Low-Field Magnetic Resonance Imaging to Visualize Chronic and Cycling Hypoxia in Tumor-Bearing Mice

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Abstract

Tumors exhibit fluctuations in blood flow that influence oxygen concentrations and therapeutic resistance. To assist therapeutic planning and improve prognosis, noninvasive dynamic imaging of spatial and temporal variations in oxygen partial pressure (pO2) would be useful. Here, we illustrate the use of pulsed electron paramagnetic resonance imaging (EPRI) as a novel imaging method to directly monitor fluctuations in oxygen concentrations in mouse models. A common resonator platform for both EPRI and magnetic resonance imaging (MRI) provided pO2 maps with anatomic guidance and microvessel density. Oxygen images acquired every 3 minutes for a total of 30 minutes in two different tumor types revealed that fluctuation patterns in pO2 are dependent on tumor size and tumor type. The magnitude of fluctuations in pO2 in SCCVII tumors ranged between 2- to 18-fold, whereas the fluctuations in HT29 xenografts were of lower magnitude. Alternating breathing cycles with air or carbogen (95% O2 plus 5% CO2) distinguished higher and lower sensitivity regions, which responded to carbogen, corresponding to chronic hypoxia and chronic hypoxia, respectively. Immunohistochemical analysis suggests that the fluctuation in pO2 correlated with pericyte density rather than vascular density in the tumor. This EPRI technique, combined with MRI, may offer a powerful clinical tool to noninvasively detect variable oxygenation in tumors. Cancer Res; 70(16); 6427–36. ©2010 AACR.

Introduction

Understanding the tumor microenvironment is crucial in the analysis of the response of solid tumor to therapy and in the augmentation of that response. Oxygen status is an important component of the tumor microenvironment. Although normal tissues display a well-regulated balance between oxygen supply and consumption, in tumors, oxygen consumption exceeds delivery, resulting in hypoxia. This situation is attributed mainly to inefficient blood supply caused by morphologic and architectural abnormalities in the tumor vasculature that develops during tumor angiogenesis (1). Tumor microvasculature is characterized by loss of hierarchy, heterogeneous vessel distribution, and tortuous vessels with elongated segments (2). Tumor blood vessels typically lack smooth muscle support, as well as an incomplete endothelial lining and basement membrane, making them leaky (3). The abnormal architecture of tumor blood vessels makes them inefficient in delivering oxygen and nutrients, resulting in longitudinal oxygen gradients within the vessels themselves (4).

Based on observations of histologic sections of human lung cancer, Thomlinson and Gray (5) hypothesized the presence of hypoxia in tumors as a potential cause of radiation treatment resistance. In the 1960s, Powers and Tolmach (6) provided direct evidence that hypoxia was present in rodent tumors. Subsequently, low oxygen levels, measured with oxygen electrodes inserted into tumors, were correlated with poor radiation treatment outcome in human head and neck cancer (7–9). Recently, many solid tumors have been shown to contain subpopulations of hypoxic cells that limit the efficacy of cancer therapy such as radiation, chemotherapy, and even surgery (8, 10). Thus, knowledge of tumor oxygen levels has both therapeutic as well as prognostic value that has prompted active research into how to monitor hypoxia in vivo and how to sensitize hypoxic regions to radiation (11, 12).

In addition to diffusion-limited chronic hypoxia, tumors were also shown to experience acute, intermittent, and cycling hypoxia (13, 14). Cycling hypoxia was suggested to contribute to tumor progression by providing repeated exposure to hypoxia reperfusion injury (15–17). Cycling hypoxia in tumors was first documented with indirect methods using dyes permitting a 110-second “snapshot” assessment of blood flow (18). Further studies with double labeling with hypoxia tracers revealed that 8% to 20% of tumor cells can experience intermittent hypoxia if they are not adjacent to blood vessels (19). Although the flow in tumor vasculature...
was aberrant, which was attributed to stasis of flow, this phenomenon was not entirely sufficient to explain cycling hypoxia (18). The first study associating erythrocyte flux with tumor pO2 was performed using a window chamber model monitoring their flux by fluorescently labeled RBC and simultaneously measuring pO2 by locally placed polarographic electrodes (20). Dewhirst and colleagues (14) established a clear relationship between erythrocyte flux and tumor pO2 fluctuations at higher frequency within the oxygen diffusion distance. Cycling hypoxia was also documented in experimental models using noninvasive approaches such as BOLD contrast magnetic resonance imaging (MRI), dynamic contrast enhanced MRI, and optical probes (21–23). Using positron emission tomography (PET) and 18F-labeled misoprostol as a hypoxia marker, tumor hypoxia was evaluated in patients with head and neck cancer every 3 days (24). These studies provided evidence that the size and distribution of hypoxic subvolumes exhibited significant changes over time. Collectively, these studies, although qualitative, reinforce the importance of studying acute hypoxia in human tumors to gain a better understanding of fluctuations in tumor physiology.

Electron paramagnetic resonance (EPR) is a spectroscopic technique similar to nuclear magnetic resonance. EPR detects paramagnetic species that have unpaired electrons. EPR spectroscopy and imaging have been implemented successfully to examine tumor reoxygenation profile, after radiation treatment of murine tumors (25, 26), to determine tumor cure dependency on hypoxic fraction (27) and examine changes in tumor oxygenation in response to vascular changes (28, 29). With the recent availability of triarylmethyl (TAM) radical probes as in vivo compatible tracers (30), EPR imaging (EPRI) is being explored for mapping tissue pO2 in live animals (31). The fundamental basis for EPR oximetry stems from the paramagnetic nature of O2 arising from its two unpaired electrons. The collisional interaction between TAM and O2 broadens the spectral line width of TAM in proportion to oxygen concentration, thereby providing a quantitative measure of tissue pO2 distribution (34). Because the operating radiofrequency of EPRI is in the same range as that of MRI, it can in principle be applied for human studies.

Although EPRI provides maps of pO2, they lack the anatomic detail available from MRI. A combined EPRI + MRI system overcomes this limitation and provides the necessary anatomic guidance to the pO2 images. We have recently shown the feasibility of using EPRI coupled with MRI operating at a common frequency of 300 MHz in both modalities with the corresponding magnetic fields at 10 mT (EPRI) and 7 T (MRI). Multiple parameters, including pO2 map, blood flow, blood volume, water diffusion coefficient, and metabolic profile were obtained from sequential scans to produce a more comprehensive assessment of tumor physiology (35). With subsequent developments in image formation and reconstruction strategies, we have made it possible to obtain three-dimensional maps of pO2 within 3 minutes in tumors implanted in mice to enable monitoring intermittent hypoxia. In this study, assessment of cycling hypoxia in two different tumor implants in mice was examined by EPRI.

Materials and Methods

Animals and tumor implantation

All animal experiments were carried out in compliance with the Guide for the care and use of laboratory animal resources (National Research Council, 1996) and approved by the National Cancer Institute (NCI) Animal Care and Use Committee. Female C3H/HeJ mice and athymic nude mice were supplied by the Frederick Cancer Research Center, Animal Production. SCCVII and HT29 solid tumors were formed by injecting 5 × 106 cells and 1 × 106 cells s.c. into the right hind legs of mice. The experiment was initiated on separate sets of animals when their individual tumors grew to approximately 600, 1,200, and 1,800 mm3 (tumor volume = length × width2 × π/6). Mice were anesthetized by isoflurane inhalation (4% for induction and 1.5% for maintaining anesthesia) and positioned prone with their tumor-bearing legs placed inside the resonator. During EPRI and MRI measurements, the breathing rate of the mouse was monitored with a pressure transducer (SA, Instruments Inc.) and maintained at 60 ± 10 breaths per minute. Core body temperature was maintained at 37°C with a flow of warm air.

EPR imaging

Technical details of the EPR scanner and oxygen image reconstruction were described in earlier reports (32, 35–38). The description of the resonator (17 mm i.d. and 17 mm long) used as an identical coil for EPRI and MRI operating at 300 MHz was previously described (39, 40). After the animal was placed in the resonator, oxygen-sensitive tracer TAM (Ox63, GE Healthcare) was injected i.v. Pharmacologic decrease of the tracer was prevented by continuous injection of TAM into mice (41). TAM was given as a 1.125 mmol/kg bolus followed by 0.04 mmol/kg/min continuous injection. The free induction decay (FID) signals were collected following the radiofrequency excitation pulses (80 ns, 80 W, 70° flip angle) under a nested looping of the x, y, and z gradients, and each time point in the FID underwent phase modulation enabling three-dimensional spatial encoding. The repetition time was 6.0 ms. Because FIDs last for a couple of microseconds, it is possible to generate a sequence of T2* maps i.e., EPR line width maps, which allowed pixel-wise estimation of pO2. The spatial resolution was 1.6 to 1.8 mm, although the pixel resolution was digitally enhanced to coregister with MRI images. Minimum signal to noise ratio used for reliable pO2 estimation is ~30.

Coregistration of pO2 images from EPRI with anatomic images from 7T MRI

MRI scans were conducted using a 7 T scanner controlled with ParaVision 5.0 (Bruker BioSpin MRI GmbH). After a quick assessment of the sample position by a fast low-angle
shot triplor sequence, T2-weighted anatomic images were obtained using a fast spin echo sequence (RARE) with an echo time of 13 ms, repetition time of 2.500 ms, 16 slices, RARE factor 8, and a resolution of 0.125 × 0.25 mm. For convenience of coregistration with EPRI, all MRI images had the same FOV of 3.2 cm and slice thickness of 2 mm. For blood volume calculation, spoiled gradient echo sequence images were collected before and 5 minutes after ultrasmall superparamagnetic iron oxide (USPIO, Molday ION, BioPAL, Inc.) injection (1.2 μL/g body weight) with the following parameters: matrix = 256 × 256; echo time = 5.4 ms; repetition time = 250 ms. Percentage of tumor blood volume was estimated by the expression 100 × (S\text{pre} − S\text{post})/ [S\text{pre} + S\text{post} (W\text{b}/W\text{t} − 1)], in which S\text{pre} and S\text{post} were the signal intensities before and after USPIO injection and W\text{b} and W\text{t} were the intravascular and extravascular water fractions (35, 42). Coregistration of EPRI and MRI images was accomplished using code written in MATLAB (Mathworks) as previously described (35, 37).

**Immunohistochemical analysis**

A hypoxia marker, pimonidazole (43), was given i.v. between EPRI and MRI scans. After MRI study, the perfusion marker Hoechst33342 was injected. Two minutes later, mice were euthanized and tumor tissues were excised at the same tilt angle and depth from the surface in coincidence with the MRI images as reported previously with slight modification (44). Tumor tissues were fixed with 4% paraformaldehyde and frozen, and 10-μm-thick sections were obtained. Tissue sections were air dried, and then the Hoechst33342 signal was recorded. After blocking nonspecific binding sites, the slides were covered by CD31 antibody (BD Biosciences; 1:250) combined with αSMA antibody (Abcam, Inc.; 1:250) or rabbit anti-pimonidazole antisera (Natural Pharmacia International, Inc.; 1:250) overnight at 4°C. The sections were incubated with Alexa Fluor 488 anti-rat and Alexa Fluor 555 anti-rabbit secondary antibody (Invitrogen 1:500). Then, they were mounted on Prolong Gold antifade reagent with 4’,6-diamidino-2-phenylindole (Invitrogen). Fluorescence microscopic observation was performed using an Axiovert 200 inverted fluorescent microscope (Carl Zeiss). Serial sections were also stained with H&E.

The quantification of CD31 and αSMA was performed according to the method described by Zhou and colleagues (45). Briefly, tissue sections were viewed at >200 magnification, and more than three fields per section were captured using the Image-Pro Plus Ver. 4.0 imaging software. Then, the quantification of vascular density and pericyte density on each image was performed using the ImageJ software package (46) and shown as the total number of positive pixels per field.

**Statistical analysis**

All results were expressed as the mean ± SEM. The differences in means of groups were determined by Mann-Whitney U test using the Stat View 5.0 J software (SAS Institute, Inc.). The minimum level of significance was set at \( P < 0.05 \).

**Results**

**Noninvasive imaging of oxygen fluctuation in SCCVII and HT29 tumors by rapid EPRI scan**

To examine the spatial distribution of tumor \( pO_2 \) and monitor temporal \( pO_2 \) fluctuations, sequential EPRI and MRI experiments on tumor-bearing mice were conducted using a resonator assembly tuned to the common frequency of 300 MHz for both modalities. Figure 1A shows eight adjacent slices of anatomic images from MRI, and Fig. 1B shows the corresponding \( pO_2 \) images from EPRI after the infusion of oxygen-sensing paramagnetic tracer TAM in the SCCVII tumor. Each MRI slice was 2 mm thick and the corresponding \( pO_2 \) image slice was selected from the three-dimensional image data of EPRI. Marked heterogeneity in \( pO_2 \) levels was observed throughout the tumor, with \( pO_2 \) levels ranging from 0 to 35 mmHg. The tumor exhibited a significant hypoxic core, surrounded by normoxic regions.
To examine the spatial and temporal changes in tumor physiology, two separate models were chosen: a SCCVII murine carcinoma and a HT29 human colon carcinoma. To visualize dynamic changes of tumor oxygenation over a time period of 30 minutes, EPRI experiments were carried out collecting nine image data sets, each taking $\sim 3$ minutes, for a three-dimensional reconstruction of $pO_2$ maps as well as the tracer distribution. Figure 2A shows the anatomic image of a SCCVII tumor obtained from MRI with four regions of interest (ROI) indicated. Figure 2B shows the corresponding time-dependent changes in $pO_2$ (top row) and the levels of TAM (bottom row) obtained by EPRI (animation is available in the Supplementary Movie S1). It should be noted that $pO_2$ values can be determined quantitatively independent of differing levels of the tracer TAM in different regions, provided the tracer EPR signals are detectable (32, 35, 36). The tumor oxygen levels displayed significant fluctuations, whereas the tracer level during this time window remained relatively stable. When tumor oxygen levels and TAM tracer levels in the four ROIs identified in the anatomic image (Fig. 2A) were plotted as a function of time, ROIs 1 and 2 displayed significant fluctuations in $pO_2$ (approximately 18-fold and 12-fold, respectively), whereas ROIs 3 and 4 displayed relatively smaller changes ($\sim 2$-fold). No significant changes in the TAM tracer level were observed across the time course (Fig. 2C, right). Comparing the $pO_2$ maps and the tracer levels as a function of time (Fig. 2B and C), it can be seen in ROI 4 that the tracer level was adequate enough for detection and imaging, yet this region displayed significant hypoxia. On the other hand, in the three remaining ROIs in which the tracer level was high and stable, the $pO_2$ fluctuations with time were significant, supporting previous observations of cycling hypoxia resulting from fluctuations in RBC flux (14).

Similar studies were conducted on the human tumor HT29 cell line. Anatomic images were obtained by T$_2$-weighted MRI, and four ROIs were identified (Fig. 3A). The spatial distribution of $pO_2$ is shown in Fig. 3B, and the $pO_2$ values against time are plotted in Fig. 3C. All four ROIs displayed time-dependent fluctuations, although of a smaller magnitude than that observed with the SCCVII tumors. Once again, TAM was distributed throughout the tumor and remained relatively stable, whereas the fluctuations of oxygen occurred (Supplementary Movie S1; Fig. 3B and C). ROI 4, which had a higher level of the TAM tracer than ROI 1 and 3, exhibited $pO_2$ levels of $<10$ mm Hg. This phenomenon was similar to that observed in SCCVII tumor, where in spite of adequate level of tracer, significant hypoxia existed in certain regions.

![Figure 2](image_url)

**Figure 2.** Noninvasive imaging of fluctuating $pO_2$ in SCCVII tumors using EPRI. A, T$_2$-weighted anatomical image of a representative SCCVII tumor–bearing mouse. Large yellow line, tumor region. Four ROIs indicated by small white line were chosen for tracing fluctuations of $pO_2$ and spin intensity with time. B, corresponding $pO_2$ maps (top) and the tracer level maps (bottom) were obtained from EPRI. Yellow line, tumor region. Time increased from left to right from 4 to 28 min. A.u., arbitrary units. C, the values of $pO_2$ and the tracer level in each ROI region, described in A, were quantified and plotted as a function of time.
of the tumor. The TAM tracer level was reported to be spatially correlated with increased tumor microvascular permeability, i.e., abnormality of tumor blood vessels, whose distribution was in turn matched with hypoxic regions (44). This behavior may be a result of longitudinal oxygen gradient, whereas plasma flow may be not significantly impeded, resulting in adequate tracer levels but with significant hypoxia. Collectively, the results shown in Figs. 2 and 3 support the use of EPRI for monitoring dynamic changes in oxygen levels in tumors.

Decrease in oxygenation and blood volume is dependent on tumor size

In general, tumor size/stage largely determines the tumor physiology and may contribute to the outcome of cancer therapy. Previous studies reported that the degree of hypoxia and inadequacy of vascularity increased with increasing tumor size (47, 48). To examine this phenomenon noninvasively, mice were divided into three groups by their tumor sizes (<600, 600–1,200, and >1,200 mm³) and were evaluated using EPRI for pO₂ and MRI for anatomy and blood volume. Figure 4A shows the tumor pO₂ maps and images of blood volume from a representative SCCVII tumor. From the EPRI maps, the averaged tumor pO₂ was found to decrease gradually with increasing tumor size. The pO₂ levels were 14.8 ± 1.1 mmHg for the smallest tumor, 12.7 ± 1.1 mmHg for the medium-size tumor, and 12.4 ± 0.7 mmHg for the largest tumor. Consistently, the fractional hypoxic volume (% volume with pO₂ < 10 mmHg) increased with tumor size and reached 41.0 ± 2.6% in the largest tumor compared with 20.9 ± 6.2% in the smallest tumor. Comparing identical regions of the corresponding MR images, we examined the blood volume as a noninvasive indicator of microvessel density using the blood pool T₂ contrast agent USPIO (42). For small tumors, the blood vessel coverage was relatively uniform, with a density of 27.7 ± 2.1%. However, blood vessels in the largest tumor displayed significant heterogeneity, and their density decreased to 8.1 ± 2.0%. Similar patterns of decreasing blood vessel density and pO₂ were observed with increasing tumor size in HT29 tumors, although in this case, the changes were not statistically significant (Fig. 4B).

Oxygen fluctuations are dependent on tumor size and tumor type

We next examined the relationship of fluctuation of tumor pO₂ with tumor size and tumor type. The intrapixel SDs of pO₂ (pO₂ SD) from the nine pO₂ images collected between 4 and 28 minutes were computed. The pO₂ SD values over the time period studied for SCCVII tumors were found to increase from 4.6 ± 0.3 mmHg in the <600 mm³ group to 6.4 ± 0.7 mmHg in the >1,200 mm³ group (correlation coefficient
Interestingly, pO2 SD in HT29 tumor underwent comparatively smaller changes as a function of tumor size (3.7 ± 0.2 mmHg for <600 mm³ group and 4.1 ± 0.5 mmHg in >1,200 mm³ group, respectively). When pO2 fluctuations were compared in two different tumor types at the same size, the pO2 SD of the SCCVII tumor was found to be larger than that of the HT29 tumor at all sizes (<600 mm³, P = 0.0318; >1,200 mm³, P = 0.0298; Fig. 5A). These results suggested that the tumor size–dependent variations in pO2 are dependent on the tumor type.

Oxygen fluctuation was not influenced by vascular density but by maturation

To investigate whether vascular density and/or maturity of the tumor vasculature contributes to tumor type–dependent differences in oxygen fluctuations, immunohistochemical analyses for CD31 and αSMA as markers of endothelial cells and pericytes, respectively, were performed (Fig. 5B). Although the tumor type–dependent difference in the vascular density (CD31, green) was negligible (3.6 ± 0.2% for SCCVII and 3.5 ± 0.2% for HT29), a difference in the pericyte coverage density (αSMA, red) between SCCVII and HT29 tumors was apparent. In the SCCVII tumor, the number of recruited pericytes was smaller (0.8 ± 0.1% for SCCVII and 13.1 ± 2.9% for HT29) and the coverage of blood vessels with pericytes was relatively less compared with the HT29 tumors (Fig. 5C). An earlier study found no correlation between vessel maturation and fluctuations in pO2 as assessed by T2*-weighted MRI; these differences with the present study may result from a different tumor model or the measurement type used (22).

Effect of carbogen breathing on tumor oxygenation

SCCVII tumor–bearing mice were subjected to an air–carbogen³–air breathing cycle to determine whether tumor

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³ 95% O₂ plus 5% CO₂.
pO₂ would change as a function of time. EPRI-derived pO₂ maps were collected during each cycle as shown in Fig. 6A. Figure 6B shows the corresponding anatomic image from MRI image from which 3 ROIs were chosen and the pO₂ difference image, which is the pO₂ image while breathing air subtracted from the pO₂ image while breathing carbogen. The pO₂ values in these three regions were plotted as a function of time (Fig. 6C). Although comparatively small fluctuations occurred during the air breathing phase (0–12 min), when subjected to carbogen breathing (12–24 min), ROIs 2 and 3 displayed a marked increase in pO₂, whereas ROI 1 displayed negligible changes (Fig. 6C). ROIs 2 and 3 again responded when the breathing gas was restored to air by displaying a steady decrease, whereas ROI 1 was unresponsive. The median pO₂ value was 7.2 mmHg during initial air breathing and increased to 13.1 mmHg at 19 minutes by carbogen breathing. Rebreathing of air moderately decreased the median pO₂ to 8.0 mmHg at 31 minutes.

Frequency histograms of the global tumor pO₂ at selected time points of the air-carbogen cycle are shown in Fig. 6D. There is a region at low pO₂ values in the histogram, which is unresponsive to changes of breathing gas, whereas a right shift of the frequency histogram resulted when carbogen was the breathing gas. Together, these data provide noninvasive experimental evidence to support the notion that diffusion limited chronic hypoxic regions, as well as regions that vary from the extremely hypoxic to the normoxic state, exist in tumors, i.e., cycling hypoxia. Supplementary Movie S2 shows representative images of pO₂ maps as a function of time during the air-carbogen-air cycle.

Discussion

Strong experimental evidence obtained using ex vivo analyses and window chamber models support the existence of cycling hypoxia in addition to chronic hypoxia in tumor implants in mice and even in human tumors (13, 14). The more malignant phenotype associated with cycling hypoxia (15, 17) makes a strong case to develop noninvasive imaging techniques to identify such behaviors in vivo. EPRI is a noninvasive low-field MRI technique, which uses paramagnetic tracers. Distribution of the tracer and quantitative pO₂ maps can be obtained by monitoring the collisional interactions between the tracer and O₂ molecule (32, 35). Sequential imaging with MRI using a common resonator permits reliable coregistration of the pO₂ map with different physiologic and metabolic images, and provides a more comprehensive assessment of the tumor microenvironment (35).

Compared with other clinically used methods of assessing tumor oxygen status, EPRI has intrinsic advantages. Unlike polarographic measurements that are invasive and are used only in accessible sites but provide quantitative pO₂ assessments from limited sampling volume, EPRI provides similar quantitative information of three-dimensional maps noninvasively. Another oxygen imaging technique, PET, in which the hypoxic tracer needs to be internalized and metabolized to bind to hypoxic regions, is not capable of absolute oxygen quantification. Additionally, unlike with EPRI in which temporal pO₂ fluctuations in order of minutes can be recognized, PET-based methods can only integrate the periods of hypoxia during the scan period.

In the present study, significant fluctuations in tumor pO₂ were seen even with a 3-minute snapshot. In the SCCVII transplant, although the fluctuations in the tracer level were minimal throughout the tumor, the pO₂ value showed significant fluctuations (~30 mmHg) in some regions, whereas other regions displayed fluctuations to a lesser extent. Some regions that were hypoxic (pO₂ <10 mmHg) remained invariant in the time window of observation, suggesting that these regions may result from diffusion-limited chronic hypoxia. In the HT29 xenograft, the extent of fluctuations in pO₂ was found to be smaller than SCCVII transplants. From EPRI studies as a function of tumor growth, it was found that both SCCVII and HT29 tumors showed increasing size of the hypoxic core with a concomitant decrease in the microvessel density.

Figure 5. A, the pO₂ SD at each pixel was calculated from nine pO₂ images obtained from 4 to 28 min after TAM injection in SCCVII (red) and HT29 (green) tumors (n = 3–4). B, representative images of double immunofluorescence staining for CD31 (green) and αSMA (red) as makers of endothelial cells and pericytes, respectively. Merged yellow cells, the vessels covered with αSMA. C, CD31 and αSMA densities were quantified as the percentage of fluorescence-positive area (n = 3–4). N.S., not significant.
Because several images were collected during the imaging time window and the pO₂ levels were determined at each time point, it was possible to obtain images of the SD of the pO₂ calculated over time, which would reflect the fluctuations. There was an increase in pO₂ SD values with increases in size, in agreement with the studies of Chaplin and colleagues (13) in which cycling hypoxia was not observed in small murine tumors, whereas larger sized tumors (>500 mg) displayed cycling hypoxic behavior. These fluctuations seem to be related to the decrease in coverage with pericytes as shown in Fig. 5.

Histologic analyses after imaging experiments were conducted to assess microvessel density, pericyte coverage, hypoxia, and cell viability to characterize the tumor noninvasively as well as at a microscopic level (Supplementary Fig. S1). The agreement between EPRI and histologic studies for hypoxia, microvessel density, and cell viability support the reliability of the pO₂ assessment capability of EPRI to monitor spatial and temporal fluctuations in pO₂ in tumor transplants. The possibility that EPRI can use the significant experience from MRI to scale up for human use makes it a promising modality for integration into radiation treatment planning such as dose painting based on oxygen maps to selectively deliver higher doses to hypoxic regions (49, 50). Because EPRI can distinguish between cycling hypoxia and chronic hypoxia, individualized treatments to tailor the dose to deliver therapeutic doses uniformly to the tumor seem to be achievable.

Figure 6. The dynamic monitoring of the effect of carbogen breathing on tumor oxygenation. A, representative time serial pO₂ maps in a SCCVII tumor. Time increased from left to right from 4 to 31 min. The breathing gas changed from air to carbogen at 12 min after the TAM injection and changed back to air at 24 min. B, anatomic image using T2-weighted MRI (left) and pO₂ difference map (right) obtained by subtracting averaged pO₂ maps while breathing air from averaged pO₂ maps while breathing carbogen. The tumor region is indicated by the contour. Three ROIs indicated by numbers were chosen for monitoring temporal pO₂ change. C, the pO₂ values in each ROI and the median pO₂ of the whole tumor region at each time point. D, temporal series of pO₂ histograms in tumor region at every 6 min. Red histograms at 13 and 19 min indicate tumor oxygen status under carbogen breathing.
Our studies described in this report suggest that the methods developed here has the capability to monitor $pO_2$ in tumor implants noninvasively and longitudinally, and examine the onset of cycling hypoxia as a function of tumor growth. Such studies along with the wealth of information available from earlier studies can provide improved understanding of the role of cycling hypoxia in treatment resistance and metastatic potential.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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