Review Article

Targeting Tumor Hypoxia in Radiotherapy: A Brief Review of Historical Background and Recent Progress

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Abstract

Tumor hypoxia is a physiologic barrier to radiotherapy and anti-tumor drug delivery. Numerous efforts have been made to overcome this barrier and to improve therapeutic outcomes. Strategies for targeting tumor hypoxia have included chemical radiosensitizers and hyperthermia, followed by combined synergic therapeutic modalities. Clinical hypoxia measurements and the development of molecular imaging agents prompted trials on dose escalation in external beam radiotherapy, which takes advantage of contemporary sophisticated radiation dose delivery techniques. Increases in our understanding of hypoxia-induced biological pathways have led to the investigation of various hypoxia targeting drugs. Radiolabeled hypoxia targeting drugs deliver radionuclides into hypoxic tumor cells and achieve highly localized cell death. In this manuscript, we briefly review the methods of targeting tumor hypoxia in radiotherapy. These include image guided dose escalation in external beam radiotherapy, radiosensitizers, and radiolabeled agents targeting hypoxia pathways and the receptors on hypoxic tumor cells. Our current understanding of tumor hypoxia is the culmination of the collective efforts of generations of researchers. New frontiers are continuing to expand, as new discoveries are being made on both the macroscopic and molecular levels.

Introduction

Tumor hypoxia is a distinct characteristic of solid tumors and results from an imbalance between the cellular oxygen consumption rate and tumor oxygen supply.[1] Primarily, available oxygen is consumed within 70 to 150 μm of tumor vasculature by rapidly proliferating tumor cells[2]; thus, the remaining tumor cells outside this range fall in a microenvironment of low oxygen. Hypoxia increases tumor resistance to radiation and is considered a poor prognostic factor in human cancers and is an important reason for the failure of radiation therapy. Accumulating evidence shows that hypoxia is responsible for inducing drug resistance[3] and correlates with increased distant metastases.[4] Therefore, hypoxia and hypoxia-related tumor microenvironments have become major targets for the development of cancer treatment.

Phenomena of tumor hypoxia

Tumor hypoxia can be quantified by the locoregional partial pressure of oxygen (pO_2). Though the pO_2 value is not a precise measure of the severity of tumor hypoxia and its biophysiological consequences, many researchers choose an arbitrary pO_2 value of 5 mmHg as the threshold of tumor hypoxia, and the fraction of tumor cells present in oxygen tension below this threshold is termed the hypoxic fraction.

Extensive work has been performed on refining the measurements of tumor hypoxia in animal models as well as in human patients. Quantitative measurements of pO_2 values can be performed using invasive probes that can provide instantaneous locoregional tumor oxygen tension *in vivo*.[5,6] Two types of pO_2 probes are frequently used to measure hypoxia: polarographic needle electrodes[5] and luminescence-based optical probes.[6] The microscopic distribution of hypoxia can be determined utilizing immunohistochemical staining with endogenous or exogenous hypoxia markers. An example of an endogenous hypoxia marker is carbonic anhydrase 9 (CAIX), a transmembrane protein that is overexpressed in hypoxic cells in a variety of tumors.[7,8] Commonly used exogenous surrogate hypoxia markers are compounds that are bioreducible within hypoxic cells, such as pimonidazole,[9] for which a commercial fluorescent antibody is available to reveal its microscopic distribution within the tumor. Positron emission tomography (PET) and single photon emission computed tomography are used to image exogenous hypoxia markers labeled with radiotracers, such as the widely used ^{18}F-fluoromisonidazole (^{18}F-MISO)[10,11] and the clinically validated ^{64}Cu-labeled agents [*e.g.*, ^{64}Cu-diacetyl-bis(N^4-methylthiosemicarbazonate) (^{64}Cu-ATSM)].[12] Fig. 1 shows the structures and bioreduction mechanisms of ^{18}F-MISO and ^{64}Cu-ATSM. Magnetic resonance spectroscopy of surrogate markers such as lactate, a metabolic product of anaerobic glycolysis,[13] or the evaluation of the tumor blood perfusion with dynamic contrast-enhanced magnetic resonance imaging has been used to noninvasively probe tumor hypoxia.[14]

Hypoxia exists in solid tumors at every growth stage. Even in its earliest phase of growth, the tumor *in situ* may develop intense

Keywords: Hyperthermia; Monoclonal antibody; Radiosensitizer; Radiotherapy; Receptor-targeted radiopharmaceutical; Simultaneous dose escalation; Tumor hypoxia.

Abbreviations: ^{18}F-MISO, ^{18}F-fluoromisonidazole; CAIX, carbonic anhydrase 9; ccRCC, clear-cell renal cell carcinoma; FDG, fluorodeoxyglucose; HIF-1α, hypoxia-inducible factor 1-alpha; IMRT, intensity-modulated radiation therapy; MTH, mild temperature hyperthermia; NSCLC, non-small cell lung cancer; PET, positron emission tomography; pO_2, partial pressure of oxygen; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau.

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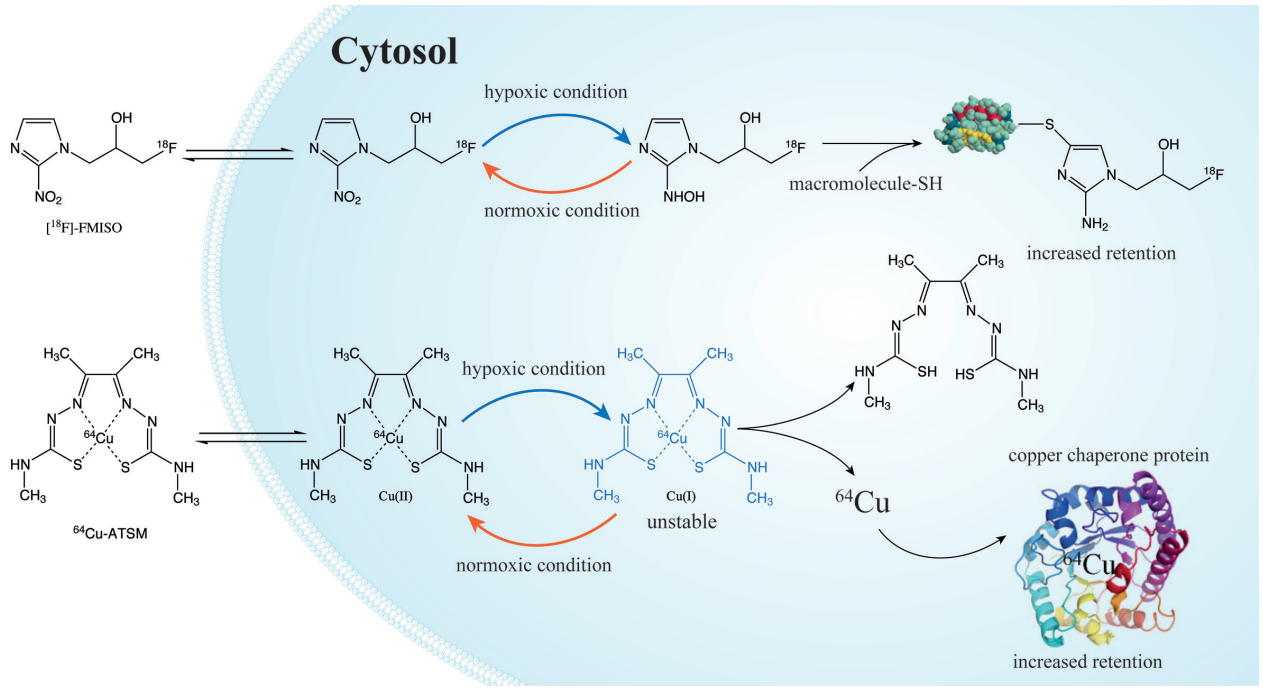

Fig. 1. The molecular structures of ^{18}F-fluoromisonidazole (^{18}F-MISO) and ^{64}Cu-diacetyl-bis(N^4-methylthiosemicarbazonate) (^{64}Cu-ATSM) and their bioreductive mechanisms in hypoxic cells.

hypoxia.[15] Hypoxic tumor cells secrete hypoxia-inducible angiogenic factors, such as vascular endothelial growth factor (VEGF), to trigger the growth of new blood vessels into the tumor, a process termed angiogenesis.[16] As the tumor grows larger, there is an abrupt decline in the hypoxic fraction, indicating the onset of angiogenesis.[15] Although hypoxia could be temporarily alleviated by the formation of new blood vessels, the tumor will soon outgrow its oxygen and nutrition. Vajkoczy *et al*. observed that the blood vessel density in transplanted C6 gliomas initially increased; but 10 days after implantation, the blood vessel density reached a maximum and did not further increase.[17] Similarly, Nöth *et al*. reported that during the first few days following GH3 tumor homogenate injection the tumor pO_2 levels increased rapidly, then

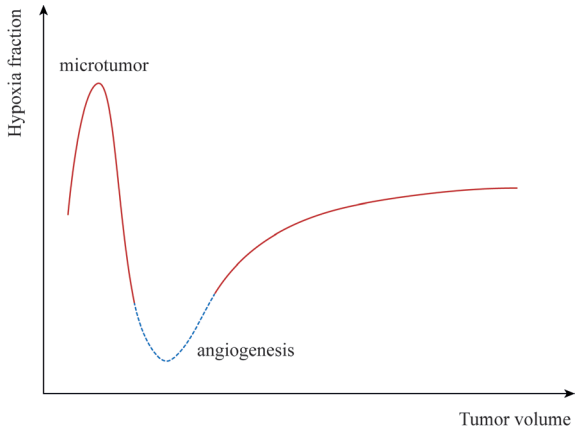

Fig. 2. Development of hypoxia during tumor growth: a conceptual diagram based on currently available data. Before the onset of angiogenesis, the microtumor depends upon diffusion for oxygen and nutrients; following angiogenesis, the hypoxic fraction steadily increases in a clinically detectable tumor.

reached a plateau, and then the pO_2 level readily decreased.[18] These findings were supplemented by clinical measurements using polarographic needle electrodes in hundreds of patients, which showed increasing tumor hypoxic fraction with increasing tumor volume.[19] The relationship between the extent of tumor hypoxia and tumor volume is conceptually illustrated in Fig. 2.

To date, three types of tumor hypoxia have been identified:[20] the diffusion-limited or "chronic" hypoxia caused by increased diffusion distance; the perfusion-limited or "acute" hypoxia caused by low blood flow; and the recently described macroscopic regional hypoxia caused by extended longitudinal blood flow gradients.[21] Tumor hypoxia is a heterogeneous phenomenon, both spatially and temporally. Spatially, observations on patient biopsies showed that in many tumors the periphery regions had a greater blood supply and, therefore, were well oxygenated, whereas elsewhere, blood vessels were less dense or less functional.[22] Temporally, studies suggest that hypoxia is a dynamic process rather than a static picture. Dewhirst *et al*. placed an oxygen electrode into R3230AC tumor masses in rats and observed, on occasion, that the pO_2 readings fluctuated around the hypoxia threshold a few times per hour.[23] Brurberg *et al*. found using optical pO_2 probes in A-07 human melanoma xenografts that the oxygen tension fluctuated at frequencies less than 0.1 cycle/min.[24] However, the extent to which these preclinical kinetic studies translate to clinical realities is still unknown. This is due to the difficulty in detecting hypoxia fluctuations in human patients and our incomplete knowledge of the underlying kinetics of acute hypoxia.

Biologic and therapeutic consequences of tumor hypoxia

Hypoxia induces a series of biological events in tumors that eventually lead to changes in the tumor microenvironment and resistance to therapy. A signature change in tumor metabolism, historically

known as the Warburg effect,[25] is due to the reliance of cancer cells on aerobic glycolysis to generate the majority of energy needed for cellular processes. The expression of genes responsible for glycolytic enzymes and glucose transporters is linked to oncogenes that include *ras*, *src*, and *c-myc*.[26,27] As a result, glycolysis is often accompanied by increased glucose uptake, a feature that is exploited in ^{18}F-fluorodeoxyglucose (FDG)-PET.[28]

Hypoxia leads to increases in hypoxia-inducible factor-1 (HIF-1), which is the central transcriptional mediator of the cellular response to hypoxia.[29] HIF-1 consists of HIF-1α and HIF-1β subunits. HIF-1β is a constitutively expressed nuclear protein; whereas the expression of HIF-1α is regulated at both the translational and posttranslational levels.[30] HIF-1α expression has been reported to be increased dramatically in conditions where pO_2 is less than 40 mmHg, and its degradation is retarded at low oxygen concentrations.[31] HIF-1α is known to induce transcription of more than 60 genes that are expressed at higher levels in cancer, particularly VEGF and enzymes of glucose metabolism. HIF-1α also induces transcription of genes involved in cell proliferation and survival, which have been shown to correlate with tumor aggressiveness and unsuccessful cancer treatments characterized by drug resistance, cancer recurrence, and poor survival rates.[32] Therefore, HIF-1α and related proteins may serve as an effective radiotherapeutic target.

The expression of VEGF in tumors is mainly driven by HIF-mediated transcription in hypoxic cells. VEGF activates VEGF receptors on endothelial cells and triggers tyrosine kinase pathways that lead to angiogenesis. In tumor cells, VEGF signaling is characterized as autocrine,[33] and it is likely established with the help of hypoxia. The functions of VEGF in solid tumors are not limited to the stimulus of vasculogenesis and angiogenesis, but it also can increase vascular permeability[34] and enhance tumor growth and metastasis. Numerous studies have linked tumor VEGF overexpression to decreased overall survival and disease-free survival in cancer patients.[35]

Carbonic anhydrases are zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide to carbonic acid. CAIX is a transmembrane protein that is expressed on the tumor cell membrane, and it is activated by HIF-1α.[36] It is overexpressed in von Hippel-Lindau (VHL) tumor suppressor gene mutated clear-cell renal cell carcinoma (ccRCC) and several other types of hypoxic solid tumors, but its expression is low in normal kidney and most other normal tissues.[7] CAIX is considered to be a biomarker of chronic tumor hypoxia,[8] and it may be involved in cell proliferation and transformation.[37] Together with the glycolysis product lactic acid, CAIX contributes to tumor extracellular acidification, a key feature of the solid tumor microenvironment that is a driving force for tumor progression and metastasis.

The influence of oxygen on the effect of radiotherapy was first recognized in the early twentieth century. Swartz observed that the reaction of skin to irradiation was less if the radiation source was pressed tight to the skin, implying that blood flow could modify the radiation response.[38] In a landmark study, Gray *et al.* observed that tumor cell viability was depressed 2.5 to three times more in air than in nitrogen by X-ray irradiation.[39] Now, it is a well-established concept that hypoxic cells are three-fold more resistant to radiation than oxygenic cells.[40,41]

With advances in methods to measure tumor hypoxia using Eppendorf polarographic electrodes[19,42] and bioreductive nitroimidazoles,[43,44] substantial insight has been gained in the role and extent of hypoxia in tumors. In both head-and-neck and cervical cancers, the probability of achieving a complete response with radiotherapy was inversely correlated with hypoxic tumor volume.[45,46] A surgical series from Höckel *et al*. correlated the overall and disease-free survival of cervical cancer patients with pO_2 levels, measured with the Eppendorf device, and found that tumor hypoxia may be correlated with the aggressiveness of the disease and may be a prognosticator for treatment outcome.[47] In high grade soft tissue sarcomas, Brizel *et al*. reported an association between tumor hypoxia and the development of metastases following multimodality treatment.[4] Furthermore, data acquired from 6,975 patients, accrued in 43 randomized trials, employing oxygen-mimicking sensitizers and hyperbaric oxygen, showed that treatment outcome was improved by reducing the influence of hypoxia.[48]

More severe tumor hypoxia has been correlated with increased distant metastases in human soft tissue sarcoma and earlier recurrence of glial brain tumors.[4,49] Graeber *et al*. reported that hypoxia induced apoptosis in oncogenically transformed cells and that further genetic alterations substantially reduced hypoxia-induced cell death.[50] This study demonstrated that tumor hypoxia provided selective pressure for an aggressive phenotype. The pathophysiologic consequences vary with the individual subtypes of acute or chronic hypoxia.[51] Classification of hypoxia subtypes may affect the radiotherapy schedule. For instance, modelling of acute and chronic hypoxia in non-small cell lung cancer (NSCLC) radiotherapy suggested that tumor control is dominated by chronic hypoxia for short hypofractionated treatments and by acute hypoxia for multifractionated treatments.[52]

Simultaneous dose escalation in radiotherapy

Progress in radiation dose delivery techniques, especially intensity-modulated radiation therapy (IMRT), makes possible sophisticated dose distribution strategies in the target volume. "Multidimensional radiotherapy" was proposed to take advantage of multimodality biological imaging and IMRT delivery in order to achieve biological conformality.[53] Using this approach, tumor hypoxia would constitute an important portion of the biological target, and this could be irradiated with escalated doses using a dose painting technique to improve the efficacy of radiotherapy. In contrast, the target volume of ordinary treatment plans receives a uniform dose. Dose painting can result in simultaneous escalated doses to the biological target, while the remainder of the target volume still receives the prescribed radiation dose. The concept of biological conformality was followed by investigating dose escalation targeting tumor hypoxia.[54,55]

Studies have shown that the uptake of hypoxia imaging agents correlated with radiation therapy outcome.[56] Dose escalation demands methods to accurately determine the spatial distribution of hypoxia within tumors that could yield valuable *a priori* information for the stratification of patients according to disease prognosis. Treatment planning studies using various positron-emitting hypoxia tracers and ^{18}F-FDG have shown the feasibility of the dose painting technique, *e.g.*, the hypoxia subvolume dose is escalated to 150% of the prescribed dose.[57] In a feasibility study on hypoxia-guided IMRT dose escalation, 20 patients with head-and-neck cancer underwent one ^{18}F-FDG PET/computed tomography (CT) scan and three subsequent ^{18}F-MISO PET/CT scans. The PET images were co-registered with CT simulation images, and the hypoxia subvolume within the gross tumor volume (GTV) was delineated based on the ^{18}F-MISO images.[11] Higher doses (20% to 50% more than the primary GTV dose) were prescribed to the hypoxia subvolume, with one patient receiving an escalated dose of 105 Gy to the hypoxia subvolume. Currently there have been several clinical trials on hypoxia guided dose escalation. In

Table 1. PET imaging agents for tumor hypoxia, metabolism and proliferation. Some have been used in completed or ongoing clinical trials on simultaneous dose escalation (https://clinicaltrials.gov, accessed November 2015)

Imaging agent	Uptake mechanism	Tumor assessment	Dose escalation
^{18}F-FDG	Diffusion/active transport	A variety of cancers	H&N; NPC; NSCLC; EC
^{18}F-MISO	Bioreduction	Brain; Cervix; H&N; Melanoma; NPC; Rectum	H&N; NPC
^{18}F-EF5	Bioreduction	Brain; Breast; Cervix; H&N; NSCLC; Prostate; Ovary; STS	
^{18}F-FLT	Monophosphorylation	H&N; Lung	H&N
^{18}F-HX4	Bioreduction	Cervix; H&N	NSCLC
^{18}F-FAZA	Bioreduction	Breast; NSCLC	
^{18}F-VM4-037	CAIX binding	H&N; Kidney; Liver; Lung; Stage 4 cancer	
^{64}Cu-ATSM	Bioreduction	Cervix; glioblastoma; NSCLC	
^{124}I-IAZGP	Bioreduction	Cervix; Rectum; Uterine	

EC, esophageal cancer; H&N, head-and-neck cancer; NPC, nasopharyngeal carcinoma; NSCLC, non-small cell lung cancer; STS, soft tissue sarcoma

a phase II clinical trial in NSCLC (clinicaltrials.gov identifier: NCT01024829), patients were randomized between dose-escalation of the entire primary tumor or the high ^{18}F-FDG uptake region inside the primary tumor. Most of the patients could be escalated to 72 Gy while observing dose constraints to normal tissues.[58] Although this registered study includes an ^{18}F-HX4 imaging component, no report was found on how the hypoxia scan was used in clinical decisions. Table 1 summarizes the clinically investigated PET imaging agents for tumor hypoxia, metabolism and proliferation, and the dose escalation clinical trials using these agents. The next step would be to utilize multimodality functional imaging in radiation therapy planning.[59]

Regarding the simultaneous dose escalation regime, questions still remain: how to accurately measure the hypoxic fraction of tumors; which are more relevant to the therapeutic outcome; how to determine the escalated radiation doses that can properly address the radioresistance of hypoxic cells. For instance, targeting acute hypoxia might be more important than chronic hypoxia in cancer treatment because there is evidence that acutely hypoxic cells have a higher metastatic potential than chronically hypoxic cells.[60–62] Similarly, it has been hypothesized that cells at intermediate oxygen levels (0.5 to 20 mmHg) and acute hypoxia are important in tumor response to fractionated radiotherapy,[63] and knowledge of acute hypoxia could help improve cancer therapy regimes and, consequently, therapeutic outcomes. To date, there have been few reports on distinguishing acute and chronic hypoxia using medical imaging.[64] Comparative studies of hypoxia tracers in the same patient demonstrated high-uptake in the same tumor with good correlations; but the regions of high hypoxic agent retention only partially overlapped.[65,66] In clinical imaging studies, segmentation of the hypoxia subvolume is often based on arbitrary thresholds,[67] which results in uncertainties in the dose escalation target. Another issue with the dose escalation regime is the optimization of dose distribution; the studies on this topic are relatively sparse.[68] Not many patients have completed clinical trials on hypoxia imaging guided dose escalation. Presently, there are no sufficient outcome data to validate the dose escalation regimes, although in theory this approach may improve local control for tumors with more severe hypoxia.

Hyperthermia in radiotherapy

Hyperthermia has a long history of use in cancer therapy. In the late nineteenth century, Dr. Coley from New York Memorial Cancer Hospital treated cancer patients with artificial systemic hyperthermia for long periods, with a positive effect on overall five-year survival rate.[69] Later, heat was tested as a solo tumoricidal agent or in combination with radiotherapy or chemotherapy. The heat was administered for short periods but at much higher temperatures, in excess of 42 °C. Subsequently, *in vitro* studies showed that the survival curves of heat-treated tumor cells were similar to that of irradiated cells,[70] and hyperthermia did not appear to be an effective tumoricidal agent on its own. When hyperthermia was used in combination with other therapy regimens, the best responses occurred at lower temperatures. Therefore, the focus of research shifted to mild temperature hyperthermia (MTH), with a temperature range of roughly 40.5 to 43.0 °C.[71]

A proper hyperthermia treatment causes dilation of local blood vessels and increases in blood perfusion. Animal studies have shown that MTH alone is capable of reducing tumor hypoxia in various types of tumors.[72,73] In treatment studies of animal tumors, hyperthermia was shown to improve median pO_2 level and reduce hypoxic fraction in dogs with spontaneous soft tissue sarcomas[74] and to enhance the effect of radioimmunotherapy in nude mice bearing human colon cancer xenografts.[75] A recent study using murine tumor models showed that a mild elevation of body temperature resulted in therapeutically beneficial changes in the tumor microvascular function by sustained reduction in tumor interstitial fluid pressure and hypoxia.[76]

Clinical trials on MTH in combination with radiotherapy and/or chemotherapy found that the local control rate could be improved with combination therapy relative to monotherapy.[77] Jones *et al*. administered 40 °C, 60 min of hyperthermia to 12 patients with locally advanced cervical carcinoma on a weekly basis, approximately 30 min after external beam radiation, and achieved an excellent clinical response.[78] In a phase I/II study, 47 patients with locally advanced breast cancer received neoadjuvant treatment with liposomal doxorubicin, paclitaxel, and 41 °C, 60 min of hyperthermia followed by surgery and radiotherapy. It was reported that the 4-year disease-free survival of 43 evaluated patients was 63%, and the 4-year overall survival was 75%.[79] A randomized study of 68 patients with post-operative local recurrent gastric carcinoma showed that abdominal hyperthermia combined with three-dimensional conformal radiotherapy yielded a higher response rate (57.2% *vs* 47.1%), prolonged median local progression-free survival time (14 months *vs* 11 months), and improved immune function for post-operative recurrent gastric cancer.[80]

MTH hardly exhibits cytotoxicity. Induction of chromosomal DNA strand breaks were not observed in cells heated at 43 °C for 30 or 60 min, which is in opposition to cells irradiated with a dose as low as 0.15 Gy.[81] However, hyperthermia is believed to interfere with radiation-induced signaling required for DNA double-strand break repair, thus resulting in increased cell death post irradiation.[82] Recently, it was reported that hyperthermia can sensitize glioma stem-like cells to radiation by inhibiting AKT signaling.[83] With the perspective of using hyperthermia with proton beam irradiation, it is hypothesized that the thermo-radiobiological advantages of hyperthermia coupled with proton dose distribution could possibly mimic carbon ion therapy.[84] One may expect that hyperthermia will gain widespread applications in combination with radiotherapy if it can be incorporated into the busy conventional radiotherapy schedule.

Chemical radiosensitizers

Early strategies for increasing the radiosensitivity of hypoxic tumor cells in radiotherapy included hyperbaric oxygen and chemical radiosensitizers. The most widely investigated and representative oxygen-mimetic radiosensitizers are the 2-nitroimidazoles, such as misonidazole and pimonidazole,[85] which undergo enzymatic- and radiation-induced redox reactions. These chemicals have high electron affinity and mimic oxygen in "fixing" radiation damage caused by free radicals in an oxygen-deficient cell. In hypoxic tumor cells, nitroimidazoles undergo a series of enzymatic reductions mediated by nitroreductase enzymes that are expressed under hypoxic conditions, leading to the generation of highly reactive anion radicals, which then bind to thiol-containing cellular molecules.[86] In preclinical studies, misonidazole had a higher electron affinity and was more effective in sensitization of tumors to radiation without concomitant effects in normal tissues than the 5-nitroimidazole, metronidazole.[87,88] However, misonidazole performed poorly clinically due to dose-limiting neural toxicity of the radiosensitizers, which necessitated the employment of inadequate therapeutic doses. Not surprisingly, a few 2-nitroimidazoles and derivatives were later successfully used as hypoxia markers.[9–11]

Nimorazole, a 5-nitroimidazole, is a less effective radiosensitizer than misonidazole but its toxicity is much less, allowing for administration of large doses.[89,90] In a Danish study, 422 patients with invasive squamous cell carcinoma of the supraglottic larynx or pharynx were randomized to receive radiotherapy with nimorazole or placebo. The nimorazole group was reported to show a significantly better locoregional control rate than the placebo group (49% *vs* 33%).[91] An international multicenter randomized trial (IAEA-HypoX) suggested that locoregional tumor control and overall survival in patients with head and neck squamous cell carcinoma could be improved by combining nimorazole and accelerated radiotherapy.[92] Currently, there are ongoing randomized trials on the addition of nimorazole to radiotherapy in patients with locally advanced head-and-neck squamous cell carcinoma (clinicaltrials.gov identifiers: NCT01950689, NCT01880359).

Efaproxiral (RSR-13) is a synthetic allosteric modifier of hemoglobin that decreases hemoglobin-oxygen binding capacity, thereby facilitating oxygen release to tissues. Efaproxiral was shown to increase systemic and iliac vascular resistance in rats and to increase tissue oxygen delivery.[93,94] Results from clinical trials with this drug, however, have demonstrated limited success. In a phase III clinical trial consisting of 515 patients with brain metastases, efaproxiral was found to mildly improve the median survival time when used as an adjunct to whole-brain radiation therapy, but the benefit was restricted to the subgroup of patients with breast cancer.[95] Thus, the use of efaproxiral as an adjunct to whole-brain radiotherapy for metastatic brain tumors was not recommended.[96]

Evofosfamide (Threshold Pharmaceuticals, Inc.), also known as TH-302, is an investigational hypoxia-activated prodrug designed to selectively target tumor hypoxia. TH-302 is a 2-nitroimidazole prodrug of the cytotoxin bromo-isophosphoramide mustard (Br-IPM). The prodrug is activated only at very low levels of oxygen by a process that involves a single-electron reduction mediated by ubiquitous cellular reductases, such as the NADPH cytochrome P450, to generate a radical anion prodrug. In the presence of oxygen, the radical anion prodrug reacts rapidly with oxygen to generate the original prodrug and superoxide. Therefore, TH-302 is relatively inert under normal oxygen conditions, remaining intact as a prodrug; when exposed to severe hypoxic conditions, however, the radical anion undergoes irreversible fragmentation, releasing the active drug Br-IPM and an azole derivative. The released cytotoxin Br-IPM alkylates DNA, inducing intrastrand and interstrand crosslinks.[97] TH-302 is being evaluated in clinical trials for the treatment of multiple tumor types as a monotherapy and in combination with chemotherapeutic agents and other targeted cancer drugs.[98] In recently reported animal studies, TH-302 enhanced the effects of VEGF-A inhibition and radiation on sarcoma xenografts;[99] the combination of TH-302 and radiotherapy further increased the growth delay of rhabdomyosarcoma R1 and H460 non-small cell lung cancer tumors than TH-302 treatment alone.[100] These results suggest that the combination of TH-302 and radiotherapy might warrant clinical testing in human patients.

Hypoxic-activated cytotoxins include mitomycins, porfiromycin, apaziquone, tirapazamine, and AQ4N. These compounds are used in order to exploit tumor hypoxia, and their use is based on the hypothesis that the oxygenation status of malignant cells can be turned into a clinical advantage. Conceptually, compounds that are converted to cytotoxic agents under low oxygen concentrations should be effective radiosensitizers for hypoxic tumor cells. Mitomycin C induces DNA cross-links through alkylation, thus inhibiting DNA and RNA synthesis, and it has been approved for the treatment of disseminated gastric cancer or pancreatic cancer. Mitomycin C lacks preferential selectivity for hypoxic cells; and, in clinical trials, it was administered once or twice during a radiotherapy course.[101] The ARO 95-06 randomized trial compared hyperfractionated accelerated chemoradiation with mitomycin C/5-fluorouracil (C-HART) with hyperfractionated accelerated radiation therapy (HART) alone in locally advanced head-and-neck cancer. Long-term results showed that C-HART remains superior to HART in terms of locoregional control and survival rates.[102]

Tirapazamine or TPZ (SR-4233) has a 1,2,4-benzotriazine-1,4 dioxide group that is susceptible to bioreduction.[103] TPZ is 15–200 fold more toxic to hypoxic than oxic cells in culture due to a one-electron reduction to a DNA-damaging free radical that is stabilized under hypoxic conditions and can act as a potent cytotoxic agent.[104] In both *in vivo* and *in vitro* settings, TPZ was found to substantially enhance radiation-induced cell death in a single-dose regimen and fractionated radiation treatment, particularly in hypoxic cells. Despite these preliminary encouraging results, several phase III trials have failed to demonstrate any benefit upon addition of TPZ to chemotherapy or radiation therapy in response rate, overall survival, or progression-free survival of NSCLC, head-and-neck cancer, or cervical carcinoma.[105]

The alkylaminoanthraquinone N-oxide AQ4N (banoxantrone) was designed as an antitumor prodrug with little intrinsic activity. Once reduced by cytochrome P450 enzymes, particularly CYP3A, it releases a stable and persistent cytotoxin, AQ4, a potent DNA

Table 2. Representative chemical radiosensitizers and therapeutic agents targeting hypoxic tumor cells

Compound	Mechanisms	Characteristics
Misonidazole	Bioreduction; fixing damage of free radicals; depletion of radioprotective thiols	2-nitroimidazole; high electron affinity; neural toxicity
Nimorazole	Same as above	5-nitroimidazole; less effective than misonidazole; much less toxic
Efaproxiral	Decreasing hemoglobin-oxygen binding capacity; facilitating oxygen release to tissues	Allosteric modifier of hemoglobin
TH-302	Activated in hypoxia; releasing potent DNA alkylating agent Br-IPM	2-nitroimidazole prodrug
Mitomycin C	Hypoxia activated cytotoxin; inducing DNA cross-links through alkylation	Lacking selectivity for hypoxic cells
Cisplatin	Producing interstrand DNA crosslinks; scavenging of hydrated electrons; formation of OH radicals	Platinum-containing chemotherapy drug; various side-effects
Tirapazamine	Activated in hypoxia; producing hydroxyl or benzotriazinyl radicals	Much more toxic to hypoxic than oxic cells
AQ4N	Reduced by cytochrome P450 enzymes; releasing cytotoxin AQ4	Antitumor prodrug; little intrinsic activity
PX-478	Suppressing hypoxia inducible factor (HIF)-1α levels and HIF-1α target genes expression; enhancing radiosensitivity	Inhibitor of HIF-1α
EZN-2968	Inhibiting HIF-1α expression	Antisense of HIF-1α
Bevacizumab	Inhibiting VEGF-A and blocking angiogenesis	Monoclonal antibody of VEGF-A; potential radioimmunotherapy agent
Girentuximab	Inhibiting CAIX; disrupting pH regulation	Monoclonal antibody of CAIX; diagnostic and therapeutic agent of ccRCC

intercalator and topoisomerase II inhibitor.[106,107] In preclinical models bearing mammary carcinoma, AQ4N was shown to interact additively with radiation, both in single-fraction irradiation and fractionated schedules.[108] AQ4N was investigated in phase I and phase II trials as a single agent or in combination with radiation and temozolomide (clinicaltrials.gov identifier: NCT00394628). However, results from the combination trials with radiation have not yet been reported.

Therapeutic agents targeting hypoxia

Monoclonal antibodies have been developed to target hypoxic cells or hypoxia pathways for tumor imaging and therapeutic purposes. Bevacizumab is a recombinant humanized monoclonal antibody that blocks angiogenesis by inhibiting VEGF-A,[109] the first clinically available angiogenesis inhibitor in the United States. It was first approved in 2004 by the U.S. Food and Drug Administration for combination use with standard chemotherapy for metastatic colon cancer. It has since been approved for use in certain lung cancers, renal cancers, ovarian cancers, and glioblastoma multiforme of the brain. Bevacizumab is on the World Health Organization's List of Essential Medicines for its use in treating certain eye diseases. Radioimmunoconjugates comprised of bevacizumab conjugated with the gamma-emitting radioisotopes bind to VEGF, allowing non-invasive detection of VEGF distribution in animal models with human ovarian tumor xenograft,[110] as well as in melanoma patients.[111] A preliminary study with the SKOV-3 ovarian cancer cell line and male mice suggested that ^{131}I-bevacizumab is a potential radioimmunotherapy agent for ovarian cancer.[112]

HIF-1 inhibitors may suppress tumor resistance to therapy through various intricate mechanisms. PX-478, an orally administered inhibitor of HIF-1α expression, was reported to suppress HIF-1α levels in human tumor xenografts and inhibit the expression of HIF-1 target genes, including VEGF and the glucose transporter-1.[113] It was reported that PX-478 inhibited HIF-1α protein levels and HIF-1 transactivating activity in a variety of cancer cell lines in both normoxic and hypoxic conditions.[114,115] PX-478 enhanced radiosensitivity of prostate carcinoma cells.[114,116] EZN-2968, an antisense oligonucleotide inhibitor of HIF-1α expression, binds HIF-1α mRNA with high affinity, causing its downregulation and a consequent reduction in HIF-1α protein level. *In vivo* and *in vitro* data showed potent downregulation of HIF-1α and VEGF mRNAs and inhibition of tumor cell growth following the administration of EZN-2968.[117] A proof of concept pilot trial with EZN-2968 demonstrated safety and potential activity in patients with refractory advanced solid tumors.[118] Radiation can induce high HIF-1α expression upon reoxygenation,[119] and it has been hypothesized that blocking HIF-1α following radiotherapy may prolong and enhance the immune effects of radiotherapy.[120] In sarcoma mouse models, trimodality therapy with radiotherapy, VEGF-A inhibition, and HIF-1α inhibition using short hairpin RNA or doxorubicin maximized the effects of radiation through destruction of tumor vasculature.[121] BAY-84-7296, an inhibitor of mitochondrial complex I and HIF-1 activity, has been shown to enhance tumor control after single-dose irradiation of human squamous cell carcinomas in nude mice.[122]

Monoclonal antibodies or CAIX-specific inhibitors are used to disrupt pH regulation by cancer cells, further impairing tumor growth and metastasis. Girentuximab (trade name Rencarex®, Wilex AG) is a chimeric monoclonal antibody that was designed for

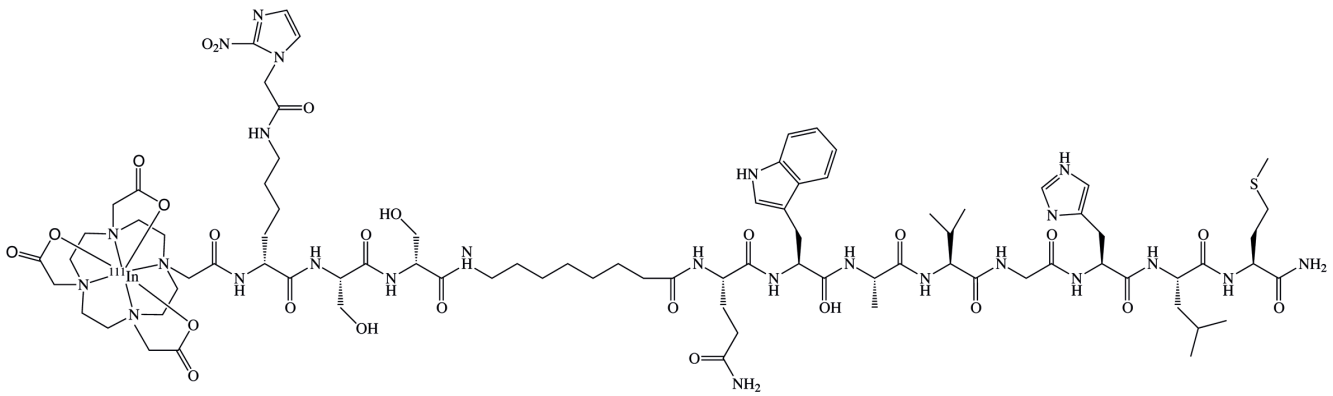

Fig. 3. Hypoxia enhanced ^{111}In-BB2r-targeted conjugate, including a chelating agent (DOTA) that forms a complex with the radiometal (^{111}In), a linker (8-Aminooctanoic acid) for the covalent attachment of the peptide, a hypoxia trapping agent (2-nitroimidazole), and a BB2r-targted peptide BN(7-14)NH_2.

the treatment of renal cell carcinoma, and it binds specifically to the protein structure of CAIX.[123,124] In a phase III trial with Rencarex to treat ccRCC, Rencarex treatment did not improve median disease-free survival compared with placebo; however, further subgroup analysis revealed that the Rencarex effect became more pronounced for higher CAIX scores (http://www.wilex.de/portfolio-english/rencarex/phase-iii-ariser/#Status, accessed June 2015). A phase I radioimmunotherapy study with ^{177}Lu-labeled girentuximab showed that the drug is well tolerated in metastatic ccRCC patients[125]; this was followed by a phase II study in patients with advanced renal cancer (clinicaltrials.gov identifier: NCT02002312). Radiolabeled girentuximab is valued in the presurgical diagnosis and characterization of ccRCC patients.[126,127] There is a significant correlation between *in vivo* PET/CT images and *in vitro* measurements of the uptake of ^{124}I-labeled girentuximab in ccRCC patients.[128] A phase III trial was designed to determine whether the combination of ^{124}I-labeled girentuximab with PET/CT could improve the diagnosis of renal masses versus CT alone. The role of ^{111}In-labeled girentuximab in the evaluation of the therapeutic effect of small renal masses is currently being investigated (clinicaltrials.gov identifier: NCT02411968). Representative chemical radiosensitizers and therapeutic agents targeting tumor hypoxia are summarized in Table 2.

Hypoxia-selective trapping agents

Receptor-targeted peptidic radiopharmaceuticals have been extensively investigated.[129,130] These radiotracers have desirable pharmacokinetics properties, including the ability to rapidly accumulate at the target site, rapidly clear from plasma, and exhibit good tumor penetration. However, the usefulness of many receptor-targeted peptides is limited by the relatively poor residence time in the tumor, due to metabolism and efflux and diffusion from the tissue. Poor retention of targeted radiotherapeutics at the tumor site might not affect uptake but may lead to a markedly lower cumulative therapeutic dose delivered to the tumor and, consequently, a reduction in the translational potential of the agents to the clinic.

Recently, efforts have been reported that take advantage of the hypoxic nature of many tumors to selectively increase the tumor residence time of receptor-targeted peptides. Garrison *et al*. incorporated 2-nitroimidazoles, which are known to be trapped bioreductively in hypoxic cells, into the bombesin (BN) peptide (Fig. 3), an amphibian peptide that targets the gastrin-releasing peptide receptor (BB2r) with nanomolar affinity.[131] The overarching idea behind this approach is depicted in Fig. 4. Specifically, this work is focused on prostate cancer, which has been shown to be among the most hypoxic types of tumors observed clinically.

Bombesin or gastrin-releasing peptide regulates numerous functions of the gastrointestinal and central nervous systems, and the effects of bombesin are mediated through the BB2r. In humans, the BB2r is expressed in the pancreas and, at much lower levels, in the stomach, adrenal glands, and brain. The BB2r has been shown to be expressed at higher densities on a variety of human tumors, such as those of the breast, lung, colon, and prostate, relative to normal tissues.[132] Upon binding of the BB2r-targeted agent to the receptor, the receptor-ligand complex becomes internalized. Under hypoxic conditions, the 2-nitroimidazole(s) attached to the targeted peptide becomes activated and capable of binding irreversibly to intracellular nucleophiles, such as proteins. Given the hypoxic burden of tumors in prostate cancer patients, the expectation is that the activation and irreversible binding to the intracellular contents of hypoxic cells will substantially and selectively increase the residence time of BB2r and other receptor-targeted peptides in hypoxic tumors, thereby leading to an increase in the diagnostic and therapeutic efficacy and clinical potential.

Based on these rationales, ^{111}In-labeled BN(7-14) conjugates, composed of 2-nitroimidazole moieties, were synthesized and evaluated both *in vitro* and *in vivo*. Internalization and efflux studies on PC-3 cells demonstrated significantly lower clearance rate of the radioconjugates containing 2-nitroimidazole relative to control under hypoxic conditions. Up to 2-fold higher macromolecule associated radioactive signals were observed for the 2-nitroimidazole-containing conjugates under hypoxic conditions, suggesting that the significant increase in retention was at least partially due to the irreversible binding of 2-nitroimidazoles to intracellular proteins. The *in vivo* biodistribution on PC-3 tumor-bearing severe combined immunodeficiency (SCID) mice demonstrated up to 20% higher tumor retention for the 2-nitroimidazole hypoxia trapping enhanced radioconjugates. These results indicate the great potential for the use of hypoxia-selective trapping agents to improve the tumor retention of various radiolabeled targeting agents.

Prospects

Tumor hypoxia is a complicated and stubborn obstacle to radiation therapy. Recently, there has been progress in the areas reviewed in this article; however, attempts to overcome tumor hypoxia and the resultant resistance to radiotherapy have only achieved lim-

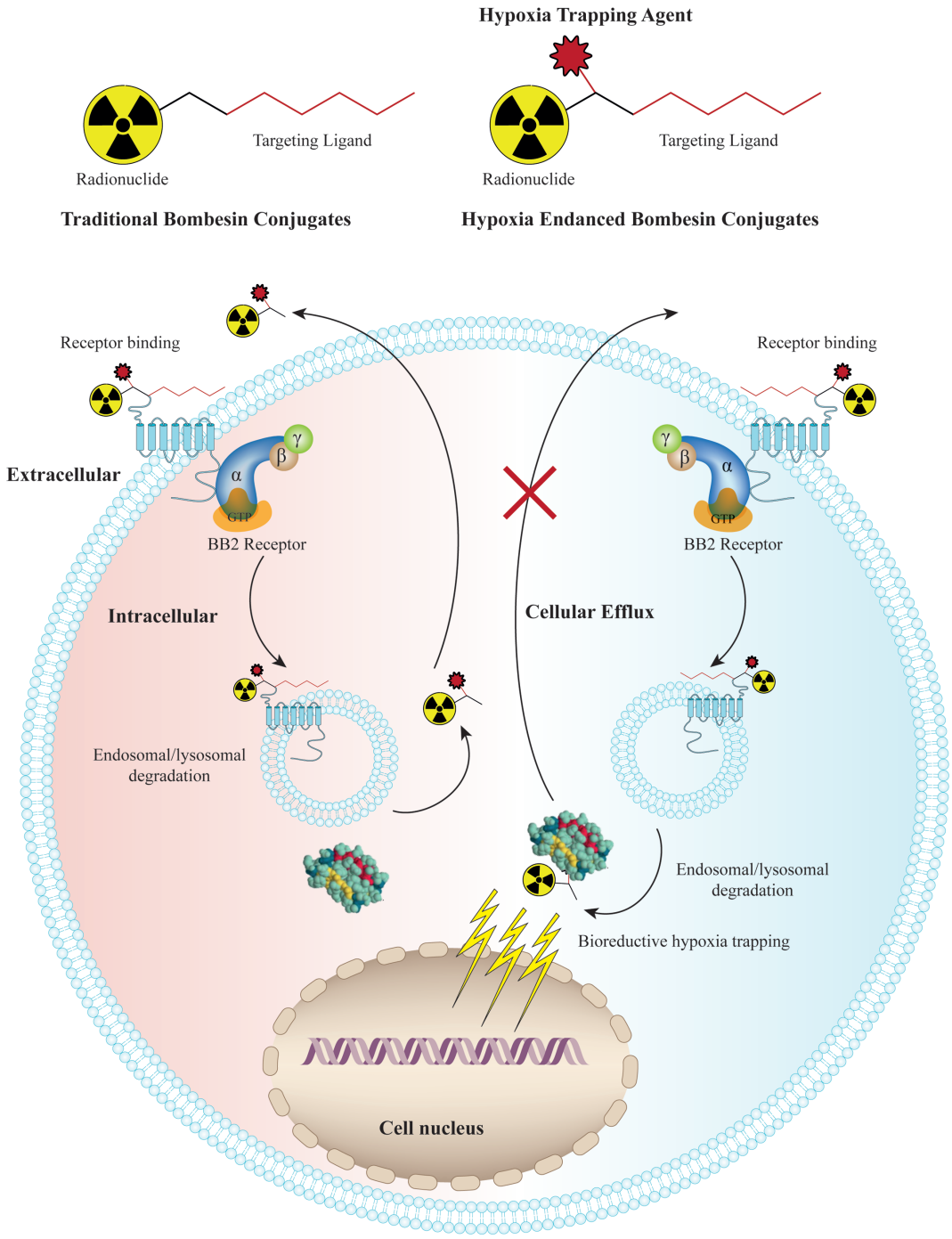

Fig. 4. Expected trapping mechanism of hypoxia enhanced ^{111}In-BB2r-targeted conjugates.

ited success. Previous experiences suggest that targeting tumor hypoxia with a single treatment modality or therapeutic agent may be insufficient.

We hypothesize that the next successful move in the battle against tumor hypoxia will be biologically guided intensity-modulated radiotherapy. This concept is not restricted to dose escalation treatment planning based on hypoxia imaging. By utilizing multidimensional molecular imaging agents and biomarkers, this approach will achieve two essential goals: first, to identify the viable and hypoxic tumor cells that are most relevant to the poor prognosis of the disease; second, to stratify patients who will most likely benefit from this treatment modality. Molecular imaging using radiolabeled hypoxia tracers or hypoxia related biomarkers will play a central role for cancer diagnosis and delineation of the tracer-specific therapeutic volume, as well as characterization of therapeutic agents *in vivo*. In this formula, the most difficult component is the identification of predictive genomic signatures.

We predict in the foreseeable future that tumor hypoxia-targeted

therapy will benefit select patients through a combination of external beam radiotherapy and molecular therapeutic agents and/or radiosensitizers. While precise delivery of escalated radiation doses to hypoxic tumor cells may be associated with better outcomes, in the long run, biological breakthroughs are expected to provide the ultimate solution to cancer therapy.

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Conflict of interest

None.

Author contributions

Designing the outline of this review (MZ, ZHZ, JG); drafting the manuscript (MZ, ZYZ, ZHZ); collecting related information and editing the manuscript (ZYZ, ZHZ, JG).

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