FORUM REVIEW ARTICLE

Imaging Tumor Hypoxia to Advance Radiation Oncology

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Abstract

Significance: Most solid tumors contain regions of low oxygenation or hypoxia. Tumor hypoxia has been associated with a poor clinical outcome and plays a critical role in tumor radioresistance. **Recent Advances:** Two main types of hypoxia exist in the tumor microenvironment: chronic and cycling hypoxia. Chronic hypoxia results from the limited diffusion distance of oxygen, and cycling hypoxia primarily results from the variation in microvessel red blood cell flux and temporary disturbances in perfusion. Chronic hypoxia may cause either tumor progression or regressive effects depending on the tumor model. However, there is a general trend toward the development of a more aggressive phenotype after cycling hypoxia. With advanced hypoxia imaging techniques, spatiotemporal characteristics of tumor hypoxia and the changes to the tumor microenvironment can be analyzed. **Critical Issues:** In this review, we focus on the biological and clinical consequences of chronic and cycling hypoxia on radiation treatment. We also discuss the advanced non-invasive imaging techniques that have been developed to detect and monitor tumor hypoxia in preclinical and clinical studies. **Future Directions:** A better understanding of the mechanisms of tumor hypoxia with non-invasive imaging will provide a basis for improved radiation therapeutic practices. *Antioxid. Redox Signal.* 21, 313–337.

Introduction

RADIATION THERAPY HAS been improved for its accuracy and safety with advanced technologies and is still one of the major therapeutic treatments for cancer. However, tumor hypoxia has been recognized as a source of radioresistance since the 1950s. The potential existence of tumor hypoxia was first theorized by Thomlinson and Gray in 1955 (217). It has been a focus for radiation oncology research since then, because molecular oxygen influences the biological effects of radiation by creating stable DNA adducts after strand breaks that cannot be easily repaired by the cell (28). Second, hypoxia-inducible factor-1 (HIF-1) activation in hypoxic tumor cells plays a critical role in tumor radioresistance (152).

Hypoxia develops in the tumor microenvironment as a result of an imbalance between oxygen supply and its consumption. For radiation therapy to overcome tumor hypoxia, several strategies have been developed, starting with the use of hyperbaric oxygen in the mid-1960s and the introduction of high linear energy transfer radiation, such as neutrons and heavy ions (86). Efforts have continued to focus on targeting

hypoxic tumor cells. Examples of strategies tested include the administration of erythropoietin (EPO) (91), carbogen breathing and nicotinamide (50, 113), artificial blood substitutes (214, 215), agents that right shift the hemoglobin saturation curve (115), hypoxia-specific cytotoxins, and hyperthermia (90, 154). In this review, we will focus on defining tumor hypoxia and its relevance in influencing tumor cell survival after radiation treatment. We will also discuss advanced imaging techniques used to detect and monitor tumor hypoxia *in vivo* in preclinical and clinical studies in order to improve the understanding of radiobiologic mechanisms and therapeutic implications.

Hypoxia Is a Unique Feature in the Tumor Microenvironment

Origins of tumor hypoxia in solid tumors

The tumor microenvironment is highly dynamic and contains heterogeneous cell populations that are exposed to different oxygen concentrations. The first device used to measure blood and tissue oxygen tensions was the Clark

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oxygen electrode. This pioneering electrochemical oxygen sensor was invented by Dr. Leland Clark in 1956 (48). The Clark electrode consists of an anode and a cathode with a thin oxygen-permeable membrane. Oxygen diffuses through the membrane and is electrochemically reduced at the indicator electrode. This electrode was a good start, but it was plagued by the self-consumption of oxygen, which led to inaccuracies in oxygen measurement, particularly at a low pressure of O_2 (pO_2). Unstable output and the need of frequent precalibration were additional limits for the Clark electrode. Nevertheless, this invention was critical to the introduction of modern oxygen analyzers (158). In the 1970s, Clark electrodes embedded in needles were used by several investigators to measure pO_2 in human tumors (53). These studies were the first to demonstrate the presence of hypoxia in human tumors.

Normal tissue pO_2 ranges, in general, between 10 and 80 mmHg, depending on the tissue type, whereas tumors often contain significant regions in which the pO_2 is <5 mmHg (19, 227). The oxygen concentration in tissues is influenced by two types of gradients: (i) radial gradients, the result of O_2 diffusion limitations, and (ii) longitudinal gradients, arising from the depletion of oxygen from hemoglobin as it traverses from the arterial input to the venous egress. Tumor hypoxia arises from limited oxygen delivery and high oxygen consumption rate of cancer cells (86, 224). The deficiencies of oxygen transport result from eight physiologic features in the tumor microenvironment: (i) a relatively sparse arterial supply that reduces the amount of oxygenated blood entering the tumor (58); (ii) inefficient orientation and geometry of microvessels that leads to an over-abundance of vasculature in some regions and insufficient density in others (188, 189); (iii) low vascular density, especially in the tumor core; (iv) extreme variations of red blood cell flux in microvessels in which some tumor microvessels contain very few to no red blood cells (56); (v) longitudinal oxygen gradient (69, 205); (vi) increased blood viscosity and sluggish flow by stiff hypoxic red blood cells; (vii) large-diameter shunt vessels, which divert blood away from the tumor bed; and (viii) unstable and cycling oxygenation state (27, 55, 179).

Tumor microvasculature is relatively immature, exhibiting insufficient basement membrane, pericyte coverage, incongruous branching, and leaky dilated vessels with irregular diameters. Tumor vasculature is under a constant state of remodeling, causing oxygen gradients to shift on a variable schedule. Normal vessels have abundance of pericyte and smooth muscle coverage, which permits adjustments of vessel diameter and information transfer in both upstream and downstream directions to form efficient network structures. However, structural abnormalities in tumor microvasculature disturb vascular communication. Deficient gap junctions are caused by hypoxia and elevated vascular endothelial growth factor (VEGF) levels. Lack of gap junctions interferes with conducted responses to the hemodynamic forces and metabolic stimuli. This leads to impaired structural adaption in tumor microvasculature. Lack of normal structural adaptation contributes to the functional shunting of blood flow, which may cause inefficient microcirculation and local hypoxia (176, 187).

Tumor pO_2 also depends on local metabolic demand. Using recessed tip oxygen electrodes, which minimizes artifacts of oxygen consumption by the electrode, our group measured

tumor pO_2 across microregions of tumors in a dorsal window chamber model. By comparing measured pO_2 values with theoretical simulations, local oxygen consumption was estimated as ranging from 0.83 to 2.22 $cm^3 O_2/100 g/min$ (59). The oxygen consumption rate of proliferating cells is three to five times higher than that of quiescent cells (230). Decreasing oxygen consumption in the tumor tissues is a strategy that is used to reduce tumor hypoxia. Several drugs that inhibit mitochondrial respiration, such as meta-iodobenzylguanidine (15), insulin (110, 111), and cyclooxygenase-2 inhibitors (49), have shown their potential to increase tumor oxygenation, thereby enhancing radiosensitivity.

Tumor hypoxia has been found in a wide range of malignancies, such as breast, cervix, vulva, head and neck, prostate, rectum, pancreas, lung, brain, soft tumor sarcoma, non-Hodgkin's lymphoma, melanoma, renal, and metastatic liver cancers (222, 225, 226). Hockel *et al.* first showed that tumor hypoxia was a poor prognostic factor for patient outcome. They analyzed 31 cervical cancer patients and found that patients with hypoxic tumors had a significantly lower overall and recurrence-free survival (96). Brizel *et al.* also showed that the presence of tumor hypoxia, before therapy, was associated with a more metastatic phenotype in humans with soft tissue sarcoma (24). In a review article, Vaupel *et al.* summarized the data from 125 studies describing the pretreatment oxygenation status as measured in the clinical setting using the computerized Eppendorf pO_2 histography system (223). The clinical prognostic significance of pretreatment tumor oxygenation status for local and distant disease control is evident across various outcome parameters (Table 1) (1, 22–26, 60, 62, 74, 75, 96–98, 100, 121, 142, 143, 149, 159–162, 173, 183, 185, 186, 209–212, 237).

Consequences of tumor hypoxia

Tumor cells often adapt to a hypoxic microenvironment by reducing their overall protein synthesis, which leads to restrained cell proliferation and cell death. This is considered a means of energy conservation (126, 127, 238). Two different pathways can lead to this inhibition of protein translation. The first is mediated by the unfolded protein response (UPR). The UPR mediates the phosphorylation of eukaryotic initiation factor 2 α (EIF2 α) by the endoplasmic reticulum kinase PERK, leading to the inhibition of mRNA translation. The second is associated with disruption of the mRNA cap-binding complex, EIF4F, which inhibits the transcript recruitment step of mRNA translation. Hypoxia also changes the cell cycle distribution, which leads to an altered response to radiation therapy or even induces cell apoptosis in p53-dependent and -independent pathways (225).

Tumor hypoxia also increases the expression and activity of HIF-1, which plays an important role in tumor progression, genetic instability (16), immune evasion (141), metastasis (40, 208), angiogenesis (137), and resistance to radiotherapy and chemotherapy (154). HIF-1 is a heterodimeric transcription factor that consists of a hypoxia-regulated α subunit and an oxygen-independent β subunit, which bind to DNA with hypoxia response elements (HREs) which control target gene transcription. HIF-1 α expression is tightly regulated by several mechanisms, but the most important one is the degradation pathway. Degradation is mediated by a family of prolyl hydroxylases that hydroxylate proline residues in the

TABLE 1. PRETHERAPEUTIC OXYGENATION STATUS OF SOLID TUMORS MEASURED USING EPPENDORF pO₂ HISTOGRAPHY SYSTEM AND PROGNOSTIC SIGNIFICANCE OF TUMOR HYPOXIA

Tumor type	Center	n	Median pO ₂ (mmHg) [range]	Endpoint	Oxygenation parameter	References
Cervical	Mainz/Leipzig	150	10 [2–34]	DFS, OS	pO ₂ < 10 mmHg	(96–98, 100)
	Toronto	135	5 [0–94]	DFS, PFS, DS	pO ₂ < 5 mmHg	(60, 74, 75, 149, 173, 237)
	Vienna	51	10 [0–60]	DFS, LC	pO ₂ < 10 mmHg	(121)
	Oslo	49	4 [1–25]	DFS, OS, LC, DS	HSV, pO ₂ < 5, 10 mmHg	(142, 143, 181, 209–212)
Head and neck	Halle/Munich	125	9 [0–59]	OS	HSV	(62)
	Durham	86	5 [0–60]	DFS, OS, LC	pO ₂ < 10 mmHg	(23, 26, 160)
	Aarhus	67	13 [0–54]	LC	pO ₂ < 2.5 mmHg	(161, 162)
	Heidelberg	44	7 [0–60]	OS	pO ₂ < 2.5 mmHg	(185, 186)
	Stanford	37	19 [0–77]	LC	Median pO ₂	(1)
Soft tissue sarcoma	Durham	45	10	DFS	pO ₂ < 10 mmHg	(22)
	Durham	34	6 [0–68]	DFS	Median pO ₂	(25)
	Aarhus	31	19 [1–58]	DFS, OS	pO ₂ < 19 mmHg	(159)
	Durham	30	10	DFS	pO ₂ < 10 mmHg	(24)

Modified table reproduced from Vaupel *et al.* (223) with permission from the authors and Mary Ann Liebert, Inc.
 n, number of patients; DFS, disease free survival; DS, distant spread; LC, local control; OS, overall survival; PFS, progression free survival; pO₂, pressure of O₂.

oxygen-dependent degradation (ODD) domain of HIF-1 α . Hydroxylated proline will then be recognized by the von Hippel-Lindau complex, which targets the protein for subsequent degradation *via* the proteasome. Under normal oxygenated conditions, HIF-1 α levels are reduced as a result of this degradation pathway. However, under hypoxic conditions, the prolyl hydroxylases cannot function properly, because they require oxygen for the hydroxylation. As a consequence, HIF-1 α accumulates and then binds to HIF-1 β as a heterodimer that regulates target gene transcription *via* HRE of DNA (194).

HIF-1 also controls the metabolic switch to aerobic glycolysis, which can maintain cell growth under hypoxic conditions (193, 194). The major metabolic changes to aerobic glycolysis in cancer cells were found by Otto Warburg in 1956 (233). He found that normal cells use glycolysis to generate about 10% of ATP, and mitochondria accounts for the remaining 90%. However, in tumor tissues, more than 50% of the energy is produced by glycolysis. It is now known that the Warburg effect is associated with HIF-1 activation to shift energy production from mitochondrial to glycolytic sources. HIF-1 stimulates glycolytic energy production by activating the genes involved in extracellular glucose import (such as GLUT1 and GLUT3) and enzymes for the glycolytic breakdown of intracellular glucose (such as phosphofructokinase 1 [PFK1]). HIF-1 also down-regulates oxidative phosphorylation within the mitochondria through activating pyruvate dehydrogenase kinase 1 (PDK1) and MAX interactor 1 (MXI1). These two effects reduce the oxygen demand of cancer cells (51). There is also an HIF-1-dependent expression of EPO and angiogenic factors (such as VEGF) that results in increased blood vessel formation for the delivery of a richer supply of oxygenated blood to the hypoxic tissue (190, 195, 197).

HIF-1 is also regulated by many factors other than hypoxia, including oncogenes, growth factors, and free radicals. Chandel *et al.* proposed that reactive oxygen species generated by mitochondria under hypoxia induced HIF-1 α stabilization. Overexpression of catalase blocked hypoxic

stabilization of HIF-1 α , whereas administration of H₂O₂ to cells increased HIF-1 expression (42). Guzy *et al.* then reported that mitochondrial complex III may be important in hypoxic signaling for HIF-1 α stabilization (84). The effects of nitric oxide (NO) on HIF-1 activity under hypoxia are complex. The initial report by Liu *et al.* suggested that NO inhibited HIF-1 α stabilization under hypoxic conditions (139). This was due to NO inhibited cytochrome c oxidase activity under hypoxia, thereby increasing PHD activity (85). Our group also showed that normoxic HIF-1 activity can be up-regulated through NO-mediated S-nitrosylation and stabilization of HIF-1 α (135). Recently, Berchner-Pfannschmidt *et al.* reported that NO induced a feedback regulatory mechanism between HIF-1 and PHD2. When cells were first exposed to NO, HIF-1 α levels transiently increased and induced PHD2 expression. PHD2 feedback can then inhibit HIF-1 α expression (14).

Hypoxic responses are also mediated by HIF-2, a heterodimer of HIF-2 α (which is also regulated by oxygen-dependent hydroxylation) and HIF-1 β . Expression of HIF-2 α is predominantly due to post-translational regulation, because mRNA levels are not significantly induced under hypoxia. HIF-2 α expression is specific to certain cell types within vertebrate species. HIF-2 α also recognizes similar HERs in the promoter region of target genes involved in erythropoiesis, angiogenesis, metastasis, and proliferation. Therefore, HIF-2 α plays a role in both development and tumorigenesis (169).

Hypoxia increases HIF-1 activity, which, in turn, up-regulates VEGF, while down-regulating the angiogenesis inhibitor thrombospondin. This leads to a pro-angiogenic environment (129). Holash *et al.* proposed a “hypoxic crisis” model for the evidence of hypoxia-driven angiogenesis initiation. They used a C6 glioma model and observed vessel cooption in the first week after tumor cell implantation. Vascular basement membrane breakdown and vessel regression followed, leading to hypoxia during the second week caused by angiopoietin-2 (ANGPT2)-mediated de-differentiation of microvessels. Once hypoxia was detected,

VEGF was up-regulated and promoted the initiation of angiogenesis (101). In contrast, our group hypothesized the “acceleration model” and proposed that hypoxia was not responsible for the initiation of angiogenesis. The initiation was driven by hypoxia-independent mechanisms, such as VEGF up-regulation by oncogenes. Once angiogenesis was initiated, tumor proliferation occurred, creating a hypoxic environment. Hypoxia then led to the up-regulation of HIF-1 and the acceleration of angiogenesis (37).

Chronic versus cycling hypoxia in tumors

It is likely that most human solid tumors contain microregions with both chronic and acute/cycling hypoxia. Both conditions have been amply described in pre-clinical models (54, 55) and in canine tumors (31). To date, however, there is scant direct evidence that cycling hypoxia exists in human tumors. There are no data refuting its existence either, in human tumors. The term chronic hypoxia refers to cells with a pO_2 below 10 mmHg. This low oxygen value can last from hours to days, which is long enough to induce changes in gene expression, such as HIF-1 activation. Cycling hypoxia results primarily from the instabilities in microvessel red blood cell flux and temporary fluctuations of perfusion (perfusion-limited hypoxia) (54, 55, 147). The pO_2 value in cycling hypoxic region fluctuates over time. The kinetics are complex and can range from a few cycles per hour to a timescale of hours to days. Our group reported that cycling hypoxia can be observed as much as 130 μ m from a microvessel. This is the distance near the maximum diffusion distance of oxygen (131).

To study the biological and therapeutic consequences of chronic and cycling hypoxia, researchers either have exposed tumor cells to variable O_2 concentrations *in vitro* or have exposed tumor-bearing mice *in vivo* to hypoxic gas mixtures to induce acute or chronic hypoxia (12). There are great variations of exposure time frames and O_2 concentrations to induce hypoxia *in vitro*. For example, to induce chronic hypoxia, tumor cells have been exposed to hypoxic conditions from as short as 4 h to as long as several weeks; whereas to induce cycling hypoxia, cells have been exposed to continuous hypoxia from 30 min to 72 h. The O_2 concentrations used for *in vitro* studies also range from 0.02% to 6%. This led to the limitation of *in vitro* hypoxia studies to realistically reflect the *in vivo* situation. There are fewer *in vivo* hypoxia studies done. Tumor-bearing mice have been allowed to breathe 7%–8% O_2 either daily for 2–4 h or continuously for approximately 28 days to induce chronic hypoxia. To induce cycling hypoxia, the mice have been exposed to 1–12 daily cycles of 1 h hypoxia/1 h air breathing (12). There is a general trend toward the development of a more aggressive phenotype after cycling hypoxia, and we will discuss this in detail throughout the next sections.

Chronic hypoxia

Chronic hypoxia is caused by limited diffusion of oxygen from tumor microvessels into the surrounding tissues. Thomlinson and Gray first described the phenomenon of chronic hypoxia in a study of bronchial carcinomas (217). This type of tumor often grew in solid cords surrounded by stroma. The centers of the large tumor area were necrotic and were surrounded by intact tumor cells. When the tumor cords grew larger, the necrotic center also enlarged; so, the thick-

ness of the sheath of viable tumors remained constant. The conclusion from this study was that tumor cells could proliferate and grow actively only if they were close to the stroma to receive a sufficient supply of oxygen and nutrients.

Chronic hypoxia can also result from hypoxemic hypoxia. One example is tumor-associated or therapy-induced anemia, causing long-lasting low oxygen content in the blood supplied to the tumor. Hypoxemic hypoxia can also be found in primary or metastatic liver tumors that are supplied with partly deoxygenated blood by branches of the portal vein. Tumor vasculature is often characterized as being leaky, dilated, elongated, and tortuous. These structural abnormalities cause spatiotemporal heterogeneity in tumor blood flow. In addition, non-functional lymphatics and high interstitial fluid pressure generated by proliferating tumor cells compresses lymphatics, and blood vessels are also present in the tumor microenvironment. Therefore, an additional source of chronic hypoxia may be caused by a compromised perfusion of leaky microvessels (11, 109).

Cycling hypoxia

Goodall *et al.* were among the first to mention that regurgitant blood flow and transient stasis occurred in tumors using the hamster cheek pouch model (80). Asaishi *et al.* then directly measured tumor vessel flow rates in melanomas growing in the hamster skin flap. They found that small tumors had very few microvessels exhibiting vascular stasis, but as the tumors grew, vascular stasis increased (9). However, the radiobiological perspective of cycling hypoxias had been studied in rats in the late 1970s using dorsal skin window chambers, which enabled direct visualization of the developing vasculature within tumors in the living animals (179, 240). By using window chambers, Reinhold *et al.* studied the changes of two naturally fluorescent coenzymes, flavin adenine dinucleotide (FAD) and NADH, and calculated the redox ratio. This ratio was derived from the relative abundance of these coenzymes involved in respiration and, therefore, reflected oxygenation status in the tumor tissues. He found fluctuations in redox ratio in tumor tissues. Yamaura and Matsuzawa observed hepatoma regrowth at the tumor periphery in window chambers after radiation treatment. They mentioned that this tumor regrowth might be due to transient hypoxia, because they observed temporary vascular stasis in this area. Subsequently, Chaplin *et al.* found that cycling hypoxia was caused by transient changes in tumor blood perfusion in the murine squamous carcinoma model (43, 44). Our group was also able to dynamically image changes in oxygen supply and demand in tumors using a combination of redox imaging, hyperspectral imaging, combined with Doppler optical coherence tomography (OCT) (Fig. 1). We found that longitudinal oxygen gradients were apparent at all of the time points. This is depicted by groups of vessels with varied Hbsat in the upper right quadrant of the tumor compared with the lower right quadrant. However, the extent of difference between the more hypoxic and less hypoxic regions varies between different time points. It should be noted that at 30-h, the upper right quadrant is more hypoxic than the same quadrant imaged at 24- or 36-h (Fig. 1a). We also demonstrated the redox ratio, which reflects that the relative extent of oxidative metabolism within the tumor is higher in areas with more oxygenated vessels

FIG. 1. Serial hemoglobin saturation and redox images from a tumor grown in the window chamber. (a) Hbsat and **(b)** redox images obtained over a 36-h time course. Longitudinal oxygen gradients **(a)** are apparent at all of the time points. This is depicted by groups of vessels with varied Hbsat in the *upper right* quadrant of tumor compared with the *lower right* quadrant. However, the extent of difference between the more hypoxic and less hypoxic regions varies between different time points. Note at 30h, the *upper right* quadrant is more hypoxic than the same quadrant imaged at 24 or 36 h. In **(b)**, the redox ratio, which reflects the relative extent of oxidative metabolism within the tumor, is higher in areas with more oxygenated vessels (3). Reproduced from Skala *et al.* (202) with permission from the authors and SPIE. Hbsat, hemoglobin saturation. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

(Fig. 1b). Our data showed a positive correlation between the blood flow, blood oxygenation, and metabolic demand in tumors using the dorsal skin fold window chamber model (201, 202). Kimura *et al.* previously demonstrated that cycling hypoxia is a relatively common phenomenon in tumors using dorsal skin window chambers. They observed fluctuations in red cell flux (RCF) in tumor microvessels, which led to changes in vascular pO_2 . Variations in RCF in the poorly vascularized region led to a greater percentage of transient hypoxia as compared with the well-vascularized tumor regions (120). Our group also demonstrated that there was a correlation between the RCF of microvessels and interstitial pO_2 (131). These results suggest that the primary determinant of cycling hypoxia is a variation in microvessel RCF.

Spatiotemporal variations in tumor perfusion have been proved as characteristics of cycling hypoxia using dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) (30, 71). The kinetics of cycling hypoxia have at least two dominant timescales: One has a frequency of a few cycles per hour, and the second ranges from many hours to days (18, 27, 39, 43–45, 63, 64, 150, 202). The higher frequency cycling hypoxia is associated with variations in RCF (120, 131, 171). The temporary changes in microvessel RCF match the fluctuations in feeding arteriolar diameter (56). Similarly, Baudalet *et al.* used blood oxygenation-level dependent magnetic resonance imaging (BOLD-MRI) and observed fluctuations in MRI signals more frequently in tumor regions containing immature blood vessels (10). These studies demonstrated that some of the fluctuations in RCF might be caused by arterial vasomotion and will be discussed in greater detail in the imaging section. Vascular intussusception, a process in which microvessels remodel to split into parallel smaller vessels, is also responsible for high-frequency hypoxia (114, 119, 168, 177). Alternatively, cycling hypoxia ranging from more than hours to days is most likely caused by changes in vascular network structures due to neoangiogenesis or blood vessel remodeling (54, 55). The slower frequency of cycling hypoxia has been verified in both human tumor xenografts and human head and neck cancer patients. Bennewith and Durand estimated that approximately 20% of tumor cells experienced cycling hypoxia over periods of several hours using human xenografts, and these cells were not immediately adjacent to tumor microvessels (13). Nehmeh *et al.* used ^{18}F -misonidazole positron-emission tomography (PET) and showed that cycling hypoxic tumor regions also existed in head and neck cancer patients (157).

The primary consequence of cycling hypoxia is the up-regulation of HIF-1 activity, which has been shown both *in vivo* and *in vitro* (170, 247). This increased HIF-1 response is more robust when cells are exposed to cycling hypoxia rather than chronic hypoxia. The mechanisms of enhanced HIF activity may include alterations in HIF-1 synthesis that is regulated by mammalian target of rapamycin and phosphorylation of the HIF-1 binding cofactor CREB-binding protein (196). Cycling hypoxia also increases the levels of free radicals as a result of hypoxia-reoxygenation injury, and these free radicals then stabilize HIF-1 (55). A major consequence of increased HIF activity may be the observed alterations in angiogenesis.

Impact of Tumor Hypoxia on Radiation Therapy

Tumor hypoxia is a well-known cause for radioresistance in pre-clinical studies. There is very strong evidence for its

importance clinically as well. There is a significant relationship between tumor hypoxia and poor clinical outcome after radiotherapy (23, 76, 97, 154). DNA is the critical target of radiation therapy through direct and/or indirect actions. In direct action, a secondary electron resulting from absorption of an X-ray photon interacts directly with the DNA. Alternatively, in indirect action, a secondary electron interacts with other atoms or molecules in the cells, such as a water molecule, to produce free radicals that can diffuse far enough to damage the DNA (86). Radiation therapy produces DNA double-strand breaks (DNA DSBs), DNA single-strand breaks (DNA SSBs), DNA base damage, and DNA-DNA and DNA-protein cross-links under normoxic condition. The oxygen effect refers to the phenomenon in which oxygen “fixes” or makes permanent damage produced by free radicals (52, 82). Cells are much more sensitive to radiation therapy in the presence of oxygen than under hypoxic condition. The ratio of doses under hypoxic to normoxic conditions necessary to produce the same level of cell killing is called oxygen enhancement ratio (OER) (94). For example, there is a dose-modifying factor of 2.5–3.0 between proliferating cells irradiated at <2.5 mmHg and those irradiated at >10 mmHg. Although 60 Gy of radiation is usually curative for tumor cell killing under normoxia, it would require >150 Gy to reach the same killing for hypoxic cells (60×2.5 or 3) (163). In addition, in the absence of oxygen, decreased DNA DSBs are produced by free radicals after radiation therapy and/or the damaged products are repairable (73, 164, 216, 231).

Hypoxic tumors commonly reoxygenate after one to a few fractions of radiation treatment (32). This effect is caused by a reduction in the oxygen consumption rate as a result of the death of radiosensitive oxygenated tumor cells, as well as an increase in perfusion (57, 184). In experimental animals, some tumors take several days to reoxygenate, but others undergo this process within 1 h. Based on tumor oxygenation studies, it is considered that fractionated radiotherapy could effectively eliminate hypoxic tumors, because these hypoxic tumor cells become reoxygenated and resensitized between individual dose fractions (86). Although tumor reoxygenation may affect the effectiveness of radiotherapy, detailed knowledge of the reoxygenation time course in certain tumors undergoing radiotherapy is still needed.

Furthermore, Jordan *et al.* demonstrated that NO can exert a similar effect to increase tumor oxygenation and radiosensitivity. They treated tumor-bearing mice with isosorbide dinitrate (a NO donor), slow infusion of insulin, or electrical stimulation of the host tissue (inducing eNOS activation) along with radiotherapy. They found an increase of NO content and oxygenation in the tumor after these treatments, which then led to an increase of radiosensitivity. They suggested that the increase in tumor oxygenation was achieved *via* alterations in blood flow and oxygen consumption through NO-mediated pathways (112). Sonveaux *et al.* also showed that radiation treatment increased eNOS production in the tumor microvasculature. This radiation-induced NO production restored vasomotion in tumor afferent arterioles and increased tumor blood flow and oxygenation. Furthermore, radiation-induced NO production benefited the effectiveness of second radiotherapy (204). These results suggest that NO can act as an intrinsic radiosensitizer.

HIF-1 is another excellent target that is used to improve radiation outcome, as high tumor HIF-1 activity is a predictor

of poor prognosis after radiotherapy (128). It is important to understand the interplay between radiotherapy and HIF-1. To study the effect of radiation on HIF-1 activity, our group used a dorsal skin window chamber model with murine and human tumor cell lines that were stably transfected with two reporter genes: red fluorescent protein under control of a constitutive CMV promoter (RFP_c) and green fluorescent protein under control of an HRE derived from the VEGF promoter (GFP_h). We found that radiation treatment induced HIF-1 activation, and this coincided with reoxygenation. This radiation-induced HIF-1 activity only occurs *in vivo*, which means that the tumor microenvironment is critical for this effect (37). Two independent mechanisms were found to explain this effect. First, radiation treatment increased free radical species, which then stabilized HIF-1. The second mechanism was related to stress granules. These are protein-mRNA complexes formed in cells during stress, such as hypoxia, to prevent protein translation. Stress granules rapidly disassemble during reoxygenation, permitting the release of HIF-1 regulated transcripts, such as VEGF. These factors then deliver survival signals and provide radioprotective effects for endothelial cells (116, 151). Our group collaborated with Chuan Li's group and showed that radiation treatment induced a second peak of HIF-1 α stabilization at 6–7 days after treatment. This second wave of HIF-1 activation correlated with macrophage infiltration into the tumors. These macrophages produced NO, causing direct nitrosylation of a single cysteine residue in the ODD domain of HIF-1 α , which prevented its proteasomal destruction (135).

To study the impact of HIF-1 activation on overall tumor radiosensitivity, our group used siRNA or a dominant-negative mutant of HIF-1 α to inhibit HIF-1 activation. We found that HIF-1 had radiosensitizing effects on tumor cells by promoting ATP metabolism, proliferation rate, and radiation-induced apoptotic potential. On the other hand, HIF-1 promoted tumor radioresistance by stimulating endothelial cell survival. Therefore, the net result of HIF-1 inhibition on tumor radiosensitivity is highly dependent on treatment sequencing. The aim will be to maximize the effects of HIF-1 blockade on the vasculature while minimizing the effects on tumor cells. This can be achieved by a specific treatment strategy of radiation first followed by HIF-1 blockade (153).

Role of Hypoxia in Tumor Progression

Tumor hypoxia and HIF-1 activation are intimately involved in tumor progression (92, 99, 191, 192). The evidence to prove that hypoxia plays an important role in tumor progression comes from studies that demonstrate a correlation between tumor oxygenation and treatment outcome in both experimental animals and cancer patients. Brizel *et al.* showed that there was a correlation between tumor hypoxia and increased risk of distant metastases in soft-tissue sarcoma patients receiving radiotherapy (24). Young *et al.* further supported the clinical data using rodent tumor cells. They exposed rodent tumor cells to hypoxia *ex vivo* or *in vivo* and observed an increase in metastases (243, 244). From these animal studies, it has been suggested that hypoxia may be enhancing metastasis by increasing genetic instability, including gene amplification, point mutation, hyper-mutagenesis, and induction of DNA strand breaks in the tumor microenvironment (20). Exposure to hypoxia also selects for cells with a

loss of p53 function or increased expression of mouse double minute 2 homolog (MDM2), which is a p53 negative regulator. This selection will lead to tumor cell resistance to apoptotic stimuli (81, 250). Additional studies using human tumor cells suggested that hypoxia could up-regulate HIF-target genes involved in the metastatic cascade, such as plasminogen activator urokinase receptor (PLAUR), the chemokine receptor CXCR4, osteopontin, lysyl oxidase (LOX), interleukin 8 (IL-8), and VEGF (40, 70, 181).

Chronic hypoxia is one of the drivers for prostate cancer development and progression (155, 156). Alqawi *et al.* developed a chronic hypoxic prostate cancer cell line (HMLL) by incubating MatLyLu cells under hypoxic condition (1% O₂ for more than 3 weeks). They found that HMLL cells had a greater expression of hypoxia-response genes, including GLUT1 and VEGF. In addition, these cells showed a higher invasive capacity relative to cells exposed to acute hypoxia (1% O₂ for 5 h) (4). Furthermore, androgen deprivation is a standard therapy for advanced prostate cancer that is used to reduce androgen receptor activation, blood flow to the prostate, and tumor hypoxia (2, 5). However, androgen-dependent prostate tumors become androgen independent after long-term androgen deprivation. Hirai *et al.* found that chronic hypoxia, but not acute hypoxia, induced androgen-independent growth and stimulated cell migration and invasion in the prostate cancer cell line LNCaP. They established this LNCaP cell line by culturing cells under chronic hypoxia (1% O₂ for more than 6 months) and demonstrated that Vav3 oncogene expression was strongly induced and associated with malignant behavior (95, 239).

In contrast, Pires *et al.* showed that hypoxia induced S-phase arrest in tumor cells and was associated with an inhibition of DNA replication during both the initiation and elongation phases. Chronic hypoxia (0.02% O₂ for 16 h) compromised the ability of the S-phase arrested cells to restart DNA replication after reoxygenation by inducing disassembly of the replisome, while acute hypoxic cells remained replication competent. These results suggest that severely chronic hypoxic cells do not contribute to continued tumorigenesis (172). The contradictory effects of chronic hypoxia on tumor progression have also been found in the animal models. Yu and Hales exposed tumor-bearing rats to chronic hypoxic conditions (10% O₂ for 14 days) and observed a significant inhibition of lung tumor growth. The underlying mechanisms for chronic hypoxia-induced inhibition of lung cancer tumor growth included inhibition of cell proliferation, induction of apoptosis, and reduction of tumor microvessel density. However, this result was highly cell-line dependent, because they observed the opposite effect of chronic hypoxia on colon cancer cells (245). Based on such inconsistent experimental data, a definite conclusion cannot be reached for the effects of chronic hypoxia on tumor progression.

There is a general trend toward the development of a more aggressive tumor phenotype after cycling hypoxia. Cycling hypoxia could contribute to tumor progression by providing repeated exposure of tumor cells to hypoxia-reoxygenation injury (34–36). Cairns *et al.* exposed KHT tumor-bearing mice to low oxygen conditions (5%–7% O₂ breathing) to induce chronic and cycling hypoxia. They demonstrated that cycling hypoxia, but not chronic hypoxia, increased the number of spontaneous micrometastases in mouse lungs and that this enhancement was due to the effect of the acute

hypoxia treatment on the primary tumors (35). Using an orthotopic cervical cancer model, the same group demonstrated that cycling hypoxia increased the frequency of metastasis to regional lymph nodes (34). This increased metastasis was correlated with increased expression of metastasis-associated genes, such as CXCR4, uPAR, VEGF, and osteopontin, in tumors of mice exposed to the cycling hypoxia treatment (46). Rofstad *et al.* used the combination of a radiobiological assay and immunohistochemical assessment of the hypoxia marker pimonidazole to distinguish between cycling and chronic hypoxia in human melanoma xenografts. They observed a positive correlation between the cycling hypoxic fraction in the primary tumors and the incidence of lung and lymph node metastasis. They even suggested that some tumor cells had more of an inherent tendency to develop cycling hypoxia (182). These results suggest that diagnostic methods which can detect tumors with a tendency toward cycling hypoxia may help identify patients with higher risks of metastasis.

Methods for Detecting Hypoxia

Understanding the influence of chronic and cycling hypoxia on the tumor microenvironment and radiation response in preclinical and clinical research studies has been advanced by hypoxia imaging techniques. These methodologies can provide information regarding the spatiotemporal characteristics of tumor hypoxia, and this information can be analyzed to investigate changes in the tumor microenvironment. Here, we will review how the imaging of chronic and cycling hypoxia has impacted or can impact the field of radiobiology.

There are general qualities that are valuable to a hypoxia imaging device. The optimal imaging system should be able to distinguish between hypoxia, anoxia, and necrosis; identify cellular pO₂ values as opposed to vascular pO₂ values; and be sensitive to pO₂ values at clinically relevant levels (0–15 mmHg). Additional aspects that will dictate the value of the modality include good spatial resolution, short acquisition time for optimal temporal resolution, ability for repeatable measurements, non-invasiveness, applicability to any tumor site (not depth limited) with a wide spatial window for measurement, and ease of use.

Multiple approaches have been developed to meet these qualities of the ideal hypoxia imaging device. The types of measurements used can vary greatly, from absolute measurements of pO₂ or oxygen concentration to indirect measurements of a related parameter, such as hemoglobin saturation. The main hypoxia imaging devices clinically used include PET and MRI, and in the laboratory setting, they include optical methods, such as optical spectroscopy, redox imaging, phosphorescence lifetime imaging, and photoacoustic tomography. We have summarized these hypoxia imaging techniques with regard to their ability to distinguish between chronic and cycling hypoxia and whether they have been used in preclinical and/or clinical radiation oncology (Table 2).

Invasive hypoxia imaging

Immunohistochemistry. One of the most extensively used techniques to detect and quantify tumor hypoxia is immunohistochemistry (IHC). The IHC methods are typically based on antibody binding to protein adducts of bioreductive

TABLE 2. ABILITY OF HYPOXIA IMAGING TECHNIQUES TO DISTINGUISH CHRONIC OR CYCLING HYPOXIA AND UTILITY IN PRECLINICAL AND/OR CLINICAL RADIATION ONCOLOGY

	IHC	PET	MRI			Optical spectroscopy	Phosphorescence lifetime imaging	Phosphoacoustic tomography
			DCE-MRI	BOLD-MRI	EPRI/MRI			
Direct/indirect	Indirect	Indirect		Indirect Indirect Indirect		Indirect	Direct	Indirect
Invasive/non-invasive	Invasive	Minimally invasive		Minimally invasive Non-invasive Minimally invasive		Non-invasive	Minimally invasive	Non-invasive
Characterization of hypoxia as chronic/cycling	Indeterminate	Distinguished by repeated imaging		Distinguished by repeated imaging Distinguished in real-time		Distinguished in real-time	Distinguished in real-time	Distinguished in real-time
Sensing depth	Not applicable	Whole body		Whole body Whole body mm-cm		mm-cm	mm-cm	mm-cm
Temporal resolution	h	min-h		s s min-h		s	s	s
Spatial resolution	< μ m	Several mm		Sub mm Sub mm-mm mm		mm-cm	Sub mm-cm	mm
Sensitivity	Moderate	Dependent on voxel spatial distribution of hypoxia		Not applicable Not applicable < 1 mmHg		High sensitivity to hemoglobin	< 1 mmHg	High sensitivity to hemoglobin
Preclinical/clinical use	Both	Both		Both Both Preclinical		Both	Preclinical	Preclinical

DCE-MRI, dynamic contrast-enhanced magnetic resonance imaging; BOLD-MRI, blood oxygenation-level dependent magnetic resonance imaging; EPRI, electron paramagnetic resonance imaging; IHC, immunohistochemistry; PET, positron-emission tomography.

nitroimidazole compounds, such as pimonidazole (221) or EF5 (140), that are systemically administered before biopsy or tumor removal is performed. At oxygen tensions below 10 mmHg, these nitroimidazoles undergo a nitroreductase-catalyzed single-electron reduction and form covalent bonds with cellular macromolecules, thus trapping the compounds inside the hypoxic cells (242). These compounds have been used to develop algorithms to provide examples of the relationship between tumor tissue pO_2 and blood vessel distribution in large-scale tumors. Evans *et al.* reported that the number and area of blood vessels, as well as the varying patterns of hypoxia relative to blood vessels, can be described quantitatively using EF5 as applied to human brain tumors, and that EF5 binding gradients away from vessels have both positive and negative values which average near zero over macroscopic tumor regions. The presence of both positive and negative EF5 gradients, in roughly equal proportions, suggests a dramatic heterogeneity of oxygen availability from blood vessels in large tumors (72). Further, this group recently identified macroscopic regions of hypoxia in rat 9L-epigastric tumors indicated by EF5 binding and described extended length longitudinal gradients of blood flow (122).

Alternatively, IHC of endogenous proteins that are overexpressed under hypoxic conditions, such as HIF-1 α or carbonic anhydrase IX (CA9), can be used as surrogates for hypoxia (33). The use of endogenous and exogenous markers to image hypoxia shows a typically more restricted distribution of bound pimonidazole than the HIF-1 target CA9. This evidence indicates that metabolic activation of nitroimidazole probes requires more severe hypoxia than does the HIF-1 response (236).

While IHC can provide important information regarding the tumor microenvironment, main limitations include invasiveness, limited sampling size, and difficulty in performing repetitive measurements to monitor changes in oxygenation due to the necessity for biopsy or surgical removal of the tumor to evaluate the tissue. These results are representative of single snapshots in time and cannot be easily used to assess the dynamics of cycling hypoxia; however, Bennewith and Durand have reported a technique to assess transient hypoxia on a global scale in tumor xenografts by sequentially administering two hypoxia markers followed by quantification of the hypoxic cells using flow cytometry. High levels of pimonidazole (the first hypoxia marker) were maintained in circulation over an 8-h period with multiple hourly injections, followed by subsequent administration of a second hypoxia marker (CCI-103F). They were able to show that a substantial number of the previously pimonidazole-labeled cells were no longer hypoxic during the circulation lifetime of the second marker, which was consistent with cycling hypoxia over an 8-h period (13).

Non-invasive hypoxia imaging

Non-invasive techniques that permit serial imaging of hypoxia provide valuable information because of the heterogeneous and cycling nature of hypoxia in tumors. Since the therapeutic implications of chronic and cycling hypoxia have been defined, imaging techniques that enable an unperturbed representation of solid tumors in patients and rodent models have proved to be instrumental in advancing radiobiology research and clinical radiation oncology.

Positron emission tomography. PET hypoxia imaging utilizes radiopharmaceuticals compounds that identify regions of tumor hypoxia. The most commonly used radiopharmaceutical to detect hypoxia is the highly stable and robust nitroimidazole derivative, ^{18}F -fluoromisonidazole (FMISO) (83). When FMISO diffuses into cells, it is first reduced by nitroreductase to a radical form. Under hypoxic conditions, these radicals will bind to intracellular macromolecules and accumulate inside the cell. Positive accumulation of FMISO is proportional to the extent of hypoxia in the pO_2 range of a few mmHg, with high FMISO uptake indicative of a low tissue O_2 concentration (123). Other frequently used compounds include ^{64}Cu -diacetyl-bis(N^4 -methylthiosemicarbazone) (Cu-ATSM) and ^{18}F -fluoroazomycinarabinofuranoside (FAZA) (134, 206). The accuracy and reliability of Cu-ATSM as a PET marker for measuring hypoxia *in vivo* was evaluated by comparing autoradiographic distributions of Cu-ATSM with the well-established hypoxia marker drug EF5 in three tumor cell lines. It was determined that the validity of Cu-ATSM uptake as a marker of hypoxia is dependent on tumor type and may involve confounding mechanisms of retention, separate from hypoxia (248). These findings have raised concerns regarding the use of Cu-ATSM to identify hypoxia with great certainty.

Clinical trials have evaluated the use of PET markers in terms of correlating hypoxia with outcome after radiotherapy (102). These studies generally involve a limited number of patients, and there are inconsistencies in the radiation treatments applied and in the PET image analyses. Further, the parameters used to distinguish between hypoxic tumors are highly variable. Horsman *et al.* performed a meta-analysis of the published studies and determined that there was a common tendency for poor radiotherapy response in those tumors showing higher uptake of the radioactive tracer, which was consistent with hypoxia (102). To highlight a specific example, it has been demonstrated that baseline hypoxia, as detected by FMISO-PET imaging, is associated with a high risk of locoregional failure in patients with head and neck squamous cell carcinoma who are treated with a platinum/fluorouracil-based chemoradiotherapy regimen (180). This study concurrently showed a striking improvement in locoregional control in patients with hypoxic tumors who are additionally treated with tirapazamine, a hypoxic cytotoxic drug, and that the absence of hypoxia with FMISO was associated with a lower risk of locoregional failure (Fig. 2) (180).

While the association has been made between tumor hypoxia and patient treatment response, an alternative use of PET imaging in radiation oncology has been recently explored. Dose escalation to hypoxic sub-volumes with conventional radiation, also known as "dose painting," has been investigated in clinical radiation oncology practice to overcome the cure-limiting effects of tumor hypoxia (178, 218). To plan for the administration of such a hypoxia radiation "boost treatment" in current clinical practice, advanced imaging, such as PET, is required. Hypoxic regions cannot be defined by standard pre-treatment computed tomography scans. Hendrickson *et al.* identified hypoxic sub-volumes of head and neck tumors in patients using FMISO-PET imaging and created example plans to simulate boosts of an additional 10 Gy on the prescribed 70 Gy to the primary tumor *via*

FIG. 2. Time to local failure (Kaplan-Meier plot) by treatment arm and hypoxia in the primary tumor. Censored times are indicated as tick marks on the curves. Cis, cisplatin; FU, fluorouracil; TPZ, tirapazamine. Reproduced from Rischin 2006 with permission from the authors and the American Society of Clinical Oncology (180). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

intensity-modulated radiation therapy (IMRT) to explore the feasibility and efficacy of this approach (93). They estimated a significant increase of 17% in tumor control probability without unacceptable increases in normal tissue complication probability with this treatment technique (Fig. 3A).

It is important to consider the dynamic properties of tumor oxygenation with cycling hypoxia when planning IMRT hypoxia imaged-guided dose painting treatment plans. A series of studies were performed to evaluate whether pre-treatment PET hypoxia images were invariant over time (132, 157). Two FMISO-PET scans, separated by 3 days, were obtained from patients with head and neck cancer. Significant changes in tumor hypoxia were observed between the sequential PET images in a subset of patients (157). Expanding on these findings, Lin *et al.* studied the influence of changes in hypoxia images on IMRT dose painting in seven patients with head and neck cancer. IMRT dose distribution derived using the first FMISO PET scan to the hypoxic region of the second FMISO PET scan was applied to evaluate the coverage of that hypoxic volume (Fig. 3B) (138). It was shown that the changes in the spatial distribution of tumor hypoxia compromised the coverage of hypoxic tumor volumes achievable by dose-painting IMRT; however, this technique still managed to consistently increase the equivalent uniform dose of the hypoxic volumes (138).

For the development of radiation treatment plans utilizing image-guided hypoxia dose painting to be most effective, through the use of PET imaging or one of the functional MRI techniques described next, serial hypoxia imaging beginning with pre-therapy and continuing throughout treatment should be performed to evaluate potential changes in the hypoxic volume as treatment progresses. Unfortunately, this is not clinically feasible at this time due to the exorbitant cost of the imaging, the increase risk of radiation exposure leading to secondary cancers from the radioisotopes required for PET

imaging (21), and the logistical challenge of performing and interpreting repeated advanced imaging in a busy radiotherapy practice.

Magnetic resonance imaging. A number of functional MRI techniques have been developed to image tumor hypoxia. We will review DCE-MRI, BOLD-MRI, and EPRI/MRI with regard to visualizing tumor hypoxia for preclinical and clinical radiation applications.

Dynamic contrast enhanced-MRI. DCE-MRI characterizes the physiological microenvironment of tumors *via* the uptake of contrast agent into tumor tissue. This imaging technique relies on fast repeated image acquisition before, during, and after the rapid intravenous administration of a low-molecular-weight, gadolinium-based (Gd-DTPA) contrast medium (219). The resulting temporal change in signal intensity within the tumor reflects a composite of tumor perfusion, vessel permeability, and the volume of extracellular space (47). DCE-MRI, therefore, enables the depiction of physiologic alterations, as well as morphologic changes. Importantly, this quantification can be carried out on a per-voxel basis, enabling the quantification of perfusion at the millimeter level.

Since perfusion is intimately associated with tissue oxygenation, the potential for Gd-DTPA-based DCE-MRI to assess tumor hypoxia has been investigated. In a recent study, human melanoma xenografts were subjected to DCE-MRI, followed by measurement of the fraction of radiobiologically hypoxic cells and pimonidazole-positive hypoxic cells. DCE-MRI parameters correlated with the extent of hypoxia in the tumors: K^{trans} (the volume transfer constant of Gd-DTPA) and v_e (the fractional distribution volume of Gd-DTPA) were high in tissues with low fractions of hypoxic cells, whereas tumors with both low K^{trans} and v_e values had high hypoxic fractions (65). This example supports clinical attempts to

FIG. 3. Hypoxia imaging with FMISO-PET in head and neck cancer patients. In (a–c) hypoxia imaging with FMISO-PET guided hypoxia-advanced radiation treatment planning with IMRT. Isodose display on axial slices for simultaneous integrated boost IMRT plan showing conformal 70 Gy dose around primary planning target volume (red) and 60 Gy dose around affected nodes (pink and blue). Hypoxic gross target volume (green) is covered by 80 Gy isodose. Parotid glands (orange and lilac) are avoided by high isodose lines. In (A–B), IMRT dose distributions in color-wash display are shown of a patient for whom the sequential hypoxia images were dissimilar. (A) Both sub-volumes of the first hypoxic sub-volume (the red contours) were prescribed 84 Gy. (B) When the same treatment plan was applied to the second hypoxic sub-volume (the green contour), a part of the hypoxic volume would not receive the intended boost dose. Reproduced from Hendrickson *et al.* (93) (a–c) and Lin *et al.* (138) (A–B) with permission from the authors and from Elsevier. FMISO, ^{18}F -fluoromisonidazole; IMRT, intensity modulated radiation therapy; PET, positron-emission tomography. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

establish DCE-MRI as a method for assessing the extent of hypoxia in tumors for treatment planning, predicting outcome, and evaluation of therapeutic response.

Changes in DCE-MRI parameters have been linked to outcome in patients with cervical cancer. These parameter changes are beneficial for this tumor type, because the efficacy of radiation and chemotherapy generally cannot be assessed reliably until after the completion of therapy due to anatomical location. DCE-MRI can provide early evidence of a positive treatment effect by demonstrating changes in contrast enhancement (47). For example, DCE-MRI parameters were quantified in 62 women with cervical carcinoma before treatment with radiation and chemotherapy and at 2–2.5 weeks after the initiation of treatment. DCE-MRI data were compared with standard clinical prognostic factors. DCE-MRI parameters reflecting heterogeneous tumor perfusion and subtle tumor volume change early during treatment were independent and better predictors of tumor recurrence and death than clinically accepted prognostic factors (*e.g.*, stage, lymph node status, and histology). The combination of

clinical prognostic factors and DCE-MRI parameters improved early prediction of treatment even further (148).

Blood oxygenation level-dependent (BOLD)-MRI. BOLD-MRI is a hypoxia imaging technique in which the primary source of contrast in images is endogenous, paramagnetic deoxyhemoglobin. As the oxygen saturation of the hemoglobin increases, the iron within the heme subunit changes from a paramagnetic high spin state under low pO_2 to a diamagnetic low spin state under high pO_2 . Deoxyhemoglobin increases the MR transverse relaxation rate (R_2^*) of water in the blood and surrounding tissues. Thus, BOLD-MRI is sensitive to pO_2 within and in tissues that are adjacent to perfused vessels (108). BOLD-MRI relies on red blood cells to be delivered to the tissue of interest to provide information regarding tissue oxygenation (103). R_2^* does not measure tissue pO_2 directly, so it is necessary to know or determine the distribution of blood volume in the tissues imaged in order to infer information regarding oxygenation. Therefore, if a tissue is perfused but has a high R_2^* relative to

FIG. 4. Mapping onto prostate gland outlines, grid arrays, and areas of high R_2^* and low relative blood volume. (a, b) Hematoxylin-eosin (H&E)- and pimonidazole-stained whole-mount pathologic slides from adjacent slices. The sites of the tumor are outlined in the H&E image. These images were used to map onto the prostate gland outlines of areas of tumor (pink) and hypoxia (brown) staining, as shown in (c) with the anatomic T_2 -weighted image. (d) The R_2^* map (8 mm; scale $0\text{--}25\text{ s}^{-1}$) at the same slice location. The arrow shows the location of the obturator internus muscle with a high R_2^* value. It should be noted that the center of the gland has intermediate R_2^* values. (e) The relative blood volume image (8 mm; scale, $0\text{--}1700\text{ AU}$) with the arrow showing the position of ischiorectal fat. The images were used to define areas of high R_2^* and low blood volume. (f) The corresponding T_2 -weighted image with the $5\times 5\text{-mm}$ grid overlay created with Adobe Photoshop. Arrowheads point to the dark line of the prostatic outline, the pink and brown color represents the position of tumor and pimonidazole staining, and the green is the area of high R_2^* values. The long arrows show the regions in which pimonidazole and tumor staining coincide with a high R_2^* . Reproduced from Hoskin 2007 with permission from the authors and Elsevier (103). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

another region in the same tissue, it can be surmised that the high R_2^* region is more hypoxic.

Hoskin *et al.* evaluated BOLD-MRI sequences as a means of identifying regional hypoxia in normal prostate glands and prostate cancers in 20 patients to validate the imaging against pimonidazole IHC of prostatectomy specimens from the same patients. The R_2^* maps from the BOLD-MR images resulted in a high sensitivity but a low specificity for defining intraprostatic tumor hypoxia. Therefore, regions of low R_2^* had a high likelihood of being non-hypoxic areas, whereas high R_2^* , although correctly identifying most of the hypoxic regions, also included some non-hypoxic areas, with greater accuracy in tumor compared with non-tumor areas (Fig. 4) (103).

Recently, clinical and preclinical studies have described correlations between BOLD-MRI and radiation and chemotherapy treatment response. Tissue oxygenation level-dependent contrast MRI correlated with significant tumor growth delay after irradiation with hyperoxic breathing in Dunning rat prostate tumors (89). Further, in a pilot study, BOLD-MRI was used to provide an early evaluation of response to neoadjuvant chemotherapy in patients with locally advanced breast cancer (251). The patients who achieved a complete pathological response exhibited a significantly higher BOLD response to oxygen breathing. These findings give confidence to the utility of BOLD-MRI as a non-invasive means of identifying hypoxic regions of cancers as well as of providing predictive capabilities for therapeutic

response, so that treatment modifications and advances can be investigated.

Electron paramagnetic resonance oximetry, EPR imaging. Measuring tissue oxygenation through the use of electron paramagnetic resonance (EPR) involves studying materials with unpaired electrons. The concepts of EPR are similar to nuclear magnetic resonance, except that it is electron spins which are excited instead of the spins of atomic nuclei.

EPR oximetry is a technique that can provide repeated assessments of average tumor pO_2 with minimal perturbation to the microenvironment (17), and it has been extensively used for pO_2 measurements in animal models (104–106). Briefly, lithium phthalocyanine (LiPc) crystals are implanted into the tissue of interest. LiPc aggregates have a single sharp EPR line, the width of which is highly sensitive to pO_2 . The EPR spectra reflect the average partial pressure of oxygen on the surface of each LiPc aggregate and enables the measurements of tumor pO_2 using only a few crystals with a weight of $\sim 30\text{--}50\text{ }\mu\text{g}$. A magnetic field gradient is used to separate the EPR spectra of the implants to determine the pO_2 of the tissue. Multisite EPR oximetry using magnetic field gradients has further expanded its *in vivo* application by enabling simultaneous pO_2 measurements at two to four sites in a tissue of interest (104–106).

EPR oximetry has been used to examine tumor reoxygenation after radiation treatment in murine tumors (78, 79) to determine tumor cure dependency on hypoxic fraction (67,

107) and to examine changes in tumor oxygenation in response to vascular changes (6, 111). Recently, it was demonstrated using EPR oximetry that ectopic C6 glioma tumors grown in mice had significantly increased pO_2 after irradiation, and this corresponded with prolonged tumor growth delay (107). In fact, clinical EPR spectrometers have been built that may permit the eventual use of EPR oximetry in clinical trials (118, 213, 234). While this technique does not provide imaging data, it is a very cost-effective method for obtaining repetitive and relatively non-invasive measurements of pO_2 from tumors over time.

EPRI imaging (EPRI) is a non-invasive, low-field MRI technique that uses paramagnetic tracers to provide quantitative high-resolution images of tumor and tissue oxygenation. The fundamental basis for EPRI oximetry stems from the paramagnetic nature of O_2 arising from its two unpaired electrons. Quantitative pO_2 maps can be obtained by monitoring the collisional interactions between a tracer and an O_2 molecule (144, 146). With the recent availability of triarylmethyl (TAM) radical probes as *in vivo* compatible tracers (8), EPRI is being explored for mapping tissue pO_2 in live animals (66, 68). The collisional interaction between TAM and O_2 broadens the spectral line width of TAM in proportion to oxygen concentration, thereby providing a quantitative measure of tissue pO_2 distribution (130). Further, sequential imaging with MRI using a common resonator permits reliable coregistration of the EPRI pO_2 map with different physiologic and metabolic images and provides a comprehensive assessment of the tumor microenvironment (146). With subsequent developments in image formation and reconstitution strategies, it is possible to obtain three-dimensional (3D) maps of pO_2 within 3 min in tumors implanted in mice to enable monitoring of intermittent hypoxia (241).

The utility of EPRI to image and study tumor hypoxia has been demonstrated in preclinical models. Elas *et al.* performed a preclinical study using fibrosarcomas grown in mice to compare sequences of tumor pO_2 values from EPRI with sequences of oxygen measurements made along a track with an Oxylite oxygen probe (66). A strong correlation was found in terms of spatial distribution pattern and pO_2 magnitude between the two modalities. Yasui *et al.* studied cycling hypoxia in two different tumor implants in mice using EPRI. It was shown that fluctuation patterns in pO_2 were dependent on tumor size and type but that it was possible to differentiate between chronic and cycling hypoxia, because real-time measurements could be performed (241).

EPRI has also been useful in studying tumor responses to treatment with regard to oxygenation. Krishna *et al.* imaged the changes in chronic and cycling tumor hypoxia pre- and 1 day after radiation in an SCCVII murine model (Fig. 5). Two regions of interest (ROIs) were selected in the tumor, and pO_2 was assessed in the ROIs over 30 min. ROI 1 represented a cycling hypoxic region showing temporal fluctuations in pO_2 , and ROI 2 indicated a chronically hypoxic region. They found that there were no significant differences in tumor volume before and 1 day after radiation (pre RT: 860 mm³; 1 day after radiation: 878 mm³). However, chronic hypoxia was increased, while cycling hypoxia was decreased by radiation (unpublished data). Further, Matsumoto *et al.* demonstrated vascular renormalization in tumor-bearing mice using EPRI + MRI by longitudinally mapping tumor pO_2 and microvessel density during treatment with the multi-tyrosine

kinase inhibitor sunitinib (145). They also showed that the radiation treatment during the period of improved oxygenation by anti-angiogenic therapy resulted in a synergistic delay in tumor growth and that sunitinib treatment suppressed cycling tumor hypoxia (145). These results suggest that longitudinal and non-invasive monitoring of tumor pO_2 may enable the identification of a window of vascular renormalization to maximize the effects of radiation in combination therapy with anti-angiogenic drugs.

Currently, EPRI is only available for preclinical applications; however, there is great interest in furthering the advancements of this technology for clinical use.

Optical spectroscopy. Optical spectroscopy enables the quantification of intrinsic sources of optical absorption, scattering, and fluorescence in tissue. There are two primary sources of intrinsic contrast for determining tissue hypoxia *in vivo*: hemoglobin saturation and fluorescence redox ratio.

Hemoglobin is the dominant tissue absorber throughout the visible spectrum. Optical spectroscopy is sensitive to hemoglobin saturation due to the differing absorption spectra of oxygenated hemoglobin (HbO₂) and deoxygenated hemoglobin (Hb) (252). Typically, a mixture of HbO₂ and Hb is present in the blood. The heme groups of hemoglobin bind oxygen, changing their absorption spectrum. It is possible to determine what fraction of the heme-binding sites of hemoglobin is bound to oxygen by characterizing the tissue absorbance as a function of wavelength. This enables the measurement of hemoglobin oxygen saturation. Once hemoglobin saturation has been determined, this value can be directly related to the tissue pO_2 by using the hemoglobin dissociation curve (133, 198).

An additional optical spectroscopy technique relevant to hypoxia is fluorescence redox imaging that utilizes the intrinsic contrast of electron carriers, nicotinamide adenine dinucleotide (phosphate) (NAD(P)) and FAD within tissue (41). NAD(P) is non-fluorescent, while its reduced form, NAD(P)H, fluoresces when excited with ultraviolet (UV) light. The reverse is true of FAD, which is fluorescent in the oxidized (FAD) state, but not the reduced (FADH₂) form (41). By measuring the fluorescence of both these parameters, a representation of the redox status of a biologic sample can be obtained. Commonly, a fluorescence “redox ratio” is calculated, defined as FAD/(FAD + NAD(P)H) or, alternatively, as NAD(P)H/FAD. The ratio of the autofluorescence contributed by these compounds can be used as a ratiometric indicator of tissue metabolic activity, an indirect measure of oxidative metabolism and, therefore, hypoxia. With this approach, it is possible to investigate the physiologic response to hypoxia; however, it does not directly reflect tissue pO_2 .

Optical spectroscopy techniques have been developed that make use of these sources of intrinsic contrast (hemoglobin saturation and fluorescence redox) to further the understanding of tissue hypoxia *in vivo*. The four main applications of optical spectroscopy principles are diffuse reflectance, hyperspectral imaging, and diffuse optic topography and tomography.

Diffuse reflectance spectroscopy. Diffuse reflectance spectroscopy enables the quantification of bulk tissue light absorption and scattering. Measuring diffuse reflectance of

FIG. 5. Three-dimensional-EPR oxygen images of chronic and cycling hypoxia before and at 1 day after radiation. EPR oxygen images were obtained pre RT and at 1 day after 3 Gy irradiation in a mouse implanted with an HT29 tumor. Two ROIs were selected in the tumor (1 and 2), and pO_2 was assessed in the ROIs every 3 min over 30 min. ROI 1 represents a cycling hypoxic region (median $pO_2 > 10$ mmHg, minimum $pO_2 < 10$ mmHg, and maximum $pO_2 > 10$ mmHg during 30 min). ROI 2 indicates a chronically hypoxic region (median $pO_2 < 10$ mmHg during 30 min). Two representative images acquired at 9 and 27 min in pre RT and at 15 and 24 min in 1 day after radiation are shown (Krishna *et al.*, unpublished data). EPR, electron paramagnetic resonance; pO_2 , pressure of O_2 ; ROI, region of interest. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

light as a function of wavelength enables the quantification of tissue absorption and scattering using quantitative models of light-tissue interaction (29). These measurements are commonly made using a fiber-optic probe that is placed in contact with the tissue surface. This enables a spot-based modality for the quantification of hemoglobin saturation and fluorescence redox ratio.

The most basic approach that is used for measuring hemoglobin saturation *in vivo* is to measure the diffuse reflectance as a function of wavelength. This is achieved by illumination of the tissue, and the backscattered light is then measured as a function of wavelength, usually with light coupled to a light source and a detector *via* a fiber optic probe. The resulting diffuse reflectance spectrum can be modeled and related to the underlying absorption and scattering properties of tissue; this can then be related to hypoxia. It is possible to obtain two-dimensional representations of tissue optical properties by acquiring an array of measurement sites or by sequentially moving the fiber optic probe to achieve spatial information. Scanning or wide-field approaches using non-contact methods or the placement of an array of sensors enables the extension of this approach to imaging (29).

As an example of the utility of this technique to monitor tumor physiology *in vivo*, Palmer *et al.* used diffuse reflectance and fluorescence spectroscopy to monitor tumor oxygenation and metabolism dynamically in response to hyperoxic gas breathing in small animal tumors (167). Vishwanath *et al.* recently demonstrated the potential clinical relevance of this technique by showing that early changes in oxygenation in response to radiation treatment are predictive of local tumor control in a xenograft model (229). Further, in another study, the non-invasive monitoring of intra-tumor drug concentration using fluorescence diffuse reflectance spectroscopy predicted the tumor response to doxorubicin *in vivo* (165).

This technique has also been used in human patients. Soliman *et al.* demonstrated that diffuse reflectance spectroscopy could be used to determine the response of breast cancer patients to pre-surgical neoadjuvant chemotherapy *via* changes in tumor functional parameters, with significant reductions in oxy- and deoxyhemoglobin measured for responders *versus* non-responders (203). An algorithm to calculate an index characterizing spatial differences in absorption spectra of tumor-containing breast tissue using diffuse reflectance spectroscopy has been developed to serve as

a biomarker of different molecular distributions within the tumor. With this algorithm, it was shown that neoadjuvant chemotherapy response is related to tumor heterogeneity, and it was possible to separate pathological complete responders from non-responders with diffuse reflectance spectroscopy (203).

Hyperspectral imaging. Hyperspectral imaging enables the quantification of transmitted or reflected light as a function of wavelength, commonly using multiple emission filters or a tunable filter. This imaging method enables the acquisition of fluorescence emission or reflectance/transmission spectra on a pixel-by-pixel basis to provide two-dimensional imaging of tumor hemoglobin saturation. High-resolution imaging of hemoglobin saturation *via* hyperspectral imaging has been achieved *in vivo* in window chamber models (174, 200, 205). Briefly, the window chamber tumor model involves the surgical attachment of a titanium frame that encloses an implanted tumor. Typically, the window chamber is placed in a dorsal skin fold (3), but fixation to orthotopic sites is also possible (199, 246). The window chamber enables direct visualization of the tumor microvasculature with a microscope. Having the microscope equipped with a tunable optic filter enables measurement of the transmitted or reflected light as a function of wavelength.

Researchers using the window chamber tumor model have used a liquid-crystal tunable filter for such work, which permits a continuously tunable, narrow band-pass transmission wavelength (205). Measurements of the transmitted or reflected light can then be related to the wavelength-dependent absorption coefficient to yield hemoglobin saturation (200, 205, 207).

The advantages of the hyperspectral imaging method to study hypoxia are that it is capable of a high resolution and that it can be used to image oxygen saturation of individual blood vessels. This level of resolution enables the modeling of oxygen transport in the microvasculature. Further, hyperspectral imaging in conjunction with imaging the fluorescence redox ratio, providing dual-modality characterization of oxygen supply and metabolism, has been recently performed in the window chamber tumor model (202).

Hyperspectral imaging has been used to study dynamic tumor microenvironmental oxygenation *via* dual emissive fluorescent/phosphorescent boron nanoparticles (BNPs), which serve as ratiometric indicators of tissue oxygen tension (166, 249). Iodide-substituted difluoroboron dibenzoylmethanepoly(lactide) (BF₂dbm(I)PLA) is a light-emitting biomaterial that offers many advantages for optical hypoxia imaging (249). It is multi-emissive, exhibiting both short-lived fluorescence (F) and long-lived fluorescence (P) after one- or two-photon excitation. Recently, dual-emissive pegylated BNPs have been fabricated, and they have been shown to have long-circulating, “stealth”-like properties for studying tumor accumulation and hypoxia imaging in preclinical studies (117).

A standard fluorescent microscope can be used for hyperspectral imaging of oxygen-sensitive nanoparticles. Specifically, a DAPI excitation filter cube is used for excitation, and a liquid-crystal tunable filter is used to isolate the emission wavelengths. Through ratiometric sensing, a simple ratio of F/P intensities at the respective emission maxima provides information about relative O₂ levels, whereas calibration enables absolute pO₂ measurements (166). Ratiometric

methods also eliminate the effects of concentration and fluctuations in light intensity or detector sensitivity. Further, since BNP serves as both the standard (F) and the oxygen sensor (P) at once, if a sensor molecule degrades, the intrinsically coupled F and P are equally affected.

Diffuse optical topography and tomography. Diffuse optical topography and tomography involve acquiring oxygenation measurements through the use of an array of light sources/detectors spanning a relatively deep (tens of mm) region of tissue (124). Diffuse optical tomography is similar to diffuse optical topography, but it uses more widely spaced sources and detectors to enable even greater penetration depth. Due to the need for deep-penetrating photons, a near infrared (NIR) light is used.

A recent clinical study demonstrates the utility of diffuse optical spectroscopic imaging. Concentrations of oxyhemoglobin, deoxyhemoglobin, total hemoglobin, and oxygen saturation in tumor and contralateral normal tissue from patients with locally advanced primary breast cancer were measured using diffuse optical spectroscopy before treatment with neoadjuvant chemotherapy (220). It was found that increased baseline tumor oxygen saturation was correlated with a pathologic complete response after treatment with a combination neoadjuvant chemotherapy regimen (Fig. 6). The combination of non-invasive diffuse optical spectroscopy with histopathology subtyping could potentially aid in a more defined characterization of individual patients with breast cancer before therapy.

FIG. 6. Baseline tumor oxygen saturation. Baseline tumor oxygen saturation as measured by diffuse optical spectroscopic imaging correlates with a pCR in breast cancer patients undergoing neoadjuvant chemotherapy. Box-and-whisker plots showing the difference in tumor oxygen saturation (stO₂) levels between pCR and non-pCR tumors (*left*; median, 77.8% vs. 72.3%; $p=0.01$, Wilcoxon) and the lack of difference in stO₂ levels between contralateral normal tissues (*right*; median, 77.7% vs. 78.1%; $p=0.98$, Wilcoxon). Reproduced from Ueda 2012 with permission from the authors and the American Association for Cancer Research (220). pCR, pathologic complete response.

The primary difficulty with the methods of diffuse optical topography and tomography is that while the absorption is low, tissue scatters light strongly, resulting in a relatively poor resolution. It enables deep-tissue imaging (~ 10 -cm depth); however, resolution is limited by tissue scattering, typically being in the order of 1 cm at a depth of about 10 cm. Regardless of this, a significant advantage of these optic techniques is that they are able to provide functional information regarding the tumor tissue. Current research has been focused on improving the functional significance of these methods, as opposed to attempting to achieve high-resolution anatomic information that is better addressed by other imaging modalities (77).

Phosphorescence lifetime imaging. Phosphorescence lifetime imaging utilizes phosphors that are encapsulated in a water-soluble dendrimer and injected into the vasculature of the tissue region of interest. The dendrimer shields and reduces the sensitivity of the phosphor to the microenvironment. These phosphors are absorbed in the NIR region of 620–1000 nm (235). The fluorescence of these phosphors is quenched by oxygen, so their lifetime is shorter at high oxygen tensions. This spectral window proves to be advantageous, because there is little absorbance from natural body pigments in this region, resulting in high specificity for this method. A light guide focuses the excited light from the phosphor to the surface of the tissue, where it is then detected by a phosphorometer. The phosphor most commonly used *in vivo* for this method is Pcl-porphyrin; other phosphors available include OxyphorG2 and Green2W (61).

Advantages for phosphorescence lifetime imaging include the ability to provide direct measurements of pO_2 throughout the entire tissue volume in absolute units with calibration. Importantly, this method has a high signal-to-noise ratio in low pO_2 environments. The NIR light used for this imaging modality results in tissue penetration depths of a few mm to a cm; therefore, the resolution of phosphorescence lifetime imaging will depend on the detection technique used. A temporal resolution of seconds or less enables almost real-time data acquisition and the ability for repeated measurements (228).

Phosphors can be detected using a variety of optical imaging modalities, enabling a wide range of multi-modality capabilities. With regard to preclinical *in vivo* applications used to study tissue hypoxia, phosphorescence lifetime imaging has become increasingly important due to the capacity for successful 3D spatial registration using confocal imaging (125, 175) and diffuse tomography (7). For example, phosphorescence lifetime imaging *via* OxyphorG₂ was used to measure fluctuations in vascular pO_2 in fibrosarcomas, gliomas, and mammary adenocarcinomas grown in dorsal skin fold window chambers every 2.5 min for 60–90 min (38). Instabilities in tumor oxygenation over time were a characteristic of the three tumor types, where oxygen delivery to the tumors was constantly changing, resulting in continuous reoxygenation events throughout the tumor. Vascular pO_2 maps revealed a significant spatiotemporal heterogeneity (38).

Photoacoustic tomography. Photoacoustic tomography combines strong optical contrast and high ultrasonic resolution in a single modality that enables deep-tissue cross-sectional or 3D imaging based on the photoacoustic effect (232).

Photoacoustic tomography involves optical irradiation by a short-pulsed laser beam and produces acoustic impulse responses. These propagate in tissue as ultrasonic waves, known as photoacoustic waves. The photoacoustic waves are detected by ultrasonic transducers that are placed outside the tissue and converted to electric signals. The electric signals are then amplified, digitized, and transferred to a computer, where an image is formed. Photoacoustic tomography can simultaneously image cross-sections of blood vessels, the concentration and oxygenation of hemoglobin, as well as blood flow *in vivo* (232). These parameters can be used to quantify oxygen metabolism at a high spatial resolution without the requirement of an exogenous contrast agent.

Li *et al.* demonstrated the use of spectroscopic photoacoustic tomography, which offers both strong optical absorption contrast and high ultrasonic spatial resolution, to determine the signal contributions of oxyhemoglobin, deoxyhemoglobin, and a molecular contrast agent, enabling simultaneous molecular and functional imaging. With this technique, human U87 glioblastoma cells grown in nude mouse brains were imaged to quantify the hemoglobin oxygen saturation and the total hemoglobin concentration of the vasculature, revealing hypoxia in tumor neovasculature (136).

Conclusion

Hypoxia is one of the most important factors influencing clinical outcome for patients treated with radiotherapy. Since hypoxia exists in tumors as both chronic and cycling, understanding the heterogeneous nature of this continuously shifting factor of the tumor microenvironment is crucial. Visualizing tumor hypoxia through non-invasive imaging techniques that can accurately and reliably depict tumor oxygenation will lead to advances in preclinical cancer research and clinical radiotherapy practices.

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Abbreviations Used

ANGPT2	= angiopoietin-2
BF ₂ dbm(I)PLA	= iodide-substituted difluoroboron dibenzoylmethane-poly lactide
BNP	= boron nanoparticle
BOLD-MRI	= blood oxygenation-level dependent magnetic resonance imaging
CA9	= carbonic anhydrase IX
CBP	= CREB-binding protein
Cu-ATSM	= ⁶⁴ Cu-diacetyl-bis(N ⁴ -methylthiosemicarbazone)
3D	= three-dimensional
DCE-MRI	= dynamic contrast enhanced magnetic resonance imaging
DNA DSBs	= DNA double-strand breaks
DNA SSBs	= DNA single-strand breaks
EIF2 α	= eukaryotic initiation factor 2 α
EPO	= erythropoietin
EPR	= electron paramagnetic resonance
EPRI	= EPR imaging
FAD	= flavin adenine dinucleotide
FAZA	= ¹⁸ F-fluoroazomycinarabinofuranoside
FMISO	= ¹⁸ F-fluoromisonidazole

Abbreviations Used (Cont.)

GFP_h = green fluorescent protein under control of an
HRE derived from the VEGF promoter
GLUT1 = glucose transporter 1
Hb = deoxygenated hemoglobin
HbO₂ = oxygenated hemoglobin
HIF-1 = hypoxia-inducible factor-1
HREs = hypoxia response elements
IHC = immunohistochemistry
IL-8 = interleukin 8
IMRT = intensity-modulated radiation therapy
LiPc = lithium phthalocyanine
LOX = lysyl oxidase
MDM2 = mouse double minute 2 homolog
MXI1 = MAX interactor 1
NAD(P) = nicotinamide adenine dinucleotide
(phosphate)
NIR = near infrared

NO = nitric oxide
OCT = optical coherence tomography
ODD = oxygen-dependent degradation
PDK1 = pyruvate dehydrogenase kinase 1
PET = positron-emission tomography
PFK1 = phosphofructokinase 1
PLAUR = plasminogen activator urokinase
receptor
pO₂ = pressure of O₂
RCF = red cell flux
RFP_c = red fluorescent protein under control
of a constitutive CMV promoter
ROI = region of interest
TAM = triarylmethyl
UPR = unfolded protein response
UV = ultraviolet
VEGF = vascular endothelial growth factor