Oxygen-Enhanced MRI Accurately Identifies, Quantifies, and Maps Tumor Hypoxia in Preclinical Cancer Models

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Abstract

There is a clinical need for noninvasive biomarkers of tumor hypoxia for prognostic and predictive studies, radiotherapy planning, and therapy monitoring. Oxygen-enhanced MRI (OE-MRI) is an emerging imaging technique for quantifying the spatial distribution and extent of tumor oxygen delivery in vivo. In OE-MRI, the longitudinal relaxation rate of protons (ΔR1) changes in proportion to the concentration of molecular oxygen dissolved in plasma or interstitial tissue fluid. Therefore, well-oxygenated tissues show positive ΔR1. We hypothesized that the fraction of tumor tissue refractory to oxygen challenge (lack of positive ΔR1, termed “Oxy-R fraction”) would be a robust biomarker of hypoxia in models with varying vascular and hypoxic features. Here, we demonstrate that OE-MRI signals are accurate, precise, and sensitive to changes in tumor pO2 in highly vascular 786-0 renal cancer xenografts. Furthermore, we show that Oxy-R fraction can quantify the hypoxic fraction in multiple models with differing hypoxic and vascular phenotypes, when used in combination with measurements of tumor perfusion. Finally, Oxy-R fraction can detect dynamic changes in hypoxia induced by the vasomodulator agent hydralazine. In contrast, more conventional biomarkers of hypoxia (derived from blood oxygenation-level dependent MRI and dynamic contrast-enhanced MRI) did not relate to tumor hypoxia consistently. Our results show that the Oxy-R fraction accurately quantifies tumor hypoxia noninvasively and is immediately translatable to the clinic.

Introduction

Hypoxia is a common feature of most solid malignancies, resulting from an imbalance between oxygen delivery and consumption (1). Tumor hypoxia is associated with the activation of angiogenesis and with metastatic potential (2). Consequently, tumor hypoxia is an important negative prognostic factor (3–5). Tumor hypoxia also mediates resistance to radiotherapy and to some chemotherapy agents and is an independent predictor of treatment failure. Strategies to counteract tumor hypoxia using either radiosensitizers or hypoxia-activated cytotoxic agents are currently being evaluated (6).

A noninvasive imaging biomarker that identifies the presence of hypoxia and measures its extent and spatial distribution within a tumor would help facilitate personalized medicine. However, no such tool is currently available. Proton (1H) MRI is used routinely in clinical medicine, making it an attractive noninvasive modality for measuring oxygen delivery and hypoxia in tumors. Current 1H MRI methods of imaging hypoxia have focused on either dynamic contrast-enhanced MRI (DCE-MRI) or R2⁎-based intrinsic susceptibility imaging, also referred to as blood oxygenation level dependent (BOLD) imaging (7).

In DCE-MRI, administration of a gadolinium-based contrast agent allows estimation of blood vessel flow and permeability, providing an indirect measurement of oxygen delivery and necrosis (8). In BOLD imaging, paramagnetic deoxyhemoglobin molecules in erythrocytes create magnetic susceptibility perturbations around blood vessels, which increase the local transverse MRI relaxation rate (R2⁎; units ms⁻¹). The value of tumor R2⁎ decreases when blood oxygen saturation increases following inhalation of hyperoxic gas (9). Unfortunately, both DCE-MRI and BOLD imaging have significant limitations that have hindered implementation as clinical biomarkers of hypoxia (10). Neither measure hypoxia directly. Further, BOLD measurements are affected by the presence of hemorrhage, by change in vessel geometry and by artifact in air/soft tissue interfaces, such as in the lungs and bowel (7).

Oxygen-enhanced MRI (OE-MRI) is a distinct 1H MRI method for quantifying tumor oxygen delivery. Here, the MRI longitudinal relaxation rate (R1; units s⁻¹) is sensitive to changes in the level of molecular oxygen (O2) dissolved in blood plasma or interstitial...
tissue fluid (11, 12). When hyperoxic gas is inhaled, excess oxygen is carried in the blood plasma in tissues with adequate perfusion. Because well-oxygenated tissue has near complete saturation of hemoglobin molecules (13), the excess delivered oxygen remains dissolved in blood plasma and interstitial tissue fluid, where it increases the R1 value (Fig. 1A). The change in R1 (ΔR1) observed is theoretically proportional to magnitude of change in dissolved O2 concentration for a given voxel (Fig. 1B; ref. 11). Several previous studies have reported positive average values of ΔR1 following oxygen inhalation in preclinical models of cancer (14–22) and in human tumors (20, 23–25). Importantly, although oxygen relaxivity, baseline R1, and signal-to-noise (SNR) ratios show some variation with field strength, the technique is feasible on both preclinical and clinical MRI platforms (22, 26).

Measuring positive ΔR1 in OE-MRI quantifies and maps oxygen delivery by identifying tissue with fully saturated hemoglobin, but does not directly identify tissue hypoxia per se. Tumor subregions refractory to oxygen challenge are considered to have low hemoglobin oxygen saturation and so excess delivered O2 molecules bind preferentially to hemoglobin but do not significantly alter plasma pO2 (Fig. 1C; ref. 10). Several preclinical OE-MRI studies have reported that some tumor subregions are refractory to hyperoxic gas challenge because they had no positive ΔR1 change (14, 19–21). If these regions are perfused yet lacking in oxygen is carried in the blood plasma in tissues with adequate perfusion.

Figure 1.
OE-MRI distinguishes between normoxic and hypoxic tissue. Each box represents an imaging voxel that contains erythrocytes (red spheres) in blood vessels (pink cylinders); tumor cells (gray ellipse); and surrounding interstitial space (white area). A, in normoxic tissue, hemoglobin molecules in erythrocytes are oxygen saturated and form oxyhemoglobin (HbO2) molecules. Dioxygen molecules (O2) are dissolved in the plasma. Inhalation of hyperoxic gas markedly increases the amount of dissolved plasma O2, but HbO2 concentration is essentially unaltered. B, increased pO2 in interstitial fluid and plasma increases tissue longitudinal relaxation rate (R1), which is detected by MRI. C, in hypoxic tissue, hemoglobin molecules are not fully oxygen saturated and many exist as deoxyhemoglobin (Hb) molecules. Inhalation of hyperoxic gas increases the HbO2 to Hb ratio but has negligible effect on plasma O2. D, because there is negligible change in pO2, the R1 remains little changed (straight black line). Since Hb has a slightly higher longitudinal relaxivity than HbO2, tissue R1 may even decrease (dotted black line).

Materials and Methods

Phantom validation of R1 measurement

In vitro validation experiments were performed on the same 7T system as used for the in vivo studies. The phantom consisted of four 5 mm test tubes (Sigma-Aldrich) with solutions of gadopentetate dimeglumine (Magnevist; Schering) serially diluted in water to yield final concentrations of 0.01, 0.05, 0.07, and 0.09 mmol/L gadolinium and an expected range of T1 between 1,400 and 2,500 ms based on the relaxivity of Magnevist at 7T at 37°C (27). Test tubes were placed inside a plastic container filled with dental paste to reduce susceptibility effects and to ensure efficient heat transfer for constant temperature. Three repeated measurements of R1 were measured for each tube on three separate days.

Tumor implantation

All experiments were performed in compliance with licenses issued under the UK Animals (Scientific Procedures) Act 1986 and following local ethical review. Studies were compliant with the United Kingdom National Cancer Research Institute Guidelines for Animal Welfare in Cancer Research (28) and with the Animals in Research: Reporting In Vivo Experiments guidelines (29).

Three cell lines exhibiting differing vascular and hypoxic phenotypes in vivo were used. Parental 786-0 renal carcinoma cells (ATCC, LCG Standards; purchased 2011; 786-0-par), and cells established from a sunitinib refractory subcutaneous xenograft (786-0; ref. 30), were cultured in RPMI supplemented with 10% (v/v) FCS (Gibco, Life Technologies). Tumors were propagated by injecting 3 × 10^6 cells in 100 µL of sterile PBS into the flanks of 8-week-old female C.B17-scid mice under isoflurane anesthesia. SW-620 colorectal carcinoma cells (ATCC; lot #8924081, purchased 2005) were cultured in DMEM supplemented with 10% FCS. Tumors were propagated by injecting 5 × 10^6 cells in 100 µL of sterile PBS into the flanks of 8-week-old female athymic NCr-Foxn1 nu mice. Immediately prior to in vivo implantation, all cells tested negative for mycoplasma infection and the number of short tandem repeats (STR) present at 7 to 10 loci were assessed by PCR to provide STR profiles, from which cell line authenticity was confirmed.

Tumor size was monitored using callipers and the formula for ellipsoid volume, (π/6) × L × W × D, where L, W, and D are the largest orthogonal dimensions of the ellipsoid. Tumors were typically used for experimentation at a volume of approximately 400 mm³.

Measurement of tissue pO2

The fiber optic oxygen sensing device OxyLite (Oxford Optonix; ref. 31) was used to measure tumor partial pressure of oxygen (pO2; units mm Hg). A heat mat maintained core body temperature, and gas delivery was at 2L/min via a nosepiece. Two dual pO2 and temperature probes were positioned within the tumor tissue. Animals initially breathed medical air, followed by 100% oxygen to mimic the gas changes induced during the MRI experiments. Data acquisition and averaging were performed using...
MRI data acquisition

Anesthesia was induced with a 10 mL/kg intraperitoneal injection of fentanyl citrate (0.315 mg/mL) plus flumazenil (10 mg/mL; Hypnorm; Janssen Pharmaceutical Ltd.), midazolam (5 mg/mL; Hypnovel; Roche), and sterile water (at 1:1:2 ratio; ref. 16). Mice were positioned in a 3 cm birdcage coil on a custom-built platform to isolate the tumor, which was surrounded by dental paste (3M) to minimize motion and susceptibility artifacts. Gas delivery (medical air or 100% oxygen) was continuous at 2 L/min through a nosepiece. Warm air maintained animal core temperature at 37°C. Lateral tail vein cannulation was performed with a heparinized 27G butterfly catheter (Venflon systems; Hospira) to enable the remote intravenous administration of gadolinium contrast agent in DCE-MRI studies or when vasomodulator was administered.

All MRI data were acquired on a 7T horizontal bore microimaging system (Bruker Instruments). Localization was performed using a multislice turboRARE T2-weighted sequence and was followed by shimming over the tumor (32). All sequences were acquired using 30 mm × 30 mm FOV (128 × 128 matrix; in plane resolution 0.234 mm) for a single 1-mm-thick slice. Experimental protocols are detailed in Supplementary Fig. S1. Sequences used were as follows:

OE-MRI: Inversion recovery (IR) True-FISP images were used to calculate R1 (TR, 2.4 ms; scan TR, 10 seconds, 48 inversion times spaced 38.8 ms apart with initial inversion time of 106.2 ms; TE, 1.2 ms; α 60°). High SNR images were used to obtain highly accurate R1; these required 8 signal averages and took 10 minutes and 40 seconds to perform. Dynamic images were used to quantify the temporal onset of R1 changes induced by switching between air and 100% oxygen. The dynamic images required 2 signal averages and took 2 minutes and 40 seconds to perform.

BOLD: Multiple gradient echo images were used to calculate R1* (TR, 200 ms; 8 echo times; TE, 6.2 to 28.2 ms with 3.1 ms echo spacing; 8 signal averages); duration 3 minutes and 25 seconds.

DCE-MRI: Data were collected using a modified True-FISP sequence (TR, 2.4 ms; scan TR, 10 seconds, 8 inversion times spaced 155 ms apart, initial inversion time 108 ms, TE, 1.2 ms; α 60°; one signal average, temporal resolution 20 seconds). After five baseline measurements, 0.1 mmol/kg bolus of the gadolinium-based contrast agent Magnevist (2 mL/kg 50 mmol/L solution) was injected intravenously at 2 mL/min using a power injector with duration of 10 minutes.

MRI analysis

Regions of interest (ROI) were drawn around the tumor on the T2-weighted images by an experienced operator (Y. Jamin). ROIs were transferred to all OE-MRI, BOLD, and DCE-MRI data. Voxelwise and median values of R1 and R1* were calculated for each map using a Bayesian maximum a posteriori approach, with inhouse software.

For OE-MRI, the voxel-wise ΔR1 was calculated, where ΔR1 = R1 (O2) − R1 (air). The initial R1 (air) was derived from the average of the first two high SNR R1 maps. The R1 (O2) was derived from the high SNR R1 map acquired during oxygen breathing. Oxygen enhancement was measured as 2 × tumor baseline R1 × cohort CoV. Voxels were then classified as enhancing (termed "Oxy-E") or refractory (termed "Oxy-R") to oxygen challenge. Voxel-wise BOLD AR1* was calculated, where AR1* = R1* (O2) − R1* (air).

Where DCE-MRI was performed, the initial area under the contrast agent concentration curve from 0 to 60 seconds (IAUC0) was calculated (16), and voxels were classified as enhancing when IAUC0 > 0. Where both OE-MRI and DCE-MRI were acquired, data were combined to distinguish three tumor subregions from one another: (i) perfused Oxy-E voxels; (ii) perfused Oxy-R voxels; and (iii) nonperfused voxels.

Hydralazine challenge

Hydralazine acts directly on vascular smooth muscle in vessels of normal tissues, causing vasodilation and reduced mean arterial blood pressure. Tumor blood vessels lacking smooth muscle do not dilate in response to hydralazine. Hence, blood is redistributed away from the tumor, reducing blood flow and increasing hypoxia within 30 minutes (33–36). Hydralazine challenge has, therefore, been used as a tool to manipulate acute hypoxia.

An initial air-to-oxygen gas challenge was performed. Then, gas delivery was switched back to air breathing, and intravenous injection of either 5 mg/kg hydralazine hydrochloride (Sigma-Aldrich Co.) or saline was performed. Finally, a second air-to-oxygen gas challenge was performed. Spatial differences between the ΔR1 defined Oxy-E and Oxy-R voxels on the two air-to-oxygen challenges were assessed by mismatch mapping. Formal randomization was not employed, rather during each day’s scanning, mice were prospectively assigned with intent to balance treatment groups according to tumor size.

Immunofluorescence analysis

Intraperitoneal injection of 60 mg/kg pimonidazole (Hypoxprobe) was performed 55 minutes before 100% O2 inhalation began to allow for maximal bioreduction of the agent in hypoxic tumor regions. Hoechst 33342 (15 mg/kg; Sigma-Aldrich Co.) was administered intravenously 1 minute prior to rapid tumor excision. Tumors were excised whole and bisected along the imaging plane so that the cut surface approximated to the MRI region of interest. Half the tumor was snap frozen, and half was formalin fixed and paraffin embedded.

Frozen tissue sections (5 μm) were obtained from snap-frozen tumor material and scanned using fluorescent microscopy on a Panoramic 250 Flash system (3DHistech) to determine the number of Hoechst-stained (perfused) vessels (excitation 350 nm/emission 480 nm). Pimonidazole binding was determined in the same sections using Hypoxprobe-1 (Hypoxprobe), a mouse-monoclonal, followed by rabbit anti–mouse-FITC conjugated secondary antibody (excitation 488 nm/emission 525 nm). Paraffin-embedded sections (5 μm) were obtained from tumor tissue dehydrated after fixation in 10% phosphate-buffered formalin. Sections were stained with hematoxylin and eosin (H&E). Data were analyzed using ImageJ software (NIH, Bethesda). The perfused vessel area, hypoxic fraction, and percentage necrosis were calculated, as described elsewhere (37).

Statistical analysis

IBM SPSS Statistics v.22 was used for all statistical analyses. In all cases, P values of <0.05 were considered significant. Comparison of median values of R1 during medical air only breathing and during oxygen challenge experiments were evaluated using a
one-way ANOVA with a post hoc Bonferroni correction for multiple comparisons.

Data comparing median values of R1, R2*, and IAUC60 and fractions of Oxy-E or Oxy-R between different tumor cohorts (786-0-R, 786-0-par, or SW620) were evaluated with independent t tests corrected for multiple comparisons. Data for different cohorts were assumed to be distributed normally and have unequal variance. Correlations between MRI and pathology were assessed by the nonparametric Spearman rho. Formal sample size calculations were not performed.

Results
OE-MRI signals are accurate, precise, stable, and sensitive to pO2 change

We sought to demonstrate OE-MRI signal precision, stability, and sensitivity to altered oxygen tension, because these factors have not been well documented. Because OE-MRI signal changes are based on the longitudinal relaxation rate, R1, we compared the expected R1 against the measured R1 in an in vitro phantom. These data showed that our R1 measurement technique is accurate (Supplementary Fig. S2).

To test measurement precision and stability in vivo, we then performed an experiment in four mice implanted with subcutaneous 786-0-R tumors (786-0-R is a fast-growing subline of the 786-0 renal cancer cell line). Mice initially breathed medical air for 40 minutes. This was followed by 100% oxygen challenge for 20 minutes. Finally, the mice breathed medical air again for 25 minutes. Multiple R1 maps were acquired (Supplementary Fig. S1A). The within-scan coefficient of variation (CoV) of baseline voxel-wise R1 was 0.41% (Supplementary Fig. S2). The within-scan CoV is shown in a sample Oxylite trace from a xenograft tumor while the mouse breathed air (top row), 100% oxygen (middle row), and back to air (bottom row). R1 measurement was stable in four mice, with 786-0-R xenografts during the initial 45 minutes of air breathing.

Challenge with 100% oxygen was then performed in the same mice, to increase tumor pO2. All four tumors showed rapid, heterogeneous and significant increase in median R1 following oxygen challenge. These changes were clearly visible in the first map after 2 minutes and 40 seconds of oxygen breathing and persisted for the duration of the oxygen challenge (P < 0.008 for all time points 8–12 compared with air breathing) showing consistent spatial arrangement (Fig. 2A and B). Across all four tumors, 89.3% of voxels were oxygen enhancing (Oxy-E), and 10.7% of voxels were oxygen refractory (Oxy-R). Sample traces of ΔR1 change are shown for Oxy-E and Oxy-R voxels (Supplementary Fig. S3). Oxylite measurement in the same tumors showed pO2 increase (beyond the limit of detection of the Oxylite equipment, namely at 100 mmHg) in 7 of 8 measured regions over the same time frame (sample trace in Fig. 2C). The Oxylite data provide independent evidence that OE-MRI detects real-time increases in the oxygen concentration in tumor interstitial tissue fluid. BOLD MRI, performed in the same mice, revealed a significant reduction in R2* during oxygen challenge (mean reduction, 39.8 ms⁻¹; SE, 11.7 ms⁻¹; P = 0.043), consistent with previous studies (38).

Tumors showed rapid reversal of R1 changes when oxygen challenge ended and air breathing was resumed (see time points 13–17 in Fig. 2A and B). There was no significant difference between baseline and end-of-study mean R1 values for the cohort. Oxylite measurement showed pO2 return to prechallenge levels over a period of 2 to 6 minutes mirroring ΔR1 change. Collectively, these data confirm that R1 measurements used for this study are accurate, precise, stable in the absence of perturbation, and sensitive to change in tumor pO2.

Oxy-R fraction detects differential levels of hypoxia in an isogenic system

We investigated whether Oxy-R fraction (the fraction of each tumor refractory to oxygen challenge) could detect different levels
of hypoxia in cancer models. We measured the Oxy-R fraction in two isogenic cell lines: the slow growing parental 786-0 cell line (786-0-par), which took 206.3 days ± 66.2 SD to reach a tumor volume of approximately 400 mm³ (n = 8 mice), and the much faster growing 786-0 subline (786-0-R), which only took only 32.1 days ± 6.9 SD to reach the same tumor volume (n = 9 mice). From these mice, we acquired OE-MRI, ΔR₂*, and DCE-MRI data along with histopathologic analysis of pimonidazole adduct formation (for hypoxia) and Hoechst 33342 uptake (for perfusion; Supplementary Fig. S1B).

Staining for pimonidazole adduct formation showed a significantly higher hypoxic fraction in 786-0-R tumors compared with the 786-0-par tumors (P = 0.008; Fig. 3A). This finding was mirrored by the Oxy-R fraction being significantly higher in the 786-0-R xenografts compared with the 786-0-par xenografts (P = 0.047; Fig. 3B). In distinction, there was no significant difference in the median values of ΔR₁, IAUC via perfusion data from the perfused portion of each tumor. These data confirm that Oxy-R fraction is sensitive to differential levels of hypoxia, but that median values of ΔR₁, IAUC via perfusion data are from the perfused portion of each tumor.

To do this, we first used the 786-0-R data already collected. We examined the within-cohort correlation between Oxy-R fraction and the pimonidazole adduct formation—based measurement of hypoxic fraction. This model is known to be highly vascularized.

Further, there was a wide dynamic range of hypoxia in this model. We then performed an equivalent experiment using SW620 tumors. This model was chosen because these tumors are relatively poorly vascularized (39).

Anticipated differences in the perfusion status of the two models were confirmed; 786-0-R xenografts had significantly greater perfused tumor area compared with SW620 xenografts (n = 8 mice), measured by DCE-MRI (P = 0.011; Table 1) and by Hoechst 33342 (P = 0.004). Pathology analysis showed that perfused vessel area measured by Hoechst 33342 (Supplementary Fig. S4A and S4B) correlated with perfused Oxy-E fraction (those voxels showing positive enhancement with both oxygen and gadolinium) and that nonperfused fraction correlated with necrosis on HE in both models (Supplementary Fig. S4C and S4D).

Oxy-R fraction correlated strongly with pathologic hypoxic fraction in 786-0-R tumors (rho, 0.810; P = 0.028; Fig. 4A), irrespective of whether the entire tumor was analyzed or if analysis was restricted to gadolinium-enhancing tumor only (perfusion Oxy-R fraction). In SW620 tumors, the Oxy-R fraction did not correlate with hypoxic fraction (measured by pimonidazole adduct formation). However, as anticipated, the perfused Oxy-R fraction and hypoxic fraction (measured by pimonidazole adduct formation) correlated strongly (rho, 0.929; P = 0.002; Fig. 4B). In both models, visual inspection revealed that perfused Oxy-R voxels were located at an interface between the peripheral perfused Oxy-E voxels and the centrally located nonperfused voxels. Collectively, these data provide evidence that Oxy-R fraction can quantify hypoxic fraction accurately, but requires perfusion data in poorly vascularized tumors.

Oxy-R fraction is sensitive to dynamic change in hypoxia

In order to provide evidence that Oxy-R fraction can detect reduced oxygen delivery to tumors, we administered the vasomodulator hydralazine to a cohort of mice bearing 786-0-R xenografts (n = 4 mice) and compared with control mice receiving saline (n = 6 mice). We performed an air-to-oxygen challenge before and after the administration of hydralazine or control (Supplementary Fig. S1C). In the initial air-to-oxygen challenge, overall ΔR₂ changes were positive, as expected in all 10 mice (Fig. 5A).

Importantly, the second air-to-oxygen challenge was initiated 15 minutes after administration of hydralazine or saline. The magnitude of ΔR₂ was reduced significantly in mice receiving hydralazine, relative to control (Fig. 5A; P < 0.01). This difference in hydralazine-treated tumors was driven by the Oxy-R fraction increasing significantly from 17.3% to 30.5% (P = 0.045; Fig. 5B).

To further understand these changes, we used “mismatch mapping” to identify tumor subregions that changed oxygen enhancement status. In control tumors, the majority of voxels remained consistently either Oxy-E or Oxy-R. The number of voxels changing from Oxy-E to Oxy-R or vice versa was equal for both directions of change and was small (Fig. 5C). In hydralazine-treated tumors, substantially more voxels turned from Oxy-E to Oxy-R than vice versa. Some of these newly Oxy-R tumor subregions were immediately adjacent to established Oxy-R regions, but other new areas of Oxy-R were detected at spatially distant subregions (Fig. 5D). These data confirm that OE-MRI can detect dynamic change in tumor hypoxia. Further, tumor regions with

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Figure 3.

Oxy-R fraction identifies differential levels of hypoxia. A, 786-0-R xenografts had significantly greater pimonidazole adduct-derived hypoxic fraction than 786-0-par xenografts, mirrored in Oxy-R fraction tumor (B). No difference was seen in median ΔR₁(C), median IAUC via perfusion data from the perfused portion of each tumor. These data confirm that Oxy-R fraction is sensitive to differential levels of hypoxia, but that median values of ΔR₁, IAUC via perfusion data are from the perfused portion of each tumor.

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appearing or resolving hypoxia can be mapped using this technique.

Discussion

Hypoxia is an important prognostic factor for solid tumors and mediates resistance to radiotherapy and to some chemotherapy agents. Noninvasive clinical imaging has potential to improve patient care by producing biomarkers that alter clinical decision making (40, 41). In the case of hypoxia, a clinically translational imaging biomarker has potential to be prognostic and/or predictive (identifying presence of hypoxia and quantifying hypoxic fraction/volume prior to therapy), to assist treatment planning (mapping targets for radiation boost or adaptive therapy; ref. 42), or to monitor therapy-induced changes in tumor hypoxia, for example following radiosensitization or hypoxia-activated cytotoxic therapy (10, 43).

To date, clinical strategies to image tumor hypoxia have been dominated by PET methods (7). Several investigational PET tracers have been evaluated, each with slightly different underlying relationship to tissue oxygen tension (44, 45). However, at present, no imaging method identifies and maps the extent of tumor hypoxia that is validated, cost-effective, widely available and used for patient benefit (7).

OE-MRI is an emerging technique that detects the presence of excess dissolved O₂ molecules in plasma and tissue fluid. Most OE-MRI studies in cancer have been small cohort-based studies, and without corresponding pathology or intervention, tending to report changes in median signal intensity or median hypoxic fraction/volume prior to therapy), to assist treatment planning (mapping targets for radiation boost or adaptive therapy; ref. 42), or to monitor therapy-induced changes in tumor hypoxia, for example following radiosensitization or hypoxia-activated cytotoxic therapy (10, 43).

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Table 1. Proportions of Oxy-E and Oxy-R tumor (with SD) in the imaging-pathology experiments, subdivided by voxel perfusion status

<table>
<thead>
<tr>
<th></th>
<th>Perfused % Oxy-E voxels</th>
<th>Not perfused % Oxy-E voxels</th>
<th>Perfused % Oxy-R voxels</th>
<th>Not perfused % Oxy-R voxels</th>
</tr>
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<tbody>
<tr>
<td>786-O-R</td>
<td>78.2 (SD 18.3)</td>
<td>7.2 (SD 5.9)</td>
<td>13.2 (SD 12.9)</td>
<td>1.2 (SD 1.6)</td>
</tr>
<tr>
<td>Parental 786-O</td>
<td>84.7 (SD 14.0)</td>
<td>11.0 (SD 12.2)</td>
<td>3.4 (SD 2.5)</td>
<td>0.9 (SD 0.8)</td>
</tr>
<tr>
<td>SW620</td>
<td>51.5 (SD 13.8)</td>
<td>25.2 (SD 14.9)</td>
<td>15.7 (SD 6.2)</td>
<td>7.6 (SD 4.3)</td>
</tr>
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</table>

Advances in OE-MRI have focused largely around providing dual ΔR₁ and ΔR₂ acquisitions (25) or in enhancing the magnitude of oxygen-induced ΔR₁, for example by developing methods sensitive to tumor lipid signals (17).

In this study, we adopted a different approach. We focused on the fact that tumors are biologically heterogeneous, composed of subregions with distinct pathophysiology (48, 49). Previous OE-MRI studies have suggested that some tumor subregions are refractory to the positive ΔR₁ induced by oxygen challenge (14, 20). We built on this observation by partitioning the OE-MRI signal into regions with enhancing (Oxy-E) or refractory (Oxy-R) signatures. We conducted a series of experiments to test the hypothesis that Oxy-R fraction tumor can identify, quantify, and map tumor hypoxia.

We first used the renal carcinoma model 786-O-R (rapid growing, vascular tumors with anticipated large oxygen-induced ΔR₁) to develop a protocol that was not only stable while breathing medical air, but also sensitive to oxygen-induced signals. This is the first study to provide detailed evidence of measurement stability and sensitivity for serial R₁ mapping in OE-MRI. The temporal evolution of oxygen-enhanced–positive ΔR₁ occurred within 3 minutes and was spatially stable. The OE-MRI data mirrored OxyLite measurement of pO₂ changes, concurring with data from a previous study where rapid increases in ΔR₁ were observed following gas challenge (15) and with data comparing OE-MRI signal changes with ¹⁹F oximetry (19).

Next, we demonstrated that Oxy-R fraction could distinguish differences in hypoxic fraction within tumors derived from two
isogenic cell lines: parental 786-0 cells and 786-0-R cells. Here, the relatively rapid growing 786-0-R tumors had significantly more hypoxia than the slow growing 786-0-par tumors. Crucially, Oxy-R fraction distinguished between cell lines, mirroring differences observed in values of hypoxic fraction quantified through pimonidazole staining.

We then tested if Oxy-R fraction could estimate hypoxic fraction. We chose the 786-0-R and SW620 models because of their differing perfusion characteristics. Significant correlation was seen between Oxy-R fraction and hypoxic fraction (defined on pimonidazole adduct formation) in the relatively well perfused 786-0-R xenografts. This association was not replicated in the poorly perfused SW620 xenografts because many Oxy-R voxels had negligible perfusion, so received insufficient oxygen gas to alter voxel $R_1$. Having accounted for these voxels using DCE-MRI, we measured Oxy-R fraction in perfused tissue and observed a significant association with hypoxia in the SW620 tumors.

We then increased the fraction of tumor hypoxia in a further cohort of 786-0-R tumors using the vasomodulator hydralazine. Oxy-R fraction increased 2-fold, providing direct evidence that OE-MRI can track acute changes in tumor oxygenation. Some signal fluctuation was observed in both directions in hydralazine-perfused SW620 tumors. This supports further evaluation of Oxy-R fraction or Oxy-R tumor volume as a prognostic and/or predictive biomarker. Third, Oxy-R voxels were located in spatially distinct subregions, suggesting that, if replicated in humans, this technique may facilitate the planning of localized boost and adaptive radiotherapy delivery strategies. Fourth, clinical tumors vary in their extent of perfusion, and this may further change with therapy. Our data suggest that subsequent studies of Oxy-R fraction or Oxy-R tumor volume as a diagnostic and/or predictive biomarker.
hindering clinical translation (7)—Oxy-R fraction can be readily quantified on clinical MRI scanners (24), making the technique suitable for rapid clinical translation.

Disclosure of Potential Conflicts of Interest
Geoff J.M. Parker is founder and CEO at and has ownership interest (including patents) in Bioxydyn Limited. He is also a consultant/advisory board member for GlaxoSmithKline. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.P.B. O’Connor, Y. Jamin, M. Babur, K.G. Finegan, K.J. Williams, R.A. Little, G.J.M. Parker, A.R. Reynolds, J.C Waterton, S.P Robinson
Study supervision: J.P.B. O’Connor, G.J.M. Parker, S.P Robinson

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