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Key Leaders' Opinion on Radiotherapy with Immuno-Targeted Therapies

Editors: Jiade J. Lu Lvhua Wang Bengt Glimelius



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AME Medical Review 2A004

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Will scholarly journals perish?

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

The introduction of online journals results 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 me thinking is the open access of clinical trials data. What would be the bigger picture if open access to clinical trials data becomes the mainstream?

It is interesting that with the primary bottleneck lying in 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 to be 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 big enough; on the other hand, for the entire population, as more as individuals (e.g., patients) are expected to be included, to contains 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 the real-world-based clinical trials so appealing? It is understandable that the results and conclusions are likely to be altered in studies targeting the same issue using the same research method with sample size changed. In addition, the probability of such a "likely" 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 more populations? If the attempts are not continuing, which should be, the "validation" is equivalent to "self-deception" in a sense.

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 arise: 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, we can easily find the constant update of database on the Internet. Simply by clicking on a button, we obtain the statistical results of the most current data. A further button click would display the validation results based on a specific population. The database would be updated at a certain period of time (e.g., 1 month or 1 day), and the statistical results would "likely" also be changed accordingly. At that time, the questions 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. Then in what way the scholarly journals would probably lead to?

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 views on the case. He encouraged us to interpret the case from different perspectives independently in what form that I found pretty practical.

The course brought 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 the rigid and cold outcomes but it would definitely cares more about the reflection on the problems, update of insights, and integration of science and arts.

AME Medical Review Series is a production of the above thinking. As an attempt, we decided to invite experts internationally to provide their views on a specific topic to share their insights with more clinicians and thus benefit more patients. The first chosen topic for the series is the currently controversial one: conventional surgery versus stereotactic body radiotherapy for

VI

the early stage lung cancer. As the first book to the series, we hope it would give you a glance at the coming changes.

The book series will be written by a group of individual experts who are willing to contribute medical reviews and comments to individuals who are interested in clinical research and medical reviews specifically. The book in your hand may possibly be on a heavy subject but we do hope it is presented in an easier way. It will be more than great if it brings you some thoughts and inspire you in some way.

Stephen D. Wang Founder and CEO, AME Publishing Company

Combining technology and biology to conquer cancer

Given the rapid advancements in technology and biology over the past decade, it has never been so exciting to be an oncologist now, particularly a radiation oncologist. In the early 2000s, the revolution in computer-driven radiotherapy technology enabled exquisitely precise direction of radiation beams to tumor targets. The advent of 4-dimensional computed tomography (CT) and on-board image-guided intensity-modulated radiotherapy, stereotactic ablative radiotherapy, intracranial gamma knife, particle therapy and other sophisticated imaging and treatment techniques have equipped radiation oncologists with novel tools to tightly conform ablative or definitive radiation doses to targets while avoiding inadvertent irradiation of surrounding critical normal structures. As a result, local control—and in some patients even survival—has been improved and treatment-related toxicity has been minimized.

However, cancer is a biological disease, not just a technologic challenge. As our ability to control local tumors improves with the use of new technology, the importance of systemic disease control grows in parallel—after all, in most cases it is metastatic disease that kills the patient. During the past decade, the development of genomic profile–based targeted therapy, immune checkpoint pathway–based immunotherapy, and chimeric antigen receptor (CAR)-T cell–mediated cancer killing has revolutionized the management of stage IV cancer of many types, particularly lung cancer, melanoma, head & neck cancer, lymphoma, leukemia, and myeloma, among others. We are now starting to think about the potential to "cure" stage IV disease, which historically has been considered incurable. Indeed, at this time we know considerably more about the biology of cancer, how it starts and how it progresses, than in the past.

In the past, we established the stage of cancer based on tumor histology, tumor location, and the degree of spread as detected by imaging such as CT, positron emission (PET)/CT, and magnetic resonance imaging. However, imaging can detect and classify cancer only when the cancer reaches a certain size, typically >5 mm. Biologically, however, "localized" disease, "advanced" disease, "metastatic" disease, and "recurrent" disease all represent ongoing biological processes, all involving a dynamic balance between the human defense system and cancer cells, that is always ongoing both before and after detection of a cancer by imaging. Circulating tumor cells (or circulating DNA or RNA) could already be present in a case judged to be "early stage" by imaging; conversely, some patients with systemically and locally controlled "stage IV" disease may survive for years, or even decades. We know now that the various stages of cancer involve different patterns of gene mutations and different levels of immunosuppression, among other biological processes. As such, recent developments in targeted therapy and immunotherapy have opened a new window for radiotherapy. For early-stage disease, immunotherapy given with a local treatment such as radiotherapy may lead to less tumor recurrence or metastasis. For metastatic disease, adding radiotherapy may overcome the resistance to targeted therapy that typically develops in most patients within 2 years after beginning targeted therapy, or could convert tumors that do not respond to immunotherapy ("cold" tumors, typically present in about 80% of common tumor types) into "hot" (responding) tumors. Moreover, cancer cells killed by radiation release tumor-associated antigens and immunoregulatory cytokines, thereby functioning as a kind of cancer-specific vaccine in situ; they further activate tumor-specific systemic immune responses to eradicate tumors even outside the radiation field (the abscopal effect). Radiation can also damage epithelial cells lining tumor blood vessels and improve the ability of immune cells, cytokines, targeted therapy agents, and chemotherapy to penetrate the tumor. These effects seem to be more prominent when the radiation used with immunotherapy involves giving high (ablative) doses, a type of therapy for which we coined the term "I-SABR" (immunotherapy and stereotactic ablative radiotherapy). I-SABR protocols are underway for both early-stage disease and locally advanced cancer worldwide.

This book provides a timely review of the details of the mechanisms underlying radiation- and immunotherapy-evoked effects, and provides updated information on clinical trials that combine biology (immunotherapy and targeted therapy) with technology (radiotherapy). Most importantly, it helps us to prepare for what comes next. Now is the era of both technology

and biology—better technology leads to better outcomes, and better biology needs better technology. By combining biology and technology, we can conquer cancer by providing both systemic and local control of disease. This is truly "precision medicine."

Joe Y. Chang, MD, PhD Professor of Radiation Oncology, Director of Stereotactic Ablative Radiotherapy, The University of Texas MD Anderson Cancer Center, Houston, USA

Preface

One of the most exciting developments in oncology has been the introduction of novel immunotherapies with many potential applications in cancer treatment. By enhancing immune response profound and long lasting antitumor effects can be achieved. These new therapeutic options do not replace but rather complement existing and proven treatment modalities, such as radiation therapy. Combination therapies have shown encouraging laboratory data and promising early clinical results. Unique toxicities are being encountered as well. Furthermore, the study of molecular biomarkers may contribute to a deeper understanding of mechanisms of action and assist in appropriate patient selection. Numerous clinical trials of radiotherapy and immunotherapy are currently ongoing. *"Radiotherapy with Immuno-Targeted Therapies"* is a collection of well written articles by recognized authors that cover several aspects of newer targeted approaches in combination with radiation. This textbook comes in a timely manner as the field is expanding and new information is rapidly accumulating.

Athanassios Argiris, MD, PhD, FACP

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Preface

The evasion of immunologic detection and clearance of neoplastic cells and prevention of the host immune system's resolution of pro-neoplastic micro-environmental conditions are among the hallmark features of cancer. Like other hallmarks (unchecked proliferation, angiogenesis, replicative immortality, etc.), the origin of these characteristics lie within the genetic and epigenetic instability of transforming cells, which then undergo an intense biologic selection process. To better understand these immunologic changes is to better fundamentally understand cancer.

Radiotherapy has a greater than 100-year track record of efficacy against nearly all forms of malignancy. Yet, even in this modern era, not all of the mechanisms that foster this efficacy have been fully elucidated. The comprehensive immunogenic effects of radiation treatment are still largely unknown. The abscopal effects of radiotherapy have been observed and reported for decades and more recent biologic investigations have revealed that radiotherapy has a profound impact on the immunologic characteristics of neoplastic cells and the host response. To better understand these radiation-induced changes is to better understand radiotherapy itself.

Therefore, a tremendous opportunity stands before the field today; to improve the therapeutic index of radiotherapy by the modulation of post-radiation tumor immunology. As mechanisms of immunologic changes are further characterized, targeted therapeutic agents and treatment strategies can be developed to augment the response of tumor cells to radiotherapy – both locally and systemically.

With this goal in mind, The AME Publishing Company has teamed with over 40 researchers, editors, and leaders in the radioimmunologic field from around the world to bring about this book, Radiotherapy with Immuno-Targeted Therapies. It puts the latest work and thought processes into one convenient source. The book is organized into two principle sections: The first provides an overview of some of the basic observations pertaining to the impact of radiotherapy on cellular immunology. Discussed are the molecular events within the innate and adaptive immune systems that occur after exposure to ionizing radiation. Central to these events being tumor antigen presentation via major histocompatibility complex (MHC) classes I and II and the elaboration of the myriad cytokines which can either enhance or diminish the tumor clearance capability of these systems. The second section highlights the disease-specific translational and clinical efforts that are ongoing. Emphasis is placed upon the different immunologic effects of ablative versus fractionated radiotherapy, mechanisms of the abscopal effect, the immunologically complicated tumors arising in brain parenchyma (primary and metastatic), and the role of EGFR in head and neck cancers.

We hope this book not only informs the reader, but inspires new questions and lines of investigation that will be incorporated into their future work.

> Jiade J. Lu, MD, MBA National Distinguished Expert Executive Vice President Shanghai Proton and Heavy Ion Center Shanghai, China

Radiotherapy with immune-targeted therapies

Not very many years ago it could well be stated that "immunotherapy has been promising for 40 years, and it is still (only) promising". Those critically expressed words about a cancer therapy that for decades struggled to reveal clinically meaningful gains for the patients in properly controlled trials are no longer true. Rather, during the past about 6 years, we have witnessed substantial gains, initially in one or a few "immunogenic" tumor types like malignant melanomas but more recently also in several other tumor types including some "difficult to treat" cancers. Abstracts at recent scientific meetings further witness about this rapid development. The efforts in basic science during those struggling decades have finally resulted in the design of specific targeted therapies in one or another way related to the immune system. Of particular relevance are the positive effects on survival of a growing number of so called checkpoint inhibitors. Starting in 2011, first ipilumumab and subsequently pembrolizumab have been approved as single therapies, and more recently different combination therapies.

The AME Publishing Company has in this book entitled "*Radiotherapy with Immune-Targeted Therapies*" collected many thoughtful and sometimes also provoking articles about the relevance of the immune system for therapy, in particular when radiotherapy is delivered and how it then could be modulated, improving therapeutic efficacy. As the title indicates, the major focus is on radiation and immunotherapy in various combinations including the immunological effects of ionizing radiation alone; radiation may have favorable effects far from the tumor volumes being irradiated. Unfortunately radiation may also have unfavorable effects mediated via the immune system. A few chapters deal with the diagnosis of the immune expression in tumors of high relevance to properly select the best patients for this type of therapy. The book brings up both basic and preclinical aspects, translational research and the results of clinical trials.

The majority of the 36 separate contributions are written by experienced scientists as an editorial text to a recently published and relevant article, often in a high-impact scientific periodical. The authors have been specially invited to give their comments providing perspectives to the work published but also giving directions, sometimes also speculative to future developments. The quality of the included articles are at least of the same magnitude as you can find in the journals where the article was first published. Not all studies or trials selected for commentaries have been positive, but negative studies/trials also need to be properly discussed and reasons why they failed examined. There are several examples of this in the different chapters.

In addition to the commentaries, a few review articles are also included. These articles focus mainly on non-small cell lung cancer, different head and neck cancers and esophageal cancer. The progress in immunotherapy in at least the first two diagnoses has been substantial clearly helping subgroups of patients. Even if the development presently is very rapid, a speed that no book or review can keep up with, these chapters are surprisingly updated. Otherwise, the commentary articles deal with virtually all cancer diagnoses also including hematological malignancies and whatever your subspecialty is you may find an article of interest to read.

Bengt Glimelius, Professor Department of Immunology, Genetics and Pathology, Experimental and Clinical Oncology; Research group Bengt Glimelius, Uppsala University, Uppsala, Sweden

Preface

All the while, surgery, radiotherapy and chemotherapy are three major means for cancer therapy. Latest data has indicated that the cure rates for cancer by surgery, radiotherapy and chemotherapy are 49%, 40% and 11% respectively. With the extensive clinical application of a variety of image-guidance technology, intensity modulation technique and radiotherapy technology such as SBRT, the accuracy of radiotherapy is scaling new heights. In order to achieve better radiotherapeutic effects, the single dose of radiotherapy and biologically equivalent doses have also been gradually enhanced.

As we all know, cancer is a systemic disease. Its occurrence and development are closely related to the immune system disorder of the body itself. As local treatments, both radiotherapy and surgery against cancer mainly through eliminating the tumors while reducing the rate of distant metastasis. Rather than eliminating the tumors locally, radiotherapy can also activate the anti-cancer immune response mechanisms and bring systemic anti-cancer activity against cancer with the assistance of immunotherapy. Moreover, radiotherapy can transform the existing immunosuppressive tumor microenvironment, further augmenting the anti-cancer immune response. It differs essentially from chemotherapy which pitches into the elimination of systemic proliferating cells. This explains why clinical research on the combined use of local radiotherapy and immunotherapy has been increasing in recent years. It is believed that the concept of cancer treatment will change essentially. The treatment for cancer will, therefore, meet with both vast development and the corresponding challenges.

In this context, the book "*Radiotherapy with Immuno-Targeted Therapies*" emerged. The book invited a number of internationally well-known experts to summarize the latest advances in the whole or certain aspect of radiotherapy and immunotherapy, or to share their unique insights based on recent clinical research published by world renowned journals. The main authors of this book are from famous universities or cancer centers of the world including the United States, England, France, Germany, Japan, Singapore and so on. This book was co-published simultaneously across the world by AME Publishing Company and Central South University Press in both Chinese and English versions. The goal of this book was to enable readers to systematically and comprehensively understand the latest research results of radiotherapy and immunotherapy.

With the progress of cancer research and the advancement of new medical technologies, there will gradually be a clear theoretical mechanism of radiotherapy and immunotherapy in conjunction with wider prospects in their joint clinical operation. Perhaps in the near future, cancer will become a chronic disease that is controllable. It is believed that these indepth studies of radiotherapy and immunotherapy will blaze the trails for cancer therapy.

Yong-Zhong Wu President of Chongqing Cancer Hospital, P.R.China

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Immunological interactions in radiotherapy—opening a new window of opportunity

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Abstract: After a span of significant developments & advances we have reached a plateau in all the oncological disciplines in last decade. Escalation of dose of radiotherapy (RT) became possible with emergence of intensity modulated radiotherapy (IMRT) and image guided radiotherapy (IGRT). Different radiosensitizing agents starting from conventional cytotoxic drugs to hypoxic radiosensitizers have been tried to increase the effect of RT. However technological advancement hasn't been translated into significant clinical benefits. Exploiting the immune system to enhance the effect of RT is a relatively new concept and a fast growing area in the field of oncology. RT cannot longer be considered as a localized treatment, but rather as a systemic weapon for solid tumors. The phenomenon of abscopal effect, meaning the action of RT upon distant 'out-of-field' foci of malignancies has been a major focus of recent research, and holds great promise for the future. In this review article we are going to discuss the immunological interactions in RT and its promising clinical implications.

Keywords: Immunology; radiotherapy (RT); cytotoxic T lymphocyte antigen-4 (CTLA-4); PD-L1

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Introduction

After the seminal paper from Hanahan and Weinberg, the hallmarks of cancer has been defined as six biological capabilities acquired during the multistep development of cancer. Since then these hallmarks are considered to be the basic principles for understanding genesis and progression of neoplastic disease. They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (1). With the advancement in research after this paper they have identified some more factors which are important to the cause and updated the list in 2011 with new hallmarks, one among them is the role of immune system (2). The major functions of immune system with regards to neoplastic process are: elimination of viruses' that drive neoplastic transformation, resolution of acute inflammatory environment identification and elimination of transformed neoplastic cells. Tumors may evolve through a Darwinianselection mechanism to circumvent the immune response, may induce local immune-suppression or a combination of both which results in tolerance by immune system. To use this as a therapeutic strategy we must break this tolerance that too carefully without eliciting an auto immune response.

Basic immunology

Immunity is the basic defense of the body against the foreign. Underlying genomic instabilities in cancer cells

make them a foreign entity rather than one's own normal cells. Understanding the basic concepts of immunology is essential in cancer immunotherapy. Broadly immunity is classified into innate and adaptive immunity with an extensive cross talk between them (3).

Innate immunity

It is the basic defense mechanism in the body and is an indispensible for normal immunity. It constitutes both cellular and acellular components which has a direct effect on the pathogens. Key players in the innate immune response are the basophils, eosinophils, mastcells, neutrophils, monocytes and macrophages. These constitute the cellular part and lactoferin, transferrin, interferons, TNF-a and lysozyme constitute the acellular part. Characteristic of innate immune response is the lack of memory (4); they will produce same response in each and every time when they encounter an antigen. The other key concept is that it is nonspecific. Even though it can differentiate between self and non-self, it cannot differentiate within the pathogens (e.g., Herpes virus from HPV). The innate arm of immunity recognize the self from non self through identification of cellular expression like pathogen associated molecular patterns (PAMPS) which are highly evolutionary conserved sequences. Toll like receptor family are one of the PAMPS and these cells are the primary sensors of pathogens. As a result of the activation of the innate immune system pathogens are either killed or are broken into peptides which help to activate the adaptive arm of immune system.

Natural killer (NK) cells

NK cells (3) are also phagocytes and have the ability to kill the cells directly. These are activated when a cell is not expressing class I major histocompatibility complex (MHC). Class I MHC is expressed in virtually all human cells however when there is a viral infection or carcinogenesis occurs which cause the down regulation of class I MHC so that cell is invisible to the immune system. Class I MHC is like a window into the cells which allows the immune cells to look inside for the viruses, mutated protein and helps in eradication of these cells (5-7). In this setting comes the importance of NK cells, it will be activated and kill the cells which are not expressing the class I MHC.

Adaptive immunity

It comprises mainly of T and B cells. Unlike the innate arm the components of adaptive immunity are activated

of antibodies. Those antibodies can directly kill the cells, activate the compliment mediated death, and it's binding to antigen results in opsonisation which leads to enhanced phagocytosis of the antigen by macrophages and neutrophils. This way adaptive and innate immunity complement each other.

T cells

Helper T cells produce cytokines for the activation of B cells and cytotoxic T cells.

T regulatory cells down regulate the function of cytotoxic T cells. Their function is to control the cytotoxic cells after its finishes action on pathogens. Once the pathogen is controlled cytotoxic T cells should be regulated otherwise it will result in chronic inflammation and leads to neoplastic transformation. The key concept concept of the adaptive immunity is the presence of memory which always leads to an exaggerated immune response when there is repeated exposure (4).

Adaptive immune response only recognizes a short sequence of peptides. That peptide has to be bound in the context of class I or class II MHC. Innate cells are the main antigen presenting cells. Class I MHC is expressed in almost all nucleated cells but class II MHC is only expressed in professional antigen presenting cells like dendritic cells and macrophages. Within the MHC there is peptide binding grooves which accommodate peptides. Triggering of the T-cell-receptor complex not only requires the antigen to be recognized on the surface of an antigen-presenting cell, but also needs a second signal to be sent in a coordinated fashion through a co-stimulatory receptor. The overall effectiveness of the interaction between the MHC, T cell receptors and the signals from the co-stimulatory molecules determines the activation process (8).

Cancer immunology

Immunotherapy has now become an important part of cancer therapy, with consistent and long lasting responses being reported for a wide range of human cancers and with the advantage of a minimal toxicity profile compared to conventional cytotoxic therapies. Cancer is characterized by accumulation of altered genetic events. These events result in the expression of neoantigens, differentiation antigens or cancer testis antigens, which results in presentation of these antigenic peptides bound to (MHC-I) molecules

by sequence specific peptides. When a B cell is activated and transformed to a plasma cell, it becomes a factory



Figure 1 Radiotherapy and its role in cancer immune cycle. CTLA-4, cytotoxic T lymphocyte antigen-4.

on the surface of cancer cells. This helps CD8+ T cells to distinguish them from normal cells.

However, even when T cell responses occur, they neither provide protective immunity to the host nor could they be used as basis for therapy. To understand these we need to look into the cancer immunity cycle (9).

Immunoediting (10)

One of the important aspects of tumor is that it develops in an immunocompetent host. It means tumors have evolved through the effects of immune system. Immune system in competent host acts as both host protecting and tumor sculpting on a developing tumor. This action of immune system on developing tumor is called as tumor immunoediting. Essentially there are three steps in tumor immune editing such as elimination, equilibrium, and escape.

Elimination

It is the earliest step of immunoediting. In this step the immune surveillance leads to removal of majority of the neoplastic cells. As complete neoplastic elimination takes place no tumor cell is going to survive but the process of immune surveillance causes Darwinian selection pressure which results in escape of some cells from immune attack. This selection pressure will result in appearance of newer

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and newer mutations to escape an immune attack so that the antigenicity is very low.

Equilibrium

It is the longest step in immune editing. In this step the host immune system and the neoplastic cells which escape the immune cell kill reach in equilibrium. Altered genetic events as a result of the Darwinian selection pressure will produce proteins that are least immunogenic. There will be equilibrium between the immunogenicity and the altered genetic events.

Escape

In this step the equilibrium is broken in favor of neoplastic cells and best genetic alteration which can survive the immune surveillance will flourish. If it gets unchecked by therapy will result in death of the host.

Cancer immunity cycle

For an effective cell killing from anticancer immune response a series of events in a systematic order should happen in the body. These events constitute the cancer immune cycle (*Figure 1*). First step in the cycle is capturing of neo antigens for processing by the dendritic cells. Next step is the presentation of this antigen by the dendritic cells on MHC I or MHC II to the T cells. Along with this the signals from costimulatory molecules lead to the activation of T cells. Effectors T cell responses are generated against the cancerspecific antigens that are identified as foreign antigens. This step is actively regulated by the balance between the T regulatory cells and the effector T cell response. Activated T cells migrate to the tumor and infiltrate into the tumor bed there they identify the tumor cells which have antigens similar to the presented one and result in tumor cell kill. Killing of the cancer cell releases additional tumor-associated antigens (first step) and the cycle continues (9).

In most of the cancer cells this cycle is not well coordinated and there is always some kind of negative regulation in each step of the cycle, e.g., tumor antigens may not be detected, dendritic cells and T cells may treat antigens as self rather than foreign, T cells may not properly migrate to tumors or inhibited from infiltrating the tumor, factors in the tumor microenvironment suppress those effector cells that are produced (9).

The ultimate aim for all the cancer therapy making use of immunology is to initiate and reinitiate and propagate and amplify this cycle and in a fashion which does not initiate an auto immune response. Till now there are several interventions aimed to improve cancer immune cycle in its most optimum way, some of which are described below.

Tumor vaccination

Effort to increase the cancer control using immunization is at targeting the first step in the cancer immunity cycle. Vaccination is an attempt to activate cancer antigen-specific T cells, as well as stimulate the proliferation of these cells. But there is uncertainties concerning the identities of antigens to use, their mode of delivery, the types of adjuvants required. Presence of the negative regulators in the tumor microenvironment (represent the final steps of cancer immunity cycle) may decrease or disable antitumor immune responses before clinically relevant tumor kill occurs. As long as there is negative regulators which are acting later in the cancer immunity cycle the prospect of cancer vaccination is limited.

Adoptive T cell therapy (11)

This is one of the exciting developments in the field of immunotherapy in which autologous T cells which are activated against tumor antigens are re-infused into the patients. This had showed substantial clinical benefit in some of the hematological malignancies.

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So a variety of approaches are in place to make use of once own immunity to clear the malignancy but none of them will offer a substantial benefit unless targeting the complete cancer immunity cycle.

Radiotherapy (RT)

Therapeutic vaccination is not the only approach by which we can introduce the cancer associated neo antigens. Other approaches are RT and chemotherapy which make use of the tumor that is already present in the system to generate an endogenous release of antigens. Since there is more systemic effect and less local cell kills per cycle of chemotherapy, RT may be more effective for liberating tumor associated antigens. Tumor itself represents a type of endogenous vaccine (9). The cell kill due to RT delivers immense amount of tumor antigens in various form and size to the system. This can act as tumor antigens thus avoiding the need for an exogenous delivery of antigens. But this approach is not fully overcoming the limitation of vaccination as it also acts proximal to the regulators in tumor micro environment.

Mechanism of radiation cell death

It is very interesting to note that RT is used both as an immunosuppressive agent and an immune stimulant in treatment of cancer. RT is considered as immunosuppressive in total body irradiation in conditioning regime of bone marrow transplant (12,13) and immune stimulant in most of the other solid tumors (14,15). Traditionally DNA double strand breaks were thought to be the sole mechanisms in radiation induced cell kill which results in tumor eradication and alter the tumor microenvironment through the apoptosis and mitotic cell death. Apart from this, cell kill due to RT has multi-dimensional effect on tumor survival but this may not be observed clinically due to the evasion and immune tolerance of tumor cells (which are distal steps in cancer immune cycle). Radiation damage to tumors results in the exposure of a large amount of tumor antigens, in the form of necrotic and apoptotic tumor cells and cellular debris to the immune system. The increased availability of released tumor-associated antigens for uptake by circulating dendritic cells and other antigen-presenting cells can result in tumor-specific immune attack (16,17).

RT also creates an inflammatory milieu by inducing the expression of several proinflammatory cytokines, including IL-1 β and TNF- α . Increased expression of these cytokines

Radiotherapy with Immuno-Targeted Therapies

has been linked to tumor regression, growth inhibition, and tumor-cell death (16,17). Furthermore, upregulation of MHC, costimulatory molecules, adhesion molecules, death receptors in tumor cells, surrounding stroma and vascular endothelium can also potentiate CD8+ T cell cytotoxic cell responses.

Similarly radiation induced cell damage results in increased expression of VCAM 1 on tumor cells which leads to increased migration of T cells to the tumor, translocation of calreticulin to the cell surface and the release of highmobility group box 1 (HMGB1) by dying tumor cells, which can activate DCs through Toll-like receptor.

Traditionally RT is delivered in 1.8-2 Gy per fractions. The fractionation has impact on the immunological effects and there is evidence from animal models that changing fractionation, more favorably hypo fractionation (18) alone results in generating robust CD8+ T cell-dependent immunity. It leads to tumor reduction, reduced relapse of primary tumor, and eradication of metastasis in some settings. Potential role of RT in this setting is untapped due to the normal tissue complications. But if we can overcome this limitation by other modes this can be a game changing strategy. Unfortunately many a time the above said effect is minimal in clinical setting as the tumor will be able to evade this immune response either by immune tolerance or by immune suppression of the host. By enhancing the frequency, magnitude, and character of the immune responses induced by RT with immune modulatory agents, cancer patients could experience further improved outcomes that is targeting the distal part of the cancer immune cycle.

Therapeutic efficacy of RT has been considered so far to be solely dependent on its capacity to induce tumor cell death either on the cancer cells themselves or on the tumor stromal and vascular microenvironment. Because of this thought process developments in RT was turning around in improving technological advances in delivery, efforts to deliver higher dose, and altering dose fractionation schedule. However the efficacy can be improved if we consider whole diseased individual as a system rather than targeting tumor only and this will guide the most effective cytotoxic therapy available for localized solid tumor into a new window of opportunity (19).

There are several mechanisms in immune tolerance by cancer cells which are acting after the neo antigens, such as loss of MHC expression and up-regulation of inhibitory molecules of immune response like PD-L1, cytotoxic T lymphocyte antigen-4 (CTLA-4). Hence there are several layers of immune regulation by which tumor escape from the immunological effects.

Cytotoxic T lymphocyte antigen-4 (CTLA-4) (20,21)

It is the key regulator of T cell response and tolerance to self-antigen. This is one of the mechanism by which body can differentiate the self from non-self-environment however the intelligent tumor cells will make use of this as an opportunity to escape from the T cell mediated cell kill. Activation of T cell requires primarily two signals. First signal is from the presentation of antigenic peptides in the context of MHC, second is from binding of CD28 co-receptor to costimulatory molecules CD80 (B7-1) and CD86 (B7-2) which results in activation, T-cell proliferation and cytokine production. CTLA-4 will compete with costimulatory molecules for the coreceptors thus leads to competitive inhibition. CTLA-4 engagement regulates integrin-dependent motility and prevents T cells from forming long-term interactions with APCs or target cells, which are necessary to sustain T-cell activation and cvtotoxic activity. CTLA-4 is constitutively expressed in T-Regs and promotes highly suppressive cytokine TGF-β.

So in the highly immune compromised tumor micro environment persistent tumor antigen exposure causes the exhaustion of T cells along with higher expression of CTLA-4 and other immune checkpoint receptors which contribute to a significantly reduced antitumor immune response.

Programmed death (22)

PD-1 is another important inhibitory receptor expressed by T cells. The activation of PD-1 plays an important role in maintenance of peripheral tolerance. There are two PD1 ligands which have been identified i.e., PD-L1 and PD-L2. Expression of PD-L2 is limited to myeloid cells. PD-1/PD-L1 axis is one of the determinants of modulation of T cell function. It regulates the T cell function through T cell receptor signal transduction and inducing apoptosis of activated T cells.

Interaction of radiation and immunology

Till now most of the effort in cancer treatment is by either targeting the tumor cell or targeting the immune system. Each of these modalities was independently thought to cause cure but it failed to deliver its purpose in many solid tumors. The combination effects are promising and can results in a magical cure not only in localized disease but also for the metastatic

	17			
Author	Diagnosis	Sample size	Treatment	Outcome
Formenti et al. (23)	Metastatic carcinoma	14	GM-CSF + RT 3.5 Gy × 10 [#]	30% patients showed abscopal effect,
	lung, bladder, breast,			five patients had metabolic response
	thymicca, eccrineca			in non-irradiated area
Chi <i>et al</i> . (24)	Hepatoma	14	8 Gy RT + intratumoral injection	12 out of 14 patients had partial
			of immature dendritic cells	response
Postow et al. (25)	Metastatic melanoma	1	Ipilimumab + RT 28.5 Gy in 3^{*}	Stable minimal disease in non-irradiated
				part after 10 months of irradiation
Hiniker et al. (26)	Metastatic melanoma	1	Ipilimumab + RT 54 Gy in 3 [#]	Complete response in primary and
				metastatic site
Slovin <i>et al</i> . (27)	Metastatic castration	50	Ipilimumab + RT	One patient had complete response,
	resistant prostate cance	r		six had stable disease and eight
				showed good biochemical response

Table 1 Clinical trials of immunoradiotherapy

[#], fractions. RT, radiotherapy.

and advanced disease. Among all the negative regulators of the cancer immune cycle the tumor microenvironment is thought to be the most important. Recent advances in clinical research aim to target these negative regulators. Most important are the PD-1/PD-L1 and CTLA-4.

When there is a strong endogenous antitumor immune response, targeting the up regulated negative regulators in the microenvironment will result in enhanced tumor control. But when there is no or reduced antitumor response, targeting inhibitory molecules will be a futile effort. In that setting, agent who can induce an anti-tumor immune response will be more effective.

PD-L1-blocking therapy reinvigorates exhausted CD8+ T cells. CTLA4-blocking therapy predominantly decreases TReg cell numbers and, together, these immune checkpoint inhibitors increase the CD8/TReg ratio and promote the peripheral clonal expansion of TILs. Role of radiation is to diversify the T cell receptor repertoire of tumor infiltrating lymphocytes. It also shapes the repertoire of the expanded peripheral clones. RT and the immune targeted agents together act synergistically and elicit an immune response locally and systemically and may results in response to even non-irradiated areas. This field seems to be promising pathway in future.

Clinical application and trials in immunotherapy and RT

Although there was evidence for contribution of immune system to the therapeutic response of radiation in preclinical setting since 1970, however it is last 10 years or so when immunotherapy concurrent with RT has turned up in clinics in a big way. There are lots of trials with experimental molecules both in preclinical and clinical settings going on. Addressing all the clinical trials are beyond the scope of this review. We are focusing on few important clinical trials (*Table 1*).

The first clinical trial combined a recombinant cancer vaccine with standard definitive RT in patients with localized prostate cancer. A randomized phase II study was conducted with patients receiving local radical RT with or without vaccine. Primary endpoint of the trial was immunologic response, with secondary endpoints of safety and clinical response. A total of 30 patients were enrolled in the study. Patients in the combination arm received a priming vaccine of recombinant vaccinia (rV) expressing prostate-specific antigen (PSA) (rV-PSA) admixed with rV expressing the costimulatory molecule B7-1 (rV-B7-1), followed by monthly booster vaccines with recombinant fowl pox (rF)-PSA. The vaccines were given with local granulocyte-macrophage colony-stimulating factor (GM-CSF, Leukine) and low dose systemic IL-2. There was no detectable increases in PSAspecific T cells in the RT-only arm but the 13 patients who completed the vaccination and radiation course had at least 3-fold increase (P<0.0005) PSA specific T cells. There was also evidence of de novo generation of T cells to prostateassociated antigens not present in the vaccine, a phenomenon described as "antigen cascade", among the patients treated in the combination arm, providing indirect evidence of immune-mediated tumor-killing (28).

The New York Group designed a "proof-of principle" clinical trial, aimed at detecting an abscopal response (a

response distant to the radiation field) after GM-CSF in metastatic cancer patients. Eligible subjects for this study were patients with at least three measurable lesions, who had stable or progressive disease during chemotherapy. The same chemotherapy was continued but RT was added to one lesion, at a dose of $3.5 \text{ Gy} \times 10$ fractions over a period of 2 weeks. After 1 week of radiation, GM-CSF, 125 µg/m², was given subcutaneously and repeated daily for 14 days. Assessment of response was performed by PET-CT. Currently 14 patients have accrued to this trial. Tumor histology was: lung cancer (6), poorly differentiated thymic carcinoma (2), breast carcinoma (4), bladder carcinoma (1), eccrine carcinoma. Twelve patients could be evaluated for response (i.e., had completed treatment and data from PET/CT before and following therapy were available): four achieved an abscopal response (30%). In five patients a decrease in standardized uptake value (SUV) of non-irradiated lesions was observed on PET scan. In three patients the response was preceded by a "flare" effect at PET (23).

After radiation exposure, the role of dying tumor cells in sensitizing dendritic cells was tested in a phase I clinical trial of fourteen patients with hepatoma (24). A single dose of 8 Gy of external-beam radiation therapy to the tumor was followed by an intra tumoral injection of immature DCs, delivered on days 2 and 24. Twelve of fourteen patients had a partial response, and most patients had increases in alphafetoprotein-specific immune responses by cytokine-release assay and ELISPOT.

Postow et al. (25) reported about a patient whose metastatic melanoma regressed with ipilimumab and concurrent palliative RT. The patient had received 28.5 Gy in 3 fractions to an area next to the spine. Post treatment CT scan revealed that masses elsewhere in the spleen and hilar lymph nodes had also regressed and eventually reached the point of stable minimal disease 10 months after radiation. This case prompted a pilot study by Hiniker et al. (26) to combine ipilimumab and concurrent RT for a patient with asymptomatic melanoma. That patient received a higher dose of 54 Gy in three fractions and showed a complete response in both the primary tumor and the metastatic lesions. In a phase I/II clinical study, Slovin et al. (27) used ipilimumab along with radiation in metastatic castration resistant prostate cancer. A total of 50 men were given ipilimumab (four 10 mg/kg doses) plus RT (8 Gy fractions to each lesion for 3 weeks), one patient had complete response, six had stable disease and eight showed good biochemical response.

With efficacy of CTLA4 blockers being proved in case

reports or phase II trials anti-PD-1/PD-L1 mAbs have drawn much interest for their potential use in lung or colon cancer (29) and in combination with CTLA-4 blockade for melanoma.

The study by Verbrugge *et al.* (30) showed neither anti-PD-1 mAb nor radiation when given alone was effective in a murine model of triple-negative breast cancer. However, the addition of anti-PD-1 mAbs enhanced the curative capacity of RT and CD137 (an agonist antibody for costimulatory molecule 4-1BB) against both established tumors and secondary tumor challenge, indicating that the combined regimen conferred antitumor immune responses and memory.

Conclusions

Radiation has been a back bone of cancer therapy since the early 20th century and is implemented in around half of latest cancer treatment plans. RT was traditionally considered as a localized form of treatment. It was thought that it has no effect on distant metastasis. With the emergence of stereotactic body radiotherapy (SBRT) and its ability to treat the oligo-metastasis there was a paradigm shift from the conventional thought process. Though SBRT is used for treating oligo metastasis but it is a tumor directed therapy only. SBRT is not the tool where the exact systemic effect of radiation has been explored. Immunotherapy concurrent with RT has opened that window for radiation to treat systemic disease with localized treatment.

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Footnote

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Chemically enhanced radiotherapy: visions for the future

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Abstract: Radiotherapy (RT) is an important part of cancer management, with more than a third of all cancer cures being attributable to RT. Despite the advances in RT over the past century, the overall outcomes in a majority of malignancies are still unsatisfactory. There has been a constant endeavor to enhance the outcome of RT, and this has been in the form of altered fractionation, oxymimetic radiosensitizers, the use of concurrent chemotherapy, anti-angiogenic therapy and anti-growth factor receptor targeted therapies. This article presents a vision for the future, with emphasis upon emerging prospects which could enhance RT outcomes. Positive speculations regarding the use of immunological aspects, the use of nanoscale technology and the adoption of metronomic concurrent chemotherapy have been presented. Also, the potential with the use of low dose hyperradiosensitivity in enhancing chemotherapy outcomes too has been discussed. In this era of evidence based clinical practise, there exists a strong obsession towards the 'present' with 'contempt towards the future'. Accepting the shortcomings of the existing modalities, there must be a strong zeal towards discovering better methodologies to enhance radiotherapeutic outcomes for the sake of a better future.

Keywords: Novel therapeutics; radiosensitizers; gold nanoparticles; hyperradiosensitivity; metronomic chemotherapy; immunoradiotherapy

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Introduction

Radiotherapy (RT) is an important aspect of cancer care, with estimates suggesting that it is currently responsible for 40% of all cancer cures (1). Ever since RT has been used for treating cancer, there has been a constant quest to improve effectiveness while reducing toxicity. The earliest methods included experimentation with radiation time-dosefractionation based on radiobiological modelling. Then, understandings about predictors of radiation response, such as anaemia and tumour hypoxia generated a quest for combating hypoxia by addition of experimental oxymimetic radiosensitizers (such as misonidazole & nimorazole) (2). That era also ushered the combination of chemotherapy with RT, earlier with agents such as hydroxyurea and methotrexate, and later with 5-FU analogs and platinum agents. The discovery of monoclonal antibodies and small molecule tyrosine kinase inhibitors against various growth factor receptors and angiogenic targets had encouraged the combination of RT with targeted therapy. The combination of hormonal therapy prior to RT in prostate cancer, the use of neoadjuvant chemotherapy prior to RT in head and neck squamous cancers have been other recent attempts intended at improving RT outcomes (3-5).

Despite the modern advances in RT such as the adoption of conformal and intensity modulated treatments, cancer patients in significant numbers continue to fall prey to the formidable malady. Thus it would be realistic to admit at present, that the current accomplishments are far from adequate, if not trivial. But there is hope for a better future. Newer understandings on cancer biology and newer perspectives in cancer therapies have opened up scope for dramatic improvements in outcomes for the future.

Recent understandings about the interactions between RT, chemotherapy and the host immune system has encouraged the development of strategies to utilize these to unlock new opportunities to improve RT outcomes. The advent of nanoscale medicine has allowed better tumour targeting of chemotherapeutic agents. The fact that RT and nanoscale medicines augment the efficacy of each other makes their concurrent use an excellent proposition worth exploration. The use of nanoscale particles with high atomic number elements as radiosensitizers is another exciting prospect worthy of investigation, as this approach can be expected to provide effects similar to heavy ion therapy, all while using a conventional photon beam. Newer perspectives in delivery of chemotherapy have explored the rather unconventional form of chemotherapy such as metronomic chemotherapy. These used with, or after RT may help enhance outcomes while sparing the patient from the toxicities of conventional chemotherapy. Also, the discovery of hyperradiosensitivity at low doses of radiation has encouraged the use of low dose RT as a chemopotentiator.

This editorial review touches upon the most promising prospects which may revolutionize radiotherapeutic treatment of cancer patients. Less emphasis has been placed on on-going trials involving newer conventional chemotherapeutics and existing molecular targeted therapies, and instead, focus has been placed upon novel ideas and technologies that are likely to be available in the foreseeable future.

Immunology, immunological interactions and prospects

While some components of the immune system, such as the cytotoxic T lymphocytes and NK cells strive hard to eliminate tumours, cancer cells enjoy the protective effect of certain immune suppressive cells and suppressive cytokines. Recently it has been understood that a variety of tumour protective immune factors exist, such as T-reg (CD4⁺CD25⁺ regulatory T-cells), MDSC (myeloid derived suppressive cells), certain cytokines (IL-10, TGF-beta) and regulatory tumor associated macrophages (TAMs) (6). It has been demonstrated that chemotherapy and RT alter tumours' immune tolerance and that this could indeed be an important, albeit less recognized mechanism of action of these modalities.

Of late, it has been established that while maximally tolerated dose (MTD) chemotherapy depletes all immune cells, low dose chemotherapy selectively depletes T-reg cells and hence enhance antitumor immune response. For example, low dose cyclophosphamide reduces suppressive cells selectively without depleting cytotoxic T cells; but at higher doses, cyclophosphamide loses this specificity (7). Other agents at lower doses such as paclitaxel and 5-FU are known to cause MDSC apoptosis. The same is discussed later under the heading dedicated to metronomic chemotherapy.

Recent research has focussed upon the function of CTLA-4 and PD-1 receptors. These receptors present on cytotoxic T cells act in a suppressive manner so as to prevent autoimmunity. However, this inhibition also allows cancer cells to survive killing by these cytotoxic T-cells. Monoclonal antibodies against CTLA-4 (ipilimumab) and PD-1 (nivolumab) have demonstrated excellent results in melanoma and lung carcinoma. RT and anti-CTLA-4 and PD-1 inhibitors are likely to be synergistic. An interesting case report described a patient who had progressive disease despite initial immunotherapy with ipilimumab and a sudden dramatic systemic response after localized RT (8).

Prospects with nanomedicine

Nanoscale technology aims to enhance drug targeting, improve biodistribution, overcome resistance mechanisms and reduce toxicity of therapeutic molecules. Various nanomedicines utilizing forms of liposomes, polymers, micelles, dendrimers and others are observed to passively accumulate in tumours owing to their vasculature which is leakier due to wider fenestrations. This enhanced accumulation at tumour sites is called 'enhanced permeability and retention' (EPR) effect (9). Though obstacles to the EPR effect do exist, most significantly in the form of reticuloendothelial system (RES) capture, this can be reduced by the use of PEGylation which by producing a hydrated barrier causes hindrance to the attachment of phagocytes. The EPR effect along with PEGylation can increase tumour drug concentration by 10-100 times in comparison to that with the use of free drugs (10). There is immense potential for the use of nanoscale technology in cancer treatment (Table 1). First of all, the availability of agents such as liposomal doxorubicin and nano-albumin bound (nab-) paclitaxel has already enhanced

Table 1 Nanoscale technologies and prospects for enhancing outcomes

Aspects of nanoscale medicine worthy of investigation for use with radiotherapy

Nanoparticle bound chemotherapy such as paclitaxel and doxorubicin may greatly reduce concurrent radiotherapy doses required to achieve tumour control

The HVGGSSV peptide bound liposomal doxorubicin binds specifically to irradiated tissues; hence, nanoparticle bound chemotherapy can be 'guided' to tumor targets painted with radiation doses

High-atomic number element nanoparticles as radiosensitizers can cause a drastic dose enhancement effect for kilovoltage range photon beams, thus enabling effectiveness comparable to that with the use of heavy ion therapy

efficacy and safety in comparison to the more traditional forms of doxorubicin and paclitaxel. Thus, the use nanoparticle bound chemotherapy in place of conventional free forms of chemotherapy will render the delivery of concurrent chemoradiotherapy much more efficacious and tolerable (11).

In a potential 'eureka moment' for oncology, it was observed that the use of poly-L glutamic acid bound paclitaxel as a radiosensitizer could reduce the TCD50 dose in a preclinical model from 53.9 Gray (Gy) to just 7.9 Gy (12). Another interesting observation has been that the tumour targeting of liposomal doxorubicin could be enhanced by the use of a peptide (HVGGSSV) which would bind selectively to irradiated tumours. Thus, this selective binding suggests that irradiation can be utilized to guide drug delivery to tumours (13).

The use of high atomic number (Z) nanoparticles as radiosensitizers is another extremely attractive approach on the horizon. The high Z atoms interact in a very different manner to ionizing radiation in comparison to low Z atoms. Since biological tissues are mostly made up of low Z atoms, the introduction of high Z nanoparticles into tumours can dramatically intensify response to RT. High Z atoms during photoelectric effect undergo inner shell ionization wherein one of the deeply bound electrons is ejected. This results in a highly unstable atomic system which stabilizes by the emission of low energy photons (fluorescence) and augur electrons. Several augur electron emissions can occur from single inner shell ionization and these augur electrons have a range of 10-100 nm and hence deposit energy very locally. Thus, a very high energy deposition which can be comparable to heavy ion therapy can be achieved with the use of a high Z nanoparticle radiosensitizer (14,15).

The most commonly studied high Z nanoparticle radiosensitizers in preclinical models include gold, iron oxide and hafnium oxide nanoparticles. An in-silico simulation showed that the use of gold nanoparticles as radiosensitizer could lead to higher dose enhancement with kilovoltage range ionizing radiation. For example, while the dose-enhancement with 2 megavolt photons was 53%, the dose enhancement with kilovoltage range photons was as high as 560% (16). While nanoparticles are known to enhance RT, the favour is returned by RT in that it enhances nanoparticle accumulation into tumours. In a mouse breast tumour model, it was observed that iron oxide nanoparticle accumulation was doubled after a single 15 Gy dose (17).

Metronomic chemotherapy to enhance RT

Metronomic chemotherapy is the chronic administration of chemotherapy at low doses which are minimally toxic, in a schedule of administration without prolonged drugfree breaks. Commonly used agents include low dose versions of conventional chemotherapy agents such as cyclophosphamide, methotrexate, capecitabine, etoposide, etc. In addition, a few non-cancer drugs such as celecoxib, metformin, valproate and such are being used (termed 'drug-repositioning', wherein drugs approved for nononcological indications are re-positioned for oncological use (18).

In contrast to conventional MTD chemotherapy, the new approach of metronomic chemotherapy is very less toxic inherently by its design. Many subtle properties of chemotherapeutic agents could possibly have been masked by the MTD approach, but are now being unravelled in the metronomic approach (19). While MTD chemotherapy solely aims towards killing of malignant cells, the metronomic approach owes its efficacy to numerous other effects. Metronomic chemotherapy has anti-angiogenic effects and this has been demonstrated experimentally in that it was able to reduce angiogenic factors such as thrombospondin-1 (20). Another study in patients undergoing thoracic irradiation for lung cancer revealed

Table 2 Advantages of metronomic chemotherapy over maximally tolerated dose chemotherapy

Attractive features of metronomic chemotherapy over conventional chemotherapy

Use of low doses over protracted periods make regimens very tolerable, thus can be used in patients unlikely to tolerate conventional dose concurrent chemotherapy

An anti-angiogenic effect possibly due to down-regulation of factors such as Thrombospondin-1 and VEGF can help reduce progression and metastases during radiotherapy

Enhanced anti-tumor immune response due to elimination of immunosuppressive cells (CD4⁺CD25⁺ T-regs & myeloid derived suppressor cells), increased MHC-1 molecule expression and increased dendritic cell maturation

that the addition of metronomic chemotherapy caused a marked reduction of VEGF (21). In addition to its antiangiogenic effects, metronomic chemotherapy also has immunoregulatory functions, wherein the elimination of immunosuppressive cells (such as T-reg & MDSC) and the increased MHC-1 molecule expression, increased dendritic cell maturation are all known to enhance anti-tumour immune response (22).

Metronomic chemotherapy has already proven beneficial in often difficult situations involving patients who were heavily treated with conventional regimens (23,24). At present there is a paucity of clinical trials combining concurrent metronomic chemotherapy with RT. If not in every patient, this approach could help improve outcomes at least in patients who are unlikely to tolerate use of conventional concurrent chemotherapy (Table 2). Lastly, it is very much feasible that the use of metronomic chemotherapy adjuvant after a course of standard treatment may help reduce distant recurrences. A study utilizing metronomic tegafur-uracil observed a very significant reduction in distant failures among nasopharyngeal carcinoma patients who had persisting plasma EBV DNA levels after completion of definitive chemoradiotherapy (25).

Prospects with antiangiogenic therapy

The use of RT can unintentionally enhance the process of angiogenesis by up-regulating factors such as VEGF in tumor cells, VEGFR in endothelial cells and $\alpha V\beta$ 3 integrin in tumor endothelial cells. Thus, the quest for combining antiangiogenic use with RT is very much rational (26). Recently, the results of the RTOG 0615 study regarding the addition of bevacizumab (monoclonal antibody targeting VEGF) with chemoradiation for nasopharyngeal carcinoma was made available. The phase II study demonstrated feasibility for adding bevacizumab to standard chemoradiation in treating nasopharyngeal carcinoma, and suggested that there may be a slight benefit in the form of delaying progression of subclinical distant disease (27). However the overall results with bevacizumab so far have been far from satisfactory, and this is despite the great zeal that existed for integrating bevacizumab into anti-cancer regimens a decade ago (28).

The toxicity and questionable efficacy of bevacizumab has now led to the quest for newer anti-angiogenic agents. The antiangiogenic effects of small molecule tyrosine kinase inhibitors such as sunitinib, sorafenib and pazopanib has roused interest, but their efficacy is yet to be established, especially in conjunction with RT.

The discovery of RGD (arginine-glycine-glutamine) as an $\alpha V\beta 3$ integrin antagonist has opened another window of opportunity. Since RT in itself up-regulates $\alpha V\beta 3$ integrin in tumor endothelial cells, despite tumour cell kill RT can unintentionally promote angiogenesis mediated progression/metastases in tumours such as glioma. The development of integrin antagonists such as the RGD peptidomimetic agent S247 and the cyclic RGD pentapeptide cilengitide may help enhance RT outcomes by antagonizing RT induced $\alpha V\beta 3$ integrin upregulation (29). However, as with all anti-angiogenic agents, optimism must be guarded until when efficacy can be proven beyond doubt.

Prospects with newer conventional chemotherapy and targeted therapy agents

The expected increments in outcomes with the combinations of RT with conventionally delivered 'newer' chemotherapy agents (e.g., docetaxel, pemetrexed, gemcitabine) and molecular targeted therapies (e.g., cetuximab, nimotuzumab, erlotinib) are small, even if statistically significant. This is in contrast to the prospects to dramatic improvements potentially feasible with

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novel agents and newer methodology available at the horizon, for example with the use of gold nanoparticles as radiosensitizers, or with the use of immunological methods of enhancing RT. This editorial review does not consider these currently existing agents as 'novel' agents, and thus, little emphasis is placed upon these.

Utilizing low dose radiation as a chemopotentiator

Conventionally, RT is delivered with dose-fraction sizes of 1.8–3 Gy per fraction. Lower doses per fraction are generally avoided given the undue prolongation of overall treatment time, as well as the prediction of lesser cell kill at lower fraction sizes. It was, however, observed that cell killing at doses <1 Gy is greater than that predicted by the linear-quadratic model, hence the nomenclature 'low dose hyperradiosensitivity'. It is postulated that this phenomenon is present below the threshold dose that would be required to initiate cellular radiation response mechanisms. In particular, a radiation inducible ATM gene dependent G2 phase checkpoint was found to have a threshold activation dose <0.4 Gy. Thus at such low doses, hyperradiosensitivity occurs due to failure of cell cycle arrest of these radiationdamaged G2-phase cells (30-32).

Despite the enhanced cell killing with low-dose fractionated radiotherapy (LDFRT), it would not be practical to utilize LDFRT alone as a treatment regimen, since the entire course of RT would be likely to be long enough to cause accelerated tumour cell repopulation to negate any cell killing. Hence, an innovative approach has been experimented, that involving the use of LDFRT to enhance chemotherapeutic outcomes.

Various studies have utilized LDFRT to potentiate various regimens of chemotherapy in difficult clinical situations. For example, LDFRT was used to potentiate chemotherapy with pemetrexed in recurrent lung carcinoma, and a dramatic enhancement in response rates was observed (33). LDFRT when used with gemcitabine in pancreatic carcinoma was associated with promising response rates (34). LDFRT was also observed to enhance response rates in head and neck carcinoma when used with neoadjuvant chemotherapy with paclitaxel and carboplatin (35). Among patients with breast carcinoma, when LDFRT was used with neoadjuvant chemotherapy, there was very good tolerability, and a good pathological complete response (pCR) rate. Among those patients who did not attain pCR, a fibrotic reaction was found to encase the residual tumour, which could potentially be inhibitory for residual cells to proliferate or metastasize (36).

Despite very good outcomes without any noticeable additional toxicity with the use of LDFRT as a chemopotentiator, it is rather unfortunate that very few clinical trials have focused upon its prospects. It is hoped that future research will help us refine the logic and the technicalities associated with the use of LDFRT as a chemopotentiator.

Summary and conclusions

While the prospects of promising modalities and methods to improve radiotherapeutic outcomes have been touched upon in this review, it must be said that there could be, and there will be many more innovations which could brighten up prospects of better outcomes for cancer patients (*Table 3*). The development very precise technologies of RT delivery, combined with the discovery of newer radiopharmaceuticals for positron emission tomography for functional imaging may enable extremely intelligent and biologically adaptive treatment delivery. The discovery of newer radioprotectors is largely welcome too, given that the existing radioprotector namely amifostine holds limited efficacy in limited sites. Also, advances in epigenetics will inevitably be attempted to augment RT outcomes.

Going by existing trends, it is unfortunate that very little interest and funding is dedicated to studies using chemotherapy to enhance RT (37). It must also be remarked that despite extremely good prospects with technologies such as high-Z radiosensitizers, and despite very promising results with initial trials with low dose hyperradiosensitivity for chemopotentiation, there has been very few trials to continue the progress (this is in sharp contrast to the amount of funding and emphasis received by futile trials involving antiangiogenic therapies). It can only be hoped that in the future, trials dedicated to chemically enhance RT will receive their due share of funding.

While clinical research follows a strict rational approach for conceptual development, occasional ideas may be found through 'out of the box, lateral thinking', too. Too much of adherence to current standards may be harmful, as potential discoveries are lost due to our staunch obsession with the present, and neglect for the future. All said and done, progress, big or small can only be expected to be achieved through efforts, and with an open mind. While the world remains obsessed with the practise of 'evidence based medicine', it can only be hoped that new innovations do get their legitimate share of optimism and emphasis.

 Table 3 'Chemical enhancement' of radiotherapy: summary of future prospects

Modality	Remarks
Nanoparticle bound chemotherapy as	Very high prospects for revolutionizing radiation based therapy
radiosensitizers	
Nanoparticle bound high-Z radiosensitizers	Groundbreaking potential can be unlocked
Radiotherapy to guide nanoparticle bound chemotherapy to tumors	A novel way to use radiotherapy to paint tumor so as to guide nanoparticle bound chemotherapy to tumor
Metronomic chemotherapy concurrently with radiotherapy	Very useful, especially in patients unable to tolerate conventional concurrent chemotherapy. May hold additional benefits due to antiangiogenic and immunomodulatory properties
Concurrent chemotherapy with newer agents such as taxanes	Already in clinical use. Small outcome improvements. Toxicity concerns
Concurrent therapy with molecular targeted monoclonal antibodies and tyrosine kinase inhibitors against growth factor receptors	Already in use. Newer agents actively being investigated
Anti-CTLA4 and anti-PD-1 monoclonal antibodies with radiotherapy	Synergy may exist. Abscopal responses can be expected
Antiangiogenic therapy with radiotherapy	Existing agents such as bevacizumab hold questionable efficacy with considerable toxicity. Newer agents awaited
Use of low dose radiation as a chemopotentiator	The phenomenon of hyperradiosensitivity at low doses to enhance chemotherapy outcomes has been experimented with promising results
Radioprotectors to widen therapeutic window	Currently, amifostine is the only approved radioprotector, however, has limited activity, confined to limited tissues. Newer radioprotectors yet to be discovered. Concerns of tumor protection must be addressed beyond doubt
Integration of newer radiopharmaceuticals for	Myriad new radiopharmaceuticals utilizing various targets have already
better radiotherapy targeting and delivery	revolutionized cancer diagnosis and treatment. Further refinements expected
Adoptive immunotherapy, viral vectors, cyclin dependent kinase inhibitors, and other novel therapies to enhance radiotherapy	Ongoing in various preclinical/early-clinical trials
Epigenetic manipulation to enhance radiotherapy outcomes	Yet to be explored

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Footnote

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Irradiation and combination immunotherapy

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Our ability to control and eradicate cancer has advanced with new combinations of surgery, chemotherapy, irradiation therapy and recently, immunotherapy. The concept of a single cancer therapy has passed with each new discovery revealing the complexity of the genetics and immunology of the tumor microenvironment. Understanding which combination of standard and emerging therapies that can provide long lasting remission of each particular cancer is paramount. Here we review the capacity of radiation therapy (RT) combined with immune checkpoint inhibitors to induce a complete response in mammary carcinoma and melanoma (1,2) and the evaluation of therapy mechanism of action using intravital microscopy. Current tools and approaches to evaluate mechanism of action of therapies have been limited, but real time intravital imaging offers prospects of enhancing our knowledge.

One standard of care cancer therapy of immunological interest is RT. Local RT is effective at killing tumor cells directly, but the effect of RT can extend beyond the treated primary tumor. The abscopal effect of RT is an anti-tumor immune response generated at sites distant (systemic) from the irradiated volume (3). Immunogenic tumor cell death caused by RT represents in effect an in situ vaccine specific for that patient (4). It provides neighboring antigen presenting cells with tumor antigens, neoantigens (nonself peptides that are generated by the mutated cancer genome), and activating danger signals, such as HMGB1 which binds to TLR4, and calreticulin which leads to priming and activation of tumor-specific T cells capable of attacking the tumor at primary or distant sites (5). RT also increases the T cell receptor (TCR) repertoire, which allows the expansion of T cells clones against the tumor with diverse TCR traits (2). Studying this process has been

limited to *in vitro* studies and "snap shot" analysis of mouse tumors and perturbed immune systems exposed to RT. The application of imaging technologies to study pathology is one of the most transformative advancements in medicine and as technology advances, it will continue to have broader applications in the future (*Figure 1*).

Previously, it has been shown via single cell microscopy that stable immunological synapse formation between DCs and T cells is required for signalling and that productive interactions can be determined by the prolonged time of contact of the membranes of each cell (6). The outcome of that interaction, whether activating or suppressing, requires the use of specific reporters or other assays. The engagement of CTLA-4 on T cells by CD80/CD86 on DC is a negative regulatory signal for T cells. Immune checkpoint antibodies, like anti-CTLA-4 and anti-PD-1, work by blocking the mechanisms that hinder the activation and function of anti-tumor T cells. Through intravital imaging, anti-CTLA-4 has been shown to increase T cell motility and reduce contact periods between T cells and antigen-presenting cells (7).

Two photon intravital imaging represents a more contemporary way to study the effects of RT and anti-CTLA-4 combination therapy. Ruocco *et al.* studied RT in combination with anti-CTLA-4 (9H10) treatment in a nonimmunogenic mouse mammary cancer model (4T1) (1). They showed that standard of care ionizing RT, is able to induce immunogenic tumor antigens and other microenvironment changes required for a robust anti-tumor response. Intravital microscopy was used to determine the efficacy of immunotherapy on tumor growth, describing the direct interaction of CXCR6⁺ CD8⁺ T cells with the CFP⁺ tumor and the behaviour of the CD8⁺ T cells within the


Figure 1 Radiation in combination with antibody immunotherapy. (A) Local radiation therapy (RT) and anti-CTLA-4 and/or anti-PD-1 antibody therapy leads to an immunogenic response to the tumor driven by DNA damage, release of HMGB1, and generation of tumor antigens and neo-antigens; (B) MHC class I, RAE-1 and ICAM-1 is upregulated on the tumor and new tumor antigens are presented to cytotoxic T cells. Dendritic cells adjacent to the tumor likewise are able to present new tumor antigens to activate the T cells against the tumor. HMGB1 can bind to TLR4 on T cells and dendritic cells to activate the NFkB pathway to initiate activation and proliferation. CTLA-4 and PD-1 targeted antibody therapy primes the T cell to be receptive to activating signals; (C) upon direct T cell contact with the tumor, RAE-1 and ICAM-1 stabilize the immunologic synapse around the T cell receptor (TCR) in contact with MHC class 1 which can lead to T cell signalling even in the absence of co-stimulation. Inhibiting the suppressive signalling of CTLA-4 and PD-1 via antibody therapy, coupled with stable TCR and TLR4 signalling leads to T cell activation and proliferation; (D) imaging reporters useful for interrogating signalling directly in the tumor or in immune cells to determine the effect of therapy within the tumor microenvironment.

tumor microenvironment after RT and/or anti-CTLA-4 therapy. RT and anti-CTLA-4 therapy in combination was shown to control the growth of established tumor and this was attributed to the enhanced infiltration of activated CD8⁺ T cells (1).

Ruocco *et al.* (1) also showed that anti-CTLA-4 antibody treatment increased T cell motility in the tumor microenvironment, whereas anti-CTLA-4 treatment with RT promoted T cell arrest in contact with tumor cells. This T cell interaction with tumor cells was an MHC class I-dependent antigen-specific event. Anti-CTLA-4 treatment increased T cell motility on ICAM-1-coated surfaces. After RT, 4T1 cells upregulated expression of MHC class I, ICAM-1, and the NKG2D ligand, RAE-1 γ . By using an NKG2D blocking antibody, DX5, with RT and anti-CTLA-4 treatment they showed that the T cell-tumor interactions were decreased and T cell velocity increased suggesting that NKG2D plays a role in stable interactions between CD8⁺ effector T cells and tumor cells. Although NKG2D does not play a role in RT-reduced primary tumor growth, the upregulation of RAE-1 does play a role in primary tumor growth in the context of RT and anti-CTLA-4 therapy, which was also shown to hold true in the experimental metastatic model. Taken together this suggests that tumor antigen recognition by the TCR of CD8⁺ effector T cells after RT is stabilized by NKG2D-RAE-1 interactions and activation is enhanced by anti-CTLA-4 treatment resulting in tumor control. In concert with these combination benefits of RT and immune checkpoint blockade immunotherapy, a subsequent study showed that RT in combination with anti-CTLA-4 had an 18% partial as best response in humans and 17% response in mice with melanoma (2). Additionally, PD-1 is a negative regulatory signal for T cells, where blocking its interaction with PDL-1 on antigen presenting cells or tumor cells has had profound therapeutic effects especially in melanoma patients resulting in an increase in activated T cells (8). Remarkably when RT and anti-CTLA-4 were combined with anti-PD-1 therapy, complete response rates in mice increased to 80% (2). Indeed, the appeal of immune checkpoint blockade therapy is that it induces long lasting anti-tumor responses in patients with advanced-stage cancers.

Intravital microscopy has been used to study the dynamic in vivo immune cell responses to infection, autoimmunity and cancer (9,10). Many of the initial tumor intravital studies focused on the development of angiogenesis and the efficacy of anti-angiogenic therapies using intravascular injection of fluorescent dyes (11). Vessel response to RT in a dorsal skin fold chamber showed that there was capillary constriction and thrombus formation from day 4 up to 20 days after treatment (12). Recently this technology has been used to determine the efficacy of a therapy, from chemotherapy penetrance to tumor apoptosis (13,14). There has been an increasing interest in immune cell interactions with tumors and other cells within the tumor microenvironment following the burgeoning field of immunotherapy. Together with development of fluorescent reporter mice to distinguish immune cell subsets and fluorescent reporter tumor cell lines, the migration, invasion and metastasis of tumors have described unexpected interactions with vessels, ECM and the bone marrow niche (9,10,15). The greatest benefit of intravital imaging is the ability to assess the early development of the tumor and interactions between small numbers of transformed cells and immune cells. A consideration of this technique is the depth of penetration where in some cases the first 150 µm of the 400 µm from the outside of the tumor is encapsulation, therefore in a heterogeneous tumor population with potentially a hypoxic or necrotic core, it is important to confirm findings using other methods such as immunofluorescent imaging of tissue sections.

The next important advancement in intravital imaging is real time signalling reporters of immune cell interactions that can be used to predict efficacy of therapies, either by reporting the signalling in immune cells, the metabolic state of cells within the microenvironment, or the apoptosis of tumor cells (14). The NFAT reporter was developed to allow the visualization of activated T cells and can be used to determine the percentage of activated cytotoxic cells within the tumor microenvironment (16). FRET reporters of calcium flux used in neuroscience have also been used to show TCR signalling and recognition of cognate antigen (17). This calcium reporter can be useful in the context of RT and immunotherapy to quantify the number of antigenspecific cytotoxic CD8⁺ T cells at the tumor site after therapy. The use of a FRET capspase-3 reporter allows the visualization of apoptotic tumor cells (18), but if multiplexed with additional information about other cells in the tumor microenvironment it could prove to be a powerful tool in dissecting the mechanism of action [reviewed in (14)]. Inhibiting cancer stem cells is a therapeutic approach of interest (15). The Confetti fluorescent construct which randomly assigns different colours to individual cells (19) is useful for lineage tracing of cancer stem cells and has shown that certain clones outcompete adjacent tumor cells. The FUCCI reporter construct allows the visualization of the different stages of cell cycle and is useful in determining whether a therapy is able to stop caaancer cell proliferation and the point of cell cycle can be determined (20). Although classical immunological assays allow us to determine the global efficacy of therapies, intravital imaging and the new reporter constructs are unique in resolution of space and time in providing insights into complex interactions within the tumor microenvironment (Figure 1D). Ruocco et al. effectively used intravital imaging to show that antigen specific recognition of tumor cells after RT is stabilized by NKG2D-RAE-1 interactions resulting in tumor control. The future of intravital imaging is in the development of new functional fluorescent reporters, specific to critical signalling pathways for the direct analysis of therapy mechanisms.

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Footnote

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Radiotherapy: killing with complement

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Radiation therapy has been used for more than 100 years to treat human disease with some of the first applications to cancers in the 1890s. Radiotherapy delivers ionizing radiation to target tissues leading to fragmentation and ionization of biomolecules, water, oxygen and other chemicals that generate reactive oxygen species (ROS). Direct and indirect ROS-mediated damage to DNA, membranes, proteins and other cellular components lead to cell death. Cancer tissues are more sensitive to this damage. The exact mechanism and mode of cell death, and the basis for selective cancer toxicity are still areas of active investigation.

Besides radiation therapy, cancer is also treated with immunotherapy, relying on provocation of the adaptive immune system to attack cancer cells. In immunotherapy, both the cellular and humoral adaptive immune response can target and eliminate abnormal cells based on antigenic differences between tumor and host. Immunotherapy activates natural killer cells or cytotoxic T cells that are specific for the tumor and attack the cancer. Taking advantage of the humoral immune response, multiple antibody treatments are available that bind to cancer antigens and activate complement-mediated cytotoxicity via the classical pathway to kill the tumor cell. This is one aspect of tumor immunity where the complement system is thought to be essential. The antibody defines the aberrant cell and the complement system lyses the tumor cell via the membrane attack complex, C5b-9. In the recent report by Surace et al. (1) a novel interaction between radiotherapy and complement is described with a different sequence of events: Radiation results in necrotic tumor cells and initiation of transient complement activation in the local tumor environment to generate anaphylatoxins, C3a and C5a, which then stimulate adaptive immunity to contribute to tumor cell killing. Surace *et al.* define C3a and C5a as complement components that are critical for recruitment of the cellular immune response and tumor cell killing following radiotherapy.

To further appreciate this finding, let's step back and think about the complement system in general. Now this system is often viewed in medical training in the same vein as the coagulation cascade: a series of numbers and letters to memorize. However, bear in mind that this is an ancient evolutionarily conserved defense system present in horseshoe crabs and sea sponges, so it has proved its worth. In the human, deficiencies in complement system components increase the likelihood of immune complex disease and serious bacterial infections. However, if complement activation is excessive or normal control mechanisms are overwhelmed, complement-mediated pathology may occur. Autoimmune diseases are a prime example of antigenantibody-mediated complement activation leading to pathology, i.e., glomerulonephritis, lupus, etc.

Some major principles of complement system should be mentioned: (I) the system is always operating at a slow steady state level of activation that can be pushed into amplification with a wide variety of stimuli; (II) the pathways produce many different biologically active products even if the pathway does not continue to lysis of the bacteria or tumor cell; (III) the system is intended to act locally and systemic activation indicates escape from local control. As seen in *Figure 1*, the complement system can be activated via 3 major pathways: the classical, the mannose binding lectin, and the alternative pathway. Each of these pathways converges on a central event—cleavage of circulating C3 into C3b and C3a. While C3a is released, C3b covalently binds to macromolecules or cells or is



Tumor Cell with abnormal surface markers

Figure 1 Radiation-induced complement activation initiates adaptive immune response and tumor cell death. The study of Surace *et al.* (1) demonstrates that radiotherapy induces cell necrosis, increases local production of C3 within the tumor and a transient local increase in complement activation with C3a and C5a generation. Increased complement activation results in C3 deposition on tumor cells. C3a and/or C5a are critically important for stimulation of dendritic cell maturation and interferon production by cytotoxic CD8⁺ T cells leading to a beneficial clinical response and tumor cell killing. Orange symbols represent activation of the innate immune response while green symbols represent activation of the adaptive immune response. Solid arrows represent data within the Surace *et al.* manuscript, while dashed arrows represent implied actions consistent with literature and existing data.

degraded into cleavage products such as iC3b, C3c and C3d. Bound C3b (termed C3 deposition), but not the C3 degradation products, participate in cleavage of C5. Both C3b and degradation products may amplify the humoral

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immune response or opsonize altered cells for phagocytosis. C3b contributes to formation of a molecular complex that then cleaves the C5 molecule resulting in generation of C5a and C5b. C3a and C5a are termed anaphylatoxins and are approximately 9,000 kD fluid phase molecules that interact with seven transmembrane G protein coupled receptors: C3aR, C5a1R (CD88) and C5a2R (2). C5b with complement components C6-9 forms a membrane attack complex creating a pore in the membrane, lysing the target cell when insufficient complement regulatory proteins are present. The inciting stimuli that initiates complement activation following radiotherapy is unclear from the study of Surace et al. (1), though they have provided some evidence that necrotic cells are involved or associated with the complement activation. What is clear is that the fluid phase activation products C3a and C5a are critically important for the radiotherapy effect in the mouse model. Radiotherapy induces local transient activation of complement, at least through C3 and C5. The generation of C3a and/or C5a and their receptor interactions are critically important for the maturation and activation of the adaptive immune response for a positive therapeutic effect of radiation therapy.

Importantly, the role of complement activation in inducing tumor specific immunity following radiotherapy is not limited to a single type of cancer. The study examined mouse models of melanoma and colon cancer as well as human skin cancer and found a transient, but clearly effective, C3a- and C5a-dependent stimulation of cellular infiltration in each. The role of C3a and C5a was evaluated using mice deficient in the specific receptors, C3aR and C5a1R (CD88) as well as blockade using antibody or small molecule antagonists of the G-protein coupled receptors. Importantly, dexamethasone treatment down-regulated the anaphylatoxin receptors and radiotherapy efficacy, suggesting a need to consider the potential loss of treatment efficacy when using dexamethasone to reduce radiation toxicity.

The location and distribution of activated C3 may also be important. Surace shows accumulation of C3 along blood vessels following 20 Gy. Vascular injury, and damage to endothelial cells in particular, has been implicated as especially important in the effects of high dose irradiation such as used by Surace (3,4). Irradiated endothelial cells increase acid sphingomyelinase activity generating ceramide that is critical to endothelial cell injury and subsequent antitumor activity of high dose irradiation. Ceramide transport proteins have been implicated in complement activation (5).

Together, these observations may describe a pathway of endothelial cell injury, ceramide activation and complement targeting leading to vascular injury, shutting down nutrient supplies to cancer and ultimately tumor cell death.

The traditional immune response to tumor cells includes antigen presenting cell activation of cytotoxic CD8⁺ T cells to produce γ -interferon. Activation of C3 by radiotherapy up-regulated C3a and C5a receptors on dendritic cells and maturation of these critical antigen presenting cells, induced γ -interferon production by cytotoxic T cells, and prevented the regulatory T cell infiltrate. Previous studies demonstrated that C3 is required for in vitro dendritic cell maturation and pro-inflammatory y-interferon CD4 and CD8 response, as well as in mouse and human graft vs. host disease (6-8). Importantly, therapeutic blockade of C3 activation with Compstatin inhibits the pro-inflammatory Th1 response without altering the regulatory T cell response (9). Additional studies demonstrated that the absence or down regulation of complement regulatory protein, Crry, induced interferon production in a mouse bladder cancer model (10). The study by Surace et al. (1) did not examine expression of complement regulatory proteins on the tumor cells. Future studies will require investigation of complement regulator expression on the tumor stroma, immune cells within the tumor and tumor cells themselves.

Direct and indirect cancer cell damage from radiotherapy can increase tumor immunogenicity. Indeed, cell disruption and death following radiotherapy have been described as an *in situ* personal vaccination against cancer (11). T cell stimulation and activation against cancer may lead to tumor responses outside the irradiated field. Such events, where one site of metastasis is irradiated and both targeted and other sites of disease respond, is termed an abscopal effect (11). Abscopal effects are relatively rare but have been described, particularly in melanoma. Despite the ability of radiotherapy to enhance an anti-cancer immune reaction, systemic anti-cancer immune activity is seen only rarely. One explanation for the limited immune response is the difficulty in determining foreign cancer cells as distinct from self. Immune checkpoint activities dampen T cell activation when antigens presented to T cells are weak or potentially self-related. The recent emergence of immune checkpoint inhibitors may offer exciting new and effective combinations of immunotherapy and radiotherapy (12). Treatments to enhance the anticancer complement response may accentuate T cell activation. Removing T cell inhibition with immune checkpoint inhibitors may provide the necessary combination treatment to unleash a truly effective anti-cancer immune response.

All complement components can be produced in the liver. However, studies have also demonstrated that many components, and C3 in particular, are produced by immune cells locally, particularly in ischemic environments (13). The Surace *et al.* manuscript demonstrated that not only immune cells but also tumor cells themselves produce C3. This is similar to findings described in gastric tumors (14). Initial investigations into the specific pathway of complement activation by Surace *et al.* indicated involvement of both the classical and alternative pathway with no evidence of lectin pathway activation. The inciting stimuli for complement activation have yet to be defined, but the data suggest that necrotic cells may be involved.

Surace et al. employed two different strategies to assess the critical importance of C3a and C5a in the effectiveness of radiation therapy. One was the use of mice deficient in C3aR, C5a1R or both. This sound strategy assesses the importance of the receptor throughout development in determining if radiation therapy will be effective. The authors also blocked C5a1R with specific antibodies and C3aR with a small molecule antagonist. These approaches add important evidence that the lack of C3a and C5a receptors during development in knockout animals is not the critical event. However, use of inhibitors is always limited by the specificity of the treatment. For example, the C3aR antagonist SB290157 also exhibits agonist activity and may stimulate C3aR and mobilize neutrophils in intestinal ischemia reperfusion injury (15,16). Literature reported doses of SB290157 range from 2-30 mg/kg with minimal data indicating that these doses are C3aR specific and lack off target effects. In fact in the rat model of placental ischemia induced hypertension, 5 mg/kg SB290157 attenuated increased circulating C3a, an unexpected finding for a simple antagonist (15). Limited solubility of SB290157 in aqueous solutions results in experimental delivery as a suspension in saline or PBS.

Although focusing on tumors implanted into mice, Surace *et al.* also demonstrates complement activation in a limited number of human skin tumors. For further translation to human, both complement and dexamethasone studies should be expanded to include more relevant tumor models that include mutations found in human melanomas, such as the Braf^{V600E} mutation which occurs in over 50% of human melanomas (17,18). As radiotherapy today frequently includes multiple doses of radiation, it will be critical to determine if multiple doses produce similar results and if radiation dosing in mice translates to dosing in humans. Radiotherapy produces inflammation in normal tissue adjacent to target cancer tissues that may lead to side effects such as nausea, emesis, brain edema, pneumonitis and other toxicities. Dexamethasone is frequently used to address these and other side effects. Based on the findings of Surace et al., dexamethasone inhibits radiation induced C3 activation and more importantly allows tumor growth. These intriguing data suggest the use of dexamethasone to protect normal tissues from radiotherapy may also protect tumor tissues. Dexamethasone was recently found to decrease short-term pain flares after radiotherapy for bone metastases without decreasing long term (6 weeks) pain control, suggesting dexamethasone may not uniformly produce tumor protection (19). Very few studies have addressed the possibility of cancer protection resulting from dexamethasone effects on tumor microenvironment, but given the widespread use of dexamethasone with radiotherapy and the findings of Surace, a closer examination of tumor protection with dexamethasone may be worthwhile.

Future studies are needed to understand the specific, and at times opposing, effects of C3a and C5a as well as the role of the second C5a receptor, known as C5a2R (C5L2). The exact role for C5a2R in the overall actions of C5a is unresolved (2). While Surace et al. examined tumor growth and dendritic cell maturation in mice deficient for either C3aR or C5a1R, the radiotherapy-induced T cell response and dexamethasone studies were performed in wild-type and double knock out (C3aR-/-C5a1R-/-) mice. Thus, future studies should examine the role of each specific anaphylatoxin and the additional receptor, C5a2R. This is particularly important as C3a and C5a have opposing actions in multiple diseases including asthma, lupus, and septic shock where C5a is pro-inflammatory and C3a is anti-inflammatory (20). The C3a-C5a dichotomy may also be due to possible interactions of either C5a or C3a with C5a2R. This soluble receptor may be a decoy receptor that interacts with C5a1R to negatively regulate the response of one or both anaphylatoxins (21-24). Delineating the important mediators and receptors may provide a mechanism to explain the excess toxicity in patients with lupus receiving radiotherapy (25).

Therapeutic intervention in the complement system creates a challenge because this system plays an essential role in host defense. The antibody to C5, eculizumab, has successfully treated paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome. Both of these diseases involve uncontrolled complement activation due to improperly functioning endogenous complement regulators. Blocking C5 activation prevents formation of the membrane attack complex and complement-mediated cell damage. Eculizumab also minimizes C5a production presenting a distinct disadvantage during radiation therapy unless preserving C3a action is sufficient. Inhibiting C5 activation also increases susceptibility of individuals to meningococcal infection so that monitoring and vaccination are essential. The therapeutic ratio in radiotherapy compares the dose of radiation that kills tumor cells with the dose that causes complications in normal tissue. Inhibiting complement activation may be an opportunity to enhance the therapeutic ratio of radiotherapy. Targeting complement inhibitors to normal tissues but not adjacent cancer tissues may contain normal tissue injury and maximize radiotherapy-induced tumor cell damage. For example, inhaled inhibitors for patients receiving radiotherapy to the esophagus or breast may provide lung protection without compromising target effects.

Traditional approaches to enhance the therapeutic ratio of radiotherapy include greater precision in delivery or use of higher doses. Indeed, high precision delivery is now technically possible for almost all sites using intensity modulated radiation therapy (IMRT), image guided radiation therapy (IGRT) and even proton irradiation. Further advances in precise delivery are limited more by our ability to identify microscopic tumor cells rather than depositing dose precisely in tissue. Dose escalation also appears to have limitations as well. As pointed out by Yamoah et al. (26) recently, several dose escalation studies have failed to show improved benefit. Therefore, improving the efficacy of radiotherapy depends on a more complete understanding of radiation effects on cancer and normal tissue and exploiting these differences for therapeutic gain. The insight provided by Surace et al. opens many new possibilities to selectively augment the action of radiation against cancer.

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Footnote

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Complementing the tumor-specific immunity in tumor radiotherapy

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The role of immune system and inflammation in tumor development has recently rekindled the attention of researchers and Douglas Hanahan and Robert A. Weinberg have listed them to one of the ten cancer hallmarks (1). Though the protective function of immune system to fight against infection was widely recognized (2), its role in tumorigenesis has been a controversial topic because while adaptive and innate immunity convincingly demonstrate anti-cancer function, certain clinical observations and animal experiments showed that the immune system could also promote the spontaneous and chemically-induced cancer development (3). Recent researches demonstrated that immune system could be manipulated as an auxiliary tool for therapeutic strategies of chemotherapy and radiotherapy to destroy cancer (4-6).

Radiotherapy is a standard treatment for cancer that triggers massive and irreversible damage to DNA. While radiotherapy was reckoned as a classical immunosuppressive treatment, accumulating evidence illustrates that it also serves a local tumor-specific immunity supporting role. This type of classical treatment strategy could induce increased presence or function of tumor-infiltrating CD8+ T cells, type I interferon (IFN) resulting in enhanced antigen cross-presentation (7), increased expression of major histocompatibility complex (MHC) class I glycoproteins and tumor-associated antigens (8), and maturation of tumorassociated dendritic cells (DCs) (9). These immunological events could further enhance the tumor-destroying effects. However, the possible upstream events that initiate these alterations remain to be elucidated.

To identify the upstream events that might be responsible for these immunological alterations of the tumor-specific immunity in tumor radiotherapy, Laura et al. conducted an unbiased analysis of immune response-related transcripts after radiotherapy in a preclinical model of melanoma and found that the local production of pro-inflammatory anaphylatoxins C3a and C5a was essential to the tumor response to radiotherapy. More interestingly, the authors identified that the microenvironmental complement was produced by local immune cells like DCs and CD8+ T cells. The complement system has been traditionally considered only to "complement" the action of the immune system in the antibody-mediated defense against pathogens. The role of complement in other pathological and physiological processes like as transplant rejection, autoimmunity, neurodegeneration, cell malignant transformation or the therapeutic process of these conditions is still a realm remained to be mined. In particular, the functions of the complement system in cancer is still controversial as the production of complement-inhibiting proteins by tumor cells or stroma has been suggested to promote tumor growth (10), whereas it is also proposed that complement in the context of chronic inflammation promotes tumor growth, migration and angiogenesis (11). Interestingly, this research coheres to a previous publication showing increased efficacy of fractionated radiotherapy when C3 was blocked (12), which further gives support to the notion that in certain context; complement system could exert tumorkilling effects. These results indicate that anaphylatoxins are critical players in radiotherapy-induced tumor-specific

immunity and subsequent clinical responses, which could be manipulated in future clinical practice for reinforcing the therapeutic efficacy of cancer treatment.

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Fractionated radiotherapy combined with PD-1 pathway blockade promotes CD8 T cell-mediated tumor clearance for the treatment of advanced malignancies

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Programmed death-1 (PD-1) is a T cell inhibitory receptor, expressed on recently activated and chronically stimulated CD4 and CD8 T cells (1,2). Through interacting with programmed death ligand-1 (PD-L1), PD-1 limits T cell receptor signaling, and maintains peripheral tolerance (1,2). PD-1 pathway blockade has the potential to restore effector function to exhausted T cells, thus boosting their antiviral and antitumor activity (2). This has prompted the development of PD-1/PD-L1 antibodies for treating cancer. Success in numerous preclinical studies (3-5) led to multicenter clinical trials, and FDA approval of anti-PD-1 agents (nivolumab or Opdivo® and pembrolizumab or Keytruda[®]) for the treatment of metastatic melanoma and non-small cell lung cancer (6-8). With as many as 31% of patients benefiting from treatment and median response duration lasting 2 years (8), it is not surprising that PD-1 pathway blockade, and interference with other T cell signaling checkpoints such as cytotoxic T lymphocyteassociated antigen 4 (CTLA-4), continues to generate excitement for cancer immunotherapy. Given that PD-1 and CTLA-4 blockade exert distinct effects on tumor control, combined blockade has been successful in mouse models of melanoma and clinical trials with 53% response rate (9). In order to further increase efficacy, it is necessary to understand how immunotherapy could complement already approved treatment modalities, such as chemotherapy and radiation.

Radiation therapy (RT) is the most widely used nonsurgical intervention for treating primary solid malignancies, and relieving cancer-associated pain stemming from bone metastases (10). Besides directly inducing tumor cell death, RT has an immunomodulatory effect. Dying tumor cells release danger-associated molecular patterns (DAMPs) such as deoxyribonucleic acid (DNA), high mobility group box 1 (HMGB-1) and adenosine triphosphate (ATP), as well as tumor-associated antigens. DAMP recognition induces a strong type I interferon (IFN) signature in dendritic cells, increases major histocompatibility complex (MHC) Class I and Class II expression, and helps prime tumor-reactive CD4 and CD8 T cells. Immune-mediated regression of the irradiated tumor and improved control of nonirradiated lesions (abscopal effect) sometimes arise in RTtreated hosts (10). However, more often than not, RT is not sufficient to override tumor-induced immunosuppression and escape (10). Several case reports pointed to a potential benefit of combining RT with immune checkpoint intervention, while a retrospective study and a phase I and II trial concluded combination therapy was safe (11-13). This has spurred extensive preclinical investigation into RT-checkpoint blockade combination therapies, with a focus on dosing, scheduling, and mechanisms underlying potential synergistic effects. RT delivered in smaller daily fractions over several days increases tumor immunogenicity compared to single-dose radiation (14). Previous work has shown that fractionated RT synergized with CTLA-4 blockade as well as other immunotherapies (e.g., Tolllike receptor 7 agonist treatment) to yield better survival than single-dose radiation, or single-dose radiation with immunotherapy (15,16).

Recently, Dovedi and colleagues published an exciting report in *Cancer Research* examining whether fractionated RT combined with PD-1 pathway inhibition could enhance survival following tumor challenge (17). The authors first noted that fractionated RT (delivered in 5 daily fractions of 2 Gy) led to increased PD-L1 expression on CT26 colon carcinoma cells. Interestingly, this effect was noted in vivo and not in vitro, suggesting that tumor-associated stroma or infiltrating T cells mediated the increase in PD-L1 expression after RT. Through elegant depletion experiments, the authors demonstrated that CD8 T cells were required for the enhanced PD-L1 expression on the tumor cells. Since IFNy can induce PD-L1 expression (18), the authors utilized anti-IFNy neutralizing antibodies and IFNyR1 shRNA to determine whether the noted increase in PD-L1 expression was IFNy-dependent. Blocking IFNy signaling abrogated RT-induced PD-L1 expression in the presence of CD8 T cells, suggesting that CD8 T cellmediated IFNy secretion is responsible for enhanced PD-L1 expression on CT26 cells (17). The increased PD-L1 expression following RT could therefore explain how some tumors evade the endogenous immune response, and provides a rationale for combining checkpoint blockade with RT for enhanced tumor control. To test the functional significance of RT-induced PD-L1 increase, Dovedi et al. combined fractionated RT with PD-1 pathway blockade. Mice bearing established CT26, 4T1 (triple negative breast) or 4,434 (melanoma) tumors exhibited significantly improved tumor control (184.3±13.5 vs. 292.8±14.3 mm² for 4T1 at day 10 post treatment) and overall survival when treated with fractionated RT and either anti-PD-1 or anti-PD-L1, than the animals treated with either monotherapy (17). Specifically, 66-80% of treated animals survived past 100 days and were protected from a subsequent recall challenge at a distinct site (17).

CD8 T cells were critical for this tumor control, as CD8 T cell depletion prior to therapy abrogated the protective effect of combined treatment (17). NK cell depletion impacted initial tumor growth, but not overall survival (17). Collectively, these findings suggest that CD8 T, but not NK cells, are necessary and sufficient for tumor control after RT and PD-1 pathway blockade. Dovedi and colleagues also depleted CD4 T cells prior to RT + anti-PD-L1 treatment, and noticed improved tumor control, albeit without a significant increase in survival (17). These data indicate that regulatory T cells (T_{regs}) might play a role in restraining antitumor immunity after combined therapy. To test this, future studies could incorporate targeted T_{reg} depletion (e.g., using Foxp3-DTR mice) or anti-CTLA-4 treatment.

The authors noted that the combination therapy was well tolerated, since body weight was not impacted (17).

However, with the use of checkpoint blockade, emergence of autoimmune-like events or overt autoimmunity is a major concern. Specifically, 9–14% of patients treated with anti-PD-1 or anti-PD-L1 developed immune-mediated grade 3 or 4 adverse events, which affected the skin, gastrointestinal tract, or thyroid (6,7). In addition, several patients developed type 1 diabetes as a result of PD-1 pathway inhibition (6,19). Future studies could evaluate serum autoantibody levels, intestinal pathology, and kidney and liver toxicity as direct readouts of immune-related adverse events following combination therapy.

Translating combination therapy into the clinic requires optimizing treatment schedule for maximizing clinical benefit, while minimizing side effects. In their report, Dovedi *et al.* investigated whether the order in which RT and PD-1 pathway blockade were administered affected treatment efficacy. Starting anti-PD-L1 treatment on the first or the last day of fractionated radiotherapy cured 57–60% of treated animals (17). However, anti-PD-L1 administered 7 days after the last dose of radiotherapy had no additive effect compared to radiation alone, and yielded no long-term survivors (17). These findings suggest that checkpoint blockade is most effective during, but not following radiation, and warrants further investigation.

In the year since Dovedi et al. published their findings, a phase I clinical trial examined the benefit of fractionated radiotherapy and CTLA-4 blockade in 22 patients with stage IV melanoma (20). Patients received fractionated RT, followed by four cycles of ipilimumab (anti-CTLA-4) treatment and were monitored for response with computed tomography (CT). Partial response (at least a 30% decrease in lesion diameter) was noted in 18% of patients (20). Another 18% had stable disease, while 64% of treated patients experienced progressive disease, suggesting that the majority of patients did not respond (20). Twyman-Saint Victor et al. then applied this treatment regimen to mice bearing B16-F10 melanoma, and similarly to Dovedi and colleagues, noted that concurrent checkpoint blockade synergized with RT, in a CD8 T cell-dependent manner. However, only 17% of animals responded to treatment. Even though combined treatment decreased the number of T_{ress} in the tumor, the number of effector CD8 T cells failed to increase (20). Importantly, transcriptional analyses of resistant tumors revealed that PD-L1 was in the top 0.2% of up-regulated genes that make up the gene signature of tumors refractory to combination therapy (20). Genetic deletion of PD-L1 by CRISPR rendered a resistant tumor cell line highly responsive to RT + anti-CTLA-4 therapy.

This prompted the authors to treat B16-F10 tumor bearing mice with anti-PD-1/PD-L1 in addition to anti-CTLA-4 and fractionated RT. In this case, 80% of animals were long-term survivors, with protective immunity against subsequent challenge (20). The authors showed that the three treatment modalities evoked non-redundant immune mechanisms. Radiotherapy led to increased CD8 T cell diversity in the tumor, CTLA-4 blockade decreased the number of tumor-infiltrating T_{regs} , while PD-L1 blockade allowed reinvigoration of exhausted intratumoral CD8 T cells (20).

Checkpoint blockade has revolutionized cancer therapy, and given hope to patient populations suffering from standard treatment-refractory tumors. Further understanding the ways in which checkpoint inhibitors complement each other and synergize with other therapies is necessary for increasing objective responses, minimizing relapse and side effects. Preclinical studies have already begun to explore optimal treatment schedules, and understand pathways driving resistance to combination therapy (17,20). Future work should focus on identifying biomarkers to predict treatment efficacy, as well as autoimmune risk screening (e.g., HLA typing, autoantibodies) to identify patients likely to develop immune-related adverse events.

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Optimizing radiation for cancer immunotherapy

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Various protocols are under investigation for the purpose of optimizing radiotherapy for cancer treatment. Due to recent advances in image guidance and radiation delivery, a new option is to treat tumors with a single intense dose of radiation, 30 Gy or more (1,2). Alternatively, radiation doses can be "fractionated" over multiple treatment periods. Radiotherapy kills tumor cells and their associated stromal and vascular cells, and in some instances can induce T cell-mediated immunity that is effective at killing tumors outside the radiated area, a phenomenon called the "abscopal effect" (3,4). Since complete tumor remission usually depends on an effective anti-tumor immune response, it is important to determine how different radiation regimens influence anti-tumor immune responses. Filatenkov and coworkers (5) investigated the effect of radiation delivery protocols on the numbers and properties of immune cells in the microenvironments of colon cancer cells in mice. Weakly immunogenic ectopic CT26 colon tumors grown in syngeneic Balb/c mice usually responded to a single 30 Gy dose of intense radiation with durable tumor remissions due to T cell-mediated tumor killing. Fractionated radiation regimens were not as effective at stimulating T cells responses or durable remissions. The authors conclude that an examination of the tumor immune response may be useful for optimizing radiation regimens applied to various tumors.

A critical role for myeloid cells in tumor immunosuppression following radiation

Solid tumors produce factors that normally evoke an immune response, but tumors also create an immunosuppressive microenvironment by producing additional anti-inflammatory factors such as adenosine (6), TGF β (7) and nitric oxide (8). Tumor cells also express indoleamine 2,3-dioxygenase (IDO) that converts L-tryptophan to L-kynurenine (9). Tryptophan depletion and kynurenine accumulation inhibit immune effector cell proliferation. These factors also stimulate the production of immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs), tolerogenic (M2) tumorassociated macrophages (TAMs), and T regulatory (Treg) cells. These suppressor cell populations influence T cells to express additional inhibitory signaling molecules (PD-1, CTLA-4, and Tim-3). Filatenkov and coworkers (5) found that high intensity radiation of tumors, as opposed to fractionated radiation, resulted in tumor remissions that were associated with an increase in tumor-associated CD8⁺ T cells and a reduction in CD11b⁺/Gr1⁺ MDSCs over 14 days following radiation. These responses were dependent on CD8⁺ dendritic cells (DCs), CD4⁺/CD40L⁺ T helper cells, CD8⁺ cytotoxic T cells and IFNy (Figure 1). Tumor remission in response to intense radiation was not observed in Batf3^{-/-} mice that lack CD8⁺ DCs (10). The findings implicate the minor CD8⁺ DC subset, as important for tumor antigen cross presentation and CD8⁺ T cell expansion in tumors and/or tumor-draining lymph nodes. They also suggest that radiation, together with T cells activation and IFNy, cooperate to reduce MDSCs in tumors, to enhance the ratio of CD8⁺ DCs/MDSCs and thereby produce anti-tumor immunity.

Effects of chemotherapeutic agents on tumor immunity

As with intense radiation, the antitumor activity of some chemotherapeutic agents is mediated in part by activation of host immunity. Gemcitabin (11) and 5-fluouracil (12) are



Figure 1 Factors that influence immune responses in tumors following intense radiation. In response to a single exposure to intense radiation (30 Gy) CT26 colon tumors rapidly release pro-inflammatory factors that stimulate the accumulation of pro-inflammatory cells shown in red. Tumors also release anti-inflammatory factors and stimulate accumulation of immunosuppressive cells or T cell exhaustion factors shown in green. Over 14 days following intense radiation there is an increase in tumor-associated CD8⁺ T cells, a decrease in myeloid-derived suppressor cells (MDSCs), and T cell dependent tumor remission. Cells and factors that are required for optimal T cell mediated tumor killing are outlined in blue. HMGB1, High mobility group box 1; NO, nitric oxide; Kyn, *L*-kynurenine.

cytotoxic to MDSCs and activate tumor T cells. Cisplatin was found to increase numbers of tumor-associated DCs, decrease MDSCs, and enhance the immune response in melanoma-bearing mice (13). Paclitaxel (14) and docetaxel (15) were also found to reduce numbers of tumorassociated MDSCs. It will be of interest to determine if these responses depend on CD8⁺ DCs, as is the case with intense radiation.

Effects of adenosine triphosphate (ATP) and adenosine of tumor immunity

ATP released from stressed or apoptotic tumor cells in response to radiation is acutely excitatory to the immune system by activating pro-inflammatory ATP-receptors (P2X and P2Y receptors) found on myeloid and lymphoid cells (16). However, certain tumors and tumor-associated suppressor cells express CD73 or CD39, which are ectoenzymes that rapidly convert pro-inflammatory ATP into anti-inflammatory adenosine (17). Myeloid-selective deletion of immunosuppressive adenosine A2A receptors (A2AR) was recently found to change the phenotype of MDSCs by greatly reducing their IL-10 production, and to suppress the growth and metastasis of 4T1-12B breast cancer cells (6). A2AR blockers in tumors stimulate the activation of T cells, much like "check point inhibitors" such as anti-PD-1 and anti-CTLA4. In fact, A2AR blockade and anti-PD-1 synergistically inhibit the growth of breast and colon cancer cells (18). Since intense radiation causes tumor cell necrosis and apoptosis, rapidly released ATP can be degraded to adenosine. It will be of interest in future studies to determine if the combination of intense radiation and adenosine receptor blockade robustly stimulates anti-tumor immunity, and the roles for CD8⁺ DCs and MDSCs in these responses.

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CD8⁺ T effector targeted elimination of regulatory tumor stroma cells for improved immunotherapy

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Mounting preclinical and clinical evidence over the past several decades implicated immune system in controlling malignancies. However, the translation of this knowledge into clinical benefit for cancer treatment has just recently been realized. A tumor-associated antigen (TAA) pulsed dendritic cell-based vaccine was the first immunotherapy product against cancer to be approved by FDA in the United States (1). This development not only overcame skepticisms about the potential of caner immunotherapy, but most importantly galvanized the field for the development of various forms of immunotherapies. A major setback for the cancer immunotherapy field has been the lack of comprehensive understanding of interactions between tumors and the immune system and how such interactions could be exploited for the development of effective immune therapies. The discovery that tumor uses a complex set of extrinsic and intrinsic mechanisms to evade the immune system paved the way for the design of effective immunotherapies with significant positive impact recently. For example, the discovery that tumors utilize the immune checkpoint receptors, such as cytotoxic T lymphocyteassociated antigen 4 (CTLA-4) and the programmed cell death protein 1 (PD-1) pathway, to effectively evade T cell responses resulted in FDA-approved therapies against various cancer types using blocking antibodies to these molecules (2,3). The checkpoint blockers, however, are only effective for certain tumor types and the efficacy is limited to a fraction (~30%) of treated patient population with the responsive tumors. Combined therapy with both checkpoint blockers in advanced melanoma was recently reported to reach an objective response rate of 61% with drug-related adverse events of grade 3-4 in 54% of patients (4). The question is if complementary approaches, particularly TAA-

based vaccine could further improve the efficacy of immune checkpoint blockers, reduce drug-related adverse effects, and broaden their application to unresponsive tumor types.

In addition to intrinsic mechanisms, tumors have also evolved to employ various extrinsic mechanisms to evade the immune system. Nonmalignant tumor stroma and the regulatory arm of the immune system represent such extrinsic mechanisms. Tumor stroma not only serves as a regulated physical barrier to intratumoral infiltration of immune effector cells, but also actively downregulates effector immune responses while promoting immune suppressive regulatory cells. For example, endothelial cells in tumor stroma were shown to upregulate FasL expression in response to prostaglandins and vascular endothelial growth factors and preferentially induce apoptosis in tumor infiltrating CD8⁺ T effector cells, but not CD4 + CD25 + FoxP3 + T regulatory (Treg) cells (5). Another regulatory cell population that has been specifically recruited into the tumor by stroma is myeloid derived suppressor cells (MDSC). Both Treg cells and MDSC represent major obstacles for the efficacy of various immunotherapies and in particular cancer vaccines. Systemic depletion of these regulatory cells may have unwanted consequences due to their roles in immune homeostasis. Therefore, targeted approaches to functionally and/or physically eliminate these cells within tumor microenvironment will have a significant clinical benefit.

A recent paper by Wu *et al.* published in *Clinical Cancer Research* has demonstrated that radiotherapy combined with intratumorally delivered TAAs as vaccine provides an effective means of priming $CD8^+$ T effector cells not only against tumor, but also stromal cells and MDSC within the tumor microenvironment (6). Intratumoral injection

of a peptide representing a dominant CD8⁺ T cell epitope for human papilloma virus E7 following radiotherapy was shown to mobilize DCs within tumor draining LNs for antigen cross-presentation, activation of CD8⁺ T cells, and effective elimination of E7 expressing TC-1 tumor in mice. The therapeutic efficacy of this approach was associated with CD8⁺ T effector cells killing not only the tumor, but also MDSC cross-presenting TAA. Sensitivity to killing by CD8⁺ T cells was limited to MDSCs as intratumoral macrophages cross-presenting TAA were not killed. Importantly, the elimination of MDSC by CD8⁺ T cells did not require antigens expressed by the tumor. Mice treated with intratumoral injection of tumor-unrelated antigens, such as OVA and influenza NS1, following radiotherapy had decreased intratumoral MDSC and showed therapeutic efficacy. This effect was limited to intratumoral, but not a distant site, injection of the peptides and required signaling via type I IFN receptor and Toll-like receptor 4. Mice deficient in one of these receptors did not show therapeutic efficacy, implicating these innate immune pathways in effector immune responses against tumor, and suggesting that the radiotherapy-induced death of a tumor has immune adjuvant effect.

Specific functional and/or physical intratumoral depletion of MDSC or Treg cells has important implications for cancer immunotherapy. First, local elimination of regulatory cells will prevent significant adverse effects associated with systemic depletion of such cells and, in particular, avoids the risk of autoimmunity. Second, this approach may provide better efficacy as it occurs at the target, where a balance favorable to immune effectors is paramount for the destruction of the tumor. It remains to be seen if the therapeutic efficacy observed with this treatment modality applies to other tumor types, particularly using a bona fide endogenous TAA as vaccine. More importantly, if effective approaches for targeted delivery of antigens can be developed for tumors that are not amenable to intratumoral injection, and if such delivery approaches will achieve a similar therapeutic efficacy to that generated by intratumoral injection of TAA. Exploiting the adjuvanticity of danger molecules released by dying tumors may have significant implications for cancer immunotherapy. Such adjuvants are expected to have localized effects, thereby lacking adverse effects associated with systemic use of exogenous adjuvants. In this context, it will be important to test if the endogenous adjuvant

activity seen with radiotherapy is also a common feature of any nonimmune therapy that target tumors for physical destruction.

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Footnote

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Expanding landscape of CDKN1A (p21) functions: CDKN1Amediated radioresistance of dermal Langerhans cells and its impact on the immune system

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The CDKN1A ($p21^{WAF1/CIP1}$) protein is the founding member of the CIP/KIP family of cyclin-dependent kinase (CDK) inhibitors. It is a p53 transcriptional target that plays a pivotal role in the DNA damage surveillance network through activating cell cycle checkpoints, promoting DNA repair, downregulating apoptosis, and triggering a senescence-like growth arrested response (premature senescence) (1-3). The anti-apoptotic property of CDKN1A is not only associated with its ability to halt cell-cycle progression and facilitate DNA repair, but also relies on its ability to inhibit the activity of proteins directly involved in the induction of apoptosis (e.g., the caspase cascade) and to control transcription, resulting in downregulation of pro-apoptotic genes and upregulation of genes with anti-apoptotic activities (1). In addition, we recently provided evidence suggesting that CDKN1A can positively regulate wild-type p53-induced phosphatase 1 (WIP1) (4), an anti-apoptotic phosphatase that inhibits p53 and its upstream kinases (e.g., ATM; CHK2). Consistent with these properties of CDKN1A, treatment of p53 wildtype solid tumor-derived cells with ionizing radiation or chemotherapeutic agents results in sustained upregulation of CDKN1A, protection against apoptotic cell death, and growth arrest through premature senescence (1).

In an elegant study recently published in *Nature Immunology* (5), Price and colleagues demonstrated a pivotal role for CDKN1A in inhibiting the apoptotic response of mouse epidermal Langerhans cells (LCs) following a total-body exposure to ionizing radiation; these LCs can subsequently migrate to the skin-draining lymph nodes and promote the expansion of regulatory T (T_{reg}) cells

(*Figure 1*). Zitvogel and Kroemer have published a News and Views article on the work done by this group in the same journal issue (6).

LCs are a subset of mononuclear phagocytes that form a dense network in the barrier surfaces, including the epidermis of the skin, and are long-lived, can divide, and replenish themselves. These cells tolerate relatively high doses of ionizing radiation and promote moderation of the immune surveillance system. Although sessile immature LCs reside in the epidermis, they are dynamic cells that can migrate to skin-draining lymph nodes where they influence the immune response. LCs require the chemokine receptor CCR7 for migrating to the lymph nodes (7).

Using a series of knockout and adoptive-transfer technologies, Price et al. revealed that the remarkable radioresistance phenotype of LCs is directly associated with high expression of CDKN1A, both endogenous and radiation-induced. Specifically, wild-type (CDKN1Aexpressing) LCs exhibited resistance toward radiationinduced apoptosis as a consequence of CDKN1A-mediated activation of the G1/S cell cycle checkpoint coupled with rapid rejoining of DNA double-strand breaks (DSBs). Instead of undergoing apoptosis following irradiation, some wild-type LCs upregulated major histocompatibility complex (MHC) class II molecules, migrated to the skindraining lymph nodes in a CCR7-dependent manner, and caused an increase in T_{reg} cell numbers, which are known to suppress the immune response through targeting effector T cells. In contrast to wild-type LCs, CDKN1A-deficient (knockout) LCs underwent apoptosis post-irradiation and were thus unable to cause the accumulation of T_{reg} cells in



Compromised anticancer immunesurveillance

Figure 1 Summary of the known roles of CDKN1A in anticancer immune surveillance. CDKN1A promotes survival of LCs following ionizing radiation exposure leading to T_{reg} cell accumulation, and inhibits effector (T_{eff}) cells by triggering their senescence. LC, Langerhans cell; DSB, double-strand break.

draining lymph nodes.

Price *et al.* (5) further demonstrated an important immunological consequence of these events. They found that the growth of subcutaneously injected malignant B16 melanoma or EL4 lymphoma cells was accelerated in irradiated (versus non-irradiated control) host mice bearing wild-type LCs, but this effect was not seen in mice bearing CDKN1A-deficient LCs or MHC class II–deficient LCs. The radiation-enhanced tumor growth was accompanied by increased numbers of T_{reg} cells in the tumor and tumor-draining lymph nodes.

The impact of CDKN1A on the immune surveillance network is not limited to LCs. Ye *et al.* (8), for example, reported that one mechanism by which T_{reg} cells suppress host immunity is by inducing CDKN1A-dependent senescence of responder naive and effector T cells. The mechanism by which CDKN1A provides a survival signal in one cell type (e.g., epidermal LCs) and a growth inhibitory (senescence) signal in another (e.g., naive/effector T cells) remains to be elucidated. However, these intriguing discoveries with hematopoietic cells, in concert with those reported previously with fibroblastic and epithelial cells (1), underscore the conclusion drawn by us (1,4) and by Warfel and El-Deiry (2) that a better understanding of the complexity of CDKN1A-mediated responses in different types of cells and tissues is crucial to determining whether modulating CDKN1A signaling might be a useful approach to the treatment of certain types of malignancies.

The findings reported by Price *et al.* (5) suggest an important role for CDKN1A in the expansion of T_{reg} cells in response to total body irradiation that results in an immune-suppressed phenotype and a growth advantage for cancer cells. Such effects have also been widely exploited for the engraftment of non-self tissues into humans and also into animals, e.g., to generate mouse models of cancer. The question of whether LC-mediated immune suppression might impact negatively on the outcome of cancer radiotherapy was also raised by Price and colleagues. However, as these authors pointed out, radiotherapy to cancer patients is given very differently than the total-body exposures used in their study, notably with the dose being highly tailored to the tumor with maximal avoidance

of normal tissue elements. We suspect that LC-mediated effects on the immune system will be much less important under such conditions, but this will require confirmation using small-animal image-guided radiotherapy platforms that better simulate the clinical situation (9).

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Footnote

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STING-cytosolic DNA sensing: the backbone for an effective tumor radiation therapy

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Local irradiation has been broadly used in the treatment of primary and metastatic tumors. It is commonly believed that treatment effect of radiation is based on a direct killing of cancer cells. However, recent studies have gradually found that tumor regression following ablative irradiation mainly depends on type I interferon signaling and CD8⁺ T cell response. In this issue of Immunity, Deng and colleagues (1) found that anti-tumor effects of radiation are contributed by both innate and adaptive immune responses. They present compelling evidence that cytosolic DNA sensing pathways bridge the known irradiation-mediated DNA damage to anti-tumor immune response.

Anti-tumor effect of radiotherapy (RT) relies on host immunity

RT has been widely used for various tumor therapy alone or in combination with surgery or chemotherapy. RT such as ionizing radiation can either damage DNA directly or indirectly through creating charged particles (free radicals) within the cells. Besides direct tumoricidal effect, radiation could also lead to transient depletion and rebound effects of resident leukocytes, which impact the ultimate therapy results.

Understanding about the mechanism of radiationmediated tumor regression has a breakthrough recently. Lee *et al.* revealed that ablative RT increases T-cell priming in draining lymphoid tissues and reduce the growth of the primary tumor or distant metastasis (2). CD8⁺ T cells utilize T-cell receptors to recognize tumor derived antigens which bind to MHCI, and mount cytolytic attack to tumor cells. Radiation could increase the peptide repertoire and MHC class I expression on tumor cells, and boosts the efficacy of adoptive CTL immunotherapy *in vivo* (3). These studies propose the essential role of CD8⁺ T cell response in RT.

Type I IFN optimizes anti-tumor adaptive immunity after radiation

Type I IFNs, comprising IFN α and IFN β proteins, are known for their unique role in inhibiting viral infection through ISG genes and critical mediators bridging innate response to adaptive immune response. Type I IFN can promote the activation and cross-presentation of DC, which is crucial to initiate the adaptive immunity. IFN- α/β directly promotes the activation, expansion and differentiation of T cell. Besides, type I IFN increases NK cell cytotoxicity by modulating the surface expression of activating and inhibitory receptors. Recently, the critical role of type I IFN in tumor immunity is gradually understood. Type I IFN can directly induce the apoptosis of tumor cell and inhibit the proliferation. Through promoting the antigen expression in neoplastic cells, type I IFN also increases the immunogenicity of tumor. Administration of exogenous IFN α have been used to treat tumors such as acute myeloid leukemia (4).

Moreover, Burnette *et al.* found that the anti-tumor efficacy of radiation depends on both the generation of host adaptive immune response and innate type I IFN response (5). However, the molecular mechanisms of type I IFN induction and details of type I IFN bridging radiation-mediated tumor damage to immune response are still unclear.

cGAS-STING sensing dying tumor DNA mediates type I IFN induction after RT

Emerging data suggest that the efficacy of various tumor therapy modalities including Ab, RT and chemotherapy depends on generation of adaptive immune response. One of critical questions is what danger signalings are triggered

by these therapy modalities and how they are recognized to initiate the adaptive response. The sensing of infection and injury can be mediated by pattern-recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (6-8). TLRs, as one kind of important PRRs, were reported to sense HMGB1 induced by antiher2 or anthracycline chemotherapy, and were essential for optimal tumor control (9,10). Surprisingly, Deng et al. found that TLR sensing is dispensable for the RT, because the deficiency of MvD88 or TRIF as TLR adaptors did not impair the tumor control. Furthermore, the fact that tumor regression could be induced by radiation after blocking of HMGB-1 indicates existing of other critical danger signaling sensing. DNA breaks generated after irradiation could be the main danger signals. Recently, STINGmediated cytosolic DNA sensing cascade was demonstrated to be the major mechanism of type I IFN induction after viral infection, which is TLR independent. Using Tmem173--- mice (STING-deficient mice), Deng et al. first found that absence of STING impaired radiation-mediated tumor regression.

Radiation treatment could induce elevated type I IFN expression in tumor microenvironment. However, radiation induced type I IFN upregulation was impaired if STING is deficient in the host but not tumor cells per se. Further qPCR experiments showed that CD11c⁺ DC cells were the main producer of type I IFN after radiation, which was impaired most significantly by STING deficiency compared with other cell populations in tumor. DCs bridge innate immunity and adaptive immunity due to its cross-priming ability. Deng et al. further explored that STING mediated type I IFN production in DC was essential to cross-prime CD8⁺ T cells. STING-mediated type I IFN production needs the engagement with TBK1 to direct IRF3 activation. IRF3-deficient DC also showed impaired cross-priming ability. Exogenous type I IFN treatment could rescue the cross-priming ability of STING deficient DC.

These results suggest that STING signaling-dependent DNA sensing is essential to trigger the adaptive immune response after radiation. It raises the question that what is the sensor of irradiation induced damaged DNA. The previous identified intracellular DNA sensors include IFN16, DAI, AIM2, DDx41 and RNA polymerase III. However, these sensors function differently and specifically depend on the cell type, expression level or DNA source. Until recently, Cai *et al.* identified cyclic-GMP-AMP (cGAMP) synthase (cGAS) as a universal cytosolic DNA sensor for STING activation (11). In this research, Deng *et al.* found that cGAS knockout or silenced by siRNA impaired the cross-priming ability of DC similar as STING deficiency. cGAS deficient DC produce significantly reduced type I IFN compared to WT DC cultured with irradiated tumor cells. This suggested that the cGAS-STING-IFN signaling is required for DC's cross-priming of irradiated tumor.

Potential questions and translational perspectives

One interesting question is how DNA from irradiated tumor cells transport into DC to trigger cytosolic DNA sensing pathway. Deng et al. found that DNA delivery in a cell contact-dependent manner but not in the free soluble form is essential to the cross-priming activity of DC. Burnette et al. have demonstrated that radiation induced DC infiltration into tumor, which was activated by type 1 IFN to enhance cross-priming capability (5). It raises one interesting scenario: irradiation induces tumor apoptosis and DC recruitment. DC may discriminate and approach apoptotic tumor cell to initiate phagocytosis for exogenous DNA harvest. Recently, Vacchelli et al. found that chemotherapy induced dving cells to release ANXA1, which promote stable contact of DCs with tumor cells through FPR1 for antigen capturing and processing (12). Whether a similar mechanism exists in the RT model needs to be explored further. Another interesting question is whether DCs activate local CD8⁺ T cells for tumor control or need to migrate into draining LN to prime de novo T cell. Does the local pre-activated CD8⁺ T cells in tumor switch to a tolerant status? If so, it will be a more efficient tumor therapy strategy to combine irradiation with anti-immune checkpoint antibody.

The studies by Deng *et al.* suggest that activating of STING signaling in DC to promote cross-priming could be one efficient strategy for tumor therapy. The 2'3'-cGAMP is synthesized by cGAS recognizing cytosolic double-stranded DNA as a secondary messenger to trigger STING signaling. Deng *et al.* found that exogenous 2'3'-cGAMP treatment could significantly improve the tumor therapeutic effect of radiation, with about 70% of mice completely rejected the tumors. Recently, Corrales *et al.* generated synthetic cyclic dinucleotides which could activate both murine and human STING, and showed impressive therapeutic effect in diverse tumor models through intratumoral injection (13). In Deng's research, cGAMP treatment alone has no therapeutic effect. Improving the ability of targeting and entering tumor will be promising for cyclic dinucleotides-

STING based tumor therapy.

It must be pointed out that, one of recent researches showed that anthracycline chemotherapeutic drug induces production of type I IFN in tumor cells through TLR3mediated RNA sensing pathway, which promotes tumor cells to release CXCL10 to attract CD8⁺ T cells and further tumor eradiation (14). Thus, in some instances, the effect of type I IFN response in tumor cells could not be ignored. It should be taken into consideration of targeting type I IFN signaling in tumor cells to attract CD8⁺ T cell for DC priming to optimize tumor control.

In this study, Deng *et al.* demonstrate a novel mechanism that the cytosolic DNA sensing for type I IFN is induced via radiation treatment for tumor control. In another study, Deng *et al.* did a combination of radiation with immune checkpoint inhibitor, anti-PD-L1, which synergistically generated durable anti-tumor responses in the mouse models (15). So far, the detailed mechanisms of this combination is still not clear. To translate these discoveries into practice, it will be necessary to further determine the toxicity and synergy of radiation with nucleic acid-sensing agonists, as well as to develop effective immunotherapies that further improve RT.

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Combination therapy with TLR7 agonist and radiation is effective for the treatment of solid cancer

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Toll like receptors (TLRs) are well-conserved pattern recognition receptors required for sensing pathogenic elements such as bacterial lipopolysaccharides, DNA, or viral RNA. TLR1, TLR2, TLR4, TLR5, and TLR6 are present on the surface of cells and recognize bacterial and fungal components. In contrast, TLR3, TLR7, and TLR9 function intracellularly by recognizing of the nucleic acids (DNA or RNA) of pathogens. The host innate immune responses are enhanced through the recognition of pathogen-associated molecules by these TLRs. Moreover, the activation of innate immunity by TLR agonists effectively drives adaptive immunity via the production of several cytokines [e.g., interleukin (IL)-12b] and the activation of antigen-presenting cells (APCs). IL-12b is a pivotal cytokine for the induction of Th1 immune response and antigen-specific cytotoxic T cells (CTLs) (1). These inductions are extremely important for cellular immune response in the host and may lead to elimination of viruses or establishment of several cancer therapies. The expression of costimulatory molecules (CD40, CD80 and CD86) on APCs is enhanced after stimulation by several TLR agonists (2,3). These costimulatory molecules are intricately involved in the induction of acquired and antigen-specific immune responses. Therefore, TLR agonists can induce cellular immune response in viral infection and cancer and have the potential to treat cancer. Indeed, the anti-tumor effects of several TLR agonists were recently evaluated in basic studies and clinical trials (4,5). In particular, one TLR7 agonist, imiquimod (Aldara 5% cream, 3M), has been approved for clinical use by the FDA. This agent is topically used for the treatment of basal cell carcinoma and other skin tumors. However, many investigators have

confirmed that monotherapy by some TLR agonists was not sufficient to completely eliminate the tumor in animal studies and clinical trials. Therefore, combination therapy with TLR agonists and other anti-cancer treatment is being evaluated. Traditional treatments for patients with cancer are surgery, chemotherapy, and radiation. Although these traditional treatments are effective for early stages of cancer, they have a limited role in advanced cancer. Recently, cancer immunotherapy has received attention as a new strategy of cancer therapy. Several studies have evaluated the anti-cancer effects of the combination of TLRs agonists and traditional cancer therapy (6).

In the study by Dovedi et al. published in Blood, the anti-tumor effect of combination therapy with a TLR7 agonist and traditional radiation therapy was evaluated in murine T cell and B cell lymphoma models (7). These data demonstrated that systemic administration of a TLR7 agonist combined with local radiation could suppress the progression of tumor growth and improve the survival rate in tumor-bearing mice. In particular, the combination of weekly administration of a TLR7 agonist and 5 fractions of 2 Gy local radiation could completely inhibit the subcutaneously established lymphoma. In many basic animal studies, it is extremely difficult to eliminate a tumor by chemotherapy, radiation, cancer immunotherapy, or any other cancer therapy in several murine lymphoma models. In general, the suppression of tumor growth but not the completely rejection of tumor is valued as cancer therapy. Therefore, this cancer treatment regimen comprising a TLR7 agonist and radiation may help establish a new strategy against chemotherapy-resistant lymphoma in clinical sites.

In previous studies, TLR agonists have been locally administrated by intratumoral injection in animal cancer models, because the anti-tumor effect of a TLR agonist was significantly higher following local administration than systemic administration (8). Topical administration of imiquimod is also approved for clinical use. However, direct intratumoral administration of TLR agonists to noncutaneous tumors is often difficult, frequently requiring image-guided delivery systems, such as ultrasound or computed tomography. Therefore, it is interesting that systemic administration of a TLR7 agonist combined with radiation therapy had greater anti-tumor activity in murine lymphoma model than intratumoral injection. As previously reported, the authors also indicated that a single systemic injection of TLR7 agonist had no antitumor effect. Repeated injection of a TLR7 agonist as monotherapy slightly improved survival rate in tumorbearing mice. The therapeutic efficacy can be dramatically increased by combination with local radiation therapy. This improvement was observed in three different murine T cell and B cell lymphoma models.

With respect to the mechanism for augmentation of anti-tumor effect by the combination of systemic administration of a TLR7 agonist and local radiation, the authors demonstrated the significance of tumor antigenspecific CD8 T cells. CD8 T cell depletion assay by anti-CD8 antibody treatment indicated that CD8 T cells were required for the enhancement of anti-tumor effect in the TLR7 agonist/radiation combination therapy. In contrast, CD4 T cells and B cells were not involved in the antitumor effect of this combination therapy. Many studies have indicated the importance of tumor antigen-specific CD8 T cells, also known as CTLs, in suppressing tumor growth by cancer immunotherapy (9). Several factors are critical in the induction and augmentation of tumor antigen-specific CD8 T cells. For example, IL-12b and IL-2 are critical cytokines for the induction of antigen-specific T cells. In particular, IL-12b is intricately involved in the establishment of Th1 response and cellular immunity in viral infection and cancer. Th1 response induces antigen-specific CD8 T cells via the production of IFN- γ . The antigen presentation by APCs such as dendritic cells is also involved in the generation of Th1 immune response and antigen-specific CD8 T cells. The authors also examined the DC function after combination therapy of systemic administration of TLR7 agonist/local radiation. Systemic administration of a TLR7 agonist up-regulates the costimulatory molecules (CD80 and CD86) on the surface of DC, and the efficacy

of phagocytosis by DC enhanced after irradiation. The up-regulation of phagocytosis of tumor antigen and costimulatory molecules on DC may also be pivotal in the induction of tumor antigen-specific CD8 T cells and the enhancement of anti-tumor effect after the combination therapy.

Recently, several other investigators have examined the anti-tumor effect of combination therapy with a TLR7 agonist and local radiation (10,11). In subcutaneous and orthotropic mouse models of colorectal and pancreatic cancer, combined treatment with local irradiation and systemic administration of a TLR7 agonist was highly effective against established tumors. These studies also demonstrated that the combination of radiation therapy and TLR7 agonist administration could stimulate the processing and presentation of locally released tumor antigens. Moreover, T cells and NK cells markedly contributed to the enhancement of anti-tumor efficacy of the combination therapy. In a spontaneous lung metastasis model, the combination therapy of primary tumors significantly reduced metastatic burden in the lung and improved survival (10). Lung metastatic lesions were never exposed to radiation directly. The reduction of lung metastasis was thought to be due to the indirect effect of the combination therapy. In the studies that evaluated the combination of systemic administration of TLR7 agonist and local radiation therapy against cancer, the combination therapy induced tumor antigen-specific CD8 T cells. Application of combination therapy to primary tumors could induce a systemic adoptive immune response to cancer cells, and the tumor antigen-specific immune response could reduce present and future metastatic lesions. Dovedi et al. also demonstrated that long-term surviving mice after the combination therapy are protected against subsequent tumor rechallenged by the induction of a tumor-specific memory immune response. The tumor specific induction of memory immune response might be involved in the improvement of survival in tumor-bearing mice or mice with any metastatic lesions.

Dovedi *et al.* demonstrated that the combination of repeated systemic injection with TLR7 agonist and fractionated radiation therapy strongly induced anti-tumor immune response and improved the prognosis of tumorbearing animals. A previous report indicated that multiple IFN-related genes were up-regulated after fractionated radiation therapy (12). The up-regulation of IFN-related genes might lead to the induction of tumor antigen-specific immune response and remission of cancer. However, no

comparable analysis was performed on the host immune response after combination therapy with repeated TLR7 agonist administration and fractionated radiation therapy. Moreover, it is unclear how the mechanism differs between single systemic injection and repeated injection of a TLR7 agonist in terms of the host immune response in tumorbearing animals. If these questions can be resolved and the mechanism of anti-cancer effect of combination therapy determined, the combination therapy with TLR7 agonists and radiation could be translated to early phase clinical trials immediately. In particular, because TLR7 agonist is systemically injected not locally administered, translation to clinical studies would be relatively easy.

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Integr(at)in(g) EGFR therapy in HNSCC

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Patients with locally advanced head and neck squamous cell carcinomas (HNSCCs) are usually addressed to surgery and/or radiotherapy. The addition of chemotherapy to radiotherapy has also been extensively investigated, but treatment outcome often remained disappointing (1). Based on high levels of epidermal growth factor (EGFR) expression detected in approximately 90% of HNSCC, and associated to worse clinical outcome and decreased response to radiotherapy (2), the anti-EGFR monoclonal antibody cetuximab has been approved for treatment of patients with HNSCC (1). A paper recently published in Journal of National Cancer Institute by Eke and colleagues (3) demonstrated that simultaneous targeting of $\beta 1$ integrin and EGFR is a promising approach to overcome radioresistance in preclinical HNSCC models. Mechanistically, radioresistance depends on pro-survival signalling transduced by a protein complex of focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK1): combined \u03b31 integrin/EGFR blocking is able to interfere with these signals.

Integrins are heterodimeric cell-surface molecules (formed by α and β subunits) that mediate cell-matrix interactions. In addition, even if not provided with intrinsic kinase activity, integrins mediate from the extracellular space into the cell through adaptor molecules such as FAK, p130Cas, Src-family kinases and GTPases of the Rho family (4,5). Via these molecules, integrin cooperatively interacts with receptor tyrosine kinase (RTK) to regulate cell survival, proliferation, adhesion, and migration (6). In HNSCC, β 1 integrin overexpression has been found and related to tumour therapy resistance (7,8).

Eke and colleagues (3) reported that β 1 integrin inhibition, by either the antibody AIIB2 or silencing with β 1 integrin siRNA, activates EGFR associated signalling in HNSCCs. Particularly, the authors observed an increase of ERK1/2 phosphorylation and a dissociation of the FAK-ERK1 protein complex, both *in vitro* and *in vivo* (*Figure 1*). It is known that the Ras/Raf/MEK/ERK pathway is one of the signalling pathways activated downstream to EGFR (9) as well as to integrins (10). Similarly, FAK transduces signal from β 1 integrins and EGFR (11) through autophosphorylation of tyrosine 397 (12), thus inducing cell motility, proliferation, and the stress response to ionizing radiation and chemotherapy (13-15). Shibue *et al.* (16) showed that β 1 integrin-FAK signalling directs the proliferation of metastatic cancer cells disseminated in the lungs: β 1 integrins regulate FAK activation in these metastatic cells, and inhibition of both proteins reduces cell proliferation (16).

A relationship between EGFR and β 1 integrin pathway has been also demonstrated in lung cancer. Morello et al. (17) reported that $\beta 1$ integrin controls EGFR signalling and tumorigenic properties of lung cancer cells. Ju et al. (18) showed that β 1 integrin over-expression is associated with acquired resistance to tyrosine kinase inhibitor gefitinib in non-small cell lung cancer (NSCLC), accompanied with increase of the cells' adhesion and migration. Moreover, the sensitivity of NSCLC cells to gefitinib is negatively correlated with levels of $\beta 1$ integrin protein expression (18). In another study (19), the integrin β 1/Src/Akt signalling pathway has been identified as a key mediator of acquired resistance to erlotinib in lung cancer: gene silencing of $\beta 1$ integrin restored sensitivity to erlotinib and reduced Src and Akt phosphorylation/activation after erlotinib treatment. In tumour samples from patients with lung cancer refractory to erlotinib and/or gefitinib, increased expression of integrin $\beta 1$, $\alpha 5$, and/or $\alpha 2$ was also observed (19). Other studies reported that EGFR inhibition is related to



Figure 1 Bypass signalling by β 1 integrin inhibition. Inhibition of β 1 integrin by the antibody AIIB2 results in dissociation of the FAK-ERK1 protein complex and activation of the EGFR pathway, with hyperphosphorylation of ERK1. EGFR, epidermal growth factor.

different negative feedback loops involving MEK1/2 and other bypass signalling, often mediated by $\beta 1$ integrin (20). Conversely, the work by Eke et al. (3) demonstrated that β1 integrin inhibition induces EGFR activation, with consequent overactivation of components of the Ras pathway. Based on these data, Eke and colleagues (3) tested the combination of β 1 integrin inhibition by AIIB2 and EGFR inhibition by cetuximab in HNSCC models. They found that the combined treatment is more effective than single agents in inducing cytotoxicity and radiosensitization of HNSCC cell lines (Figure 2). In tumour xenografts, the combination AIIB2/cetuximab/radiotherapy produced higher tumour control rates compared to single anti- β 1 integrin treatment. On the other hand, in a different tumour model, Poschau and colleagues demonstrated that both β 1 integrin and EGFR targeting are inefficient to radiochemosensitize colorectal cancer cells (21).

Ionizing radiations are able to induce damages to several sub-cellular structures, from the plasma membrane to the cell nucleus. Particularly, in cancer therapy, radiationinduced cytotoxicity is closely linked to DNA damage (22). In this respect, several studies report the involvement of nuclear EGFR in DNA repair, for both non-homologous end joining (via DNA-protein kinase) and homologous





Figure 2 Effects of simultaneous $\beta 1$ integrin/EGFR inhibition. Targeting of $\beta 1$ integrin and EGFR by the antibodies AIIB2 and cetuximab results in inhibition of cell proliferation and survival as well as in radiosensitization. EGFR, epidermal growth factor.

recombination (via Rad51) (23-25). The role of β 1 integrins in this context is less known. However, several studies have reported that β 1 integrin targeting enhances radiochemosensitivity in different tumour types (13,26-28). In fact, β 1 integrins may regulate chromatin structure by increasing acetylation of the core histone H3 and by reducing the interaction of the linker histone H1 with DNA (29). Moreover, they have been involved in the protection from bleomycin-induced DNA breakage (30). The results obtained by Eke and colleagues (3) suggest that cooperative EGFR/ β 1 integrins interactions may play a critical role in DNA damage repair; therefore, the simultaneous inhibition of both signalling pathways may significantly improve radiosensitization of HNSCC models.

In the paper by Eke *et al.* (3), an interactome analysis on deregulated phosphoproteins, followed by network Betweeness Centrality (BC) analysis (31) revealed that simultaneous EGFR/ β 1 integrin inhibition induces a stronger perturbation of signalling compared to single EGFR or β 1 integrin targeting. Particularly, the addition of cetuximab to AIIB2 prevents the AIIB2-induced hyperphosphorylation of Raf/MEK/ERK and FAK signalling. In different human cancer cell lines including ovarian, lung and HNSCC cells, FAK has been described downstream to Ras/Raf/MEK/ERK pathway (11,12,14,32). In order to evaluate the role of FAK downstream to β 1 integrin and EGFR, as well as its interaction with the Ras pathway, the authors performed modulation (downregulation/overexpression) of both FAK and ERK1 by siRNAs or by expression vectors. They found that FAK plays a key role in the radiosensitization of HNSCC cell lines. Moreover, the authors concluded that FAK operates downstream to ERK1, regulating the DNA damage and survival response controlled by β 1 integrin and EGFR (3).

Altogether, the results by Eke demonstrate the efficacy of simultaneous β1 integrin/EGFR targeting in combination with radiotherapy in HNSCC tumours and propose this strategy as a reasonable and feasible option to overcome tumour radioresistance and diminish tumour recurrence in patients. However, the feasibility of $\beta 1$ integrin targeting in cancer patients needs further evaluation. In 2014, a first-in-human clinical trial testing Fc-engineered IgG1 monoclonal antibody targeting integrin $\alpha 5\beta 1$ was performed to evaluate tolerability, maximum tolerated dose, pharmacokinetics, pharmacodynamics and preliminary anti-tumour activity in patients with advanced solid tumours. Unfortunately, the trial was prematurely terminated without reaching end-points for the high toxicity (33). Moreover, since Eke and colleagues found that two out of the ten tested models do not respond to combination therapy, further studies will be required to understand the mechanisms of nonsusceptibility for β 1 integrin/EGFR targeting. The knowledge of molecular determinants of response, i.e., FAK phosphorylation/dephosphorylation after exposition to AIIB2/cetuximab, could allow a selection of patients who will potentially benefit from this kind of therapy.

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Radiation therapy and the abscopal effect: a concept comes of age

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The concept of utilizing localized radiation therapy to elicit out-of-target tumor responses-the abscopal effect-was proposed over 50 years ago (1,2). Over the past decades, the abscopal mechanism has been elucidated by the work of many investigators, including Formenti and Demaria, who showed that this process was likely mediated by the immune system leading to immunogenic tumor cell death, a process which involves dendritic cells, T regulatory cells, and suppressor cells as critical mediators (3-5). This research was inspired by the hypothesis that targeted radiotherapy in the proper setting can produce a consistent and robust abscopal effect, thus delivering clinically meaningful anti-tumor responses and disease control, if not eradicating distal disease in patients with metastatic cancer. The recent successes of several immune check point inhibitor clinical trials in various malignancies have demonstrated wide applicability and enormous therapeutic potential of immunomodulation and have galvanized keen interest in this field (6-10). An ambitious goal of combining radiotherapy and immunotherapy in the clinic would be long term remission for cancer patients with metastatic disease, perhaps through an approach analogous to delivering an *in-situ* anti-tumor vaccine (11-13).

In this proof-of-principle clinical trial, Golden and colleagues are the first to demonstrate that abscopal responses can be consistently detected in patients with confirmed solid metastatic cancer treated with radiation therapy and immunotherapy (14). All of the enrolled 41 patients had to have stable or progressing disease to standard systemic treatments and at least three distinct sites of measurable disease. These patients would then be maintained on their previous standard systemic regimen and receive granulocyte-macrophage colony-stimulating factor (GM-CSF) administered subcutaneously. The patients would receive fractionated irradiation (3.5 Gy \times 10 daily fractions) to one of these measurable lesions. Non-irradiated lesions were then assessed either by physical examination or by CT scans 7–8 weeks from the start of treatment. An abscopal response was defined as an at least 30% reduction in size from baseline in any measurable non-irradiated lesion.

Golden et al. showed that 27% of the patients treated with this regimen demonstrated abscopal responses. Furthermore, those patients who developed an abscopal response had better overall survival (21 vs. 8 months). While there were a diverse set of solid tumor types in the study, the two best represented groups were nonsmall-cell lung cancer patients and breast cancer patients. The publication is the first reported study to date with long term results that validates the concept of synergistic interactions between radiotherapy, chemotherapy, and GM-CSF. One key interpretation of these results is that the concurrent use of GM-CSF and fractionated radiation, when combined with systemic treatment, can stimulate the patient's immune system to overcome immune tolerance. This study also showed that administering GM-CSF with the other modalities was safe and tolerable, and that further developing this combination treatment paradigm holds great promise. Building on this work and other studies, numerous centers worldwide are currently testing strategies that combine radiotherapy and immunotherapy (15,16).

A perplexing question is why the abscopal effect does not occur more frequently in patients receiving radiotherapy. A plausible explanation is that radiation treatments in different settings can be either immunosuppressive or immunostimulatory depending on tissue and tumor context, and the host anti-tumor immune response is often regulated through a tight network of opposing stimulatory and inhibitory signals (17,18). This study and other studies

suggest that a permissive tumor/host environment and an appropriate set of immunomodulatory events at the proper timing may be necessary to trigger an abscopal response (19,20). We are only beginning to realize the complexity of these pathways and their interactions and find ways to enhance the anti-tumor immune response. For example, several novel categories of targeted immunomodulators have recently been developed. They include TLR agonists, TGF- β antagonists and the immune checkpoint inhibitors, anti CTLA-4, anti PD-1, and anti PD-L1/L2 agents, which have re-kindled hope for successful cancer immunotherapy (21-24). There are numerous other potential immunomodulatory agents in the investigational pipeline. Some studies suggest that multiple immune mechanisms need to be targeted, and that dual immune checkpoint blockade together with radiotherapy might be necessary to elicit the optimal abscopal response (25). With a deeper understanding of mechanisms underlying tumor immune regulation, future strategies may be able to produce clinically meaningful abscopal responses more consistently.

The successful clinical demonstration of abscopal responses also shifts the treatment paradigm for radiation oncologists. The traditional goal of radiotherapy is to eradicate local disease by maximizing direct tumor cell killing while minimizing nearby normal tissue damage (26). Most of the research effort in radiation biology has been focused on understanding the mechanisms underlying DNA damage and repair pathways, cellular repopulation, and tissue re-oxygenation (27). In patients with metastatic disease, it has been widely accepted that the standard treatment for distant disease is from the administration of chemotherapy, hormonal therapy, or biologic targeted agents. Combining radiotherapy with immunotherapy shifts the focus from direct tumor kill to immunomodulation, which is at least in part due to broadened neoantigen exposure, thus memory T-cell repertoire expansion, T-cell infiltration into tumor and enhanced T-cell mediated tumor rejection (28-30). The optimal dosing, fractionation, and target volume determination could be quite different from classic radiotherapy paradigms.

In summary, this study by Golden and colleagues provides a foundation moving forward to explore a wider range of clinical strategies to be tested in clinical trials for metastatic cancer. Many important questions will need to be addressed. First, how can we best determine and monitor abscopal responses? Second, what impact do other modalities e.g., radioablation, electroporation, particle radiation such as protons or carbon ions, have on the abscopal effect? What systemic and immunomodulatory agents should be used and how should they be timed with the radiotherapy? What is the best disease site to irradiate in order to elicit the maximal abscopal response? Should short, large fractions of radiation be used or will longer, smaller fractions be better? These questions and others are actively being addressed in clinical studies. As the reviewed work and others in this rapidly moving field demonstrate, combination radiotherapy and immunotherapy represents an emerging treatment modality that may alter the natural history of solid malignancies.

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Immunologic response of combined interleukin-2 and stereotactic body radiotherapy

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Introduction

Cytokine therapies have been available for the treatment of several cancers since the 1980s, with variable success. Since that time, the prevalence of metastatic disease has rapidly risen in the U.S. population as result of improvements in systemic and supportive therapies (1). In particular, there has been considerable advancement in targeted inhibitors of human ligands and receptors, which generally offer reduced toxicities compared to cytotoxic chemotherapy. Recently, this approach has been applied to the field of cancer immunotherapy, which aims to enhance the native immune system for the purpose of destroying malignant cells.

The greatest historical success of immunotherapy has been in the management of lymphomas and leukemias. Although these agents were initially cytokine therapies, the targeted CD20-inhibitor rituximab was developed and approved for the treatment of hematologic malignancies. More recently, targeted immunotherapies for the treatment of metastatic solid tumors have gained FDA approval, and may offer more favorable toxicity profiles (2). For patients with metastatic melanoma and renal cell carcinoma (RCC), targeted agents offer both progression-free and overall survival benefits over cytokines (2-4). Although these agents do not necessarily offer curative potential, their efficacy exceeds that of cytokine therapies with diminished or equivalent toxicity.

As systemic therapies improve, it is clear that the prevalence of metastatic disease will continue to increase. Accordingly, utilization of radiation therapy for palliation and local control of oligometastases will necessarily rise. As a result, a new role for radiation therapy as a sensitizer to systemic therapy is actively being explored. Unfortunately, scarce data exists describing the safety and efficacy of combined radiation and targeted therapies. Drawing from a breadth of preclinical data (5), many hypothesize that radiation therapy augments the response to immunotherapy. However, it is difficult to discern whether this increased response is solely attributable to these novel agents, or whether a synergistic effect is truly present.

In the June 2012 issue of *Science Translational Medicine*, Seung and co-authors report results from a phase I study investigating combination stereotactic body radiotherapy (SBRT) and interleukin-2 (IL-2) for the treatment of metastatic RCC and melanoma (6). Twelve treatment-naive patients were enrolled and assigned to one of three doseescalated cohorts: (20–60 Gy in 1–3 fractions). Two cycles of high-dose IL-2 were administered after SBRT, with up to six total cycles for patients demonstrating an objective response. PET and CT imaging were used to assess response via modified RECIST guidelines (7). To assess for immunologic response, the authors collected peripheral T cells before SBRT and during IL-2 therapy. The frequencies of certain T cell subpopulations were predictive for response to therapy.

Seung *et al.* should be commended for their work, as it contributes valuable data to an area of great research interest. Although this is now an older paper, this study provided early data for SBRT in combination with immunotherapy before the efficacy of newer agents had been established in randomized trials. As the utilization of both targeted therapies and SBRT increase, the safety and efficacy of combined therapy remains unclear. Many
clinicians prefer to delay systemic therapy rather than administer concurrently with radiation therapy. However, many hypothesize that radiation-induced tumor antigen release augments immunogenic therapies, and thus concurrent therapy may offer an added benefit (8-10).

As a phase I trial, the principal objective of this investigation was to evaluate treatment-related toxicity and the feasibility of combined SBRT and IL-2. Although no dose-limiting adverse events attributable to SBRT occurred, several anticipated adverse events related to IL-2 were observed and resolved. Unfortunately, the overall incidence and grade of IL-2-related toxicities were not reported. As such, it is difficult to compare this series with historical toxicity rates, such as those reported by Atkins et al. (11). Late toxicities related to SBRT are certainly possible, and SBRT may have increased the rate of diarrhea, nausea, and transaminasemia attributed to IL-2, particularly among patients who underwent hepatic SBRT. Moreover, 60 Gy in 3 fractions (cohort 3) delivered peripherally or centrally is, at the least, associated with some degree of chest wall toxicity or fatigue. Of note, no patients in this cohort underwent SBRT to the hilum or mediastinum; larger samples may certainly demonstrate these toxicities.

It is unclear why the authors chose not to report more extensive toxicity data, as it would have strengthened their assertion that SBRT with IL-2 should be considered in this population. One must consider the relative risks and benefits of cytokine therapy over targeted therapy: grade \geq 3 toxicities with IL-2 are very common, including hypotension (45%), oliguria (39%), vomiting (37%), diarrhea (32%), thrombocytopenia (17%), confusion (13%), infection (11%), pulmonary edema (9%), and hepatic dysfunction (9%) (11). In addition, the incidence of treatment-related fatal toxicity is approximately 2%. In contrast, the overall incidence of grade ≥ 3 toxicity with the use of nivolumab alone, ipilimumab alone, and combination nivolumab/ipilimumab for untreated melanoma are 16%, 27%, and 55%, respectively, with significantly greater efficacy (2).

Given the morbidity and cost associated with management of these toxicities, clinicians and patients must consider whether IL-2 should be considered over novel targeted immunotherapies. With recent encouraging phase III data, it is unclear whether patients treated with combination IL-2 and SBRT will achieve superior outcomes compared with CTLA-4 or PD-1 inhibitors alone or in combination with SBRT. Although most studies report minimal toxicities associated with SBRT and targeted therapies, there have been several reports of an increased risk of radiation necrosis (12) and bowel toxicity (13,14).

The authors predominantly focused upon objective response in their study, which exceeded recent data for combination nivolumab and ipilimumab (2). The overall response rate in the intent-to-treat analysis was 67%, and was higher among patients with melanoma (71%) compared with RCC (60%). The authors assert that this 71% response rate is statistically significantly greater than the historical response rate of IL-2 monotherapy for melanoma (16%) (11). As a phase I study, it is difficult to compare this response with historical IL-2 response rates (Table 1). In the frequently-cited historical standard, Atkins et al. included 270 patients with metastatic melanoma from eight clinical trials (11). The overall response rate was 16%, similar to that observed among patients with metastatic RCC (14-20%) (15). Accordingly, we are presented with a phase I study reporting response rates of 67% compared with historical response rates of 16%, with the difference attributed to SBRT. Although this may be a real effect, one must also consider the differences in study design. First, an older set of response guidelines was utilized in Atkins et al.: a partial response required at least 50% reduction in total tumor area with stable symptomatology and laboratory abnormalities on at least two separate instances. Just 4 of 12 (33%) patients in Seung et al. achieved a 50% reduction in maximal tumor diameter. In contrast, the more recent criteria used by Seung et al. required a decrease in total maximum lesion diameter of at least 30%. Second, Seung et al. included only treatment-naïve patients, while 46% of patients in Atkins et al. had progressed on a different systemic therapy. Therefore, it is unclear whether this 67% response would remain as robust if identical response evaluation and patient eligibility were used. To address this, the authors have initiated two accruing phase II randomized trials (SBRT + IL-2 vs. IL-2 alone), which include the requisite control group to assess the research hypothesis (NCT01416831, NCT02306954).

To further evaluate these results, we can explore whether available data support an immunologic basis for radiation as a sensitizer to immunotherapy (5,17,18). Total body irradiation (TBI), for example, has been demonstrated to increase the efficacy of IL-2 in mice (19). However, a phase II trial failed to replicate this effect, with an overall response rate of just 4% (20). Seung *et al.* assert that this poor response is due to bystander irradiation (lymphocyte depletion) or inadequate dose per fraction (poor immunogenicity) (21,22). Among patients with metastatic

Study	Population	Therapy	Objective	PFS [‡]	OS [‡]	Grade ≥3
Study			$response^\dagger$	(month)	(month)	toxicity
Atkins et al. (11)	Melanoma	IL-2 (n=270)	16%	NR	11	>45%
meta-analysis						
Klapper <i>et al</i> . (15)	RCC	IL-2 (n=259)	20%	NR	19	>38%
retrospective series						
Knisely et al. (16)	Melanoma	SRS + ipilimumab (n=50)	NR	NR	21	NR
retrospective series		SRS (n=27)	NR	NR	5	NR
Seung et al. (6) phase I	RCC/Melanoma	SBRT + IL-2 (n=12)	67%	NR	>16	NR
Larkin et al. (2) phase III	Melanoma	Nivolumab (n=316)	44%	7	NR	44%
		lpilimumab (n=314)	19%	3	NR	56%
		Nivolumab + ipilimumab (n=314)	58%	12	NR	69%
Patel <i>et al</i> . (12)	Melanoma	SRS + ipilimumab (n=20)	NR	NR	8	>30%
retrospective series		SRS (n=34)	NR	NR	9	>12%

Table 1 Efficacy and safety of immunotherapies with or without radiation therapy for advanced melanoma and renal cell carcinoma

[†], complete or partial response; [‡], median PFS and OS reported. PFS, progression-free survival; OS, overall survival; RCC, renal cell carcinoma; SBRT, stereotactic body radiotherapy; IL-2, interleukin-2; NR, not reported; SRS, stereotactic radiosurgery.

RCC and melanoma, there has been some evidence suggesting a benefit with the use of immunotherapies after SRS (23) or whole-brain radiotherapy (9). In addition, ipilimumab has been demonstrated to increase survival when combined with radiation in mice (24,25). However, a lack of prospective controlled data limits the ability to draw any meaningful conclusions (12,16,26).

In addition to distant control, one must also consider local control after SBRT. Melanoma and RCC are among the most common histologies treated with SBRT and stereotactic radiosurgery (SRS), with 12-month local control ranging from 70% to 95% (22,27-34). Given this high rate of local control, an ongoing cooperative group trial (NRG-BR001) is exploring the feasibility of irradiating multiple lung, colon, or breast oligometastases over a 1-3 weeks period. If the primary endpoint of acceptable toxicity is met, this may lead to future trials exploring SBRT in the management of oligometastases. Of note, Seung et al. reported no local failures among irradiated lesions despite significant variation in dose among the three cohorts. With larger samples, one would expect to see greater durability in cohorts 2 and 3. Although the sample size was small and radiographic follow-up was not reported, this control rate is impressive and may support a synergistic local relationship between SBRT and IL-2.

Although the utilization of IL-2 has decreased given the development of targeted therapies, the results presented by Seung *et al.* are provocative given the 67% objective

response rate. In comparison, the recent Larkin *et al.* phase III trial for untreated melanoma reported a 58% response with the use of combined nivolumab and ipilimumab, with a grade \geq 3 toxicity rate of 55%. It is unknown whether either regimen is associated with a survival benefit, or whether SBRT truly augments the efficacy of immunotherapy. Several ongoing trials are exploring immunotherapies alone and in combination with SBRT. Beyond the aforementioned trials designed by Seung *et al.*, ipilimumab is being combined with SBRT (phase I, NCT01557114; phase I/II, NCT01497808; phase II, NCT01565837) and whole brain radiotherapy (phase I, NCT01703507).

Conclusions

Seung *et al.* have provided early results describing favorable safety and efficacy with combination immunotherapy and SBRT. In select situations, the available preclinical and clinical data suggest an additive benefit of SBRT without substantially increased toxicities. However, newer targeted therapies may offer similar efficacy and toxicity without SBRT, and have been studied in randomized settings. Although IL-2 with SBRT may provide encouraging local control rates, it is difficult to favor this approach as upfront therapy given the available phase III data demonstrating considerable efficacy with CTLA-4 and PD-1 inhibitors. To address this gap in the literature, currently accruing trials are exploring SBRT with these targeted agents

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to corroborate studies such as this paper. Given the encouraging preclinical and clinical results, we look forward to the results of such trials on whether SBRT can truly augment response rates.

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Footnote

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Radiation, cytokines and T-cell checkpoints: can we cure metastatic cancer?

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Miller *et al.* discuss the clinical findings of a pilot study investigating the combination of stereotactic body radiation therapy (SBRT) and interleukin-2 (IL-2) in patients with advanced melanoma and renal cancer performed by our group (1). The authors discuss our work in the context of anti-tumor efficacy and toxicity of T-cell checkpoint antibodies including anti-CTLA-4 and anti-PD1. We treated our first patient with SBRT and IL-2 in 2009. There have been remarkable and practice-changing discoveries in cancer immunotherapy since that time summarized in *Table 1*, and many more are anticipated to follow in the near term.

One of the main points raised by Miller was that our report did not extensively discuss toxicity of the combination. The focus of our report was on the immunobiology of SBRT + IL-2 and also to describe the clinical responses, which were greater than anticipated. There was a general description of toxicity in our manuscript in which we make the points that there was not dose-limiting toxicity of radiation over the dose range and schedule explored and that the toxicity from IL-2 was not greater than anticipated. In a separate report from our group we reported on IL-2 toxicities in 500 patients treated in our biotherapy program (2). It is the practice of our biotherapy program to treat each patient to their individualized maximum tolerated dose of IL-2. This practice is based the biology of IL-2. The capillary leak, which is the main underlying cause of IL-2 toxicity, is related to the activity and trafficking of T cells, which in turn is related to the anti-tumor effect of this cytokine. All of the patients who participated in our SBRT + IL-2 clinical trial had hypotension requiring pressor support, acute kidney injury with serum creatinine peaking between 4-6 mg/dL

and capillary leak with fluid retention resulting in weight gain of between 10–20 pounds. The side effects of IL-2 are indeed severe, but all of our patients recovered normal function after completing IL-2. In our report of 500 patients who had received IL-2, the incidence of death from IL-2 was less than 1% and no individuals died during the administration of SBRT + IL-2. It should also be noted that the side effects of combined T-cell checkpoint antibodies can be severe and are not always reversible. In the report by Larkin *et al.*, the probability of experiencing grade 3 or 4 toxicity with the combination of ipilimumab and nivolumab was 68.7% in patients with previously untreated melanoma (3).

As is pointed out by Miller and colleagues, the objective response of ipilimumab and nivolumab in patients with untreated melanoma is high and remarkably so compared to treatments used in the past. In the report by Larkin cited above, complete response was observed in 11.5%, partial response 46.2% and stable disease in 13.1% of patients. These findings are impressive, yet the median progression-free survival was 11.5 months. This implies that at least half the patients who receive ipilimumab and nivolumab will need a new therapy within a year. The reality for the majority of patients with melanoma and renal cancer is that they will die as a consequence of their malignancy, despite the advances that have been made in the immunotherapy of cancer in the last 5 years.

The goal for future cancer immunotherapy (or any cancer therapy) should be the cure of malignancy, and not just delayed progression. Of the 8 patients we describe in our pilot study who had regression of melanoma or renal cancer, 6 remain alive and free of malignancy, now

Agent	Year of FDA approval	Indications
Ipilimumab	2011	Advanced melanoma
Pembrolizumab	2014	Advanced melanoma, lung cancer
Nivolumab	2015	Advanced melanoma, lung cancer, renal cancer
Ipilimumab and nivolumab	2015	Advanced melanoma

Table 1 Summary of T-cell checkpoint antibody approvals and indications

greater than 6 years after SBRT + IL-2, and we believe that these patients are likely cured of their malignancy. Of the 2 initial responders who expired, one died from an unrelated pulmonary condition 7 years after SBRT + IL-2 and 1 died 3 years after treatment from melanoma brain metastases. New immunotherapy pathways or modalities with the potential to achieve cure include other T-cell checkpoints such as TIM-3 and LAG-3, T-cell costimulatory agents like OX40 and 4-1BB, bi-specific targeting antibodies, oncolvtic viruses, engineered T cells and vaccines. There will almost certainly still be a role for cytokines like IL-2, in part because IL-2 monotherapy can still cure some individuals, but it will also be needed to provide a proliferation signal for engineered T cells and tumor infiltrating lymphocytes. Radiation will be needed not only for palliation, but also because of its ability to prime immune responses. There are still many unanswered questions about the best way to amplify the clinical effects of immunotherapy with radiation, but since our initial report of SBRT + IL-2, there are now at least 21 trials listed on the cancer.gov web site investigating the combination of high dose per fraction radiation and immunotherapy. These efforts will help us to better understand the immune mechanisms of radiation, but more importantly, will help us to guide us in curing more patients with advanced cancer.

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Identification of the hidden survival advantage for anti-angiogenic therapy in glioblastoma

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Glioblastoma (GBM) remains one of the most aggressive of malignancies associated with significant morbidity and mortality for patients. Treatment has relied on surgery, radiation, and chemotherapy with emerging biologically based therapies under active investigation. A hallmark of GBM clinically and pathologically has been the intense tumor-associated angiogenesis that occurs with the disease. GBM associated angiogenesis promotes not only tumor progression but has a marked impact on a patient's neurological function due to abnormalities of the bloodbrain-barrier and dysregulation of cerebral autoregulation of blood flow, a phenomena required for neurological function.

The development of anti-angiogenic therapy in the form of VEGF neutralizing antibodies and VEGF receptor tyrosine kinase inhibitors has offered an opportunity to intervene against this GBM phenotype. While some benefit for patients is seen in the setting of recurrent disease, large scale randomized studies combining bevacizumab (VEGF neutralizing antibody) with standard chemotherapy and radiation in newly diagnosed GBM failed to demonstrate a survival advantage (1,2). Given the importance of angiogenesis to GBM this was highly disappointing. In order to better understand the mechanism behind this general failure and search out windows where VEGF inhibition would provide a clinical benefit Batchelor and colleagues examined GBM patients with advanced imaging modalities in the setting of standard therapy combined with anti-angiogenic therapy (3). The investigators posit that a "vascular normalization index" composed of factors derived from imaging to include perfusion, vessel diameter and permeability, circulating biomarkers, changes in tumor

interstitial pressure, and measures of tumor oxygenation may be utilized to identify patients likely to benefit from anti-angiogenic therapy.

In this study of 40 patients newly diagnosed with GBM undergoing standard chemoradiation with the addition of the oral pan-VEGF receptor tyrosine kinase inhibitor, cediranib, the investigators observed that patients experiencing a durable increase in tumor perfusion had improved survival compared to those having a decreased tumor perfusion or those in the standard treatment cohort. Importantly, this survival advantage was independent of the known prognostic factors of performance status and O6-methyl guanine methyl transferase (MGMT) gene promoter methylation status. In addition, it was this same subset of patients with increased tumor perfusion associated with cediranib therapy that demonstrated a decrease in the differential of arteriole and venule oxygen saturation levels suggesting enhanced delivery of oxygen to the brain and tumor tissue with cediranib. The end result of these observations the investigators state is a normalization of the tumor vasculature generating a hypothesis that this phenomena results in improved tumor oxygenation promoting enhanced cytotoxicity from therapy and/or improved drug delivery to the tumor. It was noted that the majority of patients had reductions in tumor enhancement with contrast on magnetic resonance imaging (MRI) and improvements in tumorassociated vasogenic edema; however, only the subset with increased tumor perfusion realized a survival advantage. In Figure 1, an example of a patient with a frontal GBM shows a reduction in contrast enhancement following treatment with bevacizumab and a marked improvement



Figure 1 The montage of MRIs for a patient with frontal GBM demonstrates the characteristic changes seen with the initiation of antiangiogenic therapy, in this case with bevacizumab. The top row (A-C) are the post-contrast T1 weighted sequences demonstrating an initial (A) robustly enhancing mass lesion in the right frontal lobe extending into the corpus callosum that briskly shows a response to bevacizumab after 8 weeks of therapy (B), but proves to be of poor duration as enhancement returns by 16 weeks (C). The bottom row (D-F) follows the same time sequence on the T2-weighted FLAIR sequences where significant tumor edema is seen at baseline (D) but responds well to bevacizumab (E). GBM, glioblastoma; MRI, magnetic resonance imaging; FLAIR, fluid-attenuated inversion recovery.

in vasogenic edema; however, this effect is not durable. The development of markers such as tumor perfusion quantification to select patients for which durable responses may exist is essential to the improvement of care for this population. It is important to recognize that the imaging measurement used to determine tumor perfusion was dynamic susceptibility contrast (DSC) and the values were compared to a baseline obtained prior to the initiation of therapy. This form of tumor perfusion analysis is in routine use particularly in brain cancer with findings of elevated or increased perfusion often interpreted as an imaging correlative for disease progression and activity. The findings of this study indicate an awareness of biological therapy use, such as anti-angiogenic therapy, is essential to the correct interpretation of treatment response and provide support for the use of rigorous assessment criteria such as the Response Assessment in Neuro-Oncology (RANO) (4).

The deployment of anti-angiogenic therapy in patients with GBM has been associated with a steep learning curve for clinicians and scientist. The initial euphoria caused by dramatic radiographic responses was followed by

confusion when controlled trials did not demonstrate a survival advantage for patients. The initial hypothesis that inhibition of tumor-associated angiogenesis in GBM would lead to improved patient outcomes has been modified by the realization that the clinical benefit related to survival is limited to a subset of patients. The ability to identify this subset of patients and more strategically deploy antiangiogenic therapy is highlighted by the study of Batchelor and colleagues. By using advanced but readily available imaging platform, physiological metrics of tumor perfusion could be measured and normalized to uninvolved brain generating a usable measure for in vivo activity of cediranib. Importantly, the phenotypic measure of perfusion change was not associated with convincing changes in serum biomarkers or with tumor genotype. This suggest that GBM associated behaviors exist that are the summation of potentially multiple genetic alterations and thus may serve as an endpoint measure from which clinical decisions may be made.

In summary, Batchelor and colleagues illustrate the importance of a detailed evaluation of a tumor phenotype. In this case, the behavior of tumor-associated angiogenesis and the validation of measurements reflective of the biological process resulted in the identification of a patient subset responsive to this biologically targeted therapy.

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Value of correlative biomarkers in understanding tumor biology

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We appreciate the insightful review of our article Batchelor *et al.* "Improved tumor oxygenation and survival in glioblastoma patients who show increased blood perfusion after cediranib and chemoradiation" by Dr. Burt Nabors (1,2). In his commentary, he skillfully highlights the initial enthusiasm followed by more measured interest for antiangiogenic agents in glioblastoma (GBM) (1). Based on the robust angiogenesis that characterizes GBM, there exists a strong biological rationale for targeting tumor blood vessels and, fundamentally, blood flow and nutrient delivery are essential to tumor survival. The therapeutic challenge with this class of agents has been identifying those patients likely to benefit and achieve a durable response both clinically and radiographically.

In our study of newly diagnosed GBM patients treated with radiation, temozolomide, and cediranib, an oral VEGFR inhibitor, we incorporated both blood and imaging markers to track changes in vascular structure and function. The goal was to better understand the physiological impact of anti-angiogenic therapy. As Dr. Nabors pointed out, we found that those patients with increased perfusion to the tumor had improved tissue oxygenation and lived longer-likely because of better delivery of temozolomide and oxygen (necessary for radiation efficacy) to the tumor resulting in improved tumor cell kill. Furthermore, we identified that blood biomarkers, specifically PIGF and sVEGFR2, were useful pharmacodynamic biomarkers of response whereas II-8 and sVEGFR1 were biomarkers of relapse.

Critically, the imaging and blood biomarkers we explored are noninvasive and can be performed serially to track changes in the tumor over time. A particular challenge with brain tumors is the limited access to serial tissue biopsies to shed light on how the tumor and its microenvironment evolves in response to therapeutic pressures. Having a tool such as MRI where signal changes reflect physical processes in the brain is essential to interpret responses and help guide therapeutic decisions or potential combination therapies (3). MRI also has the benefit of capturing known tumor heterogeneity since the entire volume of an individual tumor is visualized as well as separate tumors in the same patient. Consequently, a crucial step to improving the care of brain tumor patients is to optimize correlative biomarkers that shed light on biological changes and use the human as the experimental model so we can learn as much as possible about the effects of drugs being developed for this challenging disease. The more we learn from our patients, the better we can design the next wave of therapies (4).

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Sense and sensibility to early combine bevacizumab to radiation treatment of brain metastasis: reply to Lou and Sperduto

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We thank Lou and Sperduto (1) for their comments regarding the results of our REBECA trial [a phase I study designed to assess the safety of bevacizumab (BEV) in combination with whole brain radiation therapy (WBRT) for brain metastases (BM) of solid tumors (2)]. They highlighted the interest of exploring VEGF-based therapy in the context of radiation therapy of BM, as well as the issues of such an approach.

They pointed out the absence of patients with melanoma or renal cell carcinoma in our population (mostly represented by breast cancer patients). That may be explained by the fact that these tumors may now benefit from specific targeted therapies (particularly BRAF and checkpoint inhibitors) and by the lack of safety information of an approach combining these specific targeted therapies with BEV. However, considering the growing use of targeted treatments across multiple tumor types, we agree with the assumption that combinations with angiogenesis inhibitors must be explored in the future, especially in lung cancer.

The timing of the delivery of anti-angiogenic agents when combined with radiation therapy was also questioned. Preclinical data support that the synergic effect of these approaches is based on (I) the normalization of tumor vascularization, which improves oxygenation and counteracts the negative effect of hypoxia on radiation effect; and (II) the inhibition of VEGF protective effect on endothelial cells. This biological rationale explains our design with an early administration of BEV before the onset of WBRT, followed by two other injections during the course of treatment (in the intent to "provide the treatment at a peak of radiation-induced hypoxia" as proposed by Lou and Sperduto). Moreover, the results from two large randomized studies combining radiotherapy with BEV for patients with malignant gliomas (3,4) seem indicate a better outcome (both for efficacy and cognitive safety) when anti-angiogenic treatment was delivered from the start of radiotherapy (3) rather than during the fourth week of radiotherapy (4). This supports the beneficial effect of an early introduction of BEV.

Finally, we agree with the comment about the growing place of stereotactic radiotherapy (SRT) for BM. The REBECA trial was designed for BM patients eligible to WBRT only (assuming that at least 50% of lung cancer and some breast cancer patients are not eligible to SRS because of too many BM). However, many BM patients are eligible to SRS and it could be assumed that the good safety profile of BEV administration with a large radiation volume may also be expected in the context of its combination with smaller volume as in SRS. This assumption seems confirmed by some recent data of SRS combined with BEV in patients with recurrent malignant gliomas, confirming the feasibility of this approach with a good safety profile (5-8). Clinical evaluation of BEV combined with SRS through a prospective trial is urgently needed for patients with BM.

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Integrating bevacizumab and radiation treatment of brain metastasis: is there sense and sensibility in this approach?

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Abstract: The incidence of brain metastasis has increased over the past decade. Standard treatment options for brain metastases include whole brain radiation therapy (WBRT), stereotactic radiosurgery (SRS) and surgery for patients with operable lesions and either mass effect or need for histologic confirmation of the diagnosis. Patients are living longer due to improvements in systemic therapeutic approaches, included targeted therapies such as inhibition of vascular endothelial growth factor (VEGF) using the monoclonal antibody bevacizumab (Bev). A recent phase I trial (REBECA) investigated adding Bev to whole-brain radiation for patients with brain metastasis from solid tumors. In this Perspectives article, we discuss the results of the REBECA trial in context of advancements in radiation and medical oncology in the era of targeted therapies, and discuss pertinent questions of interest in this field.

Keywords: Brain metastasis; bevacizumab (Bev); whole-brain radiation; angiogenesis; vascular endothelial growth factor (VEGF)

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Introduction

The incidence of metastasis of systemic malignancies to the central nervous system (CNS) (brain metastasis) has increased significantly over the past decade. Incidence is most frequent in patients with lung cancer, breast cancer, or melanoma (1). Standard treatment options for brain metastases include whole brain radiation therapy (WBRT), stereotactic radiosurgery (SRS) and surgery for patients with operable lesions and either mass effect or need for histologic confirmation of the diagnosis. Patients are living longer due to improvements in systemic therapeutic approaches, including maturation of the field of molecular oncology and identification of rational targets for therapy. Incremental increases in overall survival have led to a new set of clinical challenges including attempts at effective therapeutic management of brain metastases and associated morbidities. Inclusion of patients with brain metastasis in clinical trials has paradoxically been extremely limited to date, but there is growing recognition of the need to

change this paradigm in the modern era (2,3). Most of the concern has centered on perception of naturally worse prognosis and inherent risk of intracranial hemorrhage in this subpopulation (3). Recent studies and strategic approaches have combined knowledge of molecular pathways from systemic malignancies with high propensity for CNS metastasis (e.g., melanoma, lung) along with examination of efficacy of treatment of primary CNS tumors. Penetration of the blood-brain barrier (BBB) has presented a particular challenge in treating patients with both primary and secondary intracranial malignancies, thus some studies are also investigating methods for negating the barrier to improve drug penetration and efficacy.

Angiogenesis is an especially prominent molecular and cellular response to hypoxia and invasion in the heterogeneous tumor microenvironment for many solid tumor malignancies. There has been a great deal of investigation into this process at the cellular level, and investigation of treatment of systemic malignancies with anti-angiogenic drugs, most prominently Bev, a monoclonal antibody that targets vascular endothelial growth factor (VEGF). To date, Bev has been FDAapproved for treatment of metastatic non-small cell lung cancers, recurrent glioblastomas (GBM), metastatic colorectal cancers, and others (4). It has also demonstrated degrees of efficacy in other forms of invasive malignancy, including atypical meningiomas (5), and found to improve progression-free survival but not overall survival in firstline treatment of glioblastoma (6,7) although there is some supportive evidence of stabilization of unresectable GBM in that setting (8,9). Success of the use of this agent in patients specifically with CNS metastasis has not been as well characterized. In 2014, Lévy et al. (10) reported results of REBECA, a phase I trial investigating the use of Bev in combination with whole-brain radiation therapy (WBRT) to treat patients with unresectable solid tumor brain metastases. This study represents one of several studies investigating the anti-angiogenic approach in this specific population, and is a first step toward designing rational clinical trials to address efficacy using this strategy in a patient with few valid therapeutic options.

REBECA: a phase I study of bevacizumab (Bev) for brain metastasis

REBECA was a single-arm phase I study with 3+3 doseescalation design.

Twenty-one patients were enrolled during a 3-year span across six cancer centers in France. Thirteen of the 21 patients had breast cancer; the remaining patients had lung, ovarian, or unknown primary malignancies (10). Two of 19 (11%) experienced intra-lesional hemorrhage but no patient experienced parenchymal brain hemorrhage. Ten of 19 (53%) showed a response at 3 months which is the expected response rate with WBRT alone. Limitations of the study included relatively small number of enrolled patients, and lack of other representative cancers that commonly metastasize to the brain (e.g., melanoma and renal cell carcinoma, both of which have a relatively high likelihood of intracranial hemorrhage compared with other types of cancer). Further investigation in safety trials with these patient populations would be warranted to make accurate conclusions of Bev safety. Bev is used in treatment of metastatic renal cell carcinomas and in NSCLC. Thus the scenario is clinically relevant for patients on Bev who develop brain metastases and undergo WBRT, and/or SRS.

Radiologic assessment of brain metastasis response: new rules for assessing efficacy of treatment in the era of bevacizumab (Bev)

Until the past few years, assessment of objective responses of intracranial tumors to treatment was made by adhering to Response Evaluation Criteria in Solid Tumors (RECIST) (v 1.1). In 2010, the Radiologic Assessment in Neuro-Oncology (RANO) Working Group formulated a set of radiologic criteria for more accurately assessing response of primary malignant brain tumors (specifically gliomas) to therapy (11). In 2015, this Working Group established a similar set of consensus guidelines for radiologic assessment of brain metastases (12). The RANO criteria has been helpful for more accurate analysis of the effects of Bev on gliomas; likewise, incorporation of the new designated criteria will be helpful for future early and late-phase trials of Bev in brain metastases.

Strategically, there is also debate regarding the ability of monoclonal antibodies, including Bev and trastuzumab, to cross the BBB. How much penetration of drug is enough to achieve a meaningful clinical and measurable response? There is a widely held view that disruption of the BBB leading to CNS metastasis renders it more permeable not only to further micrometastases, but also to administered drugs. Part of the response process includes upregulation of VEGF, which in turn induces vascular permeability that permits tumoral growth of micrometastases. Methods that have been proposed to improve efficacy of BBB penetration of drugs include liposomal delivery and non-pharmacologic methods such as induction of hyperthermia (13). Furthering the debate on utility of Bev are studies questioning its efficacy in treatment of primary gliomas. Anti-angiogenic treatment relieves peri-tumoral edema, resulting in primary relief of tumor-induced symptoms in many patients. However, Bev may stabilize the permeabilized BBB, which would be counteractive by preventing adequate delivery of concurrently administered chemotherapeutic drugs (14). Thus there is concern about use of Bev on multiple fronts in gliomas that should also be addressed in future trials assessing Bev for intracranial metastatic tumors. In this era of molecular oncology, it will be imperative to also consider genomic differences in systemic malignancies and to acknowledge that these differing profiles and driving mutations may influence response between tumor types. Known differences in invasive capacity of metastatic CNS tumors compared with primary gliomas (less invasive

at the cellular level in the former) may also require a different strategic approach (15).

When to give bevacizumab (Bev): is concurrent administration the wrong approach?

Timing of administration of Bev in relation to radiation is also an aspect of interest scientifically. The REBECA study authors concluded from their study that Bev provided the best efficacy with RT when administered at a higher dose of 15 mg/kg three times (every 14 days) concurrent with WBRT (30 Gy/10 fractions over 2 weeks). Response rates in this study were modest even with this combination, which the authors propose as a starting point for evaluation in future phase II trials. Biologically, there is a potential paradox in terms of Bev efficacy: radiationinduced cellular stress may induce angiogenesis, which can create vascularity that may improve drug delivery to tumor tissue. Striking a balance of efficacious drug delivery with disruption of angiogenesis to prevent tumor growth is vital. One recent study using an in vivo preclinical model of breast cancer brain metastasis proposed preconditioning tumors with Bev in advance of (rather than concurrent with) chemotherapy (16). Conversely, "preconditioning" with RT first, followed chronologically by administration of Bev, may be more logical and beneficial by providing treatment at a peak of radiation-induced hypoxia. As the half-life of Bev is relatively long (21 days) (17), sequential administration (in either order) should be explored further in preclinical models as a different strategic approach to its use. The authors of the REBECA study proposed early administration of Bev (2 weeks before initiation of WBRT) to induce vascular normalization to enhance the effects of radiation (10). However, this strategy remains hypothetical and speculative at this point in time; data supporting this approach in preclinical models would be needed before pursuing this in human trials.

Bevacizumab (Bev) in combination with RT: lessons from treating primary brain tumors

Assessment of the safety of Bev in combination with RT is imperative, particularly due to past or residual fear of its risk of intracranial hemorrhage in patients with brain metastases. Bev in combination with salvage SRS has been examined in recurrent (refractory to prior irradiation as well as temozolomide chemotherapy) malignant gliomas and found to be well tolerated (18). Administration of adjuvant Bev following SRS resulted in a 3.1-month improvement in progression-free survival compared to SRS alone. The incidence of grade 3 and 4 toxicities was similar between the two groups (18). The principle that Bev would be effective in suppressing angiogenesis activated by radiation-induced expression of hypoxia-inducible factor 1 (HIF-1) may translate to treatment of brain metastases regardless of the radiation modality employed (19).

Is WBRT the correct radiation-based modality for concurrent bevacizumab (Bev)?

In the era of improved radiation modalities, we should also consider alternate radiation approaches including SRS, intensity-modulated radiation therapy (IMRT) or even proton beam therapy rather than WBRT for treating brain metastases when applicable. There is increasing concern about the utility of adding WBRT to SRS, especially in terms of effects of the former on worsening neurocognition (20,21). It is now well established that the converse, adding SRS to WBRT, improves survival in patients with single brain metastases, and in patients <65 years as well as those with well-controlled systemic disease, and higher graded prognostic assessment (GPA) scores (22,23). Studies demonstrating sufficient control of limited brain metastases with SRS alone (24,25) provide impetus for future evaluation of studies adding Bev to SRS rather than to WBRT. Considering the concerns expressed from the authors of RTOG 8205 detailing worsened neurocognition in patients receiving 1st-line Bev with radiotherapy of GBM, adding this to WBRT may compound this issue (7). The REBECA study authors point out that some patients are ineligible for SRS, and thus WBRT is their next best option. For example, 3 of the 21 patients in this study had four brain metastases (all in breast cancer patients), 1 of whom had heavy systemic burden of malignancy as well, and thus high chance of further intracranial recurrence (10). Likewise, in terms of prognostic assessment of enrolled patients using Recursive Partitioning Analysis (RPA) classification, only 5 of 21 patients had RTOG prognostic group Class 1 (Karnofsky Performance status \geq 70, age <65, primary tumor controlled, absence of extracranial metastasis) (10). Thus the majority of patients truly had poor prognostic features that may have aligned with less benefit from SRS. Nonetheless, consideration could be given to trials incorporating Bev to SRS for patients with the good outcome features noted above per RPA or GPA assessments.

Lou and Sperduto. Bevacizumab and brain metastasis

Conclusions

Ongoing questions include how to best balance treatment of primary systemic malignancy with treatment of intracranial metastasis of that malignancy. Differences may arise depending on context of vascularity of the primary malignancy, heterogeneity and discordance between primary tumor and metastatic lesions that metastasize to and thrive in the CNS microenvironment, and genomic profiles include identity of the driving mutation. Regardless of these factors, there is increased recognition that spatially, intracranial disease is compartmental and that prognosis, and possible response to Bev therapy, may in fact vary based on location within the brain parenchyma. Furthermore, all other parameters being equal, prognostic assessment may diverge based on whether a single metastatic lesion is solitary (i.e., absence of active detectable systemic disease) or present with systemic activity. In these cases, creative use of welltolerated biologic agents such as Bev in combination with localized radiation modalities (focal irradiation and/or SRS) has potential as efficient approaches to improving prognostic outcome while sparing patients of potentially inefficient chemotherapies. The role of biological agents in the optimal management of brain metastases remains undefined. The REBECA study is among the first of hopefully many studies that will seek to address these issues.

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Novel radiotherapy approaches for lung cancer: combining radiation therapy with targeted and immunotherapies

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Abstract: Targeted therapies and immunotherapies have quickly become fixtures in the treatment armamentarium for metastatic non-small cell lung cancer (NSCLC). Targeted therapies directed against epidermal growth factor receptor (EGFR) mutations, anaplastic lymphoma kinase (ALK) translocations, and ROS-1 rearrangements have demonstrated improved progression free survival (PFS) and, in selected populations, improved overall survival (OS) compared with cytotoxic chemotherapy. Immunotherapies, including checkpoint inhibitor monoclonal antibodies against programmed death receptor 1 (PD-1) and programmed death ligand 1 (PD-L1), have now also demonstrated improved survival compared with chemotherapy. The use of these novel systemic agents in non-metastatic patient populations and in combination with radiation therapy is not well defined. As radiation therapy has become more effective and more conformal with fewer toxicities, it has increasingly been used in the oligometastatic or oligoprogression setting. This has allowed improvement in PFS and potentially OS, and in the oligoprogressive setting may overcome acquired drug resistance of a specific lesion(s) to allow patients to remain on their targeted therapies. Molecularly targeted therapies and immunotherapies for patients with metastatic NSCLC have demonstrated much success. Advances in radiation therapy and stereotactic body radiotherapy, radiation therapy have led to combination strategies with targeted therapies among patients with lung cancer. Radiation therapy has also been combined with immunotherapies predominantly in the metastatic setting. In the metastatic population, radiation therapy has the ability to provide durable local control and also augment the immune response of systemic agents, which may lead to an abscopal effect of immune-mediated tumor response in disease sites outside of the radiation field in select patients.

Keywords: Abscopal effect; immunotherapy; lung cancer; radiation therapy; targeted therapy

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Role of radiation in early stage and locally advanced non-small cell lung cancer (NSCLC)

Definitive radiation therapy has been part of the standard of care for patients with locally advanced NSCLC for almost 5 decades. Combined modality therapy with chemoradiation became the preferred treatment of these patients based on multiple clinical trials showing improved survival (1,2).

Conventionally fractionated radiation therapy remains the standard, and attempts at dose escalation have failed to show a benefit in this patient population (3). Newer technologies such as intensity modulated radiation (4), image guided radiation therapy, and proton therapy (5-7) are increasingly being utilized or studied to lower rates of toxicity with combined modality therapy.

 Table 1 Classes of targeted therapies in clinical use in metastatic

 non-small cell lung cancer

Target	Currently available targeted therapies
EGFR	Erlotinib
	Afatinib
	Gefitinib
	Cetuximab
ALK	Crizotinib
	Ceritinib
ROS1	Crizotinib
MET	Crizotinib
VEGF	Bevacizumab
	Ramucirumab

EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase; ROS1, ROS proto-oncogene 1; VEGF, vascular endothelial growth factor.

Surgical resection has been the standard of care for patients with stage I NSCLC with 5 years survival rates of approximately 60-70% (8,9). While patients determined to be medically inoperable have been treated in the past with standard fractionated radiotherapy, newer technologies within radiation therapy have led to the standardization of high dose, ablative hypofractionated therapy termed stereotactic body radiation therapy (SBRT) or stereotactic ablative radiotherapy (SABR) (10). SBRT has allowed for improved dose conformity, improved local tumor control, and superior overall survival (OS) when compared to conventionally fractionated radiotherapy (11,12). Based on the improved outcomes with SBRT and the increased utilization of this technology, interest in its use for medically operable patients has emerged. A recently published pooled analysis of two randomized trials comparing surgery and SBRT for stage I NSCLC demonstrated that SBRT was highly effective and had a limited toxicity profile, and that there was equipoise between the two treatment options (13).

SBRT has also begun to be used more frequently in patients with oligometastatic disease, including lung, liver, and bone metastases. Recent data has shown excellent control rates with encouraging progression free survival (PFS) in patients with oligometastatic NSCLC (14,15). Conventionally fractionated radiotherapy, in combination with chemotherapy, can also be considered in patients with oligometastatic disease not amenable to treatment with SBRT and may improve survival in a select subset of patients with minimal extrathoracic disease (16).

Targeted therapy for advanced NSCLC

With the discovery of molecular pathways that correspond with tumor progression and growth, numerous potential targets have been identified and explored for potential therapeutics for advanced NSCLC (*Table 1*).

Epidermal growth factor receptor (EGFR) is an essential part of the oncogenic growth pathway and is expressed at higher levels in some lung cancers. EGFR as a molecular target has shown promising results in advanced lung cancer. Monoclonal antibodies, such as cetuximab and panitumumab, and tyrosine kinase inhibitors (TKIs), such as gefitinib, erlotinib, and afatinib, are available. Initial trials evaluating patients treated with cytotoxic chemotherapy either in combination or followed by EGFR pathway inhibitors without prior molecular mutation analyses demonstrated mixed results, although trials have generally demonstrated at least a benefit to PFS (17-23). Further subset analysis of many of these trials showed clear correlation between the presence of EGFR driver mutations and clinical benefit of these agents. This has led to the standardization of the use of EGFR TKIs in the first line setting for patients with EGFR mutations (24-30).

Vascular endothelial growth factor (VEGF) plays an essential part in tumor angiogenesis and is often expressed at higher rates in NSCLC, thus creating another molecular pathway target for therapy. The most well studied VEGF inhibitor in NSCLC, bevacizumab, has shown increased PFS and OS in patients with non-squamous NSCLC when added to standard cytotoxic chemotherapy (31-33). Ongoing trials are evaluating bevacizumab with other platinum combinations (NCT00150657, NCT00753909), as well as with other targeted agents such as erlotinib and ramucirumab (NCT01532089, NCT00257608, NCT00553800).

One of the most promising recent areas of new drug development in treatment of NSCLC has been anaplastic lymphoma kinase (ALK) inhibitors. These are targeted agents directed at the novel fusion oncogene echinoderm microtubule associated protein like 4-anaplastic lymphoma kinase (EML4-ALK). The first available drug was crizotinib, an oral small-molecule inhibitor of ALK and c-Met tyrosine kinases. Crizotinib has shown favorable outcomes both in the second line setting, as well as in the primary treatment setting for patients that are positive for this rearrangement (34,35). Second generation TKI inhibitors of ALK include ceritinib and alectinib are undergoing investigation in national trials in ALK positive patients that have progressed, as well as the primary setting with pending

1	1	0
Monoclonal antibody	Target	FDA approved
Ipilimumab	CTLA-4 on T cells	Melanoma
Nivolumab	PD-1 on T cells	Lung cancer, melanoma
Pembrolizumab	PD-1 on T cells	Melanoma
BMS-936559	PD-L1 on tumor cells	No
MEDI4736	PD-L1 on tumor cells	No
MPDL3280A	PD-L1 on tumor cells	No
Lirilumab	Killer-cell immunoglobulin-like receptor (KIR) on NK cells	No
BMS-986016	Lymphocyte-activation gene 3 (LAG3) on tumor infiltrating lymphocytes	No

Table 2 Checkpoint inhibitors in clinical use or under development for advanced or metastatic non-small cell lung cancer

FDA, Food and Drug Administration; CTLA-4, cytotoxic T-lymphocyte antigen 4; PD-1, programmed death receptor 1; PD-L1, programmed death ligand 1; NK, natural killer.

results (NCT02292550, NCT02393625, NCT02075840, NCT02271139). ALK inhibitors have also demonstrated efficacy in patients with chromosomal rearrangements of the gene encoding ROS1 proto-oncogene receptor tyrosine kinase, which occurs in 1-2% of patients with NSCLC (36).

Immunotherapy for advanced NSCLC

Utilizing the immune system as an effective oncologic tool to fight cancer has been the subject of preclinical and clinical research for several decades (37). Immunotherapy agents allow the immune system to recognize a patient's cancer cells as foreign, prompting an immune response resulting in tumor cell death and/or inhibition of tumor growth. Newer immunotherapy agents have been developed based on improved knowledge of the molecular process of the immune response, leading to a resurgence in investigative use of these agents for patients with NSCLC. Such checkpoint inhibitors include monoclonal antibodies to cytotoxic T-lymphocyte antigen 4 (CTLA-4) such as ipilimumab, as well as antibodies to programmed death receptor 1 (PD-1), such as nivolumab and pembrolizumab (*Table 2*).

CTLA-4 is responsible for regulation of early T cell activity. It becomes upregulated after antigen exposure and competes for binding with CD28, preventing the stimulatory signal needed for T cell activation. Thus, inhibition of this receptor allows T cell activation after tumor antigen presentation. PD-1 is also upregulated on T cells, but it is thought to play a role further down the immune response pathway within the tumor microenvironment. Binding of PD-1 to programmed death ligand 1 (PD-L1) leads to T cell inactivation, and antibodies to PD-1 allow activation to proceed at the site of direct anti-tumor immune response.

The majority of data for use of these newer immunotherapy agents in NSCLC have been studied in advanced, stage IV patients. Ipilimumab was developed as an IgG1 CTLA-4 monoclonal antibody and was originally investigated in metastatic melanoma. A phase II randomized trial combining ipilimumab with standard first line chemotherapy in patients with stage IIIB-IV NSCLC showed improvement of PFS with the addition of ipilimumab (38). Subset analysis showed that patients with squamous cell histology benefitted primarily from the addition of ipilimumab, prompting an ongoing phase III trial that is comparing standard first line chemotherapy with carboplatin and paclitaxel with or without the addition of ipilimumab in patients with advanced squamous cell NSCLC. Additional trials are evaluating its effectiveness in combination with other targeted or immunotherapy agents (39).

Anti PD-1 antibody agents have been more commonly studied in patients with progressive metastatic NSCLC and showed promising results with prolonged tumor responses (40). Based on the recently published data from the CheckMate 017 and 063 trials in 2014, nivolumab has now received Food and Drug Administration (FDA) approval for treatment of advanced squamous cell NSCLC. Checkmate 063 was a single arm phase II trial in patients that had progressed after at least two prior systemic treatments. Nivolumab achieved an encouraging 1 year survival rate of 41% in these heavily pretreated patients (41). The follow up phase III trial, CheckMate 017, randomized patients with metastatic squamous cell NSCLC who had progressed after doublet chemotherapy to nivolumab or and docetaxel. The trial was stopped early due to superior OS in the nivolumab arm with a median survival of 9.2 vs. 6 months

in the docetaxel arm (P=0.00025). Nivolumab also showed a more favorable toxicity profile compared with docetaxel (42). Additional phase III trials are currently evaluating pembrolizumab monotherapy in both the first line and second line setting for advanced and metastatic NSCLC (NCT02220894, NCT02142738) (38).

Targeted therapy with radiation therapy for localized NSCLC

Many targeted therapies have been integrated into the treatment of localized NSCLC. While the data are much more limited than for the metastatic setting, targeted therapies have been used in combination with or concurrently with radiation therapy. The majority of this data are in conjunction with radiation therapy in the setting of locally advanced NSCLC classically treated with concurrent chemotherapy and radiation.

Preclinical data have shown biologic rationale for combining EGFR inhibitors and radiation therapy. Cetuximab has been combined with chemotherapy and radiation in treatment of locally advanced NSCLC in both phase II and phase III trials (3,43,44). In two sequential Radiation Therapy Oncology Group (RTOG) trials, cetuximab was combined with carboplatin/paclitaxel and radiation therapy for stage IIIA/IIIB lung cancer. While the median survival (22.7 months) and 24-month OS (49.3%) achieved in the phase II study (RTOG 0324) of cetuximab and concurrent chemoradiation were longer than any previously reported by the RTOG (43), the randomized phase III trial RTOG 0617 failed to show a benefit to the addition of cetuximab to chemoradiation in an unselected population (3). Among all patients, median OS in patients randomized to cetuximab was 25.0 vs. 24.0 months among those not receiving cetuximab (P=0.29). However, in a planned analysis of the association of EGFR expression and outcome, among patients with an EGFR H score of 200 or higher, cetuximab use was associated with improved OS (42.0 vs. 21.2 months, P=0.032) (3).

Gefitinib and erlotinib have also been integrated into both the concurrent chemoradiation setting, as well as a maintenance therapy after chemoradiation for locally advanced NSCLC (45-47). Again, phase III trials have failed to show a benefit to these agents in all subsets of patients, but they have shown improved outcomes in patients who had evidence of EGFR amplification or EGFR mutation, suggesting that in selected patients, these drugs may prolong PFS or OS in combination with chemotherapy and radiation therapy for non-metastatic patients. Newer studies are evaluating the use of these agents in patients with confirmed mutations (NCT01391260, NCT01822496, NCT02277457) (38).

Another area of clinical interest combining radiation and targeted therapy has been in the limited or oligometastatic setting. While the definition of oligometastatic has varied in the clinical literature, there has been increased use of local therapies for patients with limited sites of metastastic disease, especially as the ability to deliver effective local therapies with less morbidity has improved. Given the encouraging local control and limited toxicity profile of SBRT in both the lung and other organs commonly afflicted with metastasis from lung cancer, this remains an active area of research in treating patients with limited oligometastatic disease in combination with targeted agents. One recent published phase II trial showed encouraging results for PFS in advanced NSCLC patients with six or fewer sites of metastatic disease when they were treated with local SBRT to these sites in combination with second line erlotinib (7). Other active studies are similarly looking at this patient population in combination with other targeted as well as immunotherapeutic agents (NCT02450591, NCT0208672, NCT02444741).

As in the oligometastatic setting, the use of radiation therapy can be considered in the oligoprogression setting among patients being treated with TKIs for metastatic NSCLC. While patients with stage IV NSCLC and EGFR mutation or ALK rearrangement have achieved excellent PFS with targeted therapy, disease progression often occurs within a year of therapy initiation. While initial progression of EGFR- or ALK-directed therapy can be diffuse, many patients can have oligoprogression, or limited sites of progression, potentially due to acquired resistance from evolutionary selection on molecularly diverse tumors in which tumor clones in some sites of metastasis but not others develop resistance. Systemic options for such patients include increasing the dose of the targeted therapy they are progressing on, switching to another next-line targeted therapy, switching to cytotoxic chemotherapy, or adding chemotherapy to the targeted therapy (48). However, several groups have recently demonstrated that radiation therapy or other local therapies to sites of oligoprogression can also be considered and can achieve durable local control of the sites of progression and also allow for patients to be maintained on their existing TKI, thus saving alternative or next-line systemic therapy options for subsequent disease progression (49,50).

Anti-angiogenesis agents typically targeting VEGF have become standard treatment components of therapy for advanced NSCLC. Bevacizumab has been studied in combination with radiation therapy, but this combination has shown a high incidence of tracheoesophageal fistula formation when given concurrently, especially among patients with squamous cell carcinoma and centrally located tumors being irradiated (51).

Given the favorable results in advanced lung cancer, integration of ALK inhibitors into the setting of locally advanced NSCLC has already entered ongoing randomized phase II trials, including NRG/RTOG 1306/ NCT01822496, which is evaluating erlotinib and crizotinib as induction therapy followed by standard chemoradiation in patients with confirmed EGFR mutation or EML4-ALK fusion rearrangement, respectively (39).

Immunotherapy with radiation therapy for NSCLC

Although there is limited data to date combining radiation therapy and immunotherapy, this combination has the ability to achieve a synergistic therapeutic effect (52,53). As ionizing radiation can increase the production and presentation of tumor antigens, it can serve to augment the antitumor immune responses achieved by checkpoint inhibitors (54). Radiation therapy can augment immunomodulation by bolstering cytotoxic T-lymphocyte activity (53) and reduce myeloid-derived suppressor cells (55), allowing for synergism with checkpoint inhibitors.

SBRT may be the radiotherapy modality most optimally combined with immunotherapy since it can achieve a more robust immune response than conventionally fractionated radiotherapy. SBRT has been shown to induce cellular expression of major histocompatibility complex (MHC) I, inflammatory mediators, costimulatory molecules, heat shock proteins, immunomodulatory cytokines, adhesion molecules, and death receptors, all of which can enhance antitumor immune responses of systemic therapy (56).

There have been a number of reports in which a distant tumor mass regresses following the administration of radiation therapy before or after treatment with immunotherapy, known as the abscopal effect (57-59). In addition to the abscopal effect, radiation therapy may also allow for immune activation that leads to a more complete or accelerated clearance of the irradiated tumor, or sterilization of microscopic metastasis that were not clinically apparent at the time of irradiation. Aside from case reports, a number

of prospective clinical trials have been completed that have combined anti-CTLA-4 therapy and radiotherapy for melanoma (60) and prostate cancer (61) with promising results. A phase I/II study in metastatic castration resistant prostate cancer combining ipilimumab in combination with radiation therapy showed 50% of patients having a decline in prostate-specific antigen (PSA) with one complete response (60). A phase I trial combining ipilimumab and radiation in melanoma showed a response rate of 18% and PFS of 3.8 months prompting further investigation into this combination in the clinical setting (62). To date, no prospective study combining radiation therapy with anti-CTLA-4, anti-PD-1, or anti-PD-L1 therapy has been completed for lung cancer.

Future directions

Targeted therapy and immunotherapy have become pillars of lung cancer treatment. As we gain a greater understanding of the molecular basis of lung cancer, additional targeted agents will become part of standard practice to expand the role beyond the currently limited proportion of lung cancer patients with a known targetable mutation or translocation. Additionally, with increasing knowledge of acquired mutations, second- and third-line targeted agents will become standard options over salvage cytotoxic chemotherapy offering the promise of greater effectiveness and less toxicity. Cooperative group studies combining targeted agents and radiotherapy for nonmetastatic patients are ongoing (NCT01822496).

Similarly, immunotherapies will become more entrenched as standard therapy for second-line NSCLC and will be investigated in the first line setting. Combination therapies will increasingly be the subject of investigation, including the inhibition of both CTLA-4 and PD-1, or the use of an immunotherapy agent with a targeted therapy or with a cytotoxic chemotherapy. Toxicities to such combinations, however, may prove prohibitive.

While there is much excitement around the phenomenon of a radiotherapy-induced anticancer immune response and combining radiation therapy with immunotherapy, numerous questions remain before this combination can be exported to routine clinical practice. Additional research is needed to determine if conventionally fractionated irradiation, multi-fraction SBRT, or single fraction SBRT is most effectively combined with immunotherapy, and how radiotherapy and immunotherapy should be sequenced. Like with combination systemic therapies, combining

radiotherapy with such novel immunotherapies and systemic therapies may result in overlapping toxicities of radiation therapy and immunotherapy. In addition to the immune modulators and checkpoint inhibitors discussed in this manuscript, additional ways to provide tumor-associated antigen to the immune system that can be combined with radiotherapy are currently being investigated, including recombinant vaccines, tumor lysates, and synthetic peptides. While early results are promising, studies combining radiation therapy with immunotherapy warrant careful consideration of toxicity and safety.

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Footnote

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Molecular targeted therapy to improve radiotherapeutic outcomes for non-small cell lung carcinoma

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Abstract: Effective treatments for non-small cell lung carcinoma (NSCLC) remain elusive. The use of concurrent chemotherapy with radiotherapy (RT) has improved outcomes, but a significant proportion of NSCLC patients are too frail to be able to tolerate an intense course of concurrent chemoradiotherapy. The development of targeted therapies ignited new hope in enhancing radiotherapeutic outcomes. The use of targeted therapies against the epidermal growth factor receptor (EGFR) has offered slight but significant benefits in concurrent use with RT for certain patients in certain situations. However, despite theoretical promise, the use of anti-angiogenics, such as bevacizumab and endostatin, has not proven clinically safe or useful in combination with RT. However, many new targeted agents against new targets are being experimented for combined use with RT. It is hoped that these agents may provide a significant breakthrough in the radiotherapeutic management of NSCLC. The current review provides a brief discussion about the targets, the targeted therapies, the rationale for the use of targeted therapies in combination with RT, and a brief review of the existing data on the subject.

Keywords: Targeted therapy; monoclonal antibodies; tyrosine kinase inhibitors; radio-sensitizer; gefitinib with radiotherapy; erlotinib with radiotherapy; cetuximab with radiotherapy

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Introduction

Radiotherapy (RT) plays an important role in the management of lung cancer. RT has an established role not just as an adjuvant therapy to surgery in the curative setting, but also in the definitive setting (as in the use of stereotactic RT for early stage lesions) and in the palliative role (involving treatment of metastatic lesions as well as in facilitating relief from compressive symptomatology) (1,2).

Since the beginning of the era of RT, there has been a quest to enhance outcomes—both by increasing the efficacy of RT, and by reducing radiation associated toxicities. The earlier years witnessed the use of altered fractionation to improve therapeutic ratio, later there had been experimentation with the use of chemotherapy (both sequential and concurrent) to enhance RT. Concurrent chemoradiotherapy with agents such as carboplatin, cisplatin, paclitaxel, docetaxel are known to enhance response rates, but also while including severe toxicities. The toxicities are sometimes severe to an extent so as to make it unusable in many patients of lung cancer since a large proportion of these patients entail co-morbidities such as diminished respiratory functions, cardiac issues, and age related issues (3). In the recent years, there has been a significant breakthrough in the radiotherapeutic management of cancer. The use of targeted therapies in concurrent use with RT was seen as an effective approach, while being less toxic than the use of concurrent chemotherapy with RT. The success with the use of cetuximab in concurrent use with RT for head-neck squamous cell carcinoma ultimately led to the experimentation of a similar approach in other malignancies, including non-small cell lung carcinoma (NSCLC) (4). Almost simultaneously, there had been an interest in the use of anti-VEGF targeted therapies in concurrent use with RT (5,6). The discovery of oral tyrosine kinase inhibitors for certain types of NSCLC ushered in unprecedented convenience and efficacy (7,8).

It is hoped that evolution of targeted therapies for lung cancer can open up a new era in the radiotherapeutic management for lung cancer, with multiple experiments evaluating ways of integrating targeted therapy and RT for achieving synergy. While there is no dearth of theoretical targets, the lack of availability of clinically effective agents against these targets has been a source of frustration. This review discusses the current state of research in regards to the use of targeted therapies in concurrent use with RT for NSCLC.

A brief history of targeted therapies for lung cancer

Limitless replicative potential, growth self-sufficiency, anti-apoptotic potential, angiogenesis and the potential for invasion and metastasis are regarded as the 'hallmarks of cancer'. The mentioned abilities are the result of dysregulation of signalling pathways either due to oncogene activation, or via a loss of tumor suppressive gene function. Oncogene activation could imply gene amplification, rearrangements, and point mutations. Loss of tumor suppressor gene function could be due to loss of heterozygosity or by epigenetic transcriptional silencing. Though both 'oncogene activation' and 'loss of tumor suppressor gene function' are known to be involved in the etiopathogenesis of lung cancer, there has been a greater understanding upon the mechanisms of oncogene activation and there exist opportunities at targeting the same (9-12).

The study of the phenomenon of oncogene activation led to the discovery of 'oncogene addiction' wherein a 'driver oncogene' is crucial for the tumor cells' survival and proliferation. The commonly activated driver oncogenes in lung cancer include *EGFR*, *KRAS*, *HER2*, *MYC*, *MET*, *EML4-ALK* and *BCL2*. Since the targeting of a 'driver oncogene' would lead to specific killing of the 'oncogene addicted' tumor cells, these 'driver oncogenes' can in a way be regarded as the 'Achilles heel' of the tumor (13,14).

Clinically, EGFR mutations are the most common in lung cancer, and are of special interest due to the availability of multiple drugs to target EGFR. EGFR is a member of a family of transmembrane receptor kinases which also includes HER2, HER3 and HER4. EGFR and its associated receptor family are necessary for survival and are involved in maintenance of tissues including skin, heart, lungs and the central nervous system. Thus, it is not surprising that mutations of EGFR are oncogenic. The prevalence of EGFR mutations in lung cancer are difficult to estimate as it varies with ethnicity, sex and smoking status. Overall, EGFR mutations are expected in about 20-40% of Asian NSCLC patients. Mutations involving the kinase domain region (located from exon 18-21) of EGFR gene are 'activating mutations' since these mutations result in constitutive kinase activity of the receptor kinase, conferring ability of auto-activation (15,16).

Initial studies (such as BR.21 & INTEREST) evaluated *EGFR* tyrosine kinase inhibitors in NSCLC patients who had received prior treatment with chemotherapy, and without regards to either the patient's histopathology or the *EGFR* mutation status. Despite this, there was an evidence of benefit with the use of gefitinib/erlotinib in comparison to placebo/chemotherapy (17,18).

The phase-III OPTIMAL trial was conducted to evaluate the PFS benefit with the use of erlotinib versus chemotherapy with gemcitabine-carboplatin. When used as first-line treatment in Chinese patients with *EGFR* mutated NSCLC. The median progression free survival (PFS) was better with erlotinib in comparison to chemotherapy (13.1 *vs.* 4.6 months; P<0.0001). These results were confirmed in the EURTAC study involving European patients (19,20).

While gefitinib and erlotinib represent oral TKIs which are effective against mutated *EGFR*, there exists an older class of targeted therapy agents, namely 'monoclonal antibodies'. Monoclonal antibodies act on the extracellular aspect of the receptor, unlike the tyrosine kinase inhibitors which act on the intracellular domain. The anti-EGFR monoclonal antibody has already proven efficacy in patients of head-neck squamous cell cancers and colorectal adenocarcinomas (21-23). Their use in lung cancer has rather been an extrapolation based upon results in other sites. While cetuximab has been the most commonly used anti-EGFR monoclonal antibody in use, newer agents include panitumumab and nimotuzumab which are expected to provide similar efficacy at lesser toxicity as they have a diminished murine component in comparison to cetuximab (24-26).

Next to *EGFR*, the second mutation of particular importance happens to be the translocation mutation *EML4-ALK*, which is a lot less common in comparison to *EGFR*. Despite constituting just 3-6% of lung adenocarcinoma, it is of special interest because of the availability of an effective agent, namely crizotinib to target *EML4-ALK* mutation (27,28).

The VEGF pathway can be blocked by using monoclonal antibodies targeting VEGF, the use of VEGF receptor inhibitors (aflibercept), and by the use of small molecule tyrosine kinase inhibitors such as sunitinib and sorafenib to target the tyrosine kinase domain of VEGF receptor. The ECOG 4599 and the European AVAIL were two large phase III trials which helped gain approval for bevacizumab use in lung cancer, but strictly to be avoided in squamous cell carcinoma histology. Toxicities such as hemorrhage, esophageal toxicity could be severe. The results with aflibercept for platinum and erlotinib resistant lung cancer have been far from satisfactory in phase II trials. Small molecule tyrosine kinase inhibitors pazopanib, sunitinib, sorafenib and mosatenib are yet to be proven for safety and efficacy in phase III trials (29-32).

More targets such as *KRAS*, *BRAF*, *MET*, *ILGF-1* and others are foci of on-going research, with no major data available for drawing impressions at this time (33-38).

Rationale for combining targeted therapies & RT

The combination of EGFR inhibitors with RT for NSCLC has strong theoretical rationale, as well as the backing of a body of evidence that can be interpolated from other sites such as head-neck & colorectal (39,40). RT induced tissue damage leads to increased EGFR expression which may be contributory to the dreaded phenomenon of accelerated tumor cell repopulation. Anti-EGFR monoclonal antibodies are especially effective in situations involving EGFR overexpression, thus rationalizing their use in concurrent use with RT. The use of anti-EGFR oral tyrosine kinase inhibitors is known to inhibit radioresistance by various mechanisms involving the cell growth pathways. It has been experimentally observed that anti-EGFR tyrosine kinase inhibitors are known to inhibit radioresistance by various mechanisms involving the cell growth pathways. It has also been observed that anti-EGFR tyrosine kinase inhibitors

is known to inhibit radioresistance by various mechanisms involving the cell growth pathways including the reduction of percentage of tumor cells in the radioresistant 'S-phase' of the cell cycle, affect Rad51 expression, and reduce the radiation induced EGFR autophosphorylation (41). Also, the use of EGFR tyrosine kinase inhibitors in patients with *EGFR* activating mutations may lead to a rapid regression, hence reducing hypoxia and enhancing radiosensitivity (42-44).

The tumor vasculature is markedly disorganized in comparison to normal vasculature. The altered tumor vascular endothelium may lead to hypoxia, which not only causes increased radioresistance, but also promotes distant metastases. Also, RT is known to induce an increase in VEGF. Thus the use of anti-angiogenic therapy in concurrent use is rational, at least from a theoretical standpoint (45-47).

Existing experience on targeted therapies in use with RT

RT with anti-EGFR monoclonal antibodies

The first anti-EGFR monoclonal antibody to be used with RT is cetuximab. Cetuximab is a chimeric monoclonal antibody (partly murine, partly human), thus holding an occasional risk of allergic reaction. Newer agents include nimotuzumab and panitumumab. Nimotuzumab being a 'humanized' monoclonal antibody has modified protein sequences to increase similarity to human antibodies. Panitumumab is a fully human monoclonal antibody. While all of three agents act on the same target (the EGF receptor), the difference lies in the extent of expected toxicities. Further, cetuximab being an IgG1 may have the ability to activate complement pathway and cause antibody dependent cellular cytotoxicity, a feature which may theoretically be lacking in the IgG2 antibodies such as panitumumab. It is unknown at this time as to whether newer molecules (nimotuzumab & panitumumab) are equally effective as cetuximab, though newer molecules are likely to be less toxic (48-50).

The use of anti-EGFR monoclonal antibodies in concurrent use with RT has been summarized in *Table 1*. Though a pooled interpretation is difficult due to the varying complexity of study designs, the following inferences can be drawn at this time—that the use of anti-EGFR monoclonal antibodies in unresectable NSCLC is a good alternative to concurrent chemotherapy in patients unlikely to tolerate concurrent chemotherapy during RT;

Table I Allu-I	Table 1 Anti-ECFF K monocional antibodies for use with radiotherapy					
Study	Туре	Subjects	Design	Inference		
N0422 (51)	Phase II	n=57; poor performance	Cetuximab + RT (60 Gy/30#);	70% patients alive at 11 m (expected was		
		status; median age 77	evaluated percent of patients	50%); median survival 15.1 m; no treatment		
			alive at 11 m	related deaths; but >50% patients had		
				grade3 adverse effects (rash, dysphagia etc.)		
NEAR (52)	Phase II	n=30; patients unfit for	IMRT + cetuximab (concurrent	Response rate 63%; median OS 19.5 m;		
		chemoradiotherapy;	& maintenance)	1 yr survival 66.7%; well tolerated; use of		
		median age 71		IMRT and non-use of chemotherapy may		
				have enhanced tolerability		
RTOG	Phase II	n=93; unresectable	RT (63 Gy/35#) with concurrent	Response rate 62%; median survival		
0324 (53)		stage III NSCLC	chemotherapy + cetuximab	22.7 m; 2 yr OS 49.3%; 20% grade 4		
				toxicity; 5 grade 5 events (deaths)		
RTOG	Phase III	n=166; unresectable	Concurrent chemotherapy with	Higher dose RT potentially harmful;		
0617 (54)		stage III NSCLC	paclitaxel-carboplatin to all; RT	addition of cetuximab added no OS benefit		
			either low dose (60 Gy) or high	when patients were already on concurrent		
			dose (74 Gy); with or without	chemotherapy		
			cetuximab; 2×2 lactorial design;			
DTOC	Dhana II	On going potentially		To appear nothelesiaal complete reasonable		
NIUG 0830 (55)	Priase II	on-going, potentially	chomoradiothorapy with or	rates with papitumumab and to assess OS		
0039 (33)			without panitumumab	& toxicity rates in comparison with BTOG		
			without paintainainab	0324 which had utilized cetuximab		
SATELLITE	Phase II	n=75; stage III NSCLC	After 2 cycles of induction	1-year OS 66%; 3-year OS 29%; feasible		
(56)			chemotherapy, 3DCRT to	and tolerable; may be a valid alternative to		
			68 Gy/34# with cetuximab	concurrent chemotherapy		
SWOG	Phase II	n=24; stage III NSCLC	RT 64.8 Gy/36#; concurrent	Median survival 14 m; PFS 8 m; response		
S0429 (57)		unable to receive chemo	and maintenance cetuximab	rate 47%; well tolerated regimen even when		
		due to co-morbidities		concurrent chemotherapy not tolerable		
Choi	Phase 1	n=15; stage IIB-IV	Palliative RT 30-36 Gy in 10-	Well tolerated & feasible; response rate		
<i>et al.</i> (58)		NSCLC unsuitable for	12#; with weekly nimotuzumab	46.7%; no skin rash or allergy		
		radical therapy	(varying doses)			
Bebb	Phase I	n=18; stage IIB-IV	Palliative RT 30-36 Gy in 10-	Attractive for patients with poor		
et al. (59)		NSCLC unsuitable for	12#; with weekly nimotuzumab	performance status or co-morbidities;		
		radical therapy	(varying doses)	absence of rash; 66% response rate		

Table 1 Anti-EGFR monoclonal antibodies for use with radiotherap

EGFR, epidermal growth factor receptor; RT, radiotherapy; NSCLC, non-small cell lung carcinoma; Gy, Gray; #, Fraction; IMRT, intensity modulated RT; OS, overall survival; PFS, progression free survival; m, months; yr, years.

that the addition of anti-EGFR monoclonal antibodies when concurrent chemotherapy is already being used may not lead to additional benefit (as also observed in the scenario with head & neck squamous carcinoma); that radiation dose escalation may not translate to any benefit; and that anti-EGFR monoclonal antibodies may be effective as radiosensitizers in all NSCLC histologies, even if mutational status is not specifically known (51-59).

RT with EGFR tyrosine kinase inhibitors

While anti-EGFR monoclonal antibodies seem to be active in proportion to the level of EGFR expression, the activity of EGFR tyrosine-kinase inhibitors depend upon the presence of specific activating mutation of the *EGFR*. Gefitinib and erlotinib are the approved EGFR tyrosine kinase inhibitors in use. These orally administered

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Study	Туре	Subjects	Design	Inference
CALEB	Phase II	n=63; unresectable	Patients divided as per risk; poor	Benefit noted both for mutated and wild
CALGB		stage III NSCLC	risk received RT with gefitinib; good	type EGFR when gefitinib added to RT;
30106 (60)			risk received RT with gefitinib &	no additional benefit of adding gefitinib to
			paclitaxel-carboplatin	chemoradiotherapy
Choong	Phase I	n=17; unresectable	Complex study design involving RT,	No survival advantage; but only 21% of
<i>et al.</i> (61)		stage III NSCLC	chemotherapy & erlotinib	patients were of adenocarcinoma histology
JCOG	Phase II	n=38; unresectable	Induction chemotherapy followed	Response rate 73%; median survival
0402 (62)		adenocarcinoma	by RT 60 Gy/30# plus gefitinib	28.5 m; 2-year survival 65.4%
Okamoto	Phase I	n=9; unresectable	14-day induction gefitinib followed	Only 2 of 9 patients confirmed to harbor
<i>et al.</i> (63)		stage III NSCLC	by RT 60 Gy/30# plus gefitinib	EGFR mutations, and these two had OS
				>5 years
Center	Phase I	n=16; inoperable	RT (70 Gy/35#) plus oral gefitinib	Feasible with moderate toxicity; overall
<i>et al.</i> (64)		stage III NSCLC	and weekly docetaxel	response rate 46%; median survival 21 m
Rothschild	Phase I	n=14; unresectable	Gefitinib plus cisplatin	Feasible; toxicities caused by cisplatin and
<i>et al.</i> (65)		NSCLC	chemoradiotherapy	not gefitinib; EGFR mutation status not
				given impetus
Stinchcombe	Phase II	n=23; unresectable	Induction chemotherapy	Partial response rate of 24%;
<i>et al.</i> (66)		NSCLC stage III	(carboplatin-irinotecan-paclitaxel)	disappointing results in spite of extremely
			followed by RT (74 Gy) with	intense regimen
			carboplatin-paclitaxel and gefitinib	
SWOG	Phase III	n=243; stage III	Concurrent chemoradiotherapy	Selection of patients not done with regards
S0023 (67)		NSCLC	followed by docetaxel; maintenance	to either histology or EGFR mutation status;
			gefitinib	gefitinib not used concurrently
Wang	Phase II	n=26; stage III-IV	Individualized RT based on tumor	1 year OS 53%; 3-year OS 30%; 96%
<i>et al.</i> (68)		NSCLC	size and volume constraints; given	local tumor control rate; favorable
			either gefitinib or erlotinib; median	toxicity, reasonable outcome; no
			RT dose 70 Gy	chemotherapy used

Table 2 EGFR targeting tyrosine kinase inhibitors for use with radiotherapy

EGFR, epidermal growth factor receptor; RT, radiotherapy; NSCLC, non-small cell lung carcinoma; Gy, Gray; #, Fraction; OS, overall survival; PFS, progression free survival; m, months.

drugs offer the advantage of convenience, too. The experience with the use of oral EGFR inhibitors with RT is summarized in *Table 2*. It can be remarked at this time that unlike anti-EGFR monoclonal antibodies which can be used in all of NSCLC regardless of the *EGFR* mutation status, the use of EGFR tyrosine kinase inhibitors must be restricted to adenocarcinoma histology harboring *EGFR* activating mutations. Also, while addition of gefitinib/ erlotinib to RT may be helpful, there seems to be no benefit with the addition of gefitinib/erlotinib when concurrent chemotherapy is already being utilized. Currently, lack of large volume of data is a serious issue which hinders the drawing of confluences. Also, many of the existing data is from trials which did not provide impetus to patient selection based on histology and mutational status (60-68).

RT with anti-angiogenic agents

About a decade ago, the approval of bevacizumab as an anti-VEGF monoclonal antibody had led to the emergence of high hopes (69,70). However, it was soon realized that the use of bevacizumab had to be strictly avoided in patients with squamous cell carcinoma histology and in those with central thoracic lesions due to serious toxicity risks. Even when used for patients with adenocarcinoma histology, the use with concurrent chemotherapy and RT has led to Table 3 Promising newer targeted therapies for concurrent usewith RT

Class	Drug
Anti-HER2	Trastuzumab
Proteasome inhibitor	Bortezomib
MAPK inhibitor	Selumetinib
EML4-ALK inhibitor	Crizotinib
PARP inhibitor	Olaparib
Anti-mTOR	Sirolimus
HSP90 inhibitor	Celastrol
HDAC inhibitor	Vorinostat
DT II II	

RT, radiotherapy.

unacceptable toxicities such as esophagitis and pneumonitis, while not offering significant enhancements in outcome. At present, the use of bevacizumab in concurrent use with RT cannot be recommended for routine clinical use. While newer anti-angiogenic agents such as endostatin, sunitinib and sorafenib are now available, it must be stressed upon that they are yet unproven for safety and efficacy in the scenario of concurrent use with RT (71-73).

RT with other targeted agents

While anti-EGFR therapies have been the mainstay of effective targeted therapies for NSCLC, there are new novel agents in consideration for trials. Bortezomib is a proteasome inhibitor, already approved for use in multiple myeloma. Though was found to have demonstrated radiosensitizing properties in vitro, it was found un-safe for clinical use in a phase-I trial combining bortezomib with RT and chemotherapy (74,75). Sirolimus, a MTOR inhibitor has been tested in a phase I trial involving RT & concurrent cisplatin (76). Though safety has been evaluated, definitive results on response and survival is awaited. Finally, trials in early phases are evaluating celastrol (HSP90 inhibitor), vorinostat (HDAC inhibitor), selumetinib (MAPK inhibitor) and olaparib (PARP inhibitor) for concurrent use with RT (77-80). Though many novel agents (Table 3) have demonstrated radio-sensitizing properties in vitro, it needs to be seen if the results can be translated clinically.

Conclusions

At present, it can be concluded that the use of anti-EGFR monoclonal antibodies for concurrent use with RT may be

beneficial, and is an attractive option for NSCLC patients who are unable to tolerate concurrent chemotherapy for any reason. At the same time, it may be remarked that the addition of cetuximab when concurrent chemotherapy is already being provided with RT may not lead to any benefit. The use of EGFR tyrosine kinase inhibitors offers the convenience of the oral route of administration. However the use of EGFR tyrosine kinase inhibitors with RT is feasible only in adenocarcinoma patients with specific mutations. Anti-angiogenic therapy with RT may lead to more harm than benefit, and must be avoided at the present time. There are many newer agents against newer targets which are under investigation for concurrent use with RT. With painstaking and time consuming efforts, there will be hope for better results in the future.

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Footnote

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Cranial irradiation in patients with *EGFR*-mutant non-small cell lung cancer brain metastases

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Abstract: The most effective treatment approach for brain metastases in patients with non-small cell lung cancer (NSCLC) and activating epidermal growth factor receptor (*EGFR*) mutations is a current subject of investigation. Cranial irradiation is the standard treatment for brain metastases, but tyrosine kinase inhibitors (TKIs) such as erlotinib have also activity against brain metastases in this subset of patients. The combination of TKI and radiotherapy is a promising one, but data is lacking to indicate whether this is superior to erlotinib or whole brain radiation therapy (WBRT) alone. Retrospective data suggest that WBRT achieves more durable intracranial control compared to erlotinib alone. Randomized, prospective studies will be necessary to determine whether TKI, cranial irradiation, or both is the optimal initial treatment for brain metastases in *EGFR*-mutant NSCLC.

Keywords: Non-small cell lung cancer (NSCLC); epidermal growth factor receptor (*EGFR*); erlotinib; whole brain radiation therapy (WBRT)

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Brain metastases occur in 20% to 40% of patients with non-small cell lung cancer (NSCLC) (1). They are a common cause of morbidity and mortality and their incidence may be increasing (2). Historically, therapeutic options for brain metastases have been limited to local therapies such as whole brain radiation therapy (WBRT), stereotactic radiosurgery, surgery or a combination of the above. Due to concern for inadequate central nervous system (CNS) penetration, chemotherapy has not typically been considered a standard primary treatment for brain metastases. In a multi-institutional retrospective analysis that included 1,833 NSCLC patients with newly diagnosed brain metastases treated with radiation therapy between 1985 and 2007, a median overall survival of seven months (95% CI, 6.5–7.5 months) was reported (3).

It is now well established that NSCLC patients harboring activating epidermal growth factor receptor (*EGFR*) mutations have a different prognosis. Studies examining survival in *EGFR*-mutated patients have shown a more favorable median survival of 15-17 months from onset of brain metastases (4,5). Based on randomized trials demonstrating improved survival, EGFR tyrosine kinase inhibitors (TKIs) have replaced cytotoxic chemotherapy as first-line treatments with patients with metastatic *EGFR*-mutant NSCLC (6,7). However, whether EGFR-TKI can enhance or replace cranial irradiation in the initial treatment of brain metastases remains unclear.

In 2013, Welsh *et al.* published the results of a bicentric phase II trial in the *Journal of Clinical Oncology* (8) that examined whether the combination of erlotinib and WBRT would improve median survival in patients with NSCLC brain metastases. Erlotinib is known to possess CNS penetrability (9). In the Welsh study, 40 NSCLC patients with radiographically confirmed brain metastases between 2006 and 2010 received a loading dose of erlotinib (150 mg per day for 6 days), followed by concurrent erlotinib (150 mg per day) with WBRT, followed by maintenance erlotinib (150 mg per day) until disease progression or adverse effects. WBRT was delivered as 30 Gy in 3 Gy fractions for the first 10 patients, then changed to a regimen of 35 Gy in 2.5 Gy fractions given concerns of possible neurotoxicity in two patients. The primary endpoint was to detect an increase in median survival from a historical control of 3.9 to 6.0 months. The authors reported that the combination of erlotinib and WBRT was well tolerated, with no grade 4 or 5 treatment-related toxicity. After median followup of 28.5 months, the median survival was 11.8 months, significantly surpassing the target of 6.0 months. Of the 40 patients included in the study, 17 had known EGFR status. Subgroup analysis showed a nonsignificant improvement in median survival (19.1 vs. 9.3 months, P=0.53) and CNS progression-free survival (12.3 vs. 5.2 months, P=0.74) in the nine patients with known activating EGFR mutations compared to the eight patients with known wild-type EGFR.

While this pioneering study demonstrates the safety and promise of administering concurrent erlotinib with WBRT, it does not directly indicate whether concurrent treatment is superior to either treatment alone (or in close succession) for EGFR-mutant patients. In preclinical models, overexpression of EGFR is associated with radiation resistance (10) and EGFR signaling blockade sensitizes EGFR-mutant cells to radiation (11). The investigators of the trial hypothesized that concurrent EGFR inhibition and WBRT may therefore be synergistic and potentially improve survival. However, the single-arm design of the study and the limited number of patients with known EGFR mutations leaves open the question of whether combination therapy would have been any more effective than erlotinib or WBRT alone. Though the study cohort's survival handily exceeded the historical expectation of 3.9 months, some of this could have been attributable to general improvements in the prognosis of NSCLC patients with brain metastases, due to factors such as stage migration from the widespread use of brain MRI screening. Known EGFR-mutant patients had particularly favorable results, but such patients are now known to have relatively better prognosis when treated with erlotinib alone.

There is prospective evidence that EGFR-TKIs are an effective primary treatment for *EGFR*-mutated NSCLC brain metastases. In an open-label, single institution phase II study, 28 molecularly selected patients with activating *EGFR*-mutant NSCLC and brain metastases received either oral gefitinib (250 mg daily) or erlotinib (150 mg daily) (5). Study patients did not receive prior local therapy for brain metastases such as radiation or surgery. The median survival and CNS progression-free-survival was 15.9 and 6.6 months, respectively. In another phase II trial, 40 patients

with non-molecularly-selected NSCLC and asymptomatic brain metastases were treated with erlotinib (150 mg daily). Clinically significant improvement in OS was observed in activating *EGFR* mutation-positive patients (37.5 months, n=8) compared to EGFR wild-type patients (18.4 months, n=15; P=0.14), as well as in CNS progression-free survival (15.2 vs. 4.4 months, P=0.02). These studies not only corroborate the longer survival observed for patients with *EGFR*-mutant NSCLC and brain metastases reported by Welsh *et al.*, they also suggest that erlotinib monotherapy may be an effective primary treatment for patients with *EGFR*-mutant NSCLC brain metastases.

There is no randomized data directly comparing erlotinib to WBRT for primary treatment of EGFR-mutant NSCLC brain metastases. In a retrospective analysis, our group examined the role of cranial irradiation in patients with EGFR-mutant lung adenocarcinoma and newly diagnosed brain metastases (12). While it did not reach statistical significance, we observed longer survival in patients who received WBRT (n=32, 35 months) compared to patients who received erlotinib alone (n=63, 26 months, P=0.62) for newly diagnosed brain metastases. Our results corroborate the favorable survivals reported by Welsh et al., as well as the phase II studies of primary EGFR-TKI therapy for brain metastases discussed above. Furthermore, we found that patients who received WBRT had significantly longer time to intracranial progression compared to those who received erlotinib alone (24 vs. 16 months, P=0.04), despite having significantly greater intracranial disease burden (more patients with >3 brain metastases and larger lesions received WBRT). This study suggests that WBRT retains an important role in intracranial control in patients with EGFR-mutant NSCLC brain metastases. In a recent metaanalysis that included 12 non-comparative studies and 363 patients, upfront cranial radiation was found to improve intracranial disease control and survival outcomes compared to TKI alone (13). The majority of patients included in the study received TKI alone (n=185), 115 patients received WBRT alone, 23 patients received stereotactic radiosurgery alone, and 40 patients received concurrent WBRT and TKI. Despite significant methodological limitations, this analysis further highlights the notion that upfront radiotherapy should not be summarily abandoned in EGFR-mutant NSCLC patients even though targeted therapies have also demonstrated CNS activity.

Pre-clinical data has demonstrated that erlotinib can cause radiosensitization through cell cycle redistribution, induction of apoptosis, and inhibition of DNA repair (11). It is therefore reasonable to hypothesize that the combination of erlotinib and WBRT for EGFR-mutant NSCLC would result in significantly improved CNS disease control and potentially enhance survival. In a retrospective analysis, Gow et al. demonstrated that patients with EGFR mutations had higher response rates to WBRT compared to patients with wild-type EGFR disease. The administration of EGFR-TKI during WBRT was independently associated with response to WBRT, and response to WBRT was an independent predictor for survival (14). In a second study, concomitant administration of gefitinib and WBRT was found to result in higher treatment response and disease control rates in patients with EGFR-mutant NSCLC brain metastases compared to gefitinib alone (15). In 2014, Lee et al. reported results of a multicenter trial that included 80 non-molecularly selected NSCLC patients with newly diagnosed brain metastases randomized to WBRT alone (20 Gy in 5 fractions) or WBRT with concurrent erlotinib (16). They reported median survival of 2.9 months with WBRT alone and 3.4 months with WBRT plus erlotinib. However, only one patient had known activating EGFR-mutation status, limiting the study's relevance to current practice where EGFR mutation status is routinely verified, and erlotinib is only offered to patients with activating mutations. Overall, these studies suggest that the approach of concurrent EGFR-TKI and WBRT is a promising treatment deserving further study in patients with brain metastases and EGFR mutations. However, definitive support for this strategy is limited by the dearth of prospective randomized data, and the fact that many published studies only contained a small subset of patients with known EGFR mutations.

In summary, the phase II study of concurrent erlotinib and WBRT from Welsh et al. demonstrates the tolerability and safety of the combination in treating newly diagnosed brain metastases from NSCLC. Patients with EGFRmutated NSCLC brain metastases appear to have improved intracranial disease control and survival compared to patients with EGFR wild-type disease. Nevertheless, whether erlotinib, radiotherapy, or both is the optimal treatment for brain metastases in this population remains unanswered. Retrospective studies (12-14) indicate that upfront cranial irradiation may improve intracranial control and possibly survival compared to EGFR-TKI alone, and the combination of WBRT and EGFR-TKI may ultimately prove to be the best strategy (14,15). However, this needs to be confirmed with prospective randomized trials, one of which is ongoing: the TRACTS trial is comparing concurrent erlotinib and WBRT vs. erlotinib

alone (clinicaltrials.gov/NCT01763385). Crucially, this trial limits eligibility to patients with known activating *EGFR* mutations. Until such data are available, we suggest that patients with brain metastases from *EGFR*-mutant NSCLC should still be considered for upfront cranial irradiation, prior to or concurrent with erlotinib or other targeted therapies.

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Footnote

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Immunotherapy in locally-advanced non-small cell lung cancer: releasing the brakes on consolidation?

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Abstract: Locally-advanced non-small cell lung cancer (LA-NSCLC) is optimally treated with definitive chemoradiation or surgery in combination with chemotherapy or chemoradiation. Prognosis, however, remains poor, and attempts to improve outcomes using consolidation or maintenance chemotherapy have not improved overall survival. Given the limited success of traditional cytotoxic chemotherapies as maintenance therapy for LA-NSCLC, recent studies have investigated the role of novel agents such as maintenance or consolidation, including antiangiogenic agents and molecular targeted therapy. With multiple newly reported trials demonstrating improved outcomes with immunotherapy over cytotoxic chemotherapy for stage IV NSCLC, integrating immunotherapy with definitive chemoradiation regimens or as consolidative therapy for LA-NSCLC is an attractive option. The recently published START trial is the first to test immunotherapy in LA-NSCLC in a randomized, phase III setting. In that trial, the administration of maintenance tecemotide (L-BLP25), which induces a T-cell response to the mucin 1 (MUC1) glycoprotein, was found to be well tolerated and improve overall survival compared with placebo among patients receiving concurrent, but not sequential, chemoradiation. Despite the promising findings of this trial, numerous questions regarding immunotherapy for LA-NSCLC remain, and several additional immunotherapy trials are underway or planned in this patient population.

Keywords: Adjuvant therapy; immunotherapy; lung cancer; radiation therapy; stereotactic body radiation therapy

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Stage III locally-advanced non-small cell lung cancer (LA-NSCLC) represents approximately 30% of new NSCLC diagnoses per year. Treatment options include definitive chemoradiation (1) or surgery in combination with chemotherapy or chemoradiation (2). Despite aggressive treatment, 5-year overall survival is only 15–20%. Therefore, much work is needed to improve outcomes in this population.

Consolidation or maintenance chemotherapy given beyond what is administered concurrently with radiation therapy has not been shown to improve survival in multiple randomized trials. The use of two cycles of systemic dosing of consolidation carboplatin and paclitaxel after concurrent chemoradiation with the same agents using radiosensitizing dosing is generally recommended and commonly used in current cooperative group trials, despite lack of randomized data supporting this approach (3). Furthermore, the phase III PROCLAIM trial randomized patients to cisplatin and pemetrexed with concurrent radiotherapy followed by consolidation pemetrexed versus cisplatin and etoposide with concurrent radiotherapy followed by cytotoxic chemotherapy of choice in stage III non-squamous NSCLC and found no difference between the two arms (4); results of this trial reinforced that chemotherapy choice based on histology does not necessarily lead to better outcomes.

Given the limited success of traditional cytotoxic

chemotherapies as maintenance therapy for LA-NSCLC, recent studies have investigated the role of novel agents as maintenance or consolidation. For example, there is an ongoing trial of nanoparticle albumin-bound paclitaxel (nab-P) after nab-P plus carboplatin in stage IIIB/IV squamous cell NSCLC (NCT02027428). Antiangiogenic agents such as bevacizumab (5) and thalidomide (3) have also been assessed but have been shown to be harmful in the consolidation setting. In addition, molecular therapy targeted against the epidermal growth factor receptor (EGFR) also has not been shown to improve outcomes after definitive chemoradiation. A phase III study did not show any benefit to maintenance gefitinib, an EGFR tyrosine kinase inhibitors (TKIs) (6). Likewise, Radiation Therapy Oncology Group (RTOG) 0617 was a 2×2 randomized study investigating radiation dose (60 vs. 74 Gy) and the use of consolidation cetuximab, a monoclonal antibody to EGFR. The 60 Gy arm was found to have better overall survival, and there was no survival benefit and more toxicities with consolidation cetuximab (7). However, these studies have been critiqued for their being tested in unselected populations. RTOG 1306 is underway to define the role of molecular therapy in patients with LA-NSCLC who have known EGFR mutations or ALK translocations, although targeted therapy is given as induction therapy and not as maintenance therapy or concurrently with radiation in this trial. Additional studies are looking at other pathways such as the inhibition of MEK downstream of the RAS oncogene pathway (NCT 01912625).

Immunotherapy has recently reshaped the standard of care in metastatic NSCLC. Immunotherapy can allow a patient's immune system to recognize cancer cells as being foreign, which can trigger an immune response and resulting tumor cell death or growth inhibition (8). Broadly, immunotherapy can be categorized into checkpoint inhibitors, including antibodies to program death receptor-1 (PD-1), program death receptor ligand-1 (PD-L1), and cvtotoxic T-lymphocyte-associated protein 4 (CTLA-4). The PD-1 inhibitor, nivolumab, was first approved by the U.S. Food and Drug Administration (FDA) for second line treatment in squamous cell lung cancer after it was shown to have a significant survival advantage over docetaxel (9). Pembrolizumab, a PD-L1 inhibitor, also received FDA approval after it was shown in the KEYNOTE-001 study to have excellent antitumor efficacy, with an objective response rate of 19.4% and median duration of response 12.5 months; PD-L1 expression in at least 50% of tumor cells correlated with improved efficacy of the drug (10).

Although these drugs have predominantly been used for lung cancer as monotherapy, there is suggestion that immunotherapy may enhance the effects of radiotherapy, and vice-versa (11). As radiation causes tumor cell apoptosis and necrosis, release of tumor antigens may induce an antitumor immune reaction by upregulation of immunogenic cell surface markers (12-14). In addition, inflammation to normal tissue caused by radiotherapy may lead to either secretion of cytokines or an infiltration of tumor-specific T cells via pathways such as vascular normalization (15,16) or induction of pro-inflammatory chemokines (e.g., cxcl16) (17). Lastly, radiotherapy may also upregulate the PD-1/PDL-1 pathway, which is an inhibitor of immune activation (18). Therefore, if the PD-1/PDL-1 pathway is blocked pharmacologically, there may be enhanced antitumor response. In addition, studies have shown in other malignancies that increased tumor-infiltrating lymphocytes predict for better clinical outcomes (19).

Therefore, immunotherapy is a promising approach to combine with chemotherapy and radiation therapy in LA-NSCLC (20). A prior phase III randomized trial from the Chiba Cancer Center in patients with lung cancer who underwent resection investigated the role of IL-2 and lymphokine activated killer (LAK) cell adoptive immunotherapy in addition to cisplatin, vandesine, and mytomycin C (versus no additional therapy) and found improved overall survival (21). More modern immunotherapy trials are shown in Table 1, listing the different immunotherapy targets currently being evaluated in this setting. In addition to the targets listed, there are additional immunotherapy approaches planned but not yet underway for LA-NSCLC, including stimulating antigenspecific immune responses by vaccination, such as the MAGE-A3, belagenpumatucel-L, or TG4010 vaccines (22).

The START trial is the first to test immunotherapy in LA-NSCLC in a randomized, phase III setting (8). The drug studied in the START trial, tecemotide (L-BLP25), induces a T-cell response to the mucin 1 (MUC1) glycoprotein, which is abnormally glycosylated in NSCLC. The primary endpoint was overall survival, and block randomization was used to ensure similar baseline characteristics of both groups, including stage (IIIA versus IIIB), response to primary chemoradiotherapy (stable disease versus objective response), chemotherapy sequencing (concurrent or sequential with radiation therapy), and geographic region (North America and Australia versus Western Europe versus the rest of the world). A total of 1,513 patients were initially enrolled in a 2:1 randomization,

Table 1 Recently completed	l and currently accru	uing studies ez	camining the role of imm	unotherapy in LA-NSCLC				
	Triol nomo	NCT trial			onond D	2	Duration of	Triol ototi io
шпппипопнару кагдег	ווומו וומווופ	number	Drug name	Outcollie	LIASE	.= =	nmunotherapy	II I al status
Programmed cell death 1	NICOLAS	02434081	Nivolumab	Safety (pneumonitis)	=	43	1 year	Not yet recruiting
Programmed cell death	PACIFIC	02125461	MED14736	SO	≡	702	1 year	Recruiting
ligand-1	Hoosier	02343952	Pembrolizumab	Time to distant relapse	=	83	1 year	Recruiting
	Rutgers/Penn/Yale	02621398	Pembrolizumab	Maximum tolerated dose (MTD)	_	30	1 year	Recruiting
Cytotoxic	None							
T-lymphocyte-associated								
protein 4								
MUC1 glycoprotein	START	00409188	Tecemotide (L-BLP25)	SO	≡	,513 U	ntil progression	Completed
	INSPIRE	01015443	Tecemotide (L-BLP25)	SO	≡	500 U	ntil progression	Terminated
	START2	02049151	Tecemotide (L-BLP25)	SO	≡	,000 U	ntil progression	Terminated
MUC1 glycoprotein and	ECOG	0082800	Tecemotide (L-BLP25)	Safety	=	55 U	p to 34 courses	Recruiting
anti-VEGF			and bevacizumab					
LA-NSCLC, locally-advanc	ed non-small cell lu	ung cancer; N	AUC1, mucin 1.					

clinical hold of the drug by the study sponsor. The median overall survival for these arms were 25.6 and 22.3 months, respectively, but this was not statistically significant (adjusted HR, 0.88, 0.75-1.03, P=0.123). This effect, however, was statistically significant in patients receiving concurrent chemoradiation (30.8 vs. 20.6 months, adjusted HR 0.78, 0.64-0.95, P=0.016), the accepted standard of care for LA-NSCLC (7). The primary concern for toxicity with integrating immunotherapy with radiation therapy is pneumonitis, and dyspnea occurred in 5 and 4% and pneumonia in 2 and 3% of the groups, respectively. Overall, the drug was well-tolerated with no increase in the serious adverse events rate over placebo.

The positive results of the trial only in the concurrent but not sequential chemoradiation setting are not surprising, not only given that concurrent patients likely have better performance statuses and smaller tumors, but also given preclinical data suggesting that T-cell mediated lysis against MUC-1 is greater after concurrent therapy than either single therapy (23). In addition, the authors provided in the appendix preliminary data that the effect is greater with some chemotherapies over others (vinorelbine and taxanes versus etoposide). The START2 and INSPIRE trials investigating tecemotide alone have been terminated (11); the ECOG trial investigating tecemotide and bevacizumab is accruing, and we hope that this trial will stratify by chemotherapy regimen.

The potential of immunotherapy in LA-NSCLC lies largely in its ability to improve locoregional control or induce an abscopal effect of micrometastatic disease, and in doing so improve overall survival. The authors of the START trial should be commended for conducting the first phase III trial of immunotherapy maintenance in patients with LA-NSCLC and demonstrating safety and improved outcomes, at least in the subset of patients receiving concurrent chemoradiation. Despite the promising findings of this trial and shared optimism for future trials to prove to be efficacious, several questions regarding immunotherapy for LA-NSCLC remain. What are the appropriate immunotherapeutic agents to use? What is the optimal length of use (36% of START trial patients had >52 weeks of therapy)? Should these agents be given before, during, or after radiotherapy and/or chemotherapy? With the newfound use of immunotherapy as monotherapy following recurrence of LA-NSCLC after

and ultimately 829 in the tecemotide group and 410 in the placebo group were included in the modified intentionto-treat analysis, with this patient drop off largely due to a

chemoradiation, and thus the increasing potential for study arm crossover, what are appropriate trial endpoints? Are outcomes dependent on molecular markers (PD-1, PD-L1)? Can immunotherapy be optimized to benefit patients potentially not fit for concurrent chemoradiation? What are the optimal cytotoxic chemotherapeutic agents to use with immunotherapy, and do some enhance the effects while others hinder response? And, lastly, which radiation therapy techniques—photon therapy versus proton therapy and conventionally fractionated radiotherapy versus stereotactic body radiation therapy (SBRT)—are optimally integrated with immunotherapies?

The START trial and others are using immunotherapy to take the brakes off of the host immune response in LA-NSCLC. Global investigators will now have to step on the gas to answer the many remaining questions regarding immunotherapy in LA-NSCLC.

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Footnote

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Dose escalation for unresectable locally advanced non-small cell lung cancer: end of the line?

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Abstract: Radiation Therapy Oncology Group (RTOG) 0617 was a randomized trial that investigated both the impact of radiation dose-escalation and the addition of cetuximab on the treatment of nonsmall cell lung cancer (NSCLC). The results of RTOG 0617 were surprising, with the dose escalation randomization being closed prematurely due to futility stopping rules, and cetuximab ultimately showing no overall survival benefit. Locally advanced unresectable NSCLC has conventionally been treated with concurrent chemoradiation. Though advances in treatment technology have improved the ability to deliver adequate treatment dose, the foundation for radiotherapy (RT) has remained the same since the 1980s. Since then, progressive studies have sought to establish the safety and efficacy of escalating radiation dose to locoregional disease. Though RTOG 0617 did not produce the anticipated result, much interest remains in dose escalation and establishing an explanation for the findings of this study. Cetuximab was also not found to provide a survival benefit when applied to an unselected population. However, planned retrospective analysis suggests that those patients with high epidermal growth factor receptor (EGFR) expression may benefit, suggesting that cetuximab should be applied in a targeted fashion. We discuss the results of RTOG 0617 and additional findings from post-hoc analysis that suggest that dose escalation may be limited by normal tissue toxicity. We also present ongoing studies that aim to address potential causes for mortality in the dose escalation arm through adaptive or proton therapy, and are also leveraging additional concurrent systemic agents such as tyrosine kinase inhibitors (TKIs) for EGFR-activating mutations or EML4-ALK rearrangements, and poly (ADP-ribose) polymerase (PARP) inhibitors.

Keywords: Lung cancer; chemoradiotherapy (CRT); dose escalation; intensity modulated radiotherapy (IMRT); cetuximab

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Chemoradiotherapy (CRT) and dose escalation

The radiotherapy (RT) dose for locoregionally advanced non-small cell lung cancer (NSCLC) has been standard since the 1980s. Radiation Therapy Oncology Group (RTOG) 73-01 randomized patients to four different regimens: 40 Gy split course or 40, 50, or 60 Gy continuous courses with 2 Gy per fraction daily treatments (1,2). This study found that in the 2D era, local control and overall survival was superior for those patients receiving 60 Gy, establishing the standard RT dose regimen. Given uniformly poor outcomes despite 60 Gy thoracic RT, multiple studies established the importance of chemotherapy, first sequentially, then concurrently with RT (3-10). A meta-analysis subsequently concluded that indeed concurrent CRT had superior overall survival in comparison to a sequential regimen (11). This has since remained the foundation of our current treatment for locally advanced lung cancer.

Seeking further improvements in stage III NSCLC outcomes, many tried to leverage technologic improvements

in the planning and delivery of RT. The introduction of computerized tomography (CT) ultimately spawned three-dimensional conformal radiation therapy (3DCRT), which allowed the application of volumetric imaging to define volumes for more complex and conformal treatment planning. This enhanced the ability to deliver higher doses to tumors while limiting doses to normal tissues. Since then, there have been a number of radiation dose escalation studies leading towards the development of the experimental arm in RTOG 0617.

Initial dose escalation with 3DCRT was studied in the setting of RT alone or sequentially following chemotherapy. RTOG 93-11 was a phase I-II dose escalation study with 3DCRT that sought to take advantage of the new technology to dose escalate beyond 60 Gy (12). Patients received sequential chemoradiation with radiation dose up to 90.3 Gy based on lung V20. The 90.3 Gy cohort had two dose-related deaths and 83.8 Gy was deemed the maximum tolerated dose. This small study notably did not show any significant difference in locoregional control with dose escalation. Kong et al. published a phase I study of dose escalation at the University of Michigan, treating with doses up to 103 Gy based on effective lung volume, with 18% of patients receiving neoadjuvant chemotherapy (13). In this study, higher doses were associated with improved rates of 5-year survival in this cohort.

In the setting of concurrent chemotherapy, a modified phase I/II trial from North Carolina of 62 patients escalated the RT dose to 74 Gy (14-16). Patients in this study were treated with induction and concurrent carboplatin and paclitaxel. North Central Cancer Therapy Group (NCCTG) 0028, a phase I/II trial, thereafter confirmed the maximum tolerated dose of RT with concurrent carboplatin and paclitaxel at 74 Gy, with too many dose limiting toxicities at 78 Gy (17). RTOG 0117 was a combined phase I/II study which initially was planned to escalate RT dose from 75.25 Gy up to 80.5 Gy with increasing dose per fraction with concurrent carboplatin and paclitaxel (18,19). However, excessive toxicity at 75.25 Gy in 2.15 Gy fractions resulted in the de-escalation to 74 Gy in 2 Gy fractions, further establishing this as the maximum RT dose. CALGB 30105 randomized patients to either paclitaxel or gemcitabine-based induction chemotherapy and concurrent CRT to 74 Gy, closing the carboplatin/gemcitabine arm due to grade 4-5 pulmonary toxicity (20). Given the findings of these studies, 74 Gy was established was the dose-escalated experimental arm for RTOG 0617.

Epidermal growth factor receptor (EGFR) inhibition with CRT

In addition to studying dose escalation, RTOG 0617 also investigated the role of cetuximab in the management of stage III NSCLC. While not the initial intent of the study, the promising results of RTOG 0324, a single arm phase II trial of cetuximab with concurrent chemoradiation with 63 Gy and carboplatin and paclitaxel published by Blumenschein et al. reported 2-year survival of 49.3% in patients without selection of patients in regard to EGFR status (21). Notably, survival in this trial was the longest achieved in a study reported by the RTOG. Furthermore, the rationale was supplemented by the results of a randomized study of cetuximab in locoregionally advanced head and neck cancer patients, finding that the addition of cetuximab carried a locoregional control and overall survival benefit over RT alone (22). These promising results of cetuximab, without selection of patients based on EGFR mutational status led to its inclusion in RTOG 0617.

RTOG 0617

RTOG 0617, therefore, was a randomized phase III study that was designed to compare 74 and 60 Gy with concurrent followed by consolidation carboplatin and paclitaxel. It was subsequently amended to address the question of the role of cetuximab concurrently and with consolidation for unresectable stage III NSCLC (23). Patients were thus randomized equally among four arms: 60 or 74 Gy with or without cetuximab. Radiation was delivered in 2 Gy fractions by 3DCRT or intensity modulated radiotherapy (IMRT), with image-guided radiation therapy and planning with positron emission tomography (PET)/CT or 4D CT encouraged. Additionally, compliance with normal tissue dose constraints was encouraged, though not required. Randomization was stratified based on RT technique (3DCRT or IMRT), Zubrod performance status, use of PET in staging, and histology.

RTOG 0617 enrolled 544 patients from 185 institutions, with 464 enrolled while randomization to radiation dose was active, and 514 for cetuximab. The radiation dose randomization was closed prematurely due to futility stopping rules, although enrollment continued for cetuximab randomization. Four-hundred nineteen patients were ultimately analyzed for outcomes. When the results were presented, a surprising survival detriment was found with the 74 Gy arm in comparison to the 60 Gy arm, with 2-year overall survival rates of 45% and 58%, respectively. There was no progression-free survival or local progression differences based on radiation dose randomization.

Furthermore, the addition of cetuximab did not significantly affect overall survival, with 2-year overall survival rates of 52% in the cetuximab and 50% in the non-cetuximab arm. However, with planned retrospective EGFR expression analysis in a subgroup of patients (203 total patients), cetuximab was seen to offer survival benefit with EGFR H-score 200 or higher (high EGFR expression), with median overall survival of 42 months in comparison to 21.2 months (HR 1.72, two-sided log-rank P=0.032). There was a trend towards survival detriment with H-score less than 200 (P=0.056).

What happened?

The premature closing of the dose escalation component was unexpected to some, although to date, radiation dose escalation in the setting of concurrent chemotherapy has not been associated with improved survival (24,25). However, as these results were surprising and counterintuitive, very thorough analysis was performed. The investigators analyzed the quality of radiation delivered, finding the overall survival difference persistent even when analyzing only those cases with physician review and dosimetric requirements of 95% of the dose covering 90% of the planned treatment volume. This suggests that tighter radiation fields to avoid toxicity were not responsible for underdosing of the target. However, mean lung dose (MLD) and V20 were both significantly higher in the 74 Gy cohort. Of note, more patients completed consolidation chemotherapy in the 60 Gy arm (70%) than the 74 Gy arm (64%), although randomized studies (26,27) and meta-analyses (28) have not shown a benefit to consolidation chemotherapy following concurrent CRT. The interaction between radiation dose and cetuixmab was also non-significant. These results suggest that greater cardiopulmonary toxicity, associated with dose escalation, may have resulted in clinically meaningful differences in survival.

Furthermore, data were recently presented comparing the outcomes of patients treated with either IMRT or 3DCRT on RTOG 0617 at the 2015 World Conference on Lung Cancer and the 2015 American Society for Radiation Oncology (ASTRO) Annual Meeting. Patients treated with IMRT had more advanced disease and larger planning target volumes (PTVs). Despite this, there was a trend towards lower V20s, and significantly lower rates of grade 3+ pneumonitis (29,30). Of note, only lung V20 was predictive of grade 3+ pneumonitis. Additional analyses focused on heart dose, which demonstrated that heart V40 was significantly lower with the use of IMRT (30) and was associated with decreased overall survival. The relationship between heart dose and survival corroborates retrospective findings described by Liao *et al.*, which found that lung and heart doses are associated with worse overall survival (31).

These data suggest that potentially the broad application of dose escalation may be detrimental to overall survival, and more stringent planning parameters may be required to derive benefit from its application. In particular, dose delivered to the heart may require close attention, particularly V40, and limits on dose escalation may be required based on the ability to meet stricter heart dose constraints.

While these technical details may explain the limitations in survival with the application of dose escalation, the comparable rates of local control with dose escalation in the context of adequate radiation coverage raise questions on the outlook for the utility of higher radiation doses. This may be attributable to radiographic evaluation of tumor progression versus radiation changes or the lower rate of chemotherapy completion in the dose escalation arm. However, there remains limited prospective data to support the utility of dose escalation, mostly in the setting of RT alone, from the University of Michigan (13). RTOG pooled analysis suggested a locoregional control and survival benefit with higher biological effective dose (BED), but pools data across a significant time period, from 1988 to 2002 which may have potential confounders (32).

Of note, patients treated in the standard treatment arm had much better outcomes than anticipated based on historical data, with 2-year overall survival of 58% and median survival of 28.7 months. The authors speculated that this may be due to staging PET and PET/CT imaging which was acquired for almost all patients (about 90%) in the study. Thus, the potential for stage migration may have played a role in these improved outcomes. Indeed, there have been data using population-based datasets such as the linked Surveillance, Epidemiology, and End Results (SEER)-Medicare database, the California Cancer Registry (CCR), and institutional databases (33-35). In each of these studies, the increased use of PET over time was associated with upstaging of disease. Despite stable overall survival from lung cancer, Dinan et al. noted improved survival within stage IV patients in the SEER-Medicare cohort (34),

while Chee described the same phenomenon in stage III and IV patients in the CCR population (33). In addition to the benefits of PET in staging, the PET-START trial randomized patients to PET-CT or CT-based radiation planning, finding that patients who had PET-CT-based planning showed a near-significant trend towards an overall survival benefit (36,37).

Additionally, RTOG 0617 did not find a benefit with the use of cetuximab applied in an unselected fashion. This too was unsurprising as cetuximab was not found to improve outcomes in CALGB 30407 (38), which randomized patients to 70 Gy with concurrent carboplatin and pemetrexed with or without cetuximab. Interestingly, on subset analysis for those patients in RTOG 0617 with overexpression of EGFR, cetuximab was shown to be associated with a survival benefit. This is consistent with the mechanism of action for cetuximab as a chimerized murine monoclonal antibody to EGFR. Furthermore, the lack of benefit in unselected NSCLC cases is not surprising as only 52% of evaluable tumors on 0617 demonstrated EGFR overexpression, compared to the ubiquitous over expression in head and neck cancers. Given this, it is reasonable that cetuximab did not show a benefit in unselected patients. The secondary analysis should serve to guide future studies on the application of cetuximab.

Future directions in treatment of locoregionally advanced NSCLC

Given the hypothesis that the dose escalation in RTOG 0617 was impacted by normal tissue toxicities, ongoing studies are focused on delivering high dose RT while limiting normal tissue doses. The ongoing RTOG 1106 is randomizing patients between the standard 60 Gy versus the use of adaptive RT using PET/CT performed between 40 and 46 Gy to escalate doses up to 80.4 Gy to a smaller fludeoxyglucose (FDG)-avid volume, sparing normal tissues (39). Additionally, the study investigators took care to take into consideration the findings of 0617 with multiple strategies detailed in the protocol, including limiting radiation duration to 6 weeks, mandated motion management, individualization of radiation dose, and credentialing for radiation planning. Notably, dose escalation will be limited based on achievable MLD, which is constrained to 20 Gy.

RTOG 1308 utilizes proton therapy to achieve a similar goal of sparing normal tissues (40). In particular, proton therapy, particularly with intensity modulated proton therapy (IMPT), has been shown to reduce radiation dose to normal tissues (41,42). Given the futility of 74 Gy, the investigators selected a control arm of the study as 70 Gy [relative biological effectiveness (RBE)] delivered by photons with concurrent platinum-based doublet therapy in comparison with a 70 Gy (RBE) proton therapy arm with concurrent chemotherapy. The trial allows for adjustment of the prescription dose based on organs-at-risk (OAR) constraints, as there is currently no justification of radiation dose escalation beyond 60 Gy when given with concurrent chemotherapy.

Hypofractionation is viewed as another method to increase the BED delivered to treat lung cancer, building off of the more recent effectiveness of hypofractionated imageguided RT, also known as stereotactic body radiotherapy (SBRT) or stereotactic ablative radiotherapy (SABR) in early stage lung cancer. Several phase I studies have also had promising results using this method (43-45). A recently published phase II study investigated dose escalation in a hypofractionated style reaching 60 Gy in 15 fractions without exceeding the maximum tolerated dose (46). This study was preceded by other studies including a phase II Italian study of 60 Gy over 20 fractions with long-term follow-up showing promising disease control with acceptable toxicity (47,48). Zhu et al. also published data using a hypofractionated method with 50 Gy in 20 fractions with sequential chemotherapy (49).

In addition to altering RT approaches for dose escalation, additional systemic agents are being incorporated to improve outcomes in the management of locally advanced NSCLC. RTOG 1306 is an ongoing study incorporating the use of targeted agents for specific mutations (50). Patients with the EGFR TK mutation and EML4-ALK fusion rearrangement are being randomized to concurrent chemoradiation to 60 Gy with or without preceding induction therapy with a targeted agent (erlotinib or crizotinib, respectively). This study leverages findings from prior studies testing the addition of EGFR TK inhibitors to concurrent CRT platforms. In general these studies have found no improved, and possibly worse, survival with concurrent EGFR tyrosine kinase inhibitor (TKI) and CRT, but promising outcomes when they are given alone or sequentially with CRT (51-53).

Poly (ADP-ribose) polymerase (PARP) inhibitors have also emerged as potential agents to be given in the concurrent setting. The ongoing SWOG 1206 (NCI 8811) (54) and Alliance Foundation Trial (AFT)-07 are randomizing patients with unresectable NSCLC to concurrent chemoradiation with consolidation chemotherapy with or without the addition of ABT-888 (veliparib).

Summary

RTOG 0617 produced unexpected results to most, particularly in its dose escalation comparison, based on the preceding phase II data. Subsequent analyses have been presented to explain the results, potentially describing necessary constraints in escalating radiation dose to treat locoregional disease. Though the addition of cetuximab did not show survival benefit, analysis of EGFR overexpression in a subgroup of patients suggests that implementation in a targeted fashion may offer benefit. Given the findings of RTOG 0617, a number of modifications in dose escalation strategy and incorporation of biological agents have emerged to form ongoing trials in locally advanced NSCLC.

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None.

Footnote

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

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Immunotherapy for nasopharyngeal cancer—a review

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Abstract: Nasopharyngeal carcinoma (NPC) is associated with the Epstein-Barr virus (EBV) and characterized by peritumoral immune infiltrate. Advanced NPC has high lethality. Immunotherapy directed against EBV antigen targets has been previously explored in clinical trials, and is likely to be validated as an important target in NPC as randomized data emerges in the future. Cancer vaccines and adoptive T cell therapy have been explored in the clinic, with the latter showing the greatest success. Recent advances in gene sequencing technology now allow personalized tumor epitope mapping, whilst the advent of immune checkpoint inhibitors targeting the PD-1/PD-L1 axis offers the opportunity to activate adaptive T cell response *in vivo*. Anti-PD1 antibodies have shown promising activity in early phase clinical trials, and randomized studies against chemotherapy are underway. As immunotherapy is incorporated into standard treatment paradigms, issues of optimal combinations with targeting agents, immune adjuvants, and sequence with chemotherapy and radiation therapy will need to be addressed. Effective strategies to increase tumor antigenicity, improve immunological memory and reduce immune escape, will need to be developed to improve treatment outcomes. Here we present a brief history of the evolution of immunotherapy in NPC, and highlight key concepts relevant to its further development in the clinic.

Keywords: Nasopharyngeal cancer (NPC); Epstein-Barr virus (EBV); immunotherapy; review

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Introduction

Nasopharyngeal cancer (NPC), an Epstein-Barr virus (EBV) associated disease, has a distinct etiology and geographic distribution. It is rare in the West with incidence of less than 1 for every 100,000 people each year, but endemic in Southern China, Hong Kong, Taiwan, and Southeast Asia, where annual incidence reaches as high as 25–50 cases per 100,000 per year. Worldwide, there are 80,000 incident cases resulting in an estimated 50,000 deaths annually (1). NPC is a chemosensitive disease and 5-year survival rate in early Stage I and II disease exceeds 80%, but outcomes are very poor in stage IV disease where the 5-year survival rate

is less than 10% (2). Although the disease is highly sensitive to chemotherapy, resistance invariably develops and better treatments are urgently needed (3,4). The Epstein-Barr virus (EBV) latently infects more than 90% of the world's adult human population and its association with NPC is thought to be mediated by an interplay of environmental (dietary, smoking, co-infectious) factors and genetic predisposition (high risk HLA allotypes). In NPC, the EBV virus expresses a type II latency program and is present in virtually all poorly differentiated and undifferentiated nonkeratinising (WHO type II and III) NPC. The expression of viral antigens in NPC makes this disease an attractive target for immunotherapy strategies such as virus specific adoptive cell therapies. Here in this review, we summarize the range of EBV-related and unrelated antigenic targets, and discuss the crucial role of the immune-suppressive microenvironment in NPC. Significant clinical trial data for cancer vaccines and adoptive T cell therapy trials are outlined, and we explore the potential role of immune checkpoint inhibitors in NPC, and their potential combinations with conventional chemotherapy and radiation therapy. Given the disappointing clinical outcomes of all manner of targeted therapies in advanced NPC, rational immune oncology strategies become all the more crucial.

Targets for immunotherapy in nasopharyngeal cancer (NPC)

Epstein-Barr virus (EBV) targets

EBV is associated with a variety of malignancies including Hodgkin Disease, Burkitt's Lymphoma, and NPC, with the expression of viral proteins on the tumor cell surface. The pathophysiological mechanisms involved in EBV integration with the host cell genome, latency, and transformation—is complex and incompletely understood. From a therapeutic standpoint, NPC expresses an array (albeit a limited repertoire) of EBV antigens (5,6). Hence, immunotherapeutic strategies in NPC have historically focused on EBV specific epitopes as a means of targeting this cancer.

EBV-associated NPC expresses a type II latency program, and tumor cells typically express the latent membrane proteins 1, 2A, and 2B (LMP1, LMP2A, and LMP2B), EBV nuclear antigen 1 (EBNA1), all of which have limited immunogenicity. In addition, several EBV noncoding RNAs primarily EBER1 and EBER2, and BamHI-A rightward transcripts (BARTs) and BamHI-A rightward frame 1 (BARF1) of EBV are expressed abundantly and are detected consistently in NPC (7-12).

EBNA1 is expressed frequently in NPC and is a dominant target for CD4 T cells. LMP1 and LMP2 are expressed in approximately 50% of NPC tumors. LMP1 may be poorly immunogenic, while the LMP2 proteins sufficiently more immunogenic and hence putative targets for EBV directed immunotherapy, such as cytotoxic T cells (8,13-15). NPC occurs in immunocompetent individuals, and it is likely that immunological pressure results in the expression of a limited array of EBV antigens. These proteins maintain cellular transformation in malignant cells and their poor immunogenicity likely plays a role in promoting immune escape by EBV-positive malignant cells (16,17). Immunotherapeutic approaches employed to target EBV are dependent upon the capacity to generate an immunological response against EBV latency response antigens, however in human hosts, these antigens have also co-evolved to evade immune recognition. LMP1 and LMP2 are known to play a role in activating and transforming cells following infection, allowing proliferation and survival of latently infected cells (18,19). The LMP antigens are oncogenic. LMP1 is a major transforming protein and behaves as a classical oncogene (20,21). LMP2A and LMP2B are likely non-essential in B cell transformation in vitro (22). However, LMP2A can transform epithelial cells via activation of the PI3 kinase-Akt pathway (23). These LMP antigens, particularly LMP1, are poorly immunogenic, likely due to poor antigen processing in infected cells and the subsequent limited amount of antigen available for presentation by MHC class I molecules (17). As a consequence, the LMP antigens, particularly LMP1, generate a subdominant CTL response when compared to the responses generated against lytic cycle antigens and other latent antigens, such as EBNA3 (24).

EBNA1 can be detected in all EBV-associated malignancies (15). EBNA1 is highly stable and contains a glycine-alanine repeat sequence near its N-terminus that inhibit translation and subsequent self-replication (25-27) and as result, EBNA1 is processed poorly via the MHC class I pathway. Nevertheless, the demonstration of EBNA1-specific CD8+ CTL thought to be induced via cross-presentation by professional antigen presentation cells rather than via direct recognition of infected cells (28), has established that endogenously processed EBNA1 can be detected by CD8+ T cells (29-31).

NPC cells have preserved antigen-processing function and can be recognized by major histocompatibility complex class I-restricted virus-specific CTLs *in vitro* (32), However, downregulation of major histocompatibility complex class I peptide expression is seen in NPC tumors as an immune evasion strategy (33). NPC patients also appear to have a lower prevalence of T cells that can recognize HLA-restricted epitopes in LMP2 and EBNA (34). Epidemiological studies have suggested that certain HLA allotypes have higher associations with nasopharyngeal carcinoma. These include HLA-A*11:01 and HLA-A*02:27 (35,36). A molecular explanation that cysteine at codon 99 of the Alpha2-helix of HLA-A protein is deleterious suggests a possible locus of susceptibility to NPC (36). Hence while NPC occurs across a variety of HLA allotypes, a meaningful strategy would

be to focus initial HLA specific strategies in allotypes with demonstrated susceptibilities such as HLA-A*11:01.

Taken in entirety, NPC-related EBV antigens LMP1, LMP2A/B, EBNA1, EBER, and EBV-encoded RNA each have distinct effects on growth, differentiation, and the host immune response. Collectively, they likely contribute to the development of NPC through the promotion of transformation and angiogenesis, inhibition of apoptosis, induction of stem-cell-like phenotype, and enhancement of cell motility. EBV antigens also aid in immune escape through various mechanisms, including switching off immunodominant viral antigens, impairing the HLA I or HLA II pathway, up-regulating immune-inhibitory molecules, and recruiting T regulatory cells and inducing T-cell anergy (37). Hence, an understanding of the virus-host interaction in the NPC environment is essential for successful EBV-targeted immunotherapies. Selection pressure-driven evolution constantly stimulates the emergence of new EBV variants (38,39) which may be more oncogenic and less immunogenic than the parental strain, with for example a higher tropism for epithelial cells rather than B cells, suggesting that some EBV strains may carry an increased NPC risk (40).

It is important to note that NPC, while associated with EBV and the expression EBV proteins, is an entity that encompasses a broader range of other distinct molecular aberrations that may also represent immune targets.

Non-EBV targets

Genomic alterations in NPC represent neoantigens that may be immunogenic. Studies in this area are few given the limitation of accessible tissue for interrogation in this disease and the paucity of pre-clinical models. Nevertheless, a landmark study of comprehensive sequencing analysis of 56 NPC patients (41) has shed light on cancer mutations relevant in NPC. Nine significantly mutated genes included *BAP1*, *MLL2*, *TSHZ3*, *TP53*, *PIK3CA*, *ERBB3*, *ERBB2*, *KRAS* and *NRAS*. Copy number alterations in MAPKAPK2 have been shown to be associated with NPC risk (42). Epigenetic alteration in NPC include the CpG island methylator phenotype and a high load of hypermethylated tumor suppressor genes (43). These genomic and proteomic alterations and more, can contribute to the production of oncogenic and immunogenic alterations.

Immunogenic alterations can broadly be categorized into (I) tumor specific mutations that result in neoantigens; (II) tumor specific antigens and proteins overexpressed in tumors but not expressed or are expressed at very low levels in normal cells including proteins such as surviving; (III) lineage specific antigens expressed on tumor cells as well as on normal cells such as gp100; and (IV) cancer/testis antigens including MAGE and NY-ESO-1 (44). Emerging sequencing technologies with predictive computational algorithms now offer the possibility of developing HLA-restricted epitope maps for each tumor and the corresponding mutational landscape. These technologies will accelerate neo-antigen discovery and improve efficiency of immune targeting strategies in trials.

Immune checkpoints

PD-1 is an inhibitory receptor expressed on the surface of activated T cells. PD-1 is a known marker of T-cell exhaustion in animal models of viral infection. This manifests itself as loss of effector functions such as the secretion of cytokines (IFN- γ , IL-2, and TNF- α), production of the cytolytic effector molecules perforin and granzyme B, and eventually apoptosis (45-47). The immune infiltrates of chronic inflammation frequently employ the B7-H1/PD-1 axis. Both PD-1 ligands, B7-H1 (PD-L1) and B7-DC (PD-L2) are up-regulated in peripheral tissues during an inflammatory response to infectious agents, in response to type 1 (α , β) and type 2 (IFN- γ) interferons (48). The biologic role of this upregulation is the prevention of collateral tissue damage mediated by antigen-experienced T cells during inflammation (49-52). Other immune-checkpoint molecules such as 2B4, CD160, T cell Immunoglobulin and Mucin domain-3 (TIM3), Lymphocyte Activation Gene-3 (LAG3) are upregulated in conjunction with PD-1 on "exhausted" CD8 T cells in tumor and chronic viral models (53). Programmed cell death ligand-1 (PD-L1) is highly expressed by cancer cells and tumor-infiltrating macrophages in virus-associated malignancies including NPC (54). PD-L1 expression on tumor correlates with advanced tumor stage and lymphatic metastasis (55) while PD-1 overexpression is associated with shorter overall survival and recurrence free survival and is an independent risk factor for death, treatment failure and local recurrence of NPC (56). These early studies in NPC have added to rationale to apply immune checkpoint inhibitor antibodies to this disease.

Tumor microenvironment

NPC is characterized by substantial immune infiltrate

in the primary tumor that consists of T cells, B cells, dendritic cells, monocytes, and eosinophils. This massive lymphoid infiltrate in the primary tumor is likely favored by inflammatory cytokines produced by tumor cells (57-60). There is evidence that despite the immunogenic nature of EBV antigen expressing cancer cells, there is a marked local tolerogenic immune suppression. T regulatory cells (Treg) within the tumor site may contribute to the functional inactivation of innate cytotoxic T cell responses. Significant expansion of circulating naïve and memory CD4+CD25^{high} Foxp3+ was identified in 56 patients (61) and a smaller number was also noted to have infiltrating Treg in the tumor microenvironment. Another study of 40 untreated patients implicated the suppressive role of Treg cells with its findings of rich populations of Treg amongst tumor-infiltrating lymphocytes (TILs). A further finding in this study was that EBV-specific T cells are enriched but inactivated in the tumor microenvironment. TILs from NPC failed to produce IFN-gamma and to exert cytotoxicity when stimulated by lymphoblastoid cell lines (34). A more recent study demonstrated that both physical and pharmacologic mediated depletion of Tregs from PBMC enhances EBV-specific T cell responses in EBV-stimulated T cell lines generated from NPC (62).

A holistic immunotherapy strategy to target NPC must take into account the following:

- (I) Cancer specific factors
- Genomic and proteomic differences between cancer and host, that are both EBV specific, but otherwise cancer genome specific too;
- Presence of cancer-associated antigens, that are ordinarily poorly expressed in normal tissue, including the known cancer testis antigens;
- Presence of immune-suppressive checkpoints on cancer cells;
- Immunosuppressive factors in the tumor microenvironment such as but not limited to tumor hypoxia, immune-suppressive cytokine production, the presence of myeloid derived suppressor cells, and immunosuppressive regulatory T cells;
- (II) Host specific factors
- HLA Class I and II type and expression that determines presentation of peptide sequences of intracellular proteins to various subsets of immune cells;
- Immune cell population diversity and matching to tumor immune epitopes and other immunogenic cancer epitopes;
- Dendritic cell function, presentation of tumor

antigens, and interaction with immune cell subsets;

• Host specific tumor permissive factors that have yet to be identified.

Immunotherapy strategies against NPC overview

In our opinion, these strategies fall into two broad categories. The first category comprises strategies that aim to harness the host's pre-existing anti-tumor capability that may be suppressed by tumor, or to augment the host's innate ability to mount an immune response against tumor. This category of strategies assumes an innate pre-existing capacity to augment host immune response that the cancer may already have escaped, and aims to meaningfully directly impact the host immune system to mount an immune response against NPC, which represents an inflammatory cancer phenotype. Examples of these include immune checkpoint inhibitors anti-PD1, anti-PD-L1, anti-CTLA4, and anti-LAG3 antibodies to disinhibit the immune response against cancer, and cancer vaccines that attempt to stimulate and generate a host immune response.

The second category comprises therapeutic strategies that directly and preferentially target cancer cells. Chemotherapy and radiation can stimulate immunogenic cell death and this is increasingly being studied and understood for use with other immunotherapy strategies. Immune cells that target cancer cells directly include cytotoxic T lymphocytes and cytokine induced killer cells.

Host targeting agents

Immune checkpoint inhibitors

More than a fifth of patients with previously treated metastatic NPC showed an objective measurable response when treated with the pembrolizumab, according to a study reported at the 2015 European Cancer Congress. Pembrolizumab is a highly selective humanized monoclonal IgG4-kappa isotype antibody against PD-1 that is designed to block the negative immune regulatory signaling of the PD-1 receptor expressed by T cells (63). Two-thirds of patients in the study had some degree of reduction in target lesion size. The median duration of response was 10.8 months. The objective response rate with pembrolizumab in NPC was 22.2%, all partial responses. Another 15 patients had stable disease, resulting in a disease control rate of 77.8%. Forty one out of 44 patients screened for study had tumors that tested positive for PD-L1 expression. All but 2 of the patients had received at least 1 prior line of therapy for advanced disease, and a third of the patients had received 5 or more prior regimens. Median progression-free survival was 5.6 months. A recently opened trial uses Nivolumab (BMS), another anti-PD1 antibody, to treat patients with recurrent and/or metastatic NPC (NCT02339558). LAG3 represents another immune checkpoint that may confer immune escape. There is an ongoing Phase I clinical trial evaluating the safety and efficacy of an anti LAG3 antibody, LAG525 (Novartis), as a single agent and in combination with an anti-PD1 antibody, PDR001 (Novartis), in patients with advanced malignancies (NCCT02460224). This study includes NPC in its inclusion criteria.

Cancer vaccines

Therapeutic cancer vaccines for NPC have historically targeted EBV antigens. A study using an LMP2 vaccine has been reported. Autologous monocyte-derived dendritic cells cultured from patients with NPC and matured with cytokines were pulsed with HLA-A1101, A2402, or B40011 restricted epitope peptides from EBV-LMP2, and injected into inguinal lymph nodes. This strategy generated an expansion in the LMP2-specific response in the peripheral blood in the majority of patients, and a partial clinical response in 2 of 16 patients enrolled in the study was seen (64). A more recent Phase II study evaluated the use of dendritic cells transduced with an adenovirus-DeltaLMP1-LMP2 vector given as five biweekly intradermal injections to sixteen heavily pretreated stage 4c NPC patients. This first-in-human study demonstrated the safety of this strategy. No increase was seen in the frequency of LMP1/2specific T cells (65). Another clinical trial using the MVA-EL vaccine has provided evidence for the effectiveness of the direct administration of a poly-specific vaccine to generate LMP/EBNA1-specific CTL responses in patients. This recombinant vaccinia virus-based vaccine, which encodes a functionally inactive fusion protein containing the CD4 epitope-rich C-terminal half of EBNA1 and CD8 epitope-rich LMP2A could induce T-cell response in 80% of patients, in some cases boosting response to both CD4+ and CD8+ mediated immunity against EBNA1 and/or LMP2 (66). This vaccine is now being evaluated in a phase II trial involving patients who have detectable plasma EBV DNA after RT or who experience optimal response to palliative chemotherapy (NCT01094405).

Cancer targeting agents

Immunogenic cell death with chemotherapy and radiotherapy: concepts from studies in other cancers

Cancer cell death can be immunogenic or non-immunogenic. Immunogenic cell death (ICD) involves changes in the composition of the cell surface as well as release of soluble mediators that occurs in a defined temporal sequence. Endoplasmic reticulum stress and autophagy result in calretuculin (CRT) exposure in the outer leaflet of preapoptotic cancer cells. Additionally, these pre-apoptotic cells secrete ATP, and release nuclear protein HMGB1 as membranes become permeabilized during necrosis. CRT, ATP, and HMGB1 bind to CD91, P2RX7, and TLR4 respectively, facilitating the recruitment of dendritic cells in the tumor bed, and engulfment of tumor antigens by dendritic cells and optimal antigen presentation to T cells (67). Radiation is commonly used in NPC and is known to cause ICD accompanied by CRT exposure, ATP release, and HMGB1 release. The concept of immunogenic cell death may well underpin the rationale for strategies that combine standard treatments of chemotherapy, small molecule inhibitors, and radiation therapy with immunotherapy. Studies to characterize the capacity of these treatments to cause immunogenic cell death specifically in NPC are needed.

Cell based therapies

EBV is associated with several cancer namely, post-transplant lymphoproliferative disease (PTLD), Hodgkin lymphoma (HL), Burkitt lymphoma, tumors in HIV-infected patients, T cell lymphoma, NK/T cell lymphoma, gastric cancers, and NPC (5). Following primary infection, EBV persists for life as a latent infection which is controlled by cytotoxic T lymphocytes (CTL) (68). Adoptive immunotherapy was first developed for the treatment of PTLD and has now been successfully utilized for over ten years using autologous EBVimmortalized LCLs to stimulate the expansion of EBVspecific CTLs (69). CTL therapies in NPC were developed on the basis of this evidence.

CTL can be heterogeneous, primarily with regards to their differentiation status and homing properties. Following antigen encounter, a naïve or memory T cell will proliferate and acquire an increasing number of effector functions, resulting in fully differentiated effector cells which display the full array of effector functions (70,71). However, differentiation into effector cells significantly

alters the trafficking properties of the T cell (72). There is now evidence that this change in homing properties can be tissue-specific, whereby stimulation in different lymphoid organs can influence trafficking to particular peripheral tissues (73,74). Effective immunotherapeutic treatment of NPC may be dependent upon the capacity to generate CTL that can home in to nasopharyngeal tissue and other sites of metastatic disease. It also remains to be elucidated what impact the differentiation status of CTL has upon survival post-transfer. Although terminal differentiation may generate greater effector function, poor survival of these T cells posttransfer may reduce the number of cells accessing tumor sites. There is evidence that less differentiated T cells retain a greater capacity to expand following antigen encounter in vivo and provide greater protection following transfer (75). Therefore, treatment with non-terminally differentiated CTL may have some benefit in prolonging their survival and proliferation capacity following adoptive transfer.

Current strategies used to generate CTL that rely upon long-term in vitro cultures will generate cells with a late-stage effector phenotype. Lymphodepletion prior to adoptive transfer may provide another mechanism to enhance survival and proliferation of transferred CTL. In addition to the benefits associated with the removal of Treg cells, there is evidence that lymphodepletion can enhance the efficacy of CTL-based therapy by removing T cells which compete for homeostatic cytokines, such as IL-15 and IL-7, and thus creating 'space' in the lymphoid system to accommodate transferred T cells (76,77). However, some recent observations have suggested that whilst lymphodepletion may promote T cell engraftment (78) it may not improve the clinical outcome following T cell therapy (79). Our group had previously shown that a delayed graft-versus-NPC effect was demonstrable in three of 21 heavily-pretreated advanced NPC patients who received a conditioning regimen of subablative cyclophosphamide, in vivo T cell lymphodepletion with iv thymoglobuline and thymic irradiation followed by sibling HLA-matched and one-antigen mismatched allogeneic peripheral blood stem cells. The delayed objective responses were coincident with rising donor haematopoietic chimerism and better survivors correlated with chronic graft-versus-host disease. These results indicated to us that a potentially powerful immune alloresponse was operative against even bulky, progressing, and chemoresistant NPC disease (80).

We proceeded to conduct and complete a phase II trial exploring the role of cytoreductive chemotherapy followed by autologous CTL in previously untreated patients with advanced EBV-associated NPC. The patients received four cycles of gemcitabine and carboplatin followed by six doses of EBV-specific T cells (81). This combination therapy was well tolerated and resulted in an encouraging response rate of 71.4% with 3 complete and 22 partial responses. Moreover, the median overall survival of 29.9 months and the 2- and 3-year overall survival rates at 62.9% and 37.1%, respectively, were significantly higher than those observed in historical controls receiving chemotherapy alone (11-22 months). The study was the first in which a chemotherapy regimen followed by a planned celltherapy is given as frontline therapy for any cancer, allowing timely delivery of adequate CTL cells following chemotherapy completion. The study also had a high overall completion rate, with 35 of the 38 enrolled patients receiving the planned consolidation with EBV-specific T cells with no attendant grade III or IV toxicities with CTL therapy. A multicenter Phase III randomized control trial (NCT02578641) using this protocol is underway.

The Italian group had previously treated ten advanced NPC patients progressing after conventional therapy, using autologous EBV-specific T cells generated from EBVinfected LCLs as antigen presenting cells to stimulate a polyclonal response to latent EBV antigens. They observed partial responses in two patients and stable disease in four others (82). The Baylor group previously observed 10 responses in 15 patients treated with active disease (5 complete responses, 2 partial responses, and 3 with stable disease) (83,84). An additional eight patients were treated in their second or subsequent remission, and five remained free of disease with follow-up of six years. Both groups have attempted to improve these results by pretreating patients with lymphodepletion using either chemotherapy with cyclophosphamide and fludarabine (79) or CD45-depleting antibodies (78) but neither added approach improved the overall response rate. In the studies by both groups, the LCL-induced EBV-specific T cells contain T cell clones that target all nine latent-cycle antigens of EBV as well as some of the virus's lytic antigens. The majority of the T cells, however, are responding to the most immunogenic antigens, including EBNA3 and the lytic-cycle antigens such as BZLF1, which are not expressed by EBV-infected NPC cells. Instead, the tumor cells express antigens associated with the type II latency pattern, including LMP1, LMP2, EBNA1, and BARF, which are less immunogenic and are present at a lower frequency in polyclonal LCL-induced EBVspecific T cells. It is therefore notable that both groups have identified an association between measurable benefit

of EBV-specific T cells and the presence in the product of LMP2-reactive clones that expand in the patient after infusion (82,83). This observation was also seen in the Phase II study reported by Chia *et al.*, who showed a strong association of benefit with specificity for EBV-LMP2 in the infused line (P=0.04) (81).

Hence current studies are enriching lines for cells that recognize the EBV antigens expressed in NPC and other type II latency tumors, using either overlapping peptide pools pulsed on dendritic cells (85) or an adenoviral construct termed AdE1-LMPpoly that encodes EBNA1 fused to CD8+ T cell epitopes from LMP1 and LMP2 to stimulate T cells (86). The second approach has been tested in 16 patients with recurrent and metastatic NPC who received EBV-specific T cells generated by stimulation with AdE1-LMPpoly. After adoptive transfer, there was a transient increase in the frequency of T cells responding to LMP1, LMP2, and EBNA1. The median overall survival of these patients was 523 days, compared with 220 days in patients who did not receive T cells (86).

Currently, several novel strategies to improve the activity of CTL in NPC are being explored in clinical trials. MALTED is testing closely matched allogeneic CTL (NCT01447056), and RESIST-NPC is testing CTL cell that additionally express Dominant Negative Receptor that confers them resistance to TGFbeta, a factor secreted by cancer cells that confers immune suppression to CTL and allows immune escape (NCT02065362).

Cytokine-induced killer (CIK) cells represent a heterogeneous population of immune cells that have been expanded from peripheral blood mononuclear cells using cytokines. These have shown *in vitro* killing in a variety of cancers (87). NPC patients who received autologous CIK cell transfusion in combination with gemcitabine plus cisplatin chemotherapy had a higher overall survival and progression-free survival rates than patients with gemcitabine plus cisplatin chemotherapy (88) CIKs have also demonstrated tumor killing capacity against putative cancer stem cells of nasopharyngeal cancer, in pre-clinical models. This was demonstrated to be mediated somewhat via NKG2D–ligands as blocking by anti-NKG2D antibody significantly but partially abrogated CIK cell-mediated cytolysis against putative NPC cancer stem cells (89).

Future directions

The broad and potent responses of immune checkpoint inhibitors in a wide variety of tumors, is deepening our understanding of tumor immunogenicity and spearheading a resurgent interest in immunotherapy for NPC. As the complex interplay of EBV and NPC continues to be unraveled, it is likely that immunotherapeutic strategies will merge into mainstream clinical practice and offer durable remissions in patients with advanced NPC who are this day incurable.

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Footnote

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Targeting epidermal growth factor receptor for head and neck squamous cell carcinoma: still lost in translation?

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Abstract: The epidermal growth factor receptor (EGFR) is preferentially expressed in head and neck squamous cell carcinoma (HNSCC), and is a promising therapeutic target. Yet other than cetuximab, no agent targeting EGFR has been approved for this disease, and none has shown benefit over the standard of care. Several randomized trials of antibody and small molecule agents have found no new indication for these agents, despite their initial promise. In this review, we examine the major clinical evidence and discuss potential future developments of translational science in this area, including use of these agents in risk-stratified subgroups, inhibition of downstream/parallel targets, and combination with immunotherapy.

Keywords: Epidermal growth factor receptor (EGFR); head and neck cancer; targeted therapy

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Prognostic importance of epidermal growth factor receptor (EGFR)

For locoregionally advanced head and neck squamous cell carcinoma (HNSCC), radiation with concurrent cisplatin-based chemotherapy has been established as the standard of care (1). Despite this, the prognosis of most patients with HNSCC remains poor with overall longterm survival around 65% (2). Furthermore, cisplatin is associated with significant toxicities. In an effort to find more targeted and less toxic agents, interest has developed around the epidermal growth factor receptor (EGFR), which is highly expressed in HNSCC and is correlated with worse outcomes (3). In March 2006, the FDA approved the EGFR monoclonal antibody (MAb) cetuximab to be used in combination with radiation therapy for the definitive treatment of locoregionally advanced HNSCC, based on phase III data showing improved overall survival compared to radiation alone (4). Nearly 10 years later, despite numerous trials of agents targeting the EGFR pathway, cetuximab remains the only FDA approved targeted compound for this indication and no trial has yet identified

a regimen including targeted agents that is superior to standard chemoradiotherapy.

EGFR (also known as ErbB1) is in the ErbB family of receptor tyrosine kinases, along with ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4). Binding ligands allow members of the ErbB family to homo- or heterodimerize, autophosphorylating the intracellular domain and creating binding sites for signaling proteins. The two primary pathways activated are RAS/RAF/MEK/ERK and PI3K/ AKT/mTOR. Typical downstream effects include promotion of cell survival, mitosis, and altered adhesion (5). The ErbB network is complex, with various fine-tuning responses; activated signaling proteins and downstream effects are dependent on the involved ligands, dimeric partners, and the cellular context (6). Furthermore, EGFR can also act as a transcription factor itself. Radiation triggers translocation of EGFR to the nucleus, where it takes part in complexes related to DNA damage repair. The EGFR antibody cetuximab blocks this translocation and causes increased DNA strand breaks following radiation (7).

These preclinical findings are in agreement with clinical data showing that increased quantitative expression of

EGFR was correlated with worse local control and survival in patients treated with radiation alone (3,8). However, these analyses failed to account for the emerging factor of human papilloma virus (HPV) status, recently found to be a strong favorable prognostic factor (9). HPV-association is often measured by the surrogate marker of $p16^{Ink4A}$ (p16) protein overexpression, which has the highest concordance with HPV DNA *in situ* hybridization in oropharyngeal tumors (10). HPV-associated tumors appear to have less frequent EGFR amplification (11,12), as well as fewer genetic alterations overall (13).

Targeting of epidermal growth factor receptor (EGFR) through the extracellular domain

Initial efforts to target EGFR in HNSCC used MAbs. Results using cetuximab, a chimeric IgG1 MAb with high affinity for the extracellular domain of EGFR, were first published by Bonner et al. in 2010 (4). In this phase III trial, patients receiving cetuximab had an improvement in median survival of nearly 20 months over those receiving radiotherapy alone. However, the trial was criticized for not having a control arm of radiotherapy with a platinum agent, considered the current standard of care. Regardless, the trial confirmed the radiosensitizing effects of cetuximab, and it also confirmed previous observations that acneiform rash is a clinical marker of cetuximab response, with patients experiencing rash having median overall survival over 40 months longer than those without. In 2011, the FDA expanded the indication for cetuximab to include recurrent and metastatic HNSCC based on the European EXTREME trial (14).

In attempts to improve upon the standard definitive treatment based on chemoradiotherapy, trials were also performed adding cetuximab to platinum-based regimens concurrent with radiation. The major phase III trial using this strategy was RTOG 0522, with results originally presented at the American Society of Clinical Oncology (ASCO) conference in 2011, then published in 2014 (12). In this study, patients with locoregionally-advanced HNSCC were randomized to chemoradiotherapy with concurrent cisplatin, with or without concurrent cetuximab. No significant differences were found in 3-year locoregional failure, distant metastasis, progression-free survival, or overall survival (72.9% control vs. 75.8% cetuximab). The cetuximab arm had significantly higher rates of acute side effects, and treatment completion was lower than the control arm. However, subgroup analysis showed

improved overall survival with cetuximab in patients younger than 50 (hazard ratio for death 0.45, P=0.02). EGFR immunohistochemical expression was evaluated as a biomarker for response, but no interaction effect with treatment arm was found.

Similar studies using other EGFR antibodies met with similar results. Panitumumab was used in the CONCERT-1 trial added to cisplatin chemoradiotherapy (15). There were no significant differences in local control or survival, and more acute toxicity and treatment discontinuation occurred in the panitumumab arm. Zalutumumab was used in the DAHANCA 19 trial added to radiotherapy with concurrent cisplatin and nimorazole (16). Preliminary results were presented at the 2013 European Cancer Congress; locoregional control, disease-specific survival, and overall survival were statistically equivalent between arms. At present, the addition of EGFR antibodies to platinumbased chemoradiotherapy has only resulted in greater acute toxicity without advantages in oncologic outcome, although attempts at refinement of this approach continue. A randomized study sponsored by the National Cancer Centre of Singapore (NCT00957086) is currently enrolling patients to examine the combination of chemoradiotherapy with nimotuzumab, which has lower affinity towards EGFR than cetuximab. Preclinical data suggests this may confer selectivity for high EGFR-expressing patients, and lower toxicity rates have been reported in phase I/II trials (17).

Targeting of epidermal growth factor receptor (EGFR) by tyrosine kinase inhibition

Another strategy for targeting EGFR is orally administered tyrosine kinase inhibitors (TKIs) that directly prevent autophosphorylation of the intracellular signaling domain. In 2013, two randomized trials using TKIs were published in the same issue of the Journal of Clinical Oncology (18,19). In the study by Martins et al. (18), patients with locoregionally advanced HNSCC were randomized to cisplatin-based chemoradiotherapy with or without the TKI erlotinib. No significant difference was found between arms in the primary endpoint of complete response rate, although there was a trend towards improvement (40% control vs. 52% erlotinib, P=0.08). Adverse effects were minimal compared to those seen with the addition of MAbs, and no differences were seen as far as completion of radiotherapy or cisplatin. Tissue evaluation was performed for less than 50% of study patients, and no biomarkers for erlotinib response were identified.

Along with this trial, the Eastern Cooperative Oncology Group (ECOG) 1302 trial results were published (19). In this phase III trial, patients with recurrent or metastatic HNSCC with poor performance status or prior failure of platinum therapy were randomized to docetaxel with or without gefitinib, another oral EGFR-TKI. There was no statistically significant difference in the primary endpoint of overall response rate. In an unplanned subgroup analysis, patients younger than 65 years showed an improved median overall survival with gefitinib, but there were higher rates of infections and treatment interruption in patients over 65.

Harrington et al. examined the addition of the TKI lapatinib, publishing phase III data in 2015 (20). Lapatinib has the theoretical advantage of being a dual-TKI, inhibiting activation of both EGFR and ErbB2. Heterodimers of EGFR-ErbB2 have been shown to be more potent signaling complexes than EGFR homodimers (6). This trial was conducted in a group of high-risk postoperative HNSCC patients who would typically receive adjuvant chemoradiotherapy (20). Patients were randomized to post-operative cisplatin chemoradiotherapy with or without concurrent/maintenance lapatinib. There was no difference in the primary endpoint of 3-year diseasefree survival (62.2% control vs. 61.1% lapatinib), and no differences in secondary end points. While more acute side effects were seen in the lapatinib group, there was no significant difference in completion of chemoradiotherapy.

Future strategies to improve outcomes using epidermal growth factor receptor (EGFR) targeted therapies

This review of major randomized trials illustrates the repeated failure of EGFR-targeted agents to add benefit to standard platinum-based therapies. One reason may be the lack of maintenance EGFR inhibition after completion of the concurrent regimen; a maintenance cetuximab phase might have contributed to the improved outcome in the recurrent metastatic setting. Another reason may be that the addition of these extra agents is too toxic, particularly in elderly patients. Acute toxicity caused more treatment delays in RTOG 0522, CONCERT-1, and ECOG 1302, which may have nullified any benefit from EGFR inhibition. In RTOG 0522 and ECOG 1302, the addition of EGFR inhibition were found on post hoc analyses to be associated with survival benefits limited to younger patients. This differential effect by age may be more pronounced with MAbs than TKIs, given their higher toxicity profiles

overall. No treatment delays were seen in the Martins *et al.* gefitinib trial and the Harrington *et al.* lapatinib trial. ECOG 1302 (combined docetaxel and gefitinib) did see treatment interruptions, but this trial included poorer performing patients for whom even an added TKI may be too difficult.

Future trials might limit enrollment to younger patients to test this hypothesis, although support for additional trials of this nature may be low at this point given the risk of harm. A converse approach would be reducing platinum dose while adding EGFR-targeted therapy, to maintain therapeutic effect while limiting platinum-related toxicity. Following this concept, a phase I study examined chemoradiotherapy with reduced-dose cisplatin but with addition of cetuximab for locally advanced HNSCC (21). In this study, 87% of patients completed therapy as planned, and 2-year overall survival was a promising 80%.

Reexamining the biological mechanisms of these agents' action may help to shed light on future directions. As mentioned above, the radiosensitization effect of cetuximab appears to be related to its ability to prevent translocation of EGFR to the nucleus, limiting DNA damage repair (7,22). Cisplatin may similarly interfere with protein transcription and DNA damage repair, making any added benefit from cetuximab unneeded (23). Combining EGFR-targeting agents with chemotherapeutics that operate based on a different mechanism may therefore be more effective. Docetaxel is an anti-mitotic agent targeting microtubule activity, and has been shown in vivo to have combinatorial radiosensitizing effects with cetuximab (24). The phase II trial RTOG 0234 showed that that cetuximab/docetaxel compared favorably to cetuximab/cisplatin for postoperative high-risk HNSCC (25), and the currently recruiting RTOG 1216 will test this comparison at the phase III level (NCT01810913).

Unlike MAbs, TKIs have found no role in either the definitive or palliative setting for HNSCC. One prominent difference between the two classes is immunogenicity. MAbs are able to provoke antibody-dependent cellular cytotoxicity through interaction with Fc-gamma receptors on immune effector cells (26). It may be that this effect is more important than inhibition of EGFR activity. EGFR activating mutations are fairly rare in HNSCC (13), implying that they are not a common cause of oncogenesis. However, EGFR amplification is seen more often in HPV-negative tumors, which are more associated with tobacco use. EGFR is not amplified just in tumor cells, but also in histologically normal mucosa of HNSCC patients (27). Thus, EGFR

amplification may be a reaction to carcinogen exposure, but not necessarily an oncogenic driver. In non-small cell lung cancer, patients without EGFR activating mutations derive no benefit from TKIs (28,29). Thus, it is not surprising that TKIs also have little benefit for unselected HNSCC patients. The combination of cetuximab and an immune checkpoint inhibitor has shown activity in murine models (30), and clinical translation of this combination could be promising.

Another consideration is that EGFR is only one signaling molecule in a network of pathways ultimately promoting cell survival and mitosis (6). EGFR expression by gene copy number has not been shown to have any predictive value for response to cetuximab, indicating escape mechanisms may be in play (31). For example, recent evidence suggests that HER3 activation is induced by cetuximab exposure, bringing into consideration the use of an alternative approach to patients with de novo or acquired resistance to cetuximab (32). In support of this theory, the HER3 ligand (neuregulin) has been suggested as a possible prognostic marker in HNSCC (33). Other members of the ErbB family and related tyrosine kinases (FGFR, IGF-1) are also under active investigation as therapeutic targets (34). A recent examination of HNSCC genetics shows a diverse array of mutations (13). Activating mutations of a signaling molecule downstream to EGFR, PIK3CA (PI3K), were seen in high proportions of tumor samples, possibly bypassing effects of EGFR inhibition. Numerous stage I/II trials of agents targeting the PI3K/ AKT/mTOR pathway are underway (35).

While new agents and combinations remain to be tested in the future, selection of the appropriate population may be the appropriate priority for designing future studies of EGFR-targeting agents. Low-risk HPV-associated tumors respond excellently to platinum chemoradiotherapy, showing long term survival rates near 95% (9). However, cetuximab is also radiosensitizing, with fewer side effects than expected from cisplatin (4). Therefore, trials such as RTOG 1016 are examining whether EGFR-targeted MAbs can be used instead of cisplatin for HPV-associated cancers. In the CONCERT-2 trial, patients with locally advanced HNSCC were randomized to radiation with concurrent cisplatin or panitumumab. While outcomes were equivalent in the subset of patients with p16-positive tumors, adverse effects were not improved (36). The need for careful selection was also highlighted, as patients with p16-negative tumors did worse with panitumumab than with standard chemoradiotherapy. Maturing randomized data will provide further information (NCT00820248, NCT01302834,

NCT01855451).

While these trials hold promise for HPV-associated disease, the majority of HNSCC patients treated with concurrent chemoradiotherapy have HPV-negative cancers. In these higher risk patients, treatment intensification remains the dominant strategic approach. One such intensified approach is being tested by the ongoing TRYHARD study (RTOG 3501; NCT01711658). This study randomizes patients with non-HPV-associated locoregionally-advanced HNSCC to accelerated cisplatin chemoradiotherapy with or without concurrent/ maintenance lapatinib. Unfortunately, since the initiation of this trial the results of Harrington et al. have been released, showing no effect of added lapatinib even in a selected postoperative high-risk subgroup. In the future, other novel agents may be added to chemoradiotherapy, in the search to improve outcomes for this higher risk population.

Predictive biomarkers to select patients likely to manifest EGFR-targeted therapy response could lead to the formulation of more effective studies, but truly prognostic biomarkers remain elusive. While HPV-association is a powerful prognostic factor in HNSCC, no EGFRcontaining trial has shown any significant interaction effect with treatment. Likewise, EGFR expression has failed to be predictive of response to EGFR-targeted therapy, although the major trials in which this was evaluated used immunohistochemistry (12,20), which is dependent on staining protocol and may be less accurate than other methods. Failure of EGFR expression to predict response may ultimately be a reflection of the mutational diversity of HNSCC and the many alternative signaling pathways by which a cell may retain oncogenic drive (13). As we advance with targeted agents related to the PI3K/AKT/ mTOR pathway, individualized genetic profiling may be necessary to determine choice of drug combinations (37). Development of acneiform rash remains the strongest biomarker of EGFR-targeted therapy response as of now. Immunological mechanisms by which this rash occurs are still under investigation, however molecular markers of immune-escape pathways may help predict response to anti-EGFR therapy (38).

Conclusions

Despite early positive clinical trials, EGFR targeting has generally not had the impact on HNSCC treatment it initially promised. However, as our understanding of the underlying biology deepens, combination with other agents

and targeting escape and resistant mechanisms as part of a broader pathway-targeting strategy may provide an answer as to the causes of innate and acquired resistance to EGFR inhibition. Multiple maturing clinical trials will provide a greater opportunity to better answer these questions over the next few years. We hope that the growing scientific understanding of EGFR's role in HNSCC will someday improve outcomes for our patients and no longer be lost in translation.

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Footnote

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Non-surgical treatment for locally advanced head and neck squamous cell carcinoma: beyond the upper limit

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Introduction

The treatment for locally advanced head and neck squamous cell carcinoma (LA-HNSCC) has become dramatically more developed over the past decade. Since LA-HNSCC treatment requires a multidisciplinary approach, this success has been made possible by the efforts and collaboration of various treatment specialists.

In this editorial, we will comment on RTOG0522 (1), a large randomized controlled trial of non-surgical treatment for patients with LA-HNSCC. Although the results of this trial were negative, RTOG0522 was one of the newest challenges in developing a novel treatment paradigm. We discuss the reason with a short review of the history of the development of non-surgical treatment for LA-HNSCC over the past several decades.

Head and neck cancer (HNC)

There are approximately 600,000 new cases of head and neck cancer (HNC) in the world each year. It is the 6th most common cancer throughout the world (2). Approximately 60% of patients have stage III or IV disease at diagnosis (3), and their prognosis remains poor despite the emergence of new therapeutic options over the last few decades. Since the head and neck region contains many organs essential for vital activities such as eating, breathing, speaking and surgical resection cannot avoid jeopardizing such functions to some extent, non-surgical treatment has been developed in addition to surgical procedures. Nowadays, concurrent chemoradiotherapy (CRT) is the non-surgical treatment standard for stage III/IV HNSCC (4).

Development of treatment over the past several decades

In the 1990s, many treatment regimens that combined radiotherapy (RT) and chemotherapy (CT) have been tested. Which treatment combination and sequence is the best? That was the question at that time. In 2000 and 2001, two meta-analyses (5,6) revealed that treatment efficacy is significantly better when platinum-based CT was concurrently delivered with RT, rather than before or after RT. Subsequently, concurrent cisplatin (CDDP) and RT (CDDP-RT) has been the standard for LA-HNSCC.

However, CDDP-RT is so intensive that it was said to be at "the upper limit of human tolerance" (7). Only half to two-thirds of patients could complete concurrent administration of high-dose CDDP at that time (8-10). For this reason, several clinical trials were conducted in 2000s to look for more feasible and effective treatment options. Recently, the benefit of adding the molecular targeting agent cetuximab to RT (bioradiation; BRT) had been reported (11). In addition, the efficacy of docetaxelcontaining triplet regimen induction chemotherapy (IC) followed by RT has also been reported (12,13). Although these results had an impact on clinical practice, they were criticized because these treatments were not compared with the standard treatment, CDDP-RT, in phase III trials. However, there are three treatment choices available for LA-HNSCC in clinical practice without a head-to-head comparison: CRT, BRT, and IC followed by RT.

BRT

Cancer treatments using agents that target tumor-specific

signal pathways have been developed for many cancers during the 2000s. In HNSCC, epidermal growth factor receptor (EGFR) is abnormally activated, and almost all HNSCC tumors express high levels of EGFR. There is a relationship between higher EGFR expression and poorer survival (14). Therefore, whether inhibition of the EGFR signal pathway is associated with better clinical outcomes was investigated during this period.

Cetuximab is a monoclonal antibody that targets the human EGFR. Its clinical efficacy with CT has been reported in colorectal cancer (15) and HNC (16). Since it also has radiosensitizing effects in animal models, Bonner et al. conducted a randomized controlled trial (11) investigating the additional benefit of cetuximab with RT. In this trial, patients with stage III or IV squamous cell carcinoma of the oropharynx, hypopharynx, or larynx were recruited. Eligible patients received either therapeutic RT plus cetuximab or RT alone. The primary endpoint, median duration of locoregional control, was significantly longer among patients treated with cetuximab and RT than those treated with RT alone [24.4 vs. 14.9 months; hazard ratio (HR), 0.68; P=0.005]. This was the first trial that showed that molecular-targeting agents add a benefit to RT. On the other hand, this trial has been criticized because the treatment for the control arm was not the standard for stage III/IV LA-HNSCC. Thus, the treatment of choice for LA-HNSCC is CDDP-RT, and BRT should be considered for patients who cannot receive CDDP for some reason (e.g., renal impairment). Whether BRT is superior to CDDP-RT remains unanswered so far. Recently, results from a randomized phase II trial comparing BRT to CDDP-RT have been reported (17). In addition, head-to-head phase III trials are ongoing; the De-ESCALaTE trial (NCT01874171) compares CRT to BRT in stage III/ IVa, human papillomavirus (HPV)-positive oropharyngeal cancer and RTOG1016 (NCT01302834) compares CRT to BRT in a similar population. These trials are ongoing and we have to wait for the results.

RTOG0522 study

The RTOG0522 study (1) was planned based on the results of the two studies mentioned above, namely that (I) cetuximab is beneficial in patients with locally advanced HNSCC when concurrently delivered with RT (11); and (II) cetuximab is beneficial for patients with recurrent or metastatic HNSCC when added to platinum-based CT (16). Since the treatment of choice for LA-HNSCC is CDDP-

RT, RTOG 0522 was planned to compare the efficacy of cetuximab plus CDDP-RT and CDDP-RT. Patients with stage III or IV HNSCC were randomly allocated to receive either accelerated fractionation (AFx), RT (70 Gy over 6 weeks), two cycles of high-dose CDDP (100 mg/m^2 , on days 1 and 22) without (Arm A) or with (Arm B) cetuximab (loading dose 400 and 250 mg/m² weekly during RT). The primary endpoint was progression-free survival (PFS); other survival endpoints and adverse events were investigated as secondary endpoints. There were 940 patients enrolled in this trial. However, the results were disappointing. Arm B, the experimental group, did not have a better 3-year PFS rate [61.2% vs. 58.9%; HR, 1.08; 95% confidence interval (CI), 0.88-1.32; P=0.76], 3-year overall survival rate (72.9% vs. 75.8%; HR, 0.95; 95% CI, 0.74-1.21 P=0.32), 3-year locoregional failure rate (19.9% vs. 25.9%; HR, 1.30; 95% CI, 0.99-1.70; P=0.97), or distant metastasis rate (13.0% vs. 9.7%; HR, 0.76; 95% CI, 0.51-1.13, P=0.08). Furthermore, the frequency of grade 3 to 4 radiation mucositis, rash, fatigue, anorexia, and hypokalemia were higher in the experimental arm. p16 positivity might be prognostic, but EGFR expression and p16 were not predictive of experimental treatment efficacy.

Negative trial: why?

Although the treatment for the experimental arm was one of the most intensive treatments available for LA-HNSCC at the time, only negative results were obtained. Two reasons for these negative results were explained by the authors (1): "the toxicity burden of radiation-cisplatin is at the maximumtolerated level" and "platinum derivatives and cetuximab have similar mechanisms of radiation sensitization (i.e., inhibition of repair of radiation-induced DNA damage)".

In addition, we think there might have been several explanations for these results. First, the cumulative CDDP dose during CRT might have affected the results. Based on a recent report, at least 200 mg/m² of CDDP should be administered to obtain an additive effect with RT (18). In RTOG0522, the cumulative dose of CDDP in both arms was less than 200 mg/m² (191.9 mg/m² in Arm A and 185.7 mg/m² in Arm B). In particular, more Arm B patients received less than 160 mg/m² of CDDP (9.8% *vs.* 11.5%). Secondly, unplanned RT interruption might have had an effect as well. More than half of the patients in Arm B experienced interruptions in radiation (50.8%), compared to 42.0% in Arm A. Unplanned RT interruption worsens survival by 1.4% per day and 10–12% per week (19). This 8.8% difference

Table 1 Randomized trials of CRT plus molecular targeting agents

Authors	Year	Ν	Phase	Setting	СТ	RT	Target agent	Result
Mesía <i>et al</i> . (23)	2015	153	rP2	LA	CDDP: 75 mg/m ² , 3 courses	CFx	Panitumumab	Negative
Martins et al. (24)	2013	204	rP2	LA	CDDP: 100 mg/m ² , 3 courses	CFx	Erlotinib	Negative
Harrington et al. (26)	2015	688	P3	PO	CDDP: 100 mg/m ² , 3 courses	CFx	Lapatinib	Negative
Ang <i>et al</i> . (1)	2014	940	P3	LA	CDDP: 100 mg/m ² , 3 courses	AFx	Cetuximab	Negative

CRT, chemoradiotherapy; CT, chemotherapy; RT, radiotherapy; rP2, randomized phase 2; LA, locally advanced; CDDP, cisplatin; CFx, conventional fractionation; P3, phase 3; PO, postoperative; AFx, accelerated fractionation.

in the proportion of patients with interruption could have negatively affected survival in Arm B. Third, 70% (625 patients) of enrolled patients had oropharyngeal cancer, and tumor specimens for a p16 assay were obtained from half (321 patients) of them. Approximately 50% of tested patients were p16 positive. Patients with p16-positive oropharyngeal cancer have a good prognosis and may undergo de-escalation of treatment intensity (20). For such patients, CDDP-RT is intensive enough to achieve a treatment effect. Thus, the protocol treatments of this study could have been too intensive to demonstrate a survival benefit for p16-positive oropharyngeal cancer patients, who accounted for one-third of all patients. These points might be possible reasons for the negative results, in addition to the reasons proposed by the authors.

Future perspective

Improving treatment efficacy for LA-HNSCC by intensifying treatment through (I) adding IC to CRT and (II) adding molecular targeting agents other than cetuximab to CRT has been challenging. The former strategy has been reported in two randomized controlled trials (21,22). Although both trials were underpowered due to a low accrual rate, the benefit of additional IC on CRT was not observed. The latter strategy has also been tried. Although the benefit of adding molecular targeting agents other than cetuximab to CRT has been investigated in phase II and phase III studies (23-26), positive results have not been observed so far (*Table 1*).

The following alternative strategies seem possible: (I) patient selection; (II) optimizing treatment delivery; and (III) new paradigm.

Patient selection

HPV-positive oropharyngeal cancer is a distinct entity,

which has been confirmed genetically (27), with better survival compared to other types of HNC (20). For HPVpositive oropharyngeal cancers, de-escalation of treatment intensity or maintenance of treatment intensity and improving QOL are the objectives. Intensifying treatment might be beneficial for patients with HPV-negative HNSCC.

Optimizing treatment delivery

As already stated, compliance with CDDP-RT is not good, so optimizing treatment delivery is one possible way to improve survival. New cytotoxic agents had been developed after 2000, and whether they improve upon CDDP-RT has been studied, for example, in the RTOG0234 trial (28). This randomized phase II trial of postoperative (PO) treatment reported docetaxel and cetuximab had better efficacy than CDDP and cetuximab as well as compared to historical controls with high-risk HNSCC that received CDDP-RT and underwent resection. Based on this trial, a phase II/III trial for this population is ongoing (RTOG1216; NCT01810913). Development of RT techniques or particle beam therapy might play some role in improving treatment efficacy as well.

Immunotherapy

Immunotherapy has changed existing treatment paradigms in other cancers. This treatment has also been attempted in HNC, both in the locally advanced and recurrent or metastatic setting. The feasibility of adding the anticytotoxic T lymphocyte-associated protein 4 (CTLA4) antibody ipilimumab (NCT01860430, NCT01935921), anti-programmed death-1 (PD-1) antibody nivolumab (RTOG3504), or anti-programmed death-ligand 1 (PD-L1) antibody pembrolizumab (NCT02641093) to RT in the locally advanced setting is currently being investigated in
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phase I and II trials.

In conclusion, the challenge to overcome "the upper limit of human tolerance" failed in RTOTG0522. While treating patients with LA-HNSCC, patient selection and optimizing treatment delivery could be the keys to obtaining a sufficient treatment effect in current clinical practice. The role of molecular targeting agents remains unclear and should be further investigated.

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Anti-epidermal growth factor receptor immunotherapy in combination with cisplatin chemoradiation for patients with advanced head and neck carcinoma—biological and clinical limitations of the triple treatment

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Squamous cell carcinoma of the head and neck region (HNSCC) is the sixth important tumour entity by incidence worldwide associated with more than 300,000 HNSCC related deaths/year (1). Current standard treatment, especially in the advanced situation, comprises definitive cisplatinum based chemoradiation therapy (CRT) or adjuvant CRT after surgical resection in patients with high risk tumours (2). Prognosis, however, remains poor for the entire entity with 5-year survival rates around 50% (3).

Due to an increasing understanding of the molecular biology of HNSCC, interest has been prompted in the development of molecularly targeted therapies to improve the efficacy of standard therapeutic regimes while minimizing toxicity. Among these targeted approaches, inhibition of the epidermal growth factor receptor (EGFR) is most advanced in the clinical setting. EGFR is a transmembrane glycoprotein and member of the ErbB receptor tyrosine kinase family. Upon ligand binding [EGF, transforming growth factor (TGF)-alpha, amphiregulin], EGFR phosphorylation induces downstream activation of the Ras/Raf/mitogen-activated protein kinase (MAPK), phospho-inositide 3-kinase (PI3K)/AKT and Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) pathways finally resulting in proliferation, inhibition of apoptosis, neovascularization, and activation of an invasive and metastatic phenotype (4). From a clinical perspective, EGFR is over-expressed in approximately 80-90% of HNSCC and correlates with poor prognosis and resistance to radiation therapy (5). Moreover, preclinical

evidences revealed that blocking EGFR by means of antagonistic antibodies restores radiation sensitivity and enhances cytotoxicity (6). Consequently, for more than a decade, EGFR-targeted strategies are evaluated as integral components in the treatment of patients with advanced HNSCC including the use of the chimeric IgG1 human monoclonal antibody cetuximab (Erbitux®), the first targeting agent to demonstrate survival advantages if combined with radiation therapy (7).

Following pioneering, euphorically commented results from large randomized studies indicating a superiority of combined cetuximab and radiotherapy (RT) in a primary curative intended situation and improved overall survival in patients with recurrent or metastatic disease in combination with cisplatin-based chemotherapy (CT) (7,8), EGFR inhibition seemed to be a promising approach to further improve efficacy of RT or CT in patients with HNSCC. Based on these evidences, Radiation Therapy Oncology Group investigators launched a phase III trial (RTOG 0522) published in the Journal of Clinical Oncology in 2014 (9). In a large cohort (n=891) of eligible patients with stage III or IV HNSCC, the study aimed to test the hypothesis that adding cetuximab to an accelerated RT and cisplatinplatform (experimental arm) improves progression-free survival (PFS) in comparison to standard cisplatin-based CRT. Results, however, were highly disappointing. Addition of cetuximab did not significantly affect 3-year PFS and overall survival, locoregional tumour control and distant metastases. To the contrary, cetuximab plus CRT resulted

in more frequent interruptions in RT despite incomplete cetuximab administration in 26.4% of the patients. Moreover, elevated levels of treatment-related radiation mucositis, rash, fatigue, anorexia, and hypokalaemia were observed. The authors thus concluded that concomitant cetuximab administration does not add clinical benefit to conventional cisplatin CRT. These negative results corroborated findings of other studies that combined anti-EGFR therapy with concurrent CRT in the locally advanced setting and consistently confirmed lack of benefit of a triple modality strategy (10).

The authors discussed their negative results to originate (1) from the toxicity burden of RCT to be at the maximum tolerated level, resulting in RT interruption(s) in 26.9% of patients after adding cetuximab and (2) lack of benefit due to similar mechanisms of radiation sensitization by platinum derivatives and cetuximab such as inhibition of DNA damage repair.

In line with that, they argued that tumours having proficient repair machinery would be resistant to both modalities, while sensitive tumours would gain no additional benefit. Consequently, use of cetuximab with agents displaying different modes of action may improve sensitization. Interestingly, RTOG 0234, a phase II trial published in the same issue (11), investigated the feasibility of an antitubulin drug docetaxel-cetuximab-radiation adjuvant regimen versus cisplatin-cetuximab-radiation triplet strategy in terms of disease-free survival (DFS). The docetaxel regimen indeed showed favourable outcome, with improved 2-year DSF compared to both, the cisplatinarm of the trial (66% *vs.* 57%, respectively) and relative to a historical cisplatin-based control (RTOG-9501), thus supporting their hypothesis.

Given the dissatisfying outcome of the RTOG-0522 trial, however, additional determinants should be taken into consideration. Besides modulation of oncogenic intracellular mechanisms, cetuximab exerts its therapeutic activity by means of induction of an antibody dependent cell-mediated cytotoxicity. In line with that, there is growing evidence on a prognostic relevance of elevated levels of tumour infiltrating immune cells for RCT response in HNSCC (12). Thus, a role of both innate and adaptive immune responses (13) should be considered as relevant for cetuximab response in future preclinical and clinical investigations.

From a radiobiological point of view, lack of benefit might further arise from modulation of tumour cell cycle distribution after anti-EGFR treatment. Cetuximab is reported to induce a G1 arrest by upregulating the cyclindependent kinase inhibitors $p27^{CIP1/WaF1}$ and $p27^{KIP1}$ (14). This may augment the efficacy of RT in situations in which rapid repopulation of surviving tumour cells during fractionated schedules might counteract the radiationinduced cell eradication-a phenomenon that could well apply to the successful combination of sole RT and cetuximab for patients with HNSCC (7). The same, however, does not hold true for the triple combination of RT, chemotherapeutic drugs and cetuximab. Platinum based drugs exert their maximal radiosensitizing and cytotoxic potential when cells proliferate into the S/G2/M phases of the cell cycle. This effect might be impaired if the cells are arrested by cetuximab in the G1 phase before and during CRT resulting in diminished cytotoxicity and radiation efficacy. Furthermore, data suggested a sequence dependency of a cetuximab and platinum drug combination (15). In these studies, maximal synergy was observed when oxaliplatin was followed by cetuximab, but antagonistic effects were detected when cetuximab preceded oxaliplatin (15). Importantly, no study has yet clinically defined the best sequence of cytotoxic agents and cetuximab application for triple modality treatment.

Although a smoking history is considered to display a major risk factor for HNSCC, human papilloma virus (HPV) infection is increasingly associated with development of the disease with 36% of patients being virus-positive in a global statistical analysis in 2013 (16). HPV- or surrogate marker p16-positive patients represent a subset with better prognosis, treatment outcome and elevated average 5-year survival rates (17). Notably, HPV positivity is associated with a lower EGFR expression and lack of copy number events for EGFR ligands (18), suggesting that EGFR expression may display a negative prognostic marker in HNSCC.

In the RTOG 0522 trail, trends were evident for worse PFS (HR, 1.57; P for interaction =0.12) and OS (HR, 1.42; P for interaction =0.13) for patients with p16-positive oropharyngeal carcinomas receiving cetuximab slightly supporting this consumption. Histochemical detection of EGFR expression, on the contrary, could not support this thesis in the RTOG 0522 trial, probably due to a restricted availability of specimens from only 43% of patients.

Patients enrolled in the RTOG 0522 trial were not selected before treatment nor did the authors define subsets of patients likely to respond to cetuximab treatment. A multitude of biomarkers, including tumour EGFR expression, copy numbers and mutations in downstream signalling pathways (e.g., KRAS) have been suggested as predictive for cetuximab resistance in HNSCC (19). However, none of these markers is yet validated in prospective trials and a single marker is not expected to be sufficient for the prediction of a complex cetuximab resistance. Against this background, Lupini *et al.* very recently reported on a multigene next-generation sequencing approach in patients with colorectal cancer (20). In their analyses, mutation in coding sequences of 21 genes (e.g., KRAS, BRAF, PI3KCA, SMAD4), predicted unfavourable response to anti-EGFR antibody cetuximab and panitumumab treatment that may also be relevant in the head and neck situation.

In conclusion, treatment of patients with locally advanced HNSCC remains challenging. A combination of the EGFR antagonists' cetuximab and panitumumab with CRT, however, not only failed to show benefit over standard therapy but was associated with elevated toxicity and thus, is not a therapeutic revolution. Reasons for this failure are multifaceted and may include burden of toxicity, impaired DNA damage response, cell cycle effects, not fully understood immunologic effects and lack of selection of patients likely to benefit from EGFR inhibition. Although there are a number of ongoing randomized trials comparing the effect of cetuximab or alternative inhibitors plus RT or cisplatin based CRT in patients with HNSCC [for an overview see (19)], research activities should further focus on establishing an predictive EGFR sensitivity signature and optimizing sequences of application in a multimodal setting (21).

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Postoperative treatment for head and neck cancer: the emerging role of EGFR-targeted therapy

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Squamous cell carcinoma of the head and neck (SCCHN) commonly invades local structures and spreads to regional lymph nodes. Treatment with surgery alone is usually inadequate to achieve optimal locoregional control in locally advanced SCCHN, which has led to the use of postoperative radiotherapy in selected high-risk patients (1). Pivotal randomized trials reported in the early 90s showed that postoperative radiotherapy results in better locoregional control than preoperative radiotherapy and that the optimal dose is reached at about 63 Gy (2,3).

The addition of chemotherapy to radiotherapy was a further attempt to improve efficacy results in locally advanced SCCHN. Two phase III trials examined the added benefit of high-dose cisplatin at 100 mg/m² given every 3 weeks for 3 cycles in combination with radiotherapy as adjuvant treatment for completely resected highrisk SCCHN. The Radiation Therapy Oncology Group (RTOG) 9501 trial enrolled patients with 2 or more lymph nodes, extracapsular spread, or positive margins (4). This trial demonstrated an improvement in locoregional control and disease-free survival (DFS) but not overall survival (OS) with the addition of cisplatin to postoperative radiotherapy. An unplanned long-term analysis with a minimum follow-up of 10 years, perhaps due to the dwindling number of patients at late time points, failed to show a statistically significant benefit in any of the efficacy parameters (5). A second trial with a similar design that conducted by the EORTC (European Organisation for Research and Treatment of Cancer) had overlapping but distinct eligibility criteria. This study enrolled patients with stage III/IV disease, except T3N0 larynx; pT1-2N0-1 were required to have either perineural spread, extranodal spread, positive margins, or

vascular tumor embolism; also, patients with oral cavity or oropharyngeal tumors with spread to level IV or V lymph nodes were eligible (6). The EORTC trial showed not only an advantage in locoregional control and progressionfree survival with cisplatin but also a statistically significant survival benefit. Widely accepted high-risk features assessed at pathology review that necessitate postoperative radiotherapy are positive margins and extracapsular nodal spread, as shown in a combined analysis of the RTOG and EORTC trials (7). However, minor risk factors, such as perineural invasion, depth of invasion for tongue cancer, and number of lymph nodes, may be relevant for therapeutic decisions. Human papillomavirus (HPV) tumor positivity is a favorable prognostic factor in the postoperative setting; whether treatment strategies should be modified for patients with good prognosis HPV positive tumors remains to be determined (8).

Other non-platinum systemic agents have been investigated in combination with radiotherapy for locally advanced SCCHN. The taxanes, such as paclitaxel or docetaxel, are potent radiosensitizers, with antitumor activity in SCCHN. Nevertheless, no phase III trial with a taxane and radiotherapy in patients with locally advanced SCCHN has been reported yet. The advent of targeted agents has broadened the horizons in SCCHN therapeutics. Cetuximab, a chimeric monoclonal antibody against epidermal growth factor receptor (EGFR), significantly improved locoregional control, progression-free survival (PFS) and OS when combined with radiotherapy compared to radiation therapy alone as primary therapy in patients with locally advanced SCCHN. Notably, the addition of cetuximab to cisplatin and radiotherapy was investigated in RTOG 0522. This adequately powered phase III trial showed that cetuximab did not improve any efficacy endpoint in the primary treatment of locally advanced SCCHN, in either HPV-positive or -negative disease (9). However, the role of cetuximab as component of postoperative treatment is a subject of ongoing research. Moreover, it remains unclear which chemotherapy agent is the best to combine with cetuximab and radiotherapy in postoperative treatment.

Harari et al. reported the results of RTOG 0234, a phase II randomized trial that evaluated two cetuximab-containing doublets, cisplatin/cetuximab or docetaxel/cetuximab given concurrently with postoperative radiotherapy (10). Eligibility required completely resected pathologic stage III/IV SCCHN with positive margins, extracapsular nodal extension, or 2 or more positive lymph nodes. Patients were randomly assigned to 60 Gy radiation with cetuximab plus either cisplatin 30 mg/m² or docetaxel 15 mg/m² once per week. A total of 238 patients were enrolled in RTOG 0234. With an adequate follow-up of 4.4 years, 2-year OS was 69% for the cisplatin arm and 79% for the docetaxel arm; 2-year DFS was 57% and 66%, respectively. Similarly to analysis of other trials, patients with p16-positive oropharyngeal cancer tumors showed markedly improved survival outcome relative to patients with p16-negative oropharyngeal cancers. Toxicities in the two arms were within what expected; there was no difference in grade 3 to 4 mucositis between the two arms. The investigators compared the DFS reported in RTOG 0234 to that in the chemoradiotherapy arm of the RTOG 9501 trial. The comparison to this historical control yielded a hazard ratio of 0.76 for the cisplatin arm versus control (P=0.05) and 0.69 for the docetaxel arm versus control (P=0.01), corresponding to an absolute improvement in 2-year DFS of 2.5% and 11.1%, respectively. Therefore, DFS in both arms compared favorably to a historical control. However, the non-platinum regimen of cetuximab/docetaxel had numerically superior survival results to cetuximab/cisplatin. It is puzzling why the combination of cetuximab, cisplatin, and radiotherapy does not lead to optimal results in either the postoperative or the primary therapy setting. A plausible explanation for the lack of an added benefit with this combination may be that cetuximab and cisplatin have overlapping mechanisms of radiation sensitization, since they both inhibit the repair of DNA double strand breaks (11). It can be hypothesized that non-platinum cytotoxics may be optimal in combination with cetuximab and radiotherapy (12,13).

The NRG Oncology cooperative group is currently conducting a three-arm randomized phase II/III trial (RTOG 1216) in patients with high-risk resected SCCHN defined as extracapsular nodal spread or positive margins that compares adjuvant radiotherapy with docetaxel and cetuximab, to radiotherapy with either weekly cisplatin or docetaxel monotherapy (NCT01810913). Finally, for intermediate risk patients an ongoing phase III trial compares postoperative radiotherapy with or without cetuximab (NCT00956007).

Other EGFR-targeted agents, including the oral tyrosine kinase inhibitors lapatinib and afatinib are investigational in SCCHN. Lapatinib was evaluated in a phase III trial but did not demonstrate added benefit to standard radiotherapy and cisplatin (14). Afatinib is also being investigated as adjuvant therapy starting after completion of primary chemoradiotherapy (NCT01345669).

Postoperative treatment of SCCHN is evolving. Whether EGFR inhibitors have a role as components of combined modality approaches after curative surgery remains to be determined by ongoing trials. Other targeted agents, including immunotherapy, are worthwhile exploring as adjuvant therapy.

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Immune targeting of cancer stem cells in gastrointestinal oncology

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Abstract: The cancer stem cell (CSC) hypothesis postulates that a sub-population of quiescent cells exist within tumors which are resistant to conventional cytotoxic/anti-proliferative therapies. It is these CSCs which then seed tumor relapse, even in cases of apparent complete response to systemic therapy. Therefore, therapies, such as immunotherapy, which add a specific anti-CSC strategy to standard cytoreductive treatments may provide a promising new direction for future cancer therapies. CSCs are an attractive target for immune therapies since, unlike chemotherapy or radiotherapy, immune effector cells do not specifically require target cells to be proliferating in order to effectively kill them. Although recent advances have been made in the development of novel systemic and targeted therapies for advanced gastro-intestinal (GI) malignancies, there remains an unmet need for durable new therapies for these refractory malignancies. Novel immunotherapeutic strategies targeting CSCs are in pre-clinical and clinical development across the spectrum of the immune system, including strategies utilizing adaptive immune cell-based effectors, innate immune effectors, as well as vaccine approaches. Lastly, since important CSC functions are affected by the tumor microenvironment, targeting of both cellular (myeloid derived suppressor cells and tumorassociated macrophages) and sub-cellular (cytokines, chemokines, and PD1/PDL1) components of the tumor microenvironment is under investigation in the immune targeting of CSCs. These efforts are adding to the significant optimism about the potential utility of immunotherapy to overcome cancer resistance mechanisms and cure greater numbers of patients with advanced malignancy.

Keywords: Cancer stem cells (CSCs); aldehyde dehydrogenase (ALDH); CD24; CD44; CD133; immunotherapy; T cells; natural killer (NK) cells; vaccines; EpCAM

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Overview of cancer stem cells (CSCs)

CSCs, also known as tumor-initiating cells or tumorrepopulating cells, are a subset of cancer cells within the bulk tumor mass. This sub-population of cells exhibits a unique phenotype that mirrors that of embryonic or pluripotent stem cells, namely the capacity to self-renew, to differentiate (or repopulate bulk tumor mass), and to maintain homeostatic control (i.e., balance self-renewal and differentiation) (1,2). In extensive pre-clinical studies, the CSC phenotype has been modelled principally by colony engraftment in long term culture and by tumor formation in immune-compromised mice. Increasing studies have validated the presence of CSC subpopulations in nearly all human malignancies (3-5), and landmark tracking studies of genetically modified cells in intestinal adenomas, among other solid neoplasms, have identified a hierarchy of asymmetric cell division and tumor repopulation, providing the highest level of evidence to date that CSCs are clinically and biologically relevant (6-8).

Experimentally, the identification and characterization of CSCs has been predicated on the expression of cell surface markers such as CD24, CD44, and CD133 as well as the expression of the intracellular enzyme aldehyde dehydrogenase (ALDH) (9). CD24 is a cell surface glycoprotein anchored by a glycosyl-phosphotidylinositol tail (10). It is heavily glycosylated and is involved in both cell-cell and cell-matrix interactions. Although CD24 has been shown to have preferential expression on CSCs, it has also been identified on differentiated cancer cells (non-CSCs) in numerous malignancies as well as hematopoietic and neuronal cells (10). In addition, tissuespecific and epigenetic patterns of glycosylation suggest that CD24 affects diverse physiological functions, some of which remain incompletely characterized. These features underscore the plasticity of CSCs and the markers which identify them (9).

CD44 is a transmembrane glycoprotein which is expressed in normal cells as well as numerous cancer cells (9,10). It functions primarily as a hyaluronic acid receptor. In this way, it promotes and regulates cell migration. CD44 has also been identified as a key protein in cell adhesion, survival, differentiation, and interaction with the tumor microenvironment. Similar to CD24, CD44 has pleotropic effects, including roles in multiple signaling cascades, so the precise mechanism by which it fosters the CSC phenotype is not well defined (9).

CD133 (prominin-1) is a transmembrane glycoprotein which was initially described as a marker of human hematopoietic progenitor cells (9,11). CD133 was subsequently discovered on primitive neural tissue, and seminal studies subsequently linked CD133 expression to tumor initiation and propagation in immunodeficient mice (12). Subsequent investigations demonstrated CD133+ CSCs in diverse GI malignancies, including pancreas, biliary, gastric, and colorectal (9). Investigators have also observed that CD133 expression is critical to the production of the plasma membrane, frequently in combination with cholesterol (11). As a result, CD133 has been referred to as the "organizer" of the plasma membrane. However, a complete understanding of the ligands for CD133 nor its downstream targets has not been fully clarified, leaving some ambiguity regarding its biological functions.

ALDH represents a class of enzymes important to numerous biochemical and metabolic cellular processes, including detoxification of enzymes and retinoic acid synthesis. Elevated ALDH activity is closely linked with the CSC phenotype (9,13). Although investigators have demonstrated other cell surface markers to correlate with the CSC phenotype (notably EpCAM in pancreatic cancer) and the expression of CSC markers has been shown to vary depending on experimental conditions and tumor type, these markers have, nevertheless, been consistently identified as CSC markers in multiple gastrointestinal (GI) malignancies. Furthermore, enriched CSC populations are predictive for worse oncologic outcome in numerous cancers, including GI (4,14-19). Although the mechanism by which ALDH and other cell surface markers confers a CSC phenotype is not definitively known, overexpression of these molecules has been associated with the CSC phenotype, while knockout or inhibition has been associated with loss of the CSC phenotype in multiple preclinical cancer models, including pancreatic and upper GI malignancies (20-23).

Similarly, epithelial-to-mesenchymal transition (EMT) is a process by which cells acquire an increased invasive and mutable phenotype. In fact, accumulating evidence indicates that EMT enables tumors to acquire a metastatic phenotype. Although controversial, there is emerging evidence that CSCs may promote the development of EMT. For example, in a model of pancreatic cancer exposure to TGF-beta upregulated CSC markers, leading to decreased E-cadherin expression, increased invasion *in vitro*, and increased metastases *in vivo* (24). Similarly, Su *et al.* showed that TGF-beta exposure in pancreatic cancer increased stem cell markers and features of the CSC phenotype via the SMAD4 pathway (25). Other authors have maintained that CSCs share the activation of common pathways with EMT, but may represent two distinct phenomena (26).

Traditional anti-cancer therapeutic strategies target proliferating cells through cytotoxic effects or targeted inhibition of pro-proliferative signaling pathways. The significantly reduced proliferative state of CSCs appears to impart these cells with intrinsic chemoresistance, and anti-proliferative therapies such as chemotherapy and radiotherapy have been shown to enrich for CSCs (12,27-31). As a result, CSCs are able to survive and remain viable in a quiescent state, and ultimately, this capacity allows CSCs to promote relapse and demise at a subsequent date, even after a period of so-called remission.

CSC biology in GI malignancies

In the past decade, there have been significant advances in the field of CSC biology (2). The emerging evidence has demonstrated that CSCs play critical roles in drug resistance, invasion, and metastasis. Furthermore, although CSCs and non-CSCs within the same tumor share similar

genetic fingerprints, there are distinct transcriptional patterns observed between CSCs and non-CSCs, highlighting the importance of plasticity and epigenetic modifications in regulating CSCs and non-CSCs. Furthermore, the activation of disparate pathways, such as hedgehog, TGF- β , and Wnt/ β -catenin, between CSCs and non-CSCs suggests that effective therapy may require selective targeting of these distinct cell populations (32).

The clinical relevance of CSC populations has been demonstrated in numerous GI malignances, including pancreatic, gastro-esophageal, colon, and biliary (33-37). For example, Rasheed et al. performed a detailed analysis of pancreatic cancer xenografts (38). These authors demonstrated that ALDH-positive cells were significantly more clonogenic in vitro and in vivo compared with unsorted or ALHD-negative cells. These ALDH-positive CSCs expressed genes consistent with a mesenchymal state and had substantially greater in vitro migratory and invasive behavior. Using ALDH, as well as CD24+CD44+EpCAM+ cells, other investigators have similarly identified pancreatic cancer cells that have CSC and mesenchymal features (39,40). The enhanced clonogenic growth and migratory properties of these stem-like pancreatic cancer cells (ALDH and/or EpCAM-positive) suggest that they play a key role in the development of metastatic disease and oncologic outcome of patients with pancreatic adenocarcinoma. Although these cells have been phenotypically and functionally well characterized, we still know very little about their genetic and epigenetic aberrations. Further analyses should reveal CSC-specific oncogenes and tumor suppressor genes.

Similarly, CSC behavior has been identified in hepatocellular carcinoma (HCC), and key studies have demonstrated the plasticity and epigenetic regulation of CSCs and non-CSCs. For example, Yimlamai et al. demonstrated that inactivation of the Hippo pathway, a regulator of cell proliferation, fostered the dedifferentiation of adult hepatocytes into cells with progenitor characteristics and CSC features (41). Villaneuva et al. identified increased Notch activation in human HCC samples, suggesting that this pathway is triggered in HCC development. Furthermore, pre-clinically, the authors observed that activation of Notch signaling correlated with biliary cancer formation via insulin-like growth factor 2, and this process was inhibited by novel γ -secretase inhibitors which inhibit the Notch pathway (42). Ultimately, the high penetrance of CSCs in this tumor model will allow for a better understanding of their biological features such as the

regulation of proliferation and progression to metastases.

CSCs have been shown to be the source of treatment resistance and eventual progression of disease. Even the recent development and introduction of targeted therapies such as tyrosine kinase inhibitors (TKIs) is associated with temporary tumor response and the subsequent development of resistance (43). Furthermore, CSCs display increased levels of the DNA checkpoint kinases, such as Chk1 and Chk2, which may play a further role in their resistance to genotoxic stress (44). These findings are not surprising given that these therapies predominantly rely on DNA damage to induce mitotic cell death (45,46). Overall, the emerging data support the concept that CSCs are important to cancer biology. Therefore, it will be important to design strategies to target CSC subsets within tumors to prevent relapse and advance multidisciplinary cancer therapy.

T cell immunotherapy

T cells, particularly cytotoxic T cells, form the principal immune effector cell of the adaptive immune system. The fundamental properties of a cytotoxic T cell response (including antigen specificity, clonal expansion, and memory) have made CD8+ T cells essential features of successful immune-based strategies toward cancer (47). Since studies indicate that CSCs are the reservoir of differentiated tumor cells and the putative source of metastases, attention has focused on using T cell therapies to specifically target CSCs.

Visus et al., for example, demonstrated that ALDH^{high} cells derived from human cancer cell lines, including pancreatic, could be used to induce a CD8+ T cell response. ALDH^{high} cells were sorted by fluorescence-activated cell sorting (FACS) and exposed to CD8+ T cells in vitro along with dendritic cells isolated from HLA-A2-restricted healthy volunteers. In some experiments, an additional step of an artificial, engineered antigen-presenting cell was also used. These CD8+ T cells were then adoptively transferred into tumor-bearing mice, and the authors observed that this strategy inhibited tumor growth and metastasis formation while survival was prolonged. This study is a notable demonstration of the concept that CSCs, in general, and ALDH1A1, in particular, are potential therapeutic target for T cell immunotherapy to selectively target CSCs in solid tumors (48). Luo et al., utilizing a similar approach, sorted ALDH^{high} cells and then co-cultured them with dendritic cells to stimulate CD8+ T cells with specificity to ALDH^{high} CSCs (49). Subsequent CD8+ T cells were found to recognize and lyse ALDH^{high} CSCs. The authors further

demonstrated significant reductions in tumor growth and improvements in survival in a mouse model. It should be noted, however, that the authors did not demonstrate that the CD8+ T cells were mediating their anti-tumor effects *in vivo* by eliminating ALDH^{high} CSCs.

Huang *et al.* engineered an anti-CD3/anti-CD133 bispecific antibody (BsAb) linked to cytokine-induced killer cells (50). In both *in vitro* and *in vivo* models of pancreatic and biliary cancer, the authors observed enhanced tumor killing and loss of CD133 positive cells with their BsAb. Despite these impressive results, it remains to be seen whether this novel therapy will have similar effects in models where CD133 is not expressed at such high levels, especially in unmanipulated primary tumors where CSC populations are frequently a small minority of the overall bulk tumor population.

The tremendous advances in the treatment of hematological malignancies using engineered T cells transduced with chimeric antigen receptors (CARs) has created substantial interest in using this cell-based immunotherapy for solid cancers (51). Following the collection of a patient's T cells, the cells are genetically engineered to express CARs specifically directed towards antigens on the patient's tumor cells. These modified T cells are then infused back into the patient. Adoptive transfer of T cells expressing CARs is a promising anticancer therapeutic as CAR-modified T cells can be engineered to target virtually any tumor-associated antigen. Given the experience in hematologic malignancies, there is great potential for this approach to improve patient-specific cancer therapy in a profound way.

Given the lack of meaningful treatment options for patients with advanced/refractory GI malignances, these cancers appear to be optimal candidates for the application of CAR therapy. However, a key feature of CAR therapy is selection of the target antigen to maximize selectivity to the tumor and minimize off-target effects/toxicity. In preclinical models, CAR T cells have been designed to target CD133+ (52), chondroitin sulfate proteoglycan 4 (with structure and function similar to CD24) (53), and epidermal growth factor receptor variant III (which is preferentially expressed on glioma stem cells) (54). Although these studies demonstrated proof-of-concept that CAR T cells could be engineered and expanded to recognize CSC targets, they were limited by their reliance on *in vitro* and *ex vivo* experimental designs.

Although adoptive transfer of CAR-modified T cells is a unique and promising cancer therapeutic, there are significant safety concerns as well as questions regarding the sustainability and affordability of this technology. Particularly in solid cancers where there is overlap in the expression of target antigens between healthy and neoplastic tissue, clinical trials have revealed toxic effects of CARs, including CAR-mediated recognition of target antigens in normal tissues. In some cases, the toxicities have paralleled those observed with graft-versus-host disease, and importantly, rare cases of fatal adverse events have been reported (55). These toxicities highlight the need for welldesigned and rigorously conducted pre-clinical and early stage clinical trials to evaluate CAR therapy in the immune targeting of CSCs since these CSC markers are also present on normal stem cells in diverse tissues.

A potential solution to the toxic side effects of CAR T cells is engineering a suicide gene into the modified T cells (56). When activated, the suicide gene triggers apoptosis in the CAR T cells, thereby reining in potential immune-related toxicity. Adoption of suicide gene therapy to the clinical application of CAR-modified adoptive T cell transfer has potential to alleviate toxicity, but concerns exist about the ability to optimally control and decouple the anti-tumor effects of the treatment while minimizing the toxicity. Nevertheless, clinical trials using CAR technology have been initiated in pancreatic cancer targeting both CEA and mesothelin, and results from these trials are eagerly awaited (57).

Natural killer (NK) cell immunotherapy

Characterized by the expression of CD56 and a lack of T cell markers, such as CD3 or the T-cell receptor (TCR), NK cells are efficient effector cells of the innate immune system. They are able to recognize and kill virally-infected and malignant cells, primarily because of modulations in MHC-I and MHC-Ib molecules on target cells. Two distinct immunotherapy strategies utilizing NK cells have evolved: one which harnesses endogenous NK cells by administering NK stimulants or targeting agents, and one which uses exogenous NK cells via adoptive cell transfer. Each of these approaches is under investigation in the immune targeting of CSCs.

There are several key advantages to harnessing NK cells (58). First, NK cells are antigen non-specific and therefore do not require the expression of a specific antigen on a given HLA allotype. In contrast, therapies targeting a specific antigen are dependent on the presence of that antigen. While antigen-specific therapies may be

highly effective and achieve long-term responses in many cases, antigen-shedding and escape variants can limit the effectiveness of this approach. Second, NK cells can be easily isolated and expanded *ex vivo* which allows for their use in adoptive cell therapies. Third, NK cells have a shorter lifespan than T cells. Whereas T cell adoptive therapies, such as CARs, often require a suicide vector to prevent the sequela of over-expansion of the transferred cells, NK cells, unless genetically altered, have a lifespan on the order of one month or less which effectively eliminates the risk of chronic toxicities which has been observed with CAR T-cell therapy.

CSCs have recently been demonstrated to be highly susceptible to NK cell attack, suggesting that NK cells may be useful as part of a combined modality approach capable of targeting CSC and non-CSC populations. Tseng et al., for example, demonstrated in both human and mouse models that stage of differentiation for both malignant and embryonic cells predicted their sensitivity to NK cell lysis (59). These authors also reported that inhibition of differentiation or reversion of cells to a less-differentiated phenotype by blocking NFkappaB or targeted knock down of COX2 significantly increased NK cell effector functions. Tallerico et al. demonstrated that freshly purified allogeneic NK cells can recognize and kill colorectal carcinomaderived cancer-initiating cells (CICs) whereas the non-CIC counterpart of the tumors was less susceptible to NK cells (60). This difference in the NK cell susceptibility was correlated with higher expression on CICs of ligands for NKp30 and NKp44 in the natural cytotoxicity receptor group of activating NK receptors. In contrast, CICs were shown to express lower levels of MHC class I on their surface than do the "differentiated" tumor cells, and MHC class I molecules are known to inhibit NK recognition and function.

The results of human clinical trials using autologous NK cells as monotherapy to treat advanced cancers have largely been disappointing, leading some investigators to conclude that autologous NK cells, in the setting of active malignancy, are inherently dysfunctional and/or hyporesponsive because of the host's immune environment (61). Accordingly, recent interest has focused on the therapeutic potential of allogeneic NK cells, primarily because of increasing evidence that NK cells become maximally activated and cytotoxic when they recognize cells lacking self MHC molecules (i.e., the "missing self" hypothesis). The selective targeting of the CSC population with NK immunotherapy (after or in combination with initial tumor debulking using cytotoxic therapies) is a novel and innovative approach

which our lab and others are using to overcome the previous limitations of adoptive NK transfer. Our laboratory is actively studying the capability of *ex vivo*-activated autologous NK cells to target CSCs in a combination approach, and we hypothesize that NK targeting of CSCs in the appropriate multimodality setting will translate to durable anti-tumor effects. Although questions remain regarding how to best optimize expansion, activation, delivery, and homing of NK cells, should the targeting of CSCs by NK immunotherapy prove to be feasible for even a select subset of GI cancer patients, then this approach will have significant clinical impact

Vaccines

The best source of tumor antigens may be autologous, selfrenewing CSCs that are proliferating in cell culture (62). As they proliferate in cell culture, such cells increasingly express phenotypic markers that are associated with invasiveness and "stemness". The efficiency of such cell cultures can be enhanced by utilizing specialized culture conditions which leads to spheroid formation, a marker of stem-like cells. Yin et al. observed decreased expression of MHC class I molecules on pancreatic CSCs in culture consistent with previous observations that CSCs are able to evade antigen-specific immune attack (63). However, these authors pulsed DCs with these in vitro CSC lysates and found the DCs were able to stimulate an effective cytotoxic effect against both CSCs and bulk tumor cells in their model. Similarly, in a breast cancer model, Mine et al. pulsed immature DCs (iDCs) with a Numb-1 peptide, a membranebound protein which plays an important role in asymmetric cell division and regulates Notch, a highly conserved regulator of cell differentiation and homeostasis (64). The authors then exposed these iDCs to non-adherent peripheral blood mononuclear cells and observed an expansion of antigenspecific CD8+ cells. However, despite this novel finding, the authors did not demonstrate the translation of these immunological effects into improved anti-tumor therapy. In contrast, Duarte et al. used FACS to isolate ALDH^{high} colon cancer cells in a rat syngeneic model (65). Immediately after sorting, cells were seeded in culture, lysed by freeze-thaw, and injected intraperitoneally with CpG as an immune adjuvant. Using this CSC-based vaccine approach, animals demonstrated a significant reduction in tumor growth and metastasis.

Tanida *et al.* demonstrated notable anti-tumor effects *in vivo* including improved mouse survival using a polyvalent vaccine designed to express α -gal epitopes (66). This study

demonstrated important translational relevance since the authors' vaccines were derived and engineered from clinical samples of primary pancreatic cancers. However, an important limitation of their study was the *in vivo* evaluation of their vaccine in α 1,3-galactosyltransferase knockout mice. This approach raises concerns that the specificity of their CSC vaccine for tumor antigens may be falsely elevated in this knockout model with the potential for less efficient targeting of CSCs where non-neoplastic α -gal is expressed. In addition, the use of knockout mice deficient in homologous antigens may mask potential toxicity.

Consequently, as with all vaccine-based approaches, there remains a concern that vaccines targeting CSCs will stimulate an immune response against non-neoplastic host tissues which express comparable antigens important for host functions. As with CAR therapy, this could lead to toxicity as well as the potential for auto-immunity, particularly since CSCs share similar antigens to healthy stem cells. Some authors have also questioned the effect of the mode of delivery of vaccines on outcome. The local delivery of vaccines may be limited by the immunosuppressive nature of the tumor microenvironment, while the systemic delivery of vaccines may be limited by the ability of primed immune effector cells to home/traffic to their targets in the tumor. A combined approach using both systemic and local delivery of vaccines may produce stronger anti-tumor responses, but this raises the possibility of greater toxicity.

Nevertheless, vaccine-based approaches targeting CSCs have the potential to evoke long term antigen specific memory to both treat advanced GI malignancies and prevent their recurrence. The ideal CSC vaccine would integrate and activate both the innate and adaptive arms of the immune system.

Tumor microenvironment

It has long been recognized that not all tumor cells are capable of propagating tumors in pre-clinical models of cancer. Although CSCs have been implicated to account for the heterogeneity identified within tumors, it has also been established that the tumor microenvironment directly interfaces with developing tumors and contributes to local immunosuppression as well as the CSC phenotype (67). Increasing studies are pointing to the importance of the tumor microenvironment to CSC maintenance, EMT transition, and oncologic outcomes. For example, Yamashina *et al.* demonstrated that CSCs were a source of immunosuppressive cytokines (GM-CSF among others), and the elaboration of these cytokines generated myeloid-derived suppressor cells (MDSCs) and M2 macrophages, both of which were associated with chemoresistance (68). As noted above, Wang *et al.* introduced TGF- β into pancreatic cancer models and observed increased invasiveness, angiogenesis, and metastasis formation as well as cells with a CSC phenotype (69). Lin *et al.* observed that IL-6 promoted CSC proliferation in colon cancer CSCs through a STAT3 dependent pathway (70). They further observed that inhibition of IL-6 or its receptor was able to counteract these effects, suggesting that immune modulation of the tumor microenvironment may be an effective strategy for CSC targeting.

Zoglmeier et al. evaluated CpG treatment on MDSC phenotype and function in a mouse model of gastric neoplasia (71). Their results indicated that TLR9 activation via CpG significantly decreased MDSC suppressive function in tumor-bearing mice. Although the authors did not assess for CSC-specific effects of their MDSC-targeting strategy, the authors suggested this mechanism as an avenue for further study. Wang et al. demonstrated that IL6 ligand and receptor expression contributed to CSC growth and survival in a glioma model (72). Furthermore, they showed that inhibition of IL6 ligand and receptor expression in CSCs increased survival of mice bearing orthotopic human xenografts. Although similar studies have not been performed in GI malignancies, there is enthusiasm that CSC targeting via IL6 antagonism may offer therapeutic benefit for advanced cancer patients.

The recent development of immune checkpoint inhibitors has demonstrated the untapped, and previously unharnessed, power of the immune system to reject malignancies and lead to sustained, long term responses (73). Yet, despite the excitement surrounding immune checkpoint inhibitors, novel approaches are needed to deliver the promise of immunotherapy to greater numbers of cancer patients (74). Recent impressive results in clinical trials of PD-1 and PDL-1 inhibitors have generated notable enthusiasm surrounding these therapies. Since quiescent/dormant CSCs must develop a mechanism of immune escape to avoid elimination by immune surveillance, it is plausible to postulate that immune checkpoint inhibitors may preferentially target CSCs and the CSC niche. This hypothesis is supported by several key publications showing that mesenchymal stem cells utilize the PD-1/PDL-1 axis to suppress inflammation and inhibit the immune response (75,76). However, as yet, there is

little pre-clinical or clinical evidence to support the notion that the impressive clinical efficacy of immune checkpoint inhibitors is acting via an anti-CSC mechanism.

Potential limitations

Immune targeting of CSCs in the stem cell niche and tumor microenvironment poses inherent challenges which may limit its potential clinical translation. Studies have shown that CSCs are less immunogenic than non-CSCs, and CSCs may downregulate many tumor-associated antigens, thereby limiting the ability of the adaptive immune system to recognize and mount an antigen-specific response to CSCs. In addition, although potentially limited by using a strictly in vitro model, Volonté et al. demonstrated that colon cancer CSCs express both membrane-bound and soluble IL-4 (77). This CSC-mediated inhibitory signaling could negatively downregulate anti-tumor T cells responses designed to target CSCs in vivo. Similarly, IL-4 levels have observed to promote tumor proliferation, invasion, and metastases in pre-clinical models of cancer, suggesting an important role of this cytokine in the immunosuppressive phenotype which is potentially preferentially regulated by CSCs (78,79).

In addition, key studies have observed evasion of immunosurveillance through shedding of MICA and MICB by CSCs and apparent CSC recruitment of regulatory T cells to promote an immune privileged state (80,81). Furthermore, in a notable study with important translational implications, Kryczek et al. observed that IL-22 promoted a CSC phenotype in both pre-clinical and patient-derived models (82). These authors then determined that CD4+ T cells were a source of IL-22 secretion, and that a higher concentration of IL-22 was associated with a worse oncologic outcome. Collectively, the findings of Kryzczek et al. highlight a fundamental point of the immune system with respect to CSCs or any other target cell, namely that it can be primed both for and against immune targeting. It will be important to recognize and address these potential limitations to ensure that the optimal results from these novel approaches are achieved.

Summary and conclusions

Accumulating evidence suggests that CSCs exist as a subpopulation of quiescent cells within the dominant tumor bulk of heterogeneous tumor cells (1,2). These typically dormant cells are considered resistant to standard anti-cancer therapies such as chemotherapy and RT. They also are capable of selfrenewal and differentiation (28-31), suggesting that CSCs are responsible for tumor repopulation after bulk tumor has been destroyed (8). Targeting the CSC population will be critical to additional meaningful advances in cancer treatment, especially for difficult to treat GI malignancies. There is significant optimism that a multimodality approach using immunotherapy in combination with cytotoxic treatments to simultaneously eradicate CSCs and non-CSCs will lead to more complete and durable cancer eradication. Immune targeting of CSCs holds significant promise in the ultimate goal of overcoming cancer resistance and curing more patients with cancer.

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Footnote

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The SCOPE of definitive chemoradiotherapy in locally advanced esophageal cancer: what direction for the future?

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Exclusive chemoradiotherapy (CRT) delivering 50 Gy over 5 weeks with cisplatin and fluorouracil-based chemotherapy is a cornerstone in locally advanced esophageal cancer or non-operable patients since the results of the pivotal study of US Intergroup RTOG-8501 (1). This trial has successfully demonstrated that some patients with esophageal carcinoma may be long-term survivors so that this treatment is now definitely accepted as curative (2). Nevertheless the prognosis is still very disappointing with a 5-year overall survival rate of approximately 25%. Attempts to improve overall survival by escalating the dose of radiotherapy with concurrent cisplatin and fluorouracil has been assessed in INT 0123 trial (3). Overall survival rate after 64.8 Gy was not superior or even lower to 50.4 Gy. This result precluded dose escalation in esophageal cancer for more than a decade. However, the results of these two studies established CRT with the 50.4 Gy dose as the standard of care in esophageal cancer.

Given that, several strategies such as upfront chemotherapy or taxane-based definitive CRT have been tested in prospective randomized trials with no improvement in overall outcomes due to harmful or even lethal significant toxicities (4,5).

Crosby *et al.* published in the *Lancet Oncology* the mature results of the SCOPE1 trial which compared 50 Gy CRT with cisplatin and capecitabine with or without cetuximab, a monoclonal antibody targeted toward the Epidermal Growth Factor Receptor (EGFR) (6). The level of EGFR expression in biopsies was not used as an inclusion criterion in the trial. Inclusion criteria included patients with favorable performance status selected to receive potentially curative definitive CRT by a specialist upper gastrointestinal multidisciplinary team. Tumors had to be staged with both endoscopic ultrasound and spiral CT scan to be T1–4 N0–1 M0. Patients should be able to swallow capecitabine, and in case of severe dysphagia, they have received protracted intravenous infusion of fluorouracil 225 mg/m²/d for 84 days.

The control group consists of cisplatin (60 mg/m² IV Day 1 of 21 day cycle for 4 cycles) and capecitabine (625 mg/m² po bid days 1–84) and, from week 7, radiotherapy (50 Gy in 25 fractions over 5 weeks, 2 Gy per fraction). The experimental group consists of the above plus cetuximab (400 mg/m² day 1 of the first week, then 250 mg/m² weekly thereafter for a further 11 weeks).

Randomisation was stratified by recruiting hospital, primary reason for not having surgery, tumour stage, and tumour histology. 72% had squamous-cell carcinoma. Tumor length and stage have been well balanced between groups. However, no data are available on weight loss at inclusion which is a major prognostic factor (7,8). 86% of patients had a ¹⁸F-fluorodeoxyglucose PET-CT scan to exclude metastatic disease and to identify lymph node involvement before starting radiotherapy.

Surprisingly both study groups received neoadjuvant chemotherapy before definitive chemoradiation. The authors stated that this schedule is the most frequently used regimen in the UK, because it allows time for careful radiotherapy planning, it allows better compliance and a shrinkage of the tumour before radiotherapy (6,9). However to our best knowledge, this sequence has not been tested in randomized phase III trials versus definitive chemoradiation first which is still the standard of care for non-surgical candidates. Response rate during neoadjuvant phase has not been reported in the paper.

Non-inferiority of capecitabine as compared to infusional fluorouracil has been demonstrated in the REAL-2 study comparing three triplet therapies in advanced esophagogastric cancer (10). In this study, 34.5% of the patients had esophageal cancer, 10.5% had squamouscell carcinoma and 22.7% had a locally advanced tumour. However all tests for heterogeneity with regard to treatment effect, including the histologic type and the anatomic subsite of the tumor did not reveal any significant heterogeneity. The feasibility of concurrent CRT with capecitabine and cisplatin for patients with esophageal carcinoma was evaluated in small phase II in single-centre series (9,11).

In the SCOPE 1 trial, 258 patients were recruited, 129 in each group. The primary endpoint of the phase 3 trial was overall survival. The CRT plus cetuximab group had a significantly shorter median overall survival [22.1 months (95% CI: 15.1-24.5) vs. 25.4 months (20.5-37.9); adjusted HR 1.53 (95% CI: 1.03-2.27); (P=0.035)]. These median survivals are among the best achieved in the literature. Patients randomized to exclusive CRT with cetuximab had a lower compliance to CRT. 19% of the patients in the cetuximab group had no radiotherapy given, versus 8% in the control arm (P=0.006), may be due to progressive disease during neoadjuvant chemotherapy or increased toxicities. Patients who received CRT plus cetuximab had also more grade 3-4 non-haematological toxicity (79% vs. 63%; P=0.004) when compared to control group patients. These toxicities were mainly dermatological, biochemical, and cardiac disorders (8 grade 3-4 cardiac events in the cetuximab group versus 2 in the control arm).

In subgroup analyses with respect to baseline characteristics, no subgroup benefit from cetuximab was observed. Evidence of lower local progression-free survival (within the radiotherapy field) was observed in the experimental group (HR 1.38, P=0.051). On multivariate analysis, stage 1–2 vs. stage 3, full-dose radiotherapy and higher cisplatin dose intensity (\geq 75% vs. <75%) were associated with improved overall and progression-free survival. Patterns of recurrence were similar in both arms. The proportion of patients with salvage surgery in each arm is unknown.

Patient-reported outcomes were secondary end points

in this trial. Quality of life was assessed using the EORTC QLQ-C30, the esophageal module QLQ-OES18 and the dermatology life-quality index (DLQI). Questionnaire compliance was good throughout the study (12). After neoadjuvant chemotherapy, there was no change in the proportion of patients with severe dysphagia, but dysphagia significantly increased after chemoradiation. Significant deterioration in functional scores and more problems with fatigue, dyspnea, appetite loss and troubles with taste were reported during CRT. Patients receiving cetuximab reported was overall higher DLQI scores than those receiving CRT alone, but the difference was not significant.

The findings of the SCOPE1 trial are in line with initial report of RTOG 0436 study which also found no improvement in survival for esophageal cancer patients when adding cetuximab (400 mg/m² day 1 then weekly 250 mg/m²) to weekly concurrent cisplatin (50 mg/m²), paclitaxel (25 mg/m²), and radiation 50.4 Gy over five weeks (13).

The addition of cetuximab to CRT has been further explored in head and neck squamous cell cancers in the randomized phase II trial RTOG 0522 which showed no benefit in overall survival and increased grade 3 or higher mucositis and skin toxicities (14). Hence, the remaining question could be whether cetuximab alone combined with radiation may improve outcome with less toxicities and a better compliance? A randomized phase II study has recently compared CRT with cisplatin and 5FU-platinumbased chemotherapy *vs.* cetuximab alone in head and neck squamous cell cancers (15). Although the study was closed prematurely, toxicities were still significantly increased with even more toxic deaths (4 *vs.* 1) with more frequent nutritional support for patients treated with cetuximab monotherapy concomitantly with radiotherapy.

In parallel, another way to increase the therapeutic ratio using an efficient and less toxic chemotherapy scheme has been explored in the PRODIGE 5 trial using a FOLFOX4 regimen (16). In this phase III trial, 267 patients treated with definitive 50 Gy CRT were randomised between the RTOG regimen (four cycles (two concomitant to radiotherapy) of fluorouracil 1,000 mg/m² per day for 4 days and cisplatin 75 mg/m² on day 1) or the same radiotherapy scheme combined with FOLFOX4 [6 cycles (three concomitant to radiotherapy) of oxaliplatin 85 mg/m², leucovorin 200 mg/m², fluorouracil bolus 400 mg/m², and infusional fluorouracil 1,600 mg/m² over 46 h every 2 weeks]. Although not superior, FOLFOX4 provided similar overall survival than that with the RTOG regimen whereas less toxic deaths occurred with 50 Gy + concomitant FOLFOX4 (1% vs. 5%). Hence the American Society of Clinical Oncology (ASCO) advised toward an acceptable treatment option in keeping with the standard RTOG regimen. The widespread use of exclusive chemoradiation with FOLFOX4, a more convenient regimen, is now rapidly growing in some European countries.

From this standpoint, how can we move forward with more long-term survivors and no increased lethal or harmful toxicities with exclusive chemoradiation?

Locoregional control remains the first cause of failure so far with still roughly half of the patients who will have a persistent tumor or who will develop a local and/or regional relapse. In the RTOG 85-01 study, the patients in each arm received elective nodal irradiation from supraclavicular fossa to esogastric junction up to 30 Gy using an outdated 2D technique (1). RTOG 85-01 results (2) showed 37% of persistent disease in the radiotherapy alone group versus 25% and 28% in the CRT randomized and non-randomized group, respectively, and there were 16% of locoregional failure in the radiotherapy-alone group versus 13% and 20% in the CRT randomized and non-randomized groups, respectively (2). The low prophylactic nodal dose used, 30 Gy, might have been too low and the evaluation of nodal status less accurate than today. This hypothesis has been recently verified in the CROSS trial where 188 were randomly assigned to the surgery arm and 178 to the CRT plus surgery arm (17). A total radiation dose of 41.4 Gy was administered in 23 fractions of 1.8 Gy. The locoregional failure rate without distant metastasis was 9.3% in the surgery alone arm versus 3.3% in the CRT plus surgery arm. These results suggest that elective nodal irradiation reduces locoregional failure, which was significantly lower than that observed with surgery alone or with CRT alone. The hypothesis is that CRT plays an important role, probably by sterilizing the microscopic nodal disease, and that the total dose of radiotherapy without surgery may be too low to control macroscopic disease, suggesting that large volumes and high doses are required.

The INT 0123 study was conducted to compare a combined modality treatment with the same scheme of chemotherapy and different doses of radiotherapy (3): the control group received a standard dose (50.4 Gy) and the experimental group received a higher dose of RT (64.8 Gy). Unfortunately, because of 11 treatment-related deaths, only 67% of patients received the high radiation dose compared with 83% in the standard dose group. Despite flaws due to the high mortality of patients, the two-year survival of

31% and 40%, and a cumulative incidence of local failure of 52% and 56% in the standard-dose and high-dose group, respectively. Again, nearly half of the patients had still a locoregional persistent or recurrent disease rates. Several drawbacks surround the interpretation of the results of INT 0123. Firstly, the equivalence in survival between the two groups was influenced by a high number of intercurrent deaths among patients on the dose escalation arm (11 vs. 2 deaths). In point of fact, this may not be due to radiation dose escalation as a majority of deaths in the high-dose arm occurred before receiving a cumulative dose greater than 50 Gy. Obviously, most of the causes of deaths were related to chemotherapy. Secondly, no elective nodal irradiation was delivered compared to RTOG 85-01. Lastly, dose escalation was performed on the primary tumor only but not on positive nodes.

Given the above, the differences in local control and survival rates between RTOG 85-01 and INT 0123 were not significant so it was difficult to conclude with evidence that dose escalation could be beneficial. Conversely, the Surveillance Epidemiology and End Results (SEER) program have found a significant correlation between survival and increments of +5 Gy of radiation dose escalation up to 65 Gy in a cohort of more than 5,000 patients treated with exclusive CRT (18). Radiation dose escalation studies showed that nowadays, 60 Gy or higher can be safely delivered in routine practice in most radiotherapy centers (19).

The main problem with esophageal cancer is the close proximity of organs at risk, particularly the heart and lungs. Most of the time, the difficulty is to limit the irradiated volume or the total dose so as not to compromise the benefit/risk ratio. In this context, Intensity-Modulated Radiotherapy (IMRT) (20) or Volumetric Modulated Arctherapy (VMAT) (21) could be used to increase mediastinal irradiated volumes or dose escalation to the primary tumor while effectively protecting healthy tissues. In the MD Anderson Cancer Center retrospective experience on 676 patients with esophageal cancer treated by exclusive CRT (of whom 263 were treated by IMRT), the authors showed that loco-regional control and overall survival were significantly better for IMRT than for threedimensional radiotherapy (22). These results need to be confirmed in a prospective study. These techniques decrease the volumes of lung and heart (23) that receive a high dose, but at the cost of delivering low doses to a greater volume of lung and normal tissues. The authors found that IMRT provided a significant lower rate of non cancer-related deaths, including cardiac-related deaths, the second cause of death after cancer in esophageal cancer patients treated with CRT (22). A more accurate approach would be VMAT combined with active breathing control using moderate deep-inspiration breath-hold to reduce doses to the lung and to improve targeting (24).

In line with Crosby *et al.* (6), we strongly believe that dose escalation should be retested with modern radiotherapy techniques, such as IMRT or VMAT with image-guided radiotherapy (IGRT). Accordingly, we recommend a thorough RT Quality Assurance review to make sure this treatment modality is reproducible with an acceptable compliance.

Based on data from SCOPE1 trial, investigators have explored radiobiological modeling of dose escalation for esophageal cancer and found that a +18% increase in tumor control could be achieved with a modest increase in the risk of cardiac and lung toxicities for nearly 75% of patients. The SCOPE trialists have launched a new phase III trial (SCOPE2) that will address the issue of radiation dose escalation up to 60 Gy using modern radiotherapy with an SIB technique.

In France, we are currently investigating dose escalation up to 66 Gy (vs. 50 Gy) combined with FOLFOX4 using modern conformal radiation techniques including IMRT and VMAT in a phase II/III trial entitled Cancer of the Oesophagus, Non-resected, treated with Chemoradiotherapy combining Oxaliplatinbased chemotherapy and Radiotherapy delivering Dose Escalation (CONCORDE) (NCT01348217). An elective nodal irradiation to 40 Gy is given in both arms. The CONCORDE study sought to evaluate locoregional control using modern radiotherapy considering improvements in tumor volume definition and tumor staging combined with newer radiation technologies may finally allow delivering "the right dose to the right volume". Patients are stratified by stage, histology, weight loss and center so that the technique of radiotherapy used (IMRT vs. 3D conformal) will be assessed on the primary endpoint. An independent review committee (EQUAL-ESTRO) performs a remote RT Quality Assurance Review. We expect in the phase III trial a significant increase in 2-year locoregional progression-free survival from 50% to 65%. The phase II part is ended including 160 patients of whom 80% were treated with IMRT/VMAT (25). Toxicity and efficacy data will be available by fall quarter of 2016.

Unless robust data will be emerging soon, it is hoped that improvements in modern radiotherapy will allow improving the therapeutic ratio in locally advanced esophageal cancer with CRT. In the meantime, 50 Gy with Platinum and fluorouracil-based regimen still remains the gold standard in this setting since more than two decades.

Lastly, the results of the CROSS trial in the preoperative setting showed a significant increased in overall survival with 41.4 Gy outdated 3D conformal radiation therapy combined with Carboplatin (AUC 2) and Paclitaxel (50 mg/m² weekly). Locoregional failure rate was 3.3% compared to 9.3% with surgery alone. After the completion of accrual in the CONCORDE study, we plan to move forward by evaluating the CROSS chemotherapy regimen with exclusive CRT 50 or 66 Gy, with respect to the results of the CONCORDE study.

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Footnote

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Combination of cetuximab with radio-chemotherapy in patients with esophageal cancer: less is more!

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A main challenge in the treatment of locally advanced esophageal cancer is to improve efficacy while minimizing treatment-related toxicity. Definitive chemoradiotherapy (CRT) is used to treat patients with locally advanced esophageal cancer who are inoperable for medical reasons, in whom complete R0 resection is unlikely or who decline surgery. However, until recently the data available were predominantly for squamous cell esophageal cancer. Molecular targeted drugs are being evaluated in clinical trials for esophageal, gastric, and gastroesophageal junction cancers. EGFR is overexpressed in 60-86% of gastric or gastroesophageal tumors and in 50-70% of esophageal cancers. Preclinical studies have shown that the chimeric monoclonal antibody cetuximab can overcome an important mechanism of radioresistance, and cetuximab was shown to bear radiosensitizing properties (1). These data led to phase I/II trials evaluating the combination of cetuximab with CRT in locally advanced esophageal squamous cell carcinoma (SCC) and adenocarcinomas with encouraging preliminary results (2,3). However, these trials were of small sample size. One study, reported by Ruhstaller included both, adenocarcinomas and SCC and showed, by adding cetuximab to preoperative CRT a significantly increased histopathologic response rate without elevated toxicity and postoperative mortality (2). Another trial, conducted by Chen and coworkers evaluated a regimen of definitive CRT plus cetuximab in 29 patients with SCC, showing a good clinical response and an acceptable safety profile despite high doses of radiotherapy (59.4 Gy) in Chinese patients (3). Moreover, the addition

of EGFR inhibitors to radiotherapy significantly improved the results of radiotherapy alone in patients with SCC of the head and neck. In a landmark study by Bonner and coworkers a nearly doubled median overall survival was achieved in patients allocated to the cetuximab-radiotherapy arm (28 to 54 months) (4). In all, there was clear rationale to test the addition of cetuximab to definitive CRT in a randomized trial in patients with cancer of the esophagus.

The SCOPE-1 phase 2/3 trial, included patients scheduled to undergo definitive CRT with both, adenocarcinoma as well as SCC of the esophagus (5). A thorough staging was conducted in most of the patients, including PET in about 85%. Patients were randomized to either receive two cycles of induction chemotherapy (capecitabin + cisplatin; XP) + definitive CRT (based on XP and 50 Gy, i.e., 25×2 Gy) or the same regimen combined with standard doses of cetuximab. Patients were stratified according to center, reason for receiving definitive CRT without surgery, histology, and tumor stage. Primary endpoint of the phase 2 part of this trial was the proportion of patients without treatment failure at week 24. The study was foreseen to proceed to phase 3 provided the phase 2 portion of the trial was positive. Overall survival was the primary endpoint of the phase 3 part. After accrual of a total of 258 patients (73% SCC, 25% adenocarcinoma) the study was stopped for futility because fewer patients were free of treatment failure at the time point 24 weeks in the CRT plus cetuximab group (66.4% vs. 73.6% in the standard arm). Likewise, overall survival was shorter in the cetuximab arm (22.1 vs. 25.4 months; adjusted HR 1.53; P=0.035).

As expected, the rates of non-haematological grade 3 or 4 toxicities were significantly higher in the cetuximab arm (79% vs. 63%; P=0.004). Moreover, the addition of cetuximab to CRT resulted in less protocol treatment being delivered and significantly compared with CRT alone.

These results are in keeping with trials using anti-EGFR therapies in combination with chemotherapy in patients with metastatic gastroesophageal cancer, such as the REAL-3 (6) and the EXPAND (7) studies. Both studies failed to demonstrate a survival advantage in unselected patient populations. In the REAL-3 study, inferior survival was noted with the addition of anti-EGFR therapy (overall survival 8.8 vs. 11.3 months; P=0.13), possibly because of using lower doses of chemotherapy in the experimental arm (6). Moreover, the POWER study (ClinicalTrials.gov Identifier NCT01627379), investigating the addition of panitumumab to fluorouracil and cisplatin in metastatic SCC of the esophagus has terminated recruitment prematurely because of futility. Similarly, no benefit of adding anti-EGFR mAbs to CRT protocols were found in patients with cancer of the head& neck and the rectum (8-11).

But what are the reasons for these negative trials? Was SCOPE-1 negative just because cetuximab is ineffective in the treatment of esophageal cancer? Some possible explanations will be discussed in brief.

(I) Toxicity and treatment intensity: an obvious problem in SCOPE-1 was that the addition of cetuximab to CRT led to significantly increased toxicity resulting in a relevant decrease in treatment compliance. Compared to CRT alone, where 90% of patients received four courses of cisplatin and 85% of patients completed all 4 cycles with capecitabine, only 77% of patients treated with cetuximab received all 4 courses of cisplatin and only 69% tolerated the four preplanned cycles of capecitabine. Moreover, only 78% of the cetuximab patients received the assigned radiation dose of 50 Gy compared to 90% in the CRT alone group. Of note, more than twice the number of patients in the cetuximab arm compared to CRT alone did not receive any radiotherapy due to chemotherapy associated side effects (19% vs. 8%; P=0.006). Thus, as the data were analyzed according to an intent-to-treat analysis, the inferior overall survival and the higher rates of treatment failure might be also explained with inferior treatment intensity. Due to a limited small sample size in SCOPE-1, a robust subgroup analysis according to tumor histology was not possible;

(II) Interaction of cetuximab with backbone regimen: the REAL-3 study demonstrated that the backbone chemotherapy regimen may significantly affect the efficacy of a particular regimen when combined with a targeted agent (6). The authors of REAL-3 concluded that the capecitabine backbone therapy, as it was also used in the SCOPE-1 and the EXPAND studies and in most patients in the COIN trial (12), might have contributed to dose reductions which might have caused the worse outcome in the cetuximab groups of these trials. Furthermore, two meta-analyses conducted in patients with KRAS wildtype metastatic colorectal cancer concluded that the addition of anti EGFR mABs to capecitabine- (or bolus 5-FU-) regimens did not improve the results of chemotherapy alone (13,14). In contrast, the combination of anti-EGFR antibodies with infusional 5-FU based regimens was associated with significantly improved response rate, progression-free-survival and overall survival. It is still a matter of speculation if this negative interaction between capecitabine and anti-EGFR mAbs are due to pharmacokinetic reasons or just a consequence of overlapping toxicities and consecutive dose reductions;

(III) Lack of valid biomarker/inclusion of unselected patients: another possible explanation is the absence of selection of the right subset of patients likely to respond to cetuximab. Many biomarkers, including high tumor EGFR expression have been shown to be an adverse prognostic factor for esophageal cancer patients and have been suggested as predictive of cetuximab resistance in various tumor entities (15-17). However, in the study by Chen and coworkers, patients with EGFR expressing tumors had a higher rate of complete and better progression-free survival with combined anti-EGFR and radiotherapy (3). The results are in line with preclinical observations, showing that EGFR inhibitors might sensitize tumors to cisplatin or radiation therapy (18). As data are conflicting, selection of patients on the basis of positive EGFR expression might not be a valid option for treatment decision for an additional EGFR antibody therapy. Other biomarkers such as mutations in BRAF, KRAS, PIK3C and the expression of PTEN have been analyzed in an analysis of the REAL3 trial (19). None of the biomarkers predicted resistance to anti-EGFR therapy: Thus, to date, unfortunately no specific biomarker has been validated. With regard to blood and tissue collection in SCOPE-1 (done at baseline and at week 24), no information is provided and correlation analysis have to be awaited.

In summary, SCOPE-1 demonstrated that the addition of cetuximab to CRT in patients undergoing definitive CRT for esophageal cancer was less effective than CRT alone but increased the toxicity burden of the cisplatin/capecitabine/

radiotherapy regimen and therefore had an adverse impact on the delivery of RT. In future trials, tumor biology and the identification of mutations that predict therapeutic response or resistance should be prerequisite to resurrect the development of EGFR inhibition in gastroesophageal cancers. Nevertheless, the authors should be commended for conducting this comparably large trial in a difficult-totreat tumor entity on the one hand, and for implementing a high level of quality assurance for radiotherapy and patient selection (PET staging in about 85%) resulting in excellent survival data in the standard arm (2-year survival of 56%) on the other hand. Thus, SCOPE-1 is an example that efforts to optimize treatment quality by treating patients in centers or assuring high quality of care nation-wide may occasionally improve treatment results to a greater extent than the implementation of new drugs.

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Potential value of immunoscoring in rectal cancer patients

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Rectal cancer therapy has markedly changed during the past decades with clear improvements for the patients (1). Population-based data based on registries with high validity (2,3) show that local recurrence rates can be as low as about 5% (4,5), similar to that in dedicated centers. Multidisciplinary team discussions prior to therapy initiation have likely also contributed to the improvements (5,6).

Better loco-regional staging, preferably with magnetic resonance imaging (MRI) can adequately describe whether the tumor is clear from the mesorectal fascia (MRF) and that an R0 resection thus is likely if a total mesorectal excision (TME) is done. If MRF is threatened, usually <1 mm, or cT3 mrf+, or involved, as it is in clinical stage T4 (cT4), preoperative treatment with time for down-sizing or down-staging before surgery is most often needed (1,7,8). Chemoradiotherapy is then the best documented treatment although in elderly patients, short-course radiotherapy with a delay is an attractive option (9). These tumors constitute about 10-15% of the rectal cancer patients. Many tumors less advanced than the locally advanced (cT3mrf+ or cT4s) have a risk of local recurrence even if adequate surgery is done and preoperative radiotherapy is then indicated. Since there then is no need for down-sizing/down-staging, shortcourse radiotherapy with immediate surgery is an attractive, convenient and well-documented treatment that reduces the risk of local recurrence by about 60% (1). These tumors, often designated locally advanced by most researchers, are best named intermediate, as for example done in the ESMO guidelines (7,8).

For early tumors, the risk of local recurrence is so small (2-5%) that radiotherapy is not indicated even if it would decrease the risk even further, since radiotherapy adds to the morbidity seen after surgery (1).

Overall survival has not improved to the same extent.

The loco-regional treatments, surgery and radiotherapy have no possibilities to influence systemic disease whether already manifest at diagnosis as synchronous metastases or appearing during follow-up as metachronous metastases. Adjuvant chemotherapy is not particularly efficient and much controversy exists about whether it has any effect at all in patients pretreated with radiotherapy or chemoradiotherapy (10-13). Presently, much focus is on delivering the systemic treatment prior to the loco-regional treatment. Several trials are ongoing, among them the RAPIDO trial randomizing patients between the reference treatment chemoradiotherapy, surgery and optional adjuvant chemotherapy versus short-course radiotherapy, neo-adjuvant chemotherapy and finally surgery (14). The term "total neoadjuvant treatment, TNT" has sometimes been used to describe this most recent development.

Another trend in rectal cancer management has focused on organ preservation, i.e., to postpone surgery, potentially indefinitely in patients who respond well to chemoradiotherapy or short-course radiotherapy alone (15). If radiotherapy is indicated to loco-regionally control the disease sufficiently better than surgery alone, it is rather uncontroversial to postpone surgery if a clinical complete remission is achieved. Although some rather small distal tumors can be locally advanced since they may threaten the MRF or grow adjacent to or into the levator- or sphincter muscles, requiring preoperative therapy with a delay to surgery, most tumors requiring preoperative therapy are quite large and the probability then to achieve a durable complete remission is much smaller. Tumor size is presently the best predictor of whether a complete clinical remission will be seen or not. In order to avoid surgery, many early tumors are thus presently treated with chemoradiotherapy. If the tumor is sensitive enough, that patient may have

a clear benefit, but for most patients the additional chemoradiotherapy will only add morbidity since those patients will have both chemoradiotherapy and subsequent surgery (16).

In order to improve the outcome after rectal cancer treatments further, we need better predictors, firstly of those who will recur after adequate surgery, i.e., are at risk of having subclinical distant deposits and, secondly, of sensitivity to radiotherapy or chemoradiotherapy. The work recently published by Anitei et al. in Clinical Cancer Research (17) had the aim to determine whether tumor immune cell infiltration, as evaluated with the immunoscore methodology, could be useful as a prognostic and predictive marker in rectal cancer patients. In patients treated with surgery alone, the endpoint was risk of recurrence, either locally or systemically. In patients treated with chemoradiotherapy, the aim was to predict whether the patients will remain recurrence-free after the preoperative treatment based upon the immunoscore in the diagnostic biopsies. The results indicate that the immunoscore is both prognostic and predictive, but the strength in this is not particularly high.

In the introduction of the article, the authors refer to an assumption by many researchers that tumor progression essentially has relied upon cell autonomous processes, i.e., the genetic changes in the tumor cells. The relevance of the microenvironment has, according to the authors, been neglected. Although much knowledge how to evaluate the microenvironment, including the response of the host to the tumor has been gained during the past decade, the prognostic role of the composition of the microenvironment in colorectal cancer (CRC) has been known since at least the 1970s (18). Since then, multiple studies have revealed its prognostic impact, also in colon and rectal cancer (19-21).

In the study, a methodology named "immunoscore" was used. It was developed in a study in colon cancer (19) as a means to standardize the evaluation for routine testing and is based on the numbers of CD3+ and CD8+ lymphocytes in the center and periphery of the tumor. The use of a score that has the potential to be standardized is a strength of the study. The study with its limited number of patients, particularly in the evaluation of response after CRT, is, however, only preliminary and should be followed by a much larger validation study. The statement by the authors in the very last sentence in the discussion "an international multicenter study should now be initiated", prior to its use clinically is definitely true (22). The need for a predictor of response to (chemo) radiotherapy is as discussed above urgently needed. This is particularly the case in early tumors where (chemo) radiation is not considered needed if major surgery is planned, but where this will be given if organ preservation is aimed at. Studies with the aim to predict outcome based upon properties of the tumor in the diagnostic biopsies are notoriously difficult, not the least depending upon the small amount of cancer cells present in the biopsies, unless "big bites" are taken. So far, no study has shown any clinically

context, functional imaging may be methodologically easier to explore. The performance of the immunoscore on the pretreatment biopsies in the article (17) is not possible to judge based upon limited number of patients (n=55), no prescription of what CRT was used (presumably about 50 Gy with a fluoropyrimidine) and the limited description of what constituted ypTN downstaging. An evaluation of response using either MRI pre-surgery (24) or one of the pathological tumor regression systems is likely more relevant.

relevant predictor (23). The purpose of the diagnostic biopsy is still only to verify the cancer diagnosis. In this

While I am sceptic to that immunoscoring in the postoperative specimen will be practically valuable in the clinics to evaluate recurrence risk and in the pretreatment biopsies to predict response to CRT, I am optimistic that further studies about the interplay between the tumor cells and the environment will lead to better understanding of mechanisms of clinical value in the future. In this context, improved possibilities to measure immune reactivity in peripheral blood, beyond those that could be done using simple routinely taken tests like C-reactive protein (CRP) or the Glasgow prognostic index (25) are needed. Any new method claiming to be used clinically must be compared with what is already around, often having the advantage of being both simple and cheap.

The checkpoint PD-1 and PDL-1 inhibitors directed against the inflammatory response (26) have created greater enthusiasm for therapeutic progress than many other targeted drugs have, also in CRC. Although the first very limited series of patients with metastatic CRC treated with pembrolizumab indicated that only MSI-H tumors, where the immune reaction is more pronounced (27), responded, the study by Anitei and co-workers (17), showing that an immune reaction in rectal cancers have prognostic information, give hope also for therapeutic attempts in rectal cancer, where MSI-H tumors virtually never are seen.

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Footnote

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Hypoxic cell sensitization in chemoradiation for cervical cancer

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The integration of cisplatin into radiation regimens was first developed in locally advanced cervical cancer, a tumour where there is the added advantage that high dose intracavity radiation can be given as well as external beam therapy (1), and the concept was later extended to head and neck cancers. However, progress since then has been slow with the addition of other radiosensitising drugs like gemcitabine providing only a modest survival advantage (2). Myelosuppression and gastrointestinal toxicity also became dose limiting with the doublet. DiSilvestro and colleagues reported a phase III trial of cisplatin based chemoradiation with or without the hypoxic cell sensitizer tirapazamine in 402 predominantly Caucasian and non-Hispanic patients with stages IB, IIa, IIIB and IVa cervical cancer in 2014 (3). The GOG 219 study took 3.5 years to accrue and was closed in 2009 prematurely on account of lack of study drug.

The study was well conducted with an adequate dose and schedule of radiation and acute toxicity was within acceptable limits, although vomiting, diarrhea and neuropathy were greater in the tirapazamine arm. Node sampling was recorded as performed in >80% of cases, but the results are not reported. Similarly results have not been presented for parallel translational studies. The study was negative with no PFS or OS benefit for the addition of tirapazamine.

Tirapazamine can be described as a second generation sensitiser based on a benzotriazine backbone which is bioreductively activated in hypoxic cells (4). In cell lines it had previously been shown to be up to 450× more cytotoxic in hypoxic compared to well-oxygenated cells, producing both single strand and double strand breaks. Earlier studies with 2-nitroimidazoles in both cervix and head and neck cancer were often underpowered and no overall effect on outcome was shown. Tirapazamine also has an inhibitory effect on DNA repair (5) and in vitro and in vivo synergy with cisplatin had been demonstrated. This last finding may be of significance because GOG 219, in common with many other randomized studies of cisplatin based chemoradiation, had shown a lower distant metastatic rate. The individual patient meta-analysis carried out in 2008 showed an absolute risk reduction of death of 6% and a 7% reduction in distant metastases by the addition of cisplatin to radiation (6).

The background to the study was preclinical work suggesting synergy between tirapazamine and cisplatin confirmed by phase II studies of this combination not only in advanced cervical cancer with a response rate of the order of 30% (7,8), but also in other tumour types. There was one phase I study of 15 patients in chemoradiation in which the dose of cisplatin was halved and the tirapazamine was administered on days 8, 10, 12, 22, 24 and 26 (9) which led to the phase III study. Subsequently two randomized head and neck cancer studies recruiting 923 patients to assess the addition of tirapazamine to cisplatin based chemoradiation showed no survival gain but increased myelosuppression in the experimental arm (10,11).

The 2008 meta-analysis furthermore identified two studies employing additional chemotherapy after completion of chemoradiation in cervical cancer, and suggested this may be associated with a gain in survival. Subsequent analysis of the subgroups of the cisplatin/gemcitabine study showed that there was a survival gain associated with the continuation of chemotherapy after the chemoradiation (2). There are two confirmatory studies in progress addressing the issue of additional adjuvant chemotherapy (ANZGOG 0972/GOG 0274 and RTOG 0724).

The above studies demonstrate that additional concomitant chemotherapy to cisplatin adds to toxicity without significant benefit, and raise the question whether the activity of tirapazamine is related to its hypoxic selectivity or to sensitization of cisplatin. A further concern is the extent to which chronic or transient hypoxia exists in tumours of different size and vascularity, as well as the known heterogeneity in the oxygen tension measured



Figure 1 Redox and how it might influence the immune system. The concept presented is that radiation drives a pro-oxidant state leading to inflammation, proinflammatory cytokines, and oxidative damage. Reactive oxygen species (ROS) also promote antigen presentation by dendritic cells, cytotoxic T-lymphocytes and M1 macrophages. Reproduced with permission from Schaue and McBride, 2015 (15).

in different parts of the same tumour (12). The message emerging over the last 10 years is that unless clinically relevant biomarkers are available, translation to clinical studies will risk rejection of promising compounds and premature progression to large phase III trials will not be cost-effective.

There have also been recent advances in our understanding of the genomics of cervical cancer and of the adaptive responses to hypoxia, which are largely mediated by HIF1a leading to transcriptional activation of genes which reduce cellular oxygen demand. There is also epigenetic repression of DICER leading to an epithelial to mesenchymal transition and acquisition of stem cell and metastatic phenotypes (13). Whole exome sequencing of 115 cervical carcinomas was reported in 2014 and besides showing differences between squamous and adenocarcinomas, suggested that there was increased expression of adjacent genes as a result of HPV integration and these included the growth factor ERB2 (14). The most frequently mutated gene set was also shown to involve immune response genes.

Ionizing radiation itself may disrupt the immune system as a result of the acute inflammation produced, and reactive oxygen species (ROS) may promote antigen presentation leading to activation of a range of cytokines and cellular responses depending on the redox status of the microenvironment (Figure 1). Several preclinical and clinical observations have led to interest in combining immune checkpoint inhibitors with radiation (16). Once the initial studies are complete, combination of radiosensitisers

with immunotherapy as an adjunct to chemoradiation will be worth exploring, either concomitantly or sequentially.

There has also been renewed interest in exploiting DNA repair pathways largely as a result of the development of the PARP inhibitors as cytotoxic agents in BRCA deficient tumours. These agents produce single strand breaks and in combination with the HR defect associated with BRCA deficiency and likely other DNA repair deficiencies, can induce a state of synthetic lethality (17). Antimetabolites used as radiosensitisers including 5-FU and gemcitabine target homologous repair (HR) while cisplatin predominantly targets non-homologous end joining (18). Evidence of radiosensitisation by the PARP inhibitors has led to the initiation of a number of clinical trials in several tumour types targeting DNA repair molecules including ATM, Chk1/2 and WEE1 (19).

In summary, there are a number of therapeutic possibilities for enhancing radiation response, including hypoxic sensitization, DNA repair, growth factor inhibition and immunomodulation (20). The interactions between these processes are beginning to become clear. As these are developed, researchers will have to deal with the difficulties of drug and radiation interactions, tumour heterogeneity and stem cell plasticity. However, cervical cancer remains one of the best systems to evaluate these effects.

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Footnote

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"Boom-Boom" radioimmunotherapy of lymphomas: are two magic bullets better than one?

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Since its first human use, dated back to 1987 (1), radioimmunotherapy (RIT) of non-Hodgkin (NHL) lymphomas has undergone many vicissitudes, and we might be not too far from the truth by affirming that this is not the best time for it (2). As a matter of fact, the worldwide enthusiasm following the approval, at the beginning of this century, of the first two RIT compounds, ⁹⁰Y-ibritumomabtiuxetan (Zevalin[®]) and ¹³¹I-tositumomab (Bexxar[®]) for relapsed/refractory indolent NHL, was tempered by the success of new chemotherapy agents (3) and of the rituximab maintenance strategies (4). Moreover, in case of relapse after optimized rituximab-including treatments, RIT showed a reduced efficacy both in aggressive and indolent NHLs (5,6). In addition, the absence of randomized phase III studies comparing RIT head-to-head with other agents and the physicians' natural reluctance to refer patients to radionuclide treatments, have played in synergy against its use. At present, RIT is underused (Figure 1) and, in February 2014, this has led to the withdrawal of Bexxar® from the US market.

Yet, RIT is by far the most effective and least toxic single treatment for NHL, and it is largely preferred by patients over other therapeutic options (7-9); in fact, none of the available anti-cancer agents would be able to produce as high as 87% ORR (including 56% CR/Cru) or 95% ORR (including 75% CR/Cru) after a single infusion, as obtained with frontline Zevalin[®] or Bexxar[®], respectively (7,8).

Interestingly, nuclear medicine has the intrinsic potential of allowing pre and post-therapeutic in-vivo biodistribution studies, which might inform the therapeutic infusion of RIT. By applying computational analysis on radioactivity distribution in organs or tumor lesions over time, internal dosimetry allows for obtaining dose calculations in these body compartments (*Figure 2*). Indeed, doses to organs and target lesions can vary intra and inter-patients because of the influence of all possible physical and biological variables in RIT, such as scheme of RIT fractionation, amount of antibody preloading, changes in size and biology etc. Unfortunately, such dosimetry studies are seldom accomplished in clinical practice, thought they might help to evaluate the effect of all these variables on RIT efficacy and toxicity, pursuing patient-specific treatment optimization.

Planar dosimety using a tracing amount of ¹³¹I-labeled antibody is part of the standard protocol for Bexxar[®] infusion, where the therapeutic administered activity is planned on a single-patient basis in order to keep the resulting total-body dose (TBD) within a predetermined limit (i.e., <75 cGy or <65 cGy in patients with platelet counts \geq or \leq 150,000/mL, respectively). As a result, the range of administered therapeutic activity per single patient is wide, that is between 47 and 212 mCi (1.74–7.8 GBq), median 91 mCi (3.36 GBq) (10). Interestingly, a significantly longer duration of response was shown for patients receiving higher TBD (>65 cGy) if compared to patients receiving less than 55 cGy (11).

Conversely, the activity to be administered in RIT with the radiometal conjugate ⁹⁰Y-ibritumomab-tiuxetan takes into account patient weight and platelet blood count only, and no optimization based on pre-therapeutic dosimetry is considered.

The choice of avoiding dosimetry in case of Zevalin[®] has several reasons. First, the biodistribution of radiometal conjugates is generally thought to be better


Figure 1 Distribution over time of a total of 101 radioimmunotherapy treatments with ⁹⁰Y-ibritumomab-tiuxetan (Zevalin[®]) performed at Sant'Andrea University Hospital of Rome, Italy, between July 2006 and October 2015.



Figure 2 Isodose map at the pixel level of an axillary lymphoma lesion of follicular origin obtained by sequential planar γ -camera imaging after the injection of a tracing amount of ¹¹¹In-Zevalin. Dose values are reported in Gy.

predictable than that of radio-halogens. Second, dosimetry of ⁹⁰Y-ibritumomab is complicated by the technical impossibility of obtaining γ -camera images by means of the pure β -emitter ⁹⁰Y, which requires the labeling of ibritumomab with a γ -emitting surrogate, such as ¹¹¹In. Third, and probably of greater importance, the marketing of RIT compounds has preceded many recent technical and theoretical achievements of internal dosimetry which, when RIT was developed, was just not advanced enough to match clinical needs and expectations. In fact, radiobiological modeling has only recently been applied to radionuclide treatments and is continuously evolving as new questions arise from therapies implying different physical and biological effects (12-14). In addition, only the breakthrough of hybrid SPECT/CT cameras has allowed accounting for errors and spatial heterogeneities in dose calculations, facilitating patient-specific voxel-based dosimetry and implementing radiobiological modeling (15). As such, standard planar dosimetry is no longer a good model for optimizing RIT efficacy, and three-dimensional, voxel-based dosimetry is warranted. Only recently three dimensional dosimetry and radiobiological modeling have been applied to RIT: a few reports have been published supporting a dose-response relationship for NHL nodal lesions treated with Bexxar[®] (16), while tumor voxel-based dosimetry of Zevalin[®] is still at its beginning (17,18).

In synthesis, much room does still exist for improvement of RIT efficacy and optimization of delivery and the feeling is that RIT is not only underprescribed but also underdosed.

An excellent effort toward dose optimization in RIT is represented by a recently published paper from a cooperative international research group reporting on the efficacy and toxicity of ⁹⁰Y-ibritumomab-tiuxetan delivered in two fractions as frontline therapy in patients with follicular lymphoma (FL) (19). From the 76 recruited, a total of 72 patients entered the final protocol; fifty-five patients (76%) received both infusions. Eight and four patients did not proceed with the second RIT infusion because of bone marrow toxicity (BM) and treating physician's discretion, respectively. Additionally, 4 patients developed mouse antibodies (HAMA) after the first cycle and one patient did not undergo the second infusion for underlining psychiatric disease. Most patients (78%) were stage III/IV; 44% patients had high-risk FLIPI. Patients with more that 20% BM infiltration were pretreated with four weekly infusions of rituximab 375 mg/m² and entered the study provided that <20% BM infiltration was achieved.

RIT infusions were administered 8 weeks apart, unless otherwise indicated by slow BM recovery. ⁹⁰Y-ibritumomab-tiuxetan was given at 11.1 MBq/Kg and injected activities were capped at 888 MBq (24 mCi). Such protocol showed an excellent 95.8% ORR including 69.4% CR/Cru, and a projected 3-year PFS of 58%. Interestingly, in contrast to previous observations, there was no significant difference in PFS between patients with tumor size < or >5 cm (65.4% vs. 50.2%, P=0.47). Hematological toxicity profile was acceptable: grade 4 thrombocytopenia and neutrophenia occurred in 6.9% and 8.3% of patients after the first infusion, increasing to 21.8% and 14.5% after the second infusion, respectively. After the second RIT, 8 (14.5%) patients received platelets and

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the same number of patients received red cell transfusions. Two (2.8%) neutropenic sepses were observed in the entire cohort. It is worth reminding, however, that 8 patients (11% of the initial cohort) could not undergo the second RIT infusion because of prolonged BM suppression after the first treatment.

Dose fractionation has a strong theoretical rationale both in external beam radiation therapy (EBRT) and in RIT since, according to the classical linear-quadratic model, it makes possible to increase the total dose delivered to tumor by decreasing normal tissue toxicity. An additional advantage of dose fractionation in RIT would be the possibility to achieve more uniform dose distributions within tumors by progressively reducing tumor size and improving blood supply (20). However, the same radiobiological principles do not necessarily apply identically to both EBRT and RIT, as the latter involves heterogeneous, continuous and continuously decreasing low-dose-rate radiation, which effects on cell killing have yet to be fully understood (21). As a matter of fact, it is interesting to note that non-targeted effects, including apoptosis, mutations, cell transformation, release of stress signals, are probably prevalent in RIT as they occur after low dose or low dose-rate irradiation (21). These so called "bystander" effects might not be fitted by linear or linear-quadratic models, rather they might saturate after a certain dose threshold, questioning the superior efficacy of the dose-fractionation vs. standard, single treatment approach in RIT, which indeed has yet to be experimentally determined in patients (21). In addition, there are other non-radiation dependent immunological effects of RIT which might help to explain the excellent response of some tumor to very low radiation burden. For example, it has been suggested that the benefit of RIT would be higher in patients with preserved T cell immunity, which might complement the effect of radiation by eliciting a cell-mediated toxicity against the mouse monoclonal antibodies used in RIT (22).

Some responses to the radiobiological questions regarding efficacy of dose fractionation on tumor control in RIT might come from the study of Illidge *et al.* (19). A retrospective dosimetric analysis of 28 patients from this cohort revealed that organ absorbed doses were similar for both fractions and that an image-based, 3D method for BM dosimetry was predictive of hematological toxicity (23). Unfortunately, however, at the time of writing no data have yet been published on the results of tumor dosimetry in these patients.

Given its complexity and all the reasons we briefly

outlined above, not surprisingly dosimetry was only retrospectively analyzed and not used to inform treatment schedule in this trial. Therefore, important radiobiologic and immunologic questions still need to be addressed. Nonetheless, the study of Illidge and colleagues is encouraging and could potentially pave the way for the conception and design of future trials aiming at a radiobiological optimization of RIT delivery. With particular regard to tumor dose-effect relationships, there might be a bulk of relevant information arising from combined dosimetric, clinical and laboratory data of this study, which would be otherwise lost if not fully analyzed and discussed. In other words, this study might have still a lot to say on the effects of RIT.

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Footnote

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Radioimmunotherapy in limited-stage diffuse large B-cell lymphoma

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Patients with limited-stage aggressive B-cell lymphoma without adverse risk factors are generally cured when treated by short-term therapy, with overall survival (OS) at 10 years ranging from 94% to 97% (1,2). However, limited-stage diffuse large B-cell lymphoma (DLBCL) patients with adverse risk factors including stage II disease, age >60 years, elevated serum lactate dehydrogenase (LDH), and poor performance status have a relatively unfavorable outcome, with a 5-year OS of 50% to 77% and a 10-year OS of 0% to 50%, and treatment optimization is needed for this subtype patients (1-4).

Radiolabeled anti-CD20 antibody ibritumomab tiuxetan (Zevalin) has shown promising efficacy in the treatment of patients in the rituximab-naive patients with DLBCL (5). Persky et al. investigated that if the addition of Zevalin to cyclophosphamide hydroxydaunomycin oncovin prednisone (CHOP) (3) plus institute for fitness research and training (IFRT) can improve the outcome of patients with limited-stage aggressive B cell lymphoma having at least one high-risk factor in a prospective single-arm phase II study Southwest Oncology Group (SWOG S0313) (6). The outcome of these patients is favorable compared with historical data, with a 5-year propellent feed system (PFS) of 82% and OS of 87%, which was superior to that of patients with limited aggressive non-Hodgkin's lymphoma (NHL). While compared with SWOG S0014 in which four doses of rituximab were combined with CHOP (3) and followed by IFRT, the results of current study appeared to be similar to that of SWOG S0014 (5-year PFS 78%, 5-year OS 83%). However, with longer follow up, the relapse in current study seemed to be fewer than those of prior trials. And treatment side effects was well-managed with no secondary myeloid neoplasms, and only 2 patients truncated due to toxicity,

these data support the value of radioimmunotherapy in first-line treatment of limited-stage DLBCL patients with adverse risk factors.

However, several aspects of this study should be addressed. First of all, this was a single-arm, prospective clinical trial, only 46 patients were enrolled into this trial, and the results of this study was compared with historical data. Similar to other SWOG studies (SWOG S0014, SWOG S8736), most patients enrolled in this study were low risk with only one adverse factor who usually have a good prognosis, while stage II patients with bulky disease, which usually have a inferior outcome, are excluded from this study (7,8). Secondly, staging and evaluation using positron emission tomography computer tomography (PET-CT) scan is not required in this study, however, PET-CT scan is very import in the evaluation of the response to chemotherapy, because complete remission evaluated by fluorodeoxyglucose-PET (FDG-PET) scan post induction-chemotherapy always indicate a very favorable outcome in early stage patients treated by CHOP (3) \pm R and followed by IFRT (9). Last, the treatment schedule in this study included 40-50 Gy IFRT of radiotherapy, the potential long-term radiation-related side effects should be considered. Although there were no patients who developed treatment-related myeloid neoplasms, data of other solid tumors and long-term side effects were not mentioned.

Introduction of rituximab or Zevalin in treatment protocols has significantly improved the prognosis of limited-stage aggressive B-cell lymphoma with at least one risk factor, compared with treatment of CHOP (3) plus IFRT. The treatment protocols in this study, SWOG S0014 ($3 \times R - CHOP + IFRT$), Ricover-60 and MINT ($6 \times R$ - CHOP ± 2R) have significantly improved the outcome of

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patients with limit-stage DLBCL, and survival of patients in this study seems to be superior to those of others (10-12). However, it is difficult to draw a final conclusion, because there are no randomized controlled clinical trials to evaluate the efficacy between these different treatment strategies. In conclusion, a randomized controlled clinical trial is needed to determine whether the protocol comprising radioimmunotherapy and CHOP (3) plus IFRT is associated with a better prognosis in patients with limitstage aggressive B cell lymphoma having at least one risk factor.

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Footnote

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

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