MRI biomarkers identify the differential response of glioblastoma multiforme to anti-angiogenic therapy

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Background. Although anti-angiogenic therapy (AATx) holds great promise for treatment of malignant gliomas, its therapeutic efficacy is not well understood and can potentially increase the aggressive recurrence of gliomas. It is essential to establish sensitive, non-invasive biomarkers that can detect failure of AATx and tumor recurrence early so that timely adaptive therapy can be instituted. We investigated the efficacy of MRI biomarkers that can detect response to different classes of AATxs used alone or in combination with radiation.

Methods. Murine intracranial glioma xenografts (NOD/SCID) were treated with sunitinib, VEGF-trap or B20 (a bevacizumab equivalent) alone or in combination with radiation. MRI images were acquired longitudinally before and after treatment, and various MRI parameters (apparent diffusion coefficient, T1w + contrast, dynamic contrast-enhanced [DCE], initial area under the contrast enhancement curve, and cerebral blood flow) were correlated to tumor cell proliferation, overall tumor growth, and tumor vascularity.

Results. Combinatorial therapies reduced tumor growth rate more efficiently than monotherapies. Apparent diffusion coefficient was an accurate measure of tumor cell density. Vascular endothelial growth factor (VEGF)-trap or B20, but not sunitinib, resulted in significant reduction or complete loss of contrast enhancement. This reduction was not due to a reduction in tumor growth or microvascular density, but rather was explained by a reduction in vessel permeability and perfusion. We established that contrast enhancement does not accurately reflect tumor volume or vascular density; however, DCE-derived parameters can be used as efficient noninvasive biomarkers of response to AATx.

Conclusions. MRI parameters following therapy vary based on class of AATx. Validation of clinically relevant MRI parameters for individual AATx agents is necessary before incorporation into routine practice.

Keywords: anti-angiogenic therapies, glioblastoma multiforme (GBM), MRI biomarker.
Fig. 1. Growth rate of intracranial GBM xenografts in response to 2 anti-angiogenic therapies (AATx) used as monotherapy or as combinatorial therapy with radiation therapy (RT). (A) Mouse intracranial xenografts were generated using GSC-1 (i) and U87 (ii) cell lines, and changes in tumor volume were compared longitudinally between these tumor models. (B) U87 xenografts were treated with AATxs (sunitinib or VEGF-trap), RT alone, or AATx + RT. Tumor volumes were measured using T2w-MRI images on days 7, 10, 14, 17, and 21 post tumor implantation. Line graphs (i, ii) are representative of relative tumor volume normalized to baseline tumor (day 7) in control and experimental groups. Bar graphs (iii) compare the relative tumor volume of different treatment groups.
inhibitors such as sunitinib, which has been shown to reduce glioma tumor growth in GBM xenografts. Despite the attractive mechanistic approach of AATx therapy in a highly vascularized tumor, clinical experience to date suggests that AATx drugs have not significantly increased the survival of GBM patients. Bevacizumab, despite demonstrating initial clinical improvements and radiological response, has shown transient benefits, and GBM recurrence is inevitable, often with a much more invasive phenotype. Use of VEGF-trap has been associated with some radiological response, but without any significant improvement in recurrent GBM. Similarly, sunitinib has failed to show benefits in improving progression-free survival in GBMs.

The ability to detect recurrence early, prior to a more aggressive phenotype, is critical for initiating alternative targeted therapies. Furthermore, it is now believed that the efficacy of AATx can be potentiated if it is combined with standard chemotherapy, radiation therapy (RT), or other targeted therapies. Establishing noninvasive biomarkers that can accurately detect the response to different classes of AATx is important and may be useful for scheduling combinatorial therapy.

Multiparametric MRI provides a promising noninvasive strategy to characterize cellular and vascular properties of tumors in addition to defining response to chemotherapy, RT, and AATx therapies. For instance, parameters derived from perfusion MRI have been used to assess response to AATx therapies in patients diagnosed with GBM. Diffusion MRI parameters have shown value in predicting survival outcome in patients with recurrent GBM who are treated with bevacizumab. However, to date there has been no clinical or preclinical studies that directly compare various MRI parameters in response to different classes of AATx. Clinical studies are limited in their ability to obtain repeat multiple biopsies at the time of perceived tumor recurrence. As such, preclinical studies provide the advantage of making direct comparison between specific MRI parameters and tumor characteristics in response to various classes of AATx. In this study, we focused on establishing MRI parameters that are indicative of tumor cellularity, growth, and vascularity in response to AATx when administered as monotherapy or in combination with RT and evaluating these MRI parameters across different classes of AATxs in preclinical models of GBM. Findings from this study could be used to inform directed questions in the clinical setting using cohorts of patients followed serially with frequent MRI imaging that compares clinical outcome and tumor progression with MRI and dynamic contrast-enhanced (DCE)-based parameters.

Materials and Methods

Cells and Transfection

We used 2 different GBM cell lines for this study. One, an established human glioma stem cell line U87 (ATCC, Rockville) and the other, a glioma stem cell line (GSC-1) obtained from a human GBM tumor specimen at The University of Texas MD Anderson Cancer Center (Houston), as previously described. To generate stable U87 or GSC-1 cell lines with VEGF-trap expression, cells were transfected with VEGF-trap using a PiggyBac transposon system (provided by Dr. Nagy), as previously described.

Intracranial Xenograft Models of Glioblastoma Multiforme

All animal experiments were carried out according to institutional Animal Care Committee guidelines. Intracranial xenografts were generated, as described previously. Seven days post U87 cell implantation and 21 days post GSC-1 cell implantation, we used baseline MRI to ensure equal tumor sizes prior to instituting treatment. We ensured successful intracranial expression of VEGF-trap in vivo by imaging the sectioned brain ex vivo (Supplementary Fig. S1).

Anti-angiogenic Therapy

In order to allow direct one-to-one comparison of the effects of AATx with a sufficient number of animals for each group, we limited the time window of the comparison to 10 days of treatment (days 7–17), starting 1 week following tumor cell implantation. Each treatment arm included 8–10 mice repeated in duplicate, and the AATxs evaluated here included sunitinib, VEGF-trap, and B20, which is the mouse equivalent of bevacizumab or Avastin (Genentech). Sunitinib (Pfizer) was prepared as previously reported, and a dose of 40 mg/kg/day was administered by oral gavage. Mice implanted with U87:VEGF-trap cells were given 3 mg/mL doxycycline (Sigma) in drinking water. B20 was administered intraperitoneally (150 μg/mouse) every 3 days. The onset of treatment remained the same for all AATxs. To study the immediate effects of eliminating VEGF-trap on physiological characteristics of tumor vasculature, the supply of doxycycline was stopped between days 17 and 21 post intracranial implantation of the U87:VEGF-trap cells. The tumors were allowed to grow until the mice became moribound and were euthanized according to animal care protocols.

Radiation Therapy

A single fraction of 8 Gy radiation was delivered at day 8 following intracranial injection using a cone-beam CT image-guided small animal irradiation system (XRT225Cx, Precision X-Ray, Inc), in which mice were positioned in an in-house, custom-built stereotactic immobilization device, as previously described.

Magnetic Resonance Imaging

MRI was performed with a 7 Tesla Biospec 70/30 (Bruker Corporation), using the B-GA12 gRTient coil insert and 7.2 cm inner diameter, linearly polarized volume resonator coil for volume between all treatment groups within the 10-day treatment window. (C) Apparent diffusion coefficient (ADC) maps were generated from DWI-MRI images. Bar graphs represent percentage change in ADC values from normal contralateral brain region. (D) Bar graphs represent the number of Ki67 positive tumor cells (i) and tumor cell density at day 17 post intracranial injection (ii). In all cases, values represent mean ± SE of 8–10 independent experiments per each time point. Significant differences from control are represented as *P < .05, **P < .01, and ***P < .0001.
Fig. 2. Change in contrast enhancement of GBM xenografts using contrast MRI images in response to anti-angiogenic therapies (AATx) and AATx + radiation therapy (RT). (A) MRI images examining contrast enhancement of U87 and GSC-1 intracranial xenografts. Representative $T_2w$ and $T_1w$ + contrast images demonstrate MRI differences between U87 and GCS-1 tumors, with GSC-1 showing a more invasive tumor but with less contrast enhancement than U87 (i). MRI images acquired on day 17 following U87 tumor implantation (10 days following treatment with AATx, RT or AATx + RT). $T_2w$ images outline tumor region and $T_1w$ + contrast plus DCE images represent contrast enhancement in different experimental groups (ii). (B) Bar graphs represent extent and percentage change in contrast enhancement of the tumor region compared with adjacent normal brain on $T_1w$ contrast MRI images. Values are shown as mean ± standard error, and significant differences between each treatment arm and control group are depicted as *$P < .05$ and **$P < .01$. (C) Line graphs show relative changes in tumor volume (measured on $T_2w$-MRI) and contrast enhancement (measured on $T_1w$ contrast MRI) for control and each treatment arm. There is a lack of correlation between tumor growth and change in contrast enhancement.
radiofrequency transmission, as detailed previously. Multiparametric MRI was used to detect real-time changes in physiological properties of a tumor in response to single-agent or combinatorial therapy with AATx at days 7, 10, 14, and 17 after intracranial tumor implantation. The acquisition of multiparametric MRI images and analysis of cerebral blood flow (CBF) are described in Supplementary Material.

Magnetic Resonance Image Analysis

MIPAV software (National Institutes of Health) was used to analyze the MRI images. Tumor region of interest (ROI) on T2-weighted (T2w) images was manually defined and used to measure the tumor volume. For contrast enhancement analysis, the percentage increase in contrast enhancement of the tumor ROI on T1-weighted (T1w) + contrast images was normalized to adjacent nontumor region. Apparent diffusion coefficient (ADC) and dynamic contrast enhanced (DCE) maps were generated from diffusion (DWI) or DCE-MRI images. Tumor ROIs on T1w + contrast or T2w images were transposed to corresponding diffusion and DCE maps. For diffusion analysis, percentage change in ADC value of tumor, as compared with similar contralateral region, was calculated based on evidence of improved motion sensitivity. Contrast enhancement of the regions that were overlapping with ventricles was excluded from analysis. For DCE analysis, tumor ROI and arterial input function were selected, propagated to all time points on each slice, and used for extraction of contrast enhancement. Average contrast enhancement data and DCE tool v1.04 (www.TheDCETool.ca) were used to calculate the initial area under the contrast enhancement curve (IAUC). The concentration of contrast agent in tumor region over time was calculated using modified Tofts model kinetic analysis.

Immunostaining

Immunostaining was performed on cryopreserved frozen brain sections of control and treated animals using anti-human primary antibodies including anti-CD31 (1:500, BD Pharmingen), anti-Ki67 (1:25, Dako), anti-angiopoietin-1 (Ang-1) (1:100, Santa Cruz Biotechnology), and anti-angiopoietin 2 (Ang-2) (1:100, Santa Cruz Biotechnology) per manufacturer’s protocol. Cell density and microvascular density (MVD) were calculated by counting the number of cell nuclei on hematoxylin-and-eosin stained sections and CD31-positive vessels per 10 high power field (40X), respectively. Vessel diameter was measured using Mirax scan software.

Laser Capture Microscopy

Frozen sections (5 μm) were dehydrated through alcohol and xylene and air-dried for 15 minutes. Laser capture microscopy (LCM) slide was loaded into the microscope (MMI Cellcut system mounted on a Zeiss Axiocvert 200 M microscope with a 340 nm laser and a Sony 3-CCD camera) holder. CD31 immunostaining was used to delineate tumor vessels. In the bright field channel, the LCM cap was lowered onto the sample. Vessels were circled on the software, and then the laser was used to cut through the tissue twice. A minimum of 15 vessels was collected for each sample.

PicoPure RNA Isolation, cDNA Synthesis and Quantitative Real-Time PCR Analysis

RNA was extracted from LCM-isolated vessels using PicoPure RNA extraction kit (Applied Biosciences). cDNA synthesis and quantitative real-time PCR (qPCR) analysis were performed using SuperScript VILO kit (Invitrogen) and Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) according to the manufacturer’s protocol.

Statistical Analysis

Data were represented as mean± standard error. The Student’s paired t test was used to analyze the significant difference at each time point from baseline between 2 experimental groups. Analysis of variance (ANOVA) was used for comparisons between multiple treatment groups, and P<.005 was considered as significant difference.

Results

GBM Growth in Response to Different Classes of AATxs Used as Monotherapy or Combinatorial Therapy

We compared tumor growth rate of U87 and GSC-1 xenografts using longitudinal MRI. The MRI characteristics of GSC and U87 tumors showed a number of distinct differences. GSC-1 cells demonstrated a latency period of 3 weeks, after which they developed rapidly growing tumors. In contrast, U87 tumors showed a steady increase in tumor volume, starting 1 week post tumor implantation (Fig. 1A). GSC-1 tumors, compared with U87, were more invasive and showed a more heterogeneous enhancement pattern on MRI (T1w + contrast). Therefore, given the long dormancy and very rapid growth of GSC-1, longitudinal therapeutic response to different classes of AATx was first examined in U87 models, and results were subsequently confirmed in GSC-1 xenografts.

We first examined the effects of sunitinib and VEGF-trap as monotherapy and in combination with RT on tumor growth using volumetric analysis on T2w-MRI images. Treatment with RT, sunitinib or VEGF-trap, used as monotherapy, reduced the growth of the GBM xenografts compared with the vehicle-treated control groups (Fig. 1B). Combinatorial therapy using sunitinib + RT or VEGF-trap + RT decreased tumor growth compared with monotherapy and resulted in even further significant growth inhibition compared with untreated control groups (P<.05 for sunitinib + RT and P<.01 for VEGF-trap + RT) (Fig. 1B). Using combinatorial therapies, VEGF-trap + RT restricted tumor growth significantly more than sunitinib + RT (P<.05), suggesting a more potent antitumor activity for VEGF-trap than sunitinib. Also, comparison of the GSC-1 tumor volume between control and VEGF-treated tumors at day 42 showed a significant reduction in tumor volume in VEGF-trap-treated animals (Fig. 5A). Moreover, the mean survival for U87 tumors treated with sunitinib was 30 days, whereas VEGF-trap treated animals on average survived for 45 days. Mean survival for GSC-1 control animals was 45 days and 55 days for GSC-1 tumors treated with VEGF-trap.
MRI Biomarkers

Apparent Diffusion Coefficient

To allow one-to-one direct comparison between effects of sunitinib and VEGF-trap as monotherapy or as combinatorial therapy with RT, we selected a 10-day treatment time window. Normalized baseline (day 7) ADC values were similar for all treatment groups ranging from 3%–13% higher values than contralateral brain parenchyma. Treatment with RT or AATx + RT resulted in a significant rise in ADC from baseline compared with control. ADC values at day 17 were above baseline as follows; RT: 35% ± 5%; sunitinib + RT: 35% ± 5%; VEGF-trap + RT: 43% ± 8%; (P < .01). While the magnitude of antioxidant above control was greater with VEGF-trap + RT compared with sunitinib + RT, this difference was not statistically significant. ADC values in response to sunitinib or VEGF-trap monotherapy were similar to the control group (day 17 sunitinib: 23% ± 3%; VEGF-trap: 24% ± 2%) (Fig. 1C).

We used Ki67 as a marker for cell proliferation to study whether reduced tumor growth rate and alterations in ADC are associated with a reduction in tumor cell proliferation. We found a statistically significant reduction in the number of proliferating cells in all treatment arms compared with the control group at day 17 (Fig. 1D; P < .0001). We examined tumor cell density in response to different treatment regimens at day 17. Tumor cell density did not change significantly in response to sunitinib or VEGF-trap but was significantly reduced with RT or combinatorial therapies (Fig. 1Dii; P < .001). The percentage reduction in cell density was 34% in RT, 32.9% in sunitinib + RT, and 48.7% in VEGF-trap + RT.

Contrast enhancement on T1w + contrast and DCE-MRI images

Contrast enhancement on T1w + contrast MRI images is used in clinical practice to determine tumor growth and overall volume. We first examined contrast enhancement of the U87 and GSC-1 tumors on contrast MRI images at early and late stages of tumor development. The pattern of contrast enhancement was significantly different between the 2 models. U87 tumors showed early and almost uniform signal enhancement throughout at all stages of tumor development. The pattern of contrast enhancement in GSC-1 tumors was detected only at later stages of tumor development, with significantly heterogeneous enhancement (Fig. 2Ai). Therefore, analysis for longitudinal changes in contrast enhancement was carried out on U87 tumors on days 7–17, and analysis for GSC-1 tumors focused to days 40–50, during the period in which contrast enhancement was evident in this model.

Monotherapy with VEGF-trap or combinatorial therapy with VEGF-trap + RT resulted in the most significant reduction in contrast enhancement on T1w images compared with other treatment groups. Most notably, there was a distinct loss of enhancement on DCE images, while T2w images continued to show the presence of tumor in these groups (Fig. 2Ai). The loss of DCE enhancement was very specific to VEGF-trap treatment, as sunitinib-treated animals showed no significant change on DCE enhancement. Quantification analysis on T1w + contrast images indicated a significant reduction in contrast enhancement with VEGF-trap or VEGF-trap + RT treatment (P < .05 for VEGF-trap and P < .001 for VEGF-trap + RT). The reduction occurred early in response to treatment and remained decreased throughout therapy. Importantly, contrast enhancement did not significantly change in response to sunitinib or sunitinib + RT as compared with control groups (Fig. 2B). We confirmed similar results in GSC-1 with VEGF-trap treated tumors showing significant reduction in signal enhancement (P < .05) on T1w + contrast and DCE images (Fig. 5B and C).

We further investigated the correlation of contrast enhancement to changes in tumor growth and found no correlation between these 2 parameters (Fig. 2C).

Alteration in Tumor Vessel Permeability With Use of Monotherapy or Combinatorial Therapy with AATxs

To evaluate longitudinal changes in vessel permeability in response to monotherapy or combinatorial therapy with AATxs, we measured IAUC, which is considered to be an indicator of both tumor vessel permeability and vessel perfusion. In both control and RT-treated U87 tumors, the percentage change in IAUC was not significantly different from baseline at each stage of tumor growth (Fig. 3A). Monotherapy with sunitinib reduced IAUC from baseline (35% reduction) at day 10; however, levels returned back to control at later stages of tumor growth (beyond day 12). Combinatorial therapy with sunitinib + RT reduced IAUC level from baseline (40% reduction) at day 10, and this reduction persisted on all treatment days (Fig. 3Aii). With use of VEGF-trap or VEGF-trap + RT, there was a significantly greater reduction in IAUC from baseline (between 70%–85% reduction, P < .05) on all treatment days (Fig. 3Aiii). Comparison of contrast agent

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Fig. 3. Dynamic contrast-enhanced (DCE)-MRI parameters in response to monotherapy and combinatorial therapies using 2 classes of anti-angiogenic therapies (AATxs). (A) Line graphs represent logarithmic scale of percentage change in initial area under the contrast enhancement curve (IAUC) from baseline for each treatment group (i, ii). Significant differences from baseline are represented as *P < .01 and **P < .0001. Representative plots show dynamics of contrast agent concentration for the tumors treated with sunitinib (iii) or VEGF-trap (iv) treatment regimens. (B) Mice were given doxycycline (+Dox) to induce VEGF-trap expression starting 7 days post tumor implantation; doxycycline was discontinued (-Dox) on days 17–21 to turn off VEGF-trap expression and resumed on days 21–35 to turn VEGF-trap on again. T1w-MRI images show continuous tumor growth, and DCE and T1w + contrast MRI images show the contrast enhancement at each time point (white arrows). (C) Mice were treated with B20 (150 µg/mouse) for 10 days, and then the drug administration was discontinued. MRI images were acquired during B20 treatment at day 17 (+B20) and after drug discontinuation at days 21 and 31 (-B20). T1w images represent the presence and growth of the tumor, and DCE and T1w + contrast images show contrast enhancement at the tumor region (white arrows) (i). Bar graphs (ii) show percentage change in contrast enhancement at tumor region compared with adjacent normal brain region. Due to limited survival of control mice, contrast enhancement at day 31 was calculated only for B20 treated animals that survived longer. Significant differences in contrast enhancement between control and B20-treated animals are represented as *P < .05 and **P < .001.
Fig. 4. Histological analysis of tumor vascularity in response to anti-angiogenic therapies (AATx therapy). (A) Immunohistochemical staining shows CD31-positive tumor microvessels in control and treated animals at day 17 post intracranial injection (i). Bar graphs represent microvessel density (MVD) per 10 high power field (40X) (ii,iii). Significant difference between control and treatment groups at each time point is represented as *P < .0001 and **P < .00001. Mice treated with B20 for 10 days; MVD was determined during drug treatment (day 17, +B20) and 2 weeks after drug discontinuation (-B20) (iv). Bar graph (v) represents MVD, and significant difference from control group is represented as *P < .0001. Scale bar:
concentration-time curves between different treatment groups indicated a complete inhibition of contrast agent accumulation following VEGF-trap treatment (Fig. 3Aii and iv). Similarly, quantitative analysis of vessel permeability indicated a significant decrease ($P < .01$) in iAUC in VEGF-trap treated GSC-1 tumors compared with control group (Fig. 5D).

Loss of Tumor Vessel Permeability in Response to VEGF-trap Treatment Is a Transient and Reversible Effect

We examined the duration by which the reduced contrast enhancement persisted upon discontinuation of VEGF-trap to better understand the kinetics of significant reduction or complete loss of contrast enhancement on $T_1$w + contrast and DCE images, respectively. Three days following discontinuation of doxycycline, contrast enhancement on $T_1$w + contrast and DCE-MR images increased back to levels comparable to those seen in the control animals. With return of enhancement, tumor volume remained unchanged and continued similar growth rate on $T_1$w (Fig. 3B). These results confirmed that reduction of contrast enhancement was directly dependent on VEGF-trap.

We repeated the experiments with another AATx, B20. Similar to VEGF-trap, B20 treatment also significantly reduced contrast enhancement (Fig. 3Ci), supporting the loss of MRI enhancement being a direct consequence of VEGF inhibition. Similarly, the loss of contrast enhancement was reversed 3 days following B20 discontinuation (Fig. 3Ci).

Alteration in Tumor Vascularity With Use of Monotherapy or Combinatorial Therapy with AATxs

To determine whether alteration in contrast enhancement is associated with changes in tumor vascularity, we determined MVD in addition to the CBF parameter on MRI (Fig. 4A). In all treatment groups, there was a significant reduction in MVD at day 10 following therapy compared with control ($P < .0001$). This reduction in MVD returned to control levels at later stages of tumor growth in the sunitinib or RT-alone groups but remained decreased in the VEGF-trap, sunitinib + RT, or VEGF-trap + RT groups (Fig. 4Ai, ii & iii). Similarly, tumor MVD significantly reduced in the groups treated with B20 compared with control (Fig. 4Aiv, v) ($P < .0001$). There was no change in MVD within 3 days after discontinuation of VEGF-trap or B20, indicating that MVD alone does not explain the return of enhancement witnessed on $T_1$w + contrast and DCE images. Furthermore, there was no relationship between the extent of contrast enhancement on $T_1$w + contrast and MVD on any treatment day, eliminating the possibility that reduced contrast enhancement is mediated by a reduction in MVD.

We next investigated whether reduced contrast enhancement with VEGF inhibition was explained by a decrease in CBF. We found a significant decrease in CBF in VEGF-trap treated tumors compared with control nontreated tumors (control: 124.25 ± 36.35 vs VEGF-trap: 67 ± 11.2; $P < .05$) (Fig. 4Bi). Associated with a decrease in CBF was an apparent compensatory dilation of GBM vasculature (Fig. 4Bii). Vessel dilatation was seen predominantly at the tumor periphery, as defined by the junction between tumor and normal brain.

Characterization of Angiogenic Factors in Tumor Vasculature of VEGF-trap Treated Animals

We investigated change in expression of angiopoietins as an additional angiogenic class of molecules that closely interacts with VEGF during normal physiological and pathological vessel formation.27,32 There was an increased expression of Ang-1 in the center of the U87 tumors treated with VEGF-trap compared with control using both immunofluorescent and qPCR analysis (Fig. 4C). Increased expression of Ang-1 was seen in both tumor cells and tumor vasculature (Fig. 4Ci, ii). There was also an increased expression of Ang-2 following VEGF inhibition that was seen predominantly in dilated vessels evident in tumor periphery (Fig. 4Civ, v).

Discussion

The use of AATx alone or in combination with RT for treatment of GBMs has been advocated for the past decade, with combinatorial therapy holding promise as a strategy that can improve response to either therapy used alone.8,18,33 However, for effective use of combinatorial therapy in clinical practice, we need to establish proper noninvasive biomarkers that can predict response to therapy and may help with designing the precise scheduling of treatment strategies.

In this study, combinatorial therapy with VEGF-trap + RT showed a statistically significant reduction in tumor growth compared with sunitinib + RT. The level of growth suppression is clearly dependent on the class of AATx used, and the difference in effect of AATx can be explained by differences in mechanism of action of the 2 AATxs.10,35,17,34 The difference could also be a reflection of the efficiency of sunitinib in crossing the blood brain barrier, which may also explain in part why sunitinib fails to demonstrate benefit in GBM clinical trials as a single AATx.15,17

MRI parameters can provide noninvasive methods for assessing tumor growth and response to therapy. For instance, ADC maps, created based on DWI images, signify tumor cell density since high-cellular density restricts water diffusion.35 We demonstrated a significant increase in ADC values for the groups treated...
with RT or sunitinib + RT (similar to our previous report)\textsuperscript{29} or VEGF-trap + RT compared with control. Increased ADC values in response to RT have been reported in human glioma tumor xenografts and also in other tumor types such as hepatic carcinoma.\textsuperscript{29,36,37} Interestingly, AATx alone or addition of AATx therapy to RT did not significantly affect ADC. This is in contrast with previously published data showing an early increase in ADC in response to sunitinib treatment in a U118 glioma mouse model,\textsuperscript{38} or a late change in ADC in response to sorafenib therapy in nude rats with implanted U87 tumors.\textsuperscript{39} These differing results can be explained by differences in tumor models and class of AATx used.

Associated with an increase in ADC, we found a parallel significant reduction in tumor cell density in the RT alone, sunitinib + RT, or VEGF-trap + RT groups as compared with control, with no significant change in cell density in response to AATx; this suggests that the increase seen in ADC is predominantly a reflection of reduced tumor cell density, which appears to be a consequence of RT alone. On the other hand, our results showed a reduction in cell proliferation index, Ki67, in all treatment groups. Therefore, we believe our results support that ADC is an accurate indicator of tumor cell density rather than tumor cell proliferation.

In addition to MRI parameters that reflect tumor growth and proliferation, we explored MRI parameters that reflect alterations in tumor vascularity. Using $T_1w$ contrast and DCE-MRI, we observed a statistically significant reduction in contrast enhancement in tumors treated with VEGF-trap or VEGF-trap + RT without any association between changes in MVD and contrast enhancement. Although MVD was reduced in sunitinib, VEGF-trap, and B20-treated tumors, the reduction in contrast enhancement was seen only in VEGF-trap and B20-treated tumors. Therefore, alternative parameters beyond MVD, such as vessel blood flow,
perfusion, or permeability may explain reduced or complete loss of enhancement on \( T_{1w} \) + contrast or DCE images. Most notably, we found that loss of contrast enhancement did not correlate with decrease in tumor growth or overall tumor volume. This finding is in agreement with previous data indicating that a reduction in contrast enhancement in response to AATx limits the interpretation of the extent of contrast enhancement on \( T_{1w} \) + contrast MRI images as a reliable predictor of tumor growth while on treatment with AATx.\(^{16,40,41}\)

The differences seen in contrast enhancement more closely paralleled the MRI parameters indicative of vessel permeability. DCE-MRI is considered to reflect physiological characteristics of tumor vasculature such as perfusion and permeability\(^ 1\) and, combined with measurements of arterial input function, is used to assess physiological quantities of vessel permeability including \( K_{trans} \), using Tofts analysis.\(^{21,31,42}\) Here, a key change observed with use of either VEGF-trap or B20 (but not sunitinib) was complete loss of enhancement on DCE-MRI images within 3 days of treatment. A previous report using a rat model of intracranial glioma tumor also shows that VEGF-trap results in a significant reduction in DCE-MRI parameters including \( K_{trans} \), \( K_{ep} \), and \( K_{v} \).\(^ {43} \)

As a result of the complete shutdown of DCE enhancement with VEGF inhibition, \( K_{trans} \) could not be analyzed accurately. Consequently, we used an alternate MRI biomarker, iAUC, which is model-independent and provides information about the blood flow, extracellular extravascular space, and vessel permeability.\(^ 31 \) To date, iAUC has principally been used as an MRI biomarker that distinguishes between treatment-induced necrosis and tumor recurrence in patients with GBMs.\(^ {44} \) Our finding of significant reduction in iAUC was linked with a significant inhibition in kinetics of contrast agent accumulation in the VEGF-trap treated tumors. iAUC also showed temporary or sustained reduction in response to sunitinib or sunitinib + RT respectively; however, the extent of iAUC reduction in response to sunitinib was not as pronounced as with VEGF-trap. Therefore, iAUC can be considered as an effective MRI biomarker reflective of changes seen in vessel permeability/perfusion in response to AATx. Previously, we have shown that \( K_{trans} \), used as a parameter of vessel permeability in response to sunitinib \(^{41} \), and in this study we similarly confirmed that iAUC is an additional reliable biomarker of vessel permeability. Moreover, in this study it appears that the reduction in iAUC was consistent with increased survival of the animals. Given that mice treated with VEGF-trap or VEGF-trap + RT survived longer than sunitinib-treated groups, iAUC also appears to correlate with overall survival, suggesting that these MRI parameters can be used as predictors of survival in response to AATx. Angiopoietins (Ang-1 and Ang-2) are involved in vascular homeostasis and maturation through their specific endothelial cell receptors Tie-1 and Tie-2. While Ang-1 reduces vessel permeability, Ang-2 exerts opposing effects resulting in dilated, destabilized, and leaky vasculature.\(^ {45} \) Following VEGF-trap treatment, we demonstrated an increased expression of Ang-1 in the center of the tumors, which could account for the diminished vascular permeability/perfusion and consequent reduction in enhancement seen in DCE and \( T_{1w} \) + contrast.

In summary, we extend our prior findings by demonstrating that ADC accurately represents tumor cell density and as such can be used as a reliable biomarker of efficient cytotoxic therapy. Our results confirm that reduction in contrast enhancement is not an accurate reflection of tumor growth in response to AATx; rather than being an accurate measure of MVD, contrast enhancement is a reflection of an alteration in physiological parameters of tumor vascularity such as vessel perfusion and permeability. We showed that DCE-derived parameters, specifically iAUC, can differentiate the response to different classes of AATxs and predict survival of GBMs and therefore potentially predict effectiveness of AATx. We demonstrated that MRI biomarkers are specific to individual AATx agents and therefore, the use and interpretation of noninvasive MRI biomarkers to monitor the therapeutic response to AATx should be tailored in the clinical setting to the specific individual agents. Further studies are required to validate these MRI parameters in the clinical setting, where cohorts of similarly treated patients with rigorous follow-up imaging are needed.

**Supplementary Material**

Supplementary material is available online at Neuro-Oncology (http://neuro-oncology.oxfordjournals.org/).

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