not been definitively determined. In this commentary, we discuss the prospect of minimizing surgical CTC increases using less invasive techniques as well as the need for more aggressive perioperative targeting of CTCs.

One area of research that may have far reaching implications in cancer treatment is the relationship between surgical technique and CTC count. Many studies have shown that common methods used for diagnosis (biopsy) and treatment (resection) of cancer can lead to bloodborne tumor cell dissemination. In one study, The Zharov lab showed that while mechanical palpation of breast tumors did not increase CTC counts in mice, tumor biopsy and resection did (8). Moreover, lung resection was shown to increase CTC count, where the presence of CTC clusters correlated with worse prognosis (9). Bayarria-Lara et al. found that CTC counts decrease 1 month after lung resection, though the presence of CTC after surgery was associated with early recurrence (10).

In a recent study published in Investigative Urology (11), Kauffman and associates investigated whether robotic assisted laparoscopic radical prostatectomy (RALRP) reduced CTC introduction in comparison to past studies conducted on open prostatectomy. They showed that RALRP did not significantly increase CTC numbers in patients, whereas past studies of open prostatectomy based on RT-PCR amplification of epithelial markers in blood were consistent with CTC increases. In the study, blood samples were drawn from 25 patients preoperatively as well as intraoperatively. Using EpCAM-positive selection, 48% of patients were shown to be CTC-positive preoperatively while 52% of patients were CTC-positive after surgery (11). Perioperative increases and decreases in CTC count were observed at the same frequency, and increases were found to never exceed 1 CTC per 8 mL blood (11). It is suggested...
that RALRP may hold an advantage to open prostatectomy due to the lack of CTC introduction (11).

Similar results have been obtained in studies focused on other cancer types. In esophageal cancer, minimally invasive esophagectomy showed lower intra- and postoperative CTC counts than open esophagectomy (12). Video-assisted lobectomy also yielded fewer CTCs than open thoracic surgery for the resection of lung cancer (13). However, the impact of the additional CTCs introduced is debated. Several reports have demonstrated a correlation between increased CTC numbers postoperatively and worse prognosis in lung, colon, and stomach cancers (14-16), while one study in pancreatic cancer found no such relationship (17). In fact, reports show that the increase of CTC after surgical procedures normalizes over time, sometimes resulting in lower CTC counts than preoperatively (2,8,10,18). The eventual fate of these observed CTCs is of course unknown. Reports of this nature compel the need for further analysis of the correlation between surgical technique and cancer progression. In addition, methods to decrease CTC frequency during surgery should be investigated, including therapeutic agents to target CTCs.

Most methods for cancer treatment focus on the eradication and shrinking the primary tumor, even though 90% of cancer fatalities arise from metastasis. Recently, our group developed a therapeutic approach that directly targets CTCs (19). This nanomedicine construct is comprised of phosphatidylcholine liposomes conjugated with E-selectin, a natural endothelial cell adhesion molecule, as well as TNF-related apoptosis-inducing ligand (TRAIL), a pro-apoptotic ligand whose receptors are upregulated on many cancer cells. The drug acts by adhering to leukocytes within a patient's blood. These cells then interact with CTCs, inducing apoptosis through TRAIL signaling (19). In preclinical studies, E-selectin/TRAIL liposomes were shown to significantly reduce CTC number in colon and prostate cancer models. When introduced into the bloodstream of mice containing colon cancer cells, the TRAIL liposomes decreased CTC count by over 90% (19). In an orthotopic prostate cancer model, CTC counts were found to be 94% lower in mice treated with ES/TRAIL liposomes compared to control mice (7). A therapeutic of this type could hold great promise as an adjuvant treatment when used perioperatively, by preventing the operative increase of CTCs and therefore any adverse downstream effects.

While CTC count surrounding surgical procedures has not been directly implicated in metastasis, it is hypothesized that the introduction of CTCs during surgery may promote cancer progression. This motivates further research to elucidate the correlation between type and timing of surgical intervention, and cancer progression. Moreover, since over 90% of cancer fatalities result from metastasis, a greater emphasis on treatments that target CTCs or disseminated tumor cells is also warranted. It is possible that by minimizing the surgically-induced CTC burst through minimally invasive surgical techniques, as well as by targeting CTCs perioperatively, we may one day decrease the occurrence of metastasis and achieve improved patient outcomes.

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Footnote

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