THE COMPLETE PATIENT QA SYSTEM

3D PATIENT PLAN QA
3D IMRT/VMAT PRE TREATMENT QA
3D IN VIVO DAILY TREATMENT QA
ONLINE PATIENT POSITIONING QA

Upgrade your patient safety by bridging the gap between patient QA and machine QA:
DoseLab, the complete TG-142 solution, is now integrated into Mobius3D!

Visit mobiusmed.com/mobius3d to learn more or register for a bi-weekly webinar at mobiusmed.com/webinars
Assessment of tumor response to radiation and vascular targeting therapy in mice using quantitative ultrasound spectroscopy

Ahmed El Kaffas, Ali Sadeghi-Naini, Omar Falou, and William Tyler Tran
Department of Radiation Oncology, Sunnybrook Health Sciences Centre, Toronto, Ontario M4N 3M5, Canada; Imaging Research and Physical Sciences, Sunnybrook Health Sciences Centre, Toronto, Ontario M4N 3M5, Canada; and Departments of Medical Biophysics and Radiation Oncology, Faculty of Medicine, University of Toronto, Toronto, Ontario M5G 1L7, Canada

Stephanie Zhou
Imaging Research and Physical Sciences, Sunnybrook Health Sciences Centre, Toronto, Ontario M4N 3M5, Canada

Amr Hashim
Department of Radiation Oncology, Sunnybrook Health Sciences Centre, Toronto, Ontario M4N 3M5, Canada and Imaging Research and Physical Sciences, Sunnybrook Health Sciences Centre, Toronto, Ontario M4N 3M5, Canada

Jason Fernandes and Anoja Giles
Imaging Research and Physical Sciences, Sunnybrook Health Sciences Centre, Toronto, Ontario M4N 3M5, Canada

Gregory J. Czarnota
Department of Radiation Oncology, Sunnybrook Health Sciences Centre, Toronto, Ontario M4N 3M5, Canada; Imaging Research and Physical Sciences, Sunnybrook Health Sciences Centre, Toronto, Ontario M4N 3M5, Canada; and Departments of Medical Biophysics and Radiation Oncology, Faculty of Medicine, University of Toronto, Toronto, Ontario M5G 1L7, Canada

(Received 7 October 2014; revised 27 June 2015; accepted for publication 29 June 2015; published 31 July 2015)

Purpose: It is now recognized that the tumor vasculature is in part responsible for regulating tumor responses to radiation therapy. However, the extent to which radiation-based vascular damage contributes to tumor cell death remains unknown. In this work, quantitative ultrasound spectroscopy (QUS) methods were used to investigate the acute responses of tumors to radiation-based vascular treatments.

Methods: Tumor xenografts (MDA-MB-231) were treated with single radiation doses of 2 or 8 Gy alone, or in combination with pharmacological agents that modulate vascular radiosensitivity. The midband fit, the slope, and the 0-MHz intercept QUS parameters were obtained from a linear-regression fit to the averaged power spectrum of frequency-dependent ultrasound backscatter and were used to quantify acute tumor responses following treatment administration. Power spectrums were extracted from raw volumetric radio-frequency ultrasound data obtained before and 24 h following treatment administration. These parameters have previously been correlated to tumor cell death. Staining using \textit{in situ} end labeling, carbonic anhydrase 9 and cluster of differentiation 31 of tumor sections were used to assess cell death, oxygenation, and vasculature distributions, respectively.

Results: Results indicate a significant midband fit QUS parameter increases of 3.2 ± 0.3 dBr and 5.4 ± 0.5 dBr for tumors treated with 2 and 8 Gy radiation combined with the antiangiogenic agent Sunitinib, respectively. In contrast, tumors treated with radiation alone demonstrated a significant midband fit increase of 4.4 ± 0.3 dBr at 8 Gy only. Preadministration of basic fibroblast growth factor, an endothelial radioprotector, acted to minimize tumor response following single large doses of radiation. Immunohistochemical analysis was in general agreement with QUS findings; an $R^2$ of 0.9 was observed when quantified cell death was correlated with changes in midband fit.

Conclusions: Results from QUS analysis presented in this study confirm that acute tumor response is linked to a vascular effect following high doses of radiation therapy. Overall, this is in agreement with previous reports suggesting that acute tumor radiation response is regulated by a vascular-driven response. Data also suggest that Sunitinib may enhance tumor radiosensitivity through a vascular remodeling process, and that QUS may be sensitive to changes in tissue properties associated with vascular remodeling. Finally, the work also demonstrates the ability of QUS methods to monitor response to radiation-based vascular strategies. © 2015 American Association of Physicists in Medicine. [http://dx.doi.org/10.1118/1.4926554]

Key words: quantitative ultrasound, ultrasound spectroscopy, QUS, tumor morphology, 3D high-frequency ultrasound, SU11248, bFGF, vascular normalization, vascular remodeling, treatment monitoring, radiation response
1. INTRODUCTION

The mechanism by which tumors respond to radiation therapy (XRT) varies as a function of the dose delivered. At conventional radiation doses (1.8–4 Gy) administered as part of a fractionated regimen, radiation is thought to predominantly act on epithelial and tumor cells by inducing direct or indirect DNA damage. Cells ultimately undergo cell death or senescence after accumulating sufficient genomic damage. However, such doses have also been suggested to elicit other tumor vascular and endothelial-based physiological alterations resulting in increased hypoxia and tumor reperfusion. These in turn commonly lead to poor treatment response.

At higher single radiation doses (equal to or greater than 8–10 Gy), host-derived endothelial cells present in tumor vasculature are suggested to be main target. These have been demonstrated to undergo rapid cell death due to an elevated release of the apoptosis messenger ceramide. This is followed by rapid vascular destruction and an acute wave of subsequent secondary tumor cell death. At lower radiation doses (<6–8 Gy), it is speculated that insufficient ceramide is released to induce this form of rapid endothelial cell death. Researchers have postulated this to be an important mechanism of radiation-induced tumor kill in vivo. Various radiation-based vascular targeting treatment strategies have also been proposed. These generally entail combining antivascular agents (i.e., antiangiogenic agents) and radiation therapy with the ultimate aim of increasing overall tumor radiosensitivity and enhancing treatment response.

The antiangiogenic agent Sunitinib (SU) is a small molecule tyrosine-kinase inhibitor that prevents the activation of a wide-spectrum of angiogenesis-regulating receptors. It is one of the few FDA-approved antiangiogenic agents for the treatment of renal cell carcinoma and gastrointestinal tumors. Sunitinib has been reported to induce a multitude of effects in tumor blood vessels and tumor cells ideal for use in conjunction with radiation therapy. Various radiation-based vascular targeting treatment strategies have been demonstrated to have the ultimate aim of increasing overall tumor radiosensitivity and enhancing treatment response.

The antiangiogenic agent Sunitinib (SU) is a small molecule tyrosine-kinase inhibitor that prevents the activation of a wide-spectrum of angiogenesis-regulating receptors. It is one of the few FDA-approved antiangiogenic agents for the treatment of renal cell carcinoma and gastrointestinal tumors. Sunitinib has been reported to induce a multitude of effects in tumor blood vessels and tumor cells ideal for use in conjunction with radiation therapy. Various radiation-based vascular targeting treatment strategies have been demonstrated to have the ultimate aim of increasing overall tumor radiosensitivity and enhancing treatment response.

Volumetric power Doppler ultrasound has recently been used to study vascular effects in radiation therapy. Results confirmed that single high doses of radiation predominantly target tumor blood vessels and that agents such as Sunitinib can be used to increase radiosensitivity by remodeling blood vessels. These findings suggest that the overall acute response of tumors to radiation therapy is directly linked to the tumor vasculature and its response to radiation therapy. Consequently, there remain numerous questions in regards to how blood vessels regulate the dose-dependent response of tumors to radiation therapy, and how an agent such as Sunitinib should be used in conjunction with radiation therapy. Here, we use quantitative ultrasound spectroscopy (QUS) along with confirmatory histological analysis to investigate acute dose-dependent tumor response to radiation alone, or in combination with Sunitinib.

QUS generally refers to methodologies that examine frequency-dependent backscatter and power spectrum-based statistical estimates of backscatter contributors from ultrasound radio-frequency (RF) raw data (i.e., before envelope detection, log amplification, and B-mode image formation) to characterize tissue acoustic properties. By conducting a Fourier-based spectroscopic analysis of ultrasound RF data obtained from tissue, with data normalized against a reference phantom or a calibration pulse, one can determine quantitative spectral parameters derived from a linear-regression approximation or more complex fittings of scattering models to the normalized power spectrum. General parameters of interest include the spectral slope (SS), the 0-MHz spectral intercept (SI), and the midband fit (MBF). These can be related to the effective scatterer size and/or the acoustic scatterer concentration. Various studies have demonstrated that these parameters are sensitive to subtle microstructural characteristics and alterations occurring in tissue due to biological responses. A number of existing and potential applications of QUS techniques in cancer have been reported in the literature and include cancer diagnosis, lesion localization, and treatment response monitoring.

The focus of this work is the use of QUS for monitoring cancer therapy. An up to 16-fold increase in the ultrasound backscatter signal intensity accompanied by considerable changes in QUS spectral parameters has been observed in some applications of cancer treatment response monitoring, when compared to nontreated tumors. Changes in observed backscatter parameters have been linked to morphological changes (cell size and distribution) occurring when cells undergo cell death after therapy. These changes include nuclear condensation and fragmentation amongst others. Parameters obtained from QUS do not rely on exogenous contrast agents since the principal source of contrast is the alteration in the physical and consequently the acoustical properties of cancer cells responding to treatment. Emerging studies have recently validated the efficacy of QUS techniques for treatment monitoring in the clinic. In this context, ultrasound is presently a relatively inexpensive and accessible medical imaging modality compared to other imaging technologies proposed for cancer treatment monitoring such as PET, SPECT, and MRI.

In the study presented here, QUS was used to measure noninvasively the dose-dependent acute radiation response of human xenograft breast cancer tumors implanted in the hind leg of mice and treated with radiation alone, or in conjunction with Sunitinib. The MDA-MB-231 cell line is a triple negative (ER-, PR-, no HER2 overexpression) breast cancer line that is highly aggressive and is highly vascularized. Patients with triple negative disease may benefit from antiangiogenic therapy in addition to radiation as they have high recurrence rates. Radiation is already used as a primary treatment modality on recurrent breast cancer and with locally advanced breast cancer. Furthermore, approaches such as accelerated partial-breast irradiation, hypofractionated whole-breast irradiation, and intensity modulated radiotherapy may...
allow radiation to be delivered to both early and advanced breast cancer at higher doses. We anticipate greater breast cancer therapy response when combining agents that act through fundamentally different physiological mechanisms (i.e., antiangiogenics with radiation), different cellular targets, and which have nonoverlapping toxicities. The extent of cell death occurring as a function of vascular destruction was evaluated by minimizing or enhancing the radiation-based endothelial cell death in subsets of animals. In order to do so, basic fibroblast growth factor (bFGF) was used to modulate endothelial cell radiosensitivity. Numerous studies have demonstrated bFGF’s ability to diminish radiation damage on endothelial cells, thus minimizing tumor cell death as a function of vascular destruction. Sunitinib was used to enhance responses to radiation. Immunohistochemistry was used as a gold standard treatment assessment method to verify QUS-based results.

Findings here reveal that vascular response to high doses of radiation is linked to acute tumor cell death. Sunitinib acted by significantly increasing the overall tumor response to radiation therapy at lower radiation doses. Immunohistochemistry results were in agreement with QUS findings and further suggest that Sunitinib may be enhancing tumor responses to radiotherapy by remodeling tumor blood vessels, leading to enhanced tumor oxygenation and direct tumor cell damage. The research demonstrates the potential of QUS in preclinical assessment of tumor response to novel vascular targeting treatments.

2. METHODS

2.A. Animal preparation

All animal experiments were conducted in compliance with guidelines approved by the Sunnybrook Health Science Centre Institutional Animal Care Committee. Breast cancer cells (MDA-MB-231) were cultured in RPMI 1600 culture medium (ATCC, Manassas, VA), 5% fetal bovine serum (FBS) with antibiotics (penicillin and streptomycin) (Life Technologies, Gaithersburg, MD), 5% fetal bovine serum (FBS) with antibiotics (penicillin and streptomycin) (Life Technologies, Gaithersburg, MD), and 5% fetal bovine serum (FBS) with antibiotics (penicillin and streptomycin) (Life Technologies, Gaithersburg, MD). Animals were anesthetized with ketamine, xylazine, and acepromazine (1 mg/kg, 5 mg/kg, and 1 mg/kg). Mice bearing xenograft tumors were treated with single radiation treatment period, control animals were reaching the protocol size end-point by the 14th day of the treatment period. These control animals, in addition to a subset (n = 4) of Sunitinib-treated animals, were imaged at baseline and on the 14th day with volumetric ultrasound for QUS analysis and were also sacrificed at 14 days for immunohistochemistry staining.

2.C. Ultrasound imaging and QUS analysis

All animals in the radiation conditions were imaged with 3D high-frequency ultrasound immediately before treatment and 24 h after treatment. Tumor-bearing animals used for assessing the effects of Sunitinib (control and Sunitinib-treated) were imaged at baseline and at 14 days following the start of Sunitinib delivery. Data were acquired using a Vevo 770 high-frequency ultrasound-imaging device (Visualsonics, Toronto, ON) with a ~25 MHz center frequency transducer (RMV-710B: ~70 µm axial resolution, ~140 µm lateral resolution, focal length of 15 mm, Visualsonics, Toronto, ON). A motorized scan stage (Visualsonics, Toronto, ON) was used to acquire 3D B-mode images and RF data, at a step size of 0.1 mm. For each of the tumors, the focal zone was placed in the center of the middle volumetric plane. Raw RF data were recorded digitally (sampled at 420 MHz) with a 12-bit dynamic range. Analysis of RF data was performed using previously validated QUS methodologies. In short, rectangular regions of interest (ROIs) encompassing the center of the tumor were selected at 10–15 different tumor planes throughout the 3D tumor volume.

The ROIs were positioned near the ultrasound focus (which was set at the center of the middle tumor plane) and were maximized in size while ensuring to exclude the skin of the tumor and far-field artifacts. A Fourier transform was applied to the RF data along each scan line within the ROI in order to estimate the power spectrum. This was done by taking the square of the magnitude of the fast Fourier transform (FFT) of the Hamming-gated RF echo segment $e_s(t,x_i)$, as a function of time ($t$) and lateral position ($x_i$) throughout the ROI. Obtained power spectra were consequently averaged over the ROI. The average power spectrum obtained from tumor tissue was then divided by a calibration spectrum obtained from a flat quartz plate acting as a reference to normalize data as described in Ref. 28. Normalization was necessary to make
the analysis method independent of system properties and instrument settings.\textsuperscript{27} Power spectra were similarly computed from the quartz echo segment $e_p(t,x_i)$. The final normalized power spectrum $S(f)$ can be expressed as

$$
S(f) = \frac{\sum_{i=\text{M}}^{\text{N}} |\text{FFT}(e_p(t,x_i))|^2}{\sum_{i=\text{M}}^{\text{N}} |\text{FFT}(e_x(t,x_i))|^2}
$$

(1)

and results from taking the square magnitude of the FFT of $e_x$ divided by $e_p$ across $i = M, M + 1, \ldots, N$ RF lines in the window. As described in Refs. 31–33, a linear-regression analysis was performed to generate a best-fit line for the normalized power spectrum (in logarithmic scale) within a −6 dB window, in order to obtain MBF, SS, and SI parameters using

$$
S(f) = \text{SS} \times f + \text{SI},
$$

$$
\text{MBF} = S(f_c).
$$

From these, SS and SI are related to the slope and the 0-MHz intercept of the line of best fit, respectively. The MBF is the solution of the line-approximated power spectrum at the center frequency $S(f_c)$ of the −6 dB bandwidth window.\textsuperscript{33} For this study, tumor responses to radiotherapy were primarily characterized using the MBF parameter obtained from the ultrasound frequency, as it has been demonstrated to be a more representative parameter reflecting tissue changes in response to treatment.\textsuperscript{38,44} In addition, spatial parametric images of MBF were also generated by displaying the results of a sliding window analysis on a pixel-by-pixel basis within the ROI using a Hamming function. Parametric images were produced using a sliding window with a time bandwidth product of approximately seven.

2.D. Histological analysis

Mice were sacrificed immediately after imaging and tumors were resected for immunohistochemistry analysis.\textsuperscript{5} Sections of tumor xenografts were stained for DNA breaks using \textit{in situ} end labeling (ISEL) as a cell death marker and cluster of differentiation (CD31) (Santa Cruz Biotechnology, Santa Cruz, CA) to evaluate vascular density. A subset of animals treated with Sunitinib only (along with control tumor-bearing animals left untreated in parallel) were stained for carbonic anhydrase 9 (CA9) as a marker for hypoxia at 14 days after the start of Sunitinib treatment. Slides with histologically stained tumor sections were digitized using a Zeiss MIRAX slide scanner (Carl Zeiss; Oberkochen, Germany). Areas of cell death were evaluated for each of the tumor ISEL-stained cross sections. This was carried out using custom \textsc{matlab} (The MathWorks, Natick, MA) routine that allows computing the number of brown pixels (ISEL-stain or CA9-stain) over the total number of pixels per tumor cross section selected as a region of interest. A similar parameter was computed for CA9 staining to estimate the tumor hypoxic levels using \textsc{matlab} (MathWorks, Natick, MA).\textsuperscript{5}

2.E. Statistical analysis

Quantified midband fit and ISEL staining were evaluated for statistical significance using a Mann–Whitney test (two-tailed, assuming unequal variances; $\alpha = 0.05*$). Each treatment condition was compared independently to the 0 Gy control condition. Statistical tests were conducted using \textsc{prism} (GraphPad Software, La Jolla, CA).

3. RESULTS

Responses of tumors to radiation therapy alone (0, 2, or 8 Gy), or in combination with bFGF or Sunitinib administration, were assessed 24 h after therapy using QUS in addition to standard histological analysis. Representative B-mode images acquired from a tumor before and after 2 Gy radiation alone, or in combination with Sunitinib treatments, are presented in Fig. 1(A). Normalized power spectrum obtained before and after treatment administration for representative animals treated with 0, 2, or 8 Gy alone or in combination with Sunitinib is exhibited in Fig. 1(B). A general increase in the MBF was noted in the case of 8 Gy radiation alone, as well as for 2 and 8 Gy doses when combined with Sunitinib. Average relative changes in the MBF at 24 h after therapy for all treatment conditions are shown in Fig. 1(C). A significant increase in the MBF when tumors are treated with 8 Gy radiation only ($p < 0.05$), compared to the untreated control tumors, was observed. However, this effect was absent when tumors were pretreated with bFGF where no significant difference compared to 0 Gy was noted. Two weeks of treatment with Sunitinib before irradiation significantly enhanced acute tumor responses to radiation treatments (2 Gy—$p < 0.01$; 8 Gy—$p = 0.01$). For example, for the 8 Gy condition administered alone, or in combination with bFGF or Sunitinib, we observed MBF increases of −4.4 dBr, 0.5, and 5.5, respectively. A relative enhancement of 3.2 dBr was observed for the 2 Gy radiation dose when combined with Sunitinib. In contrast, 2 Gy radiation alone, or 2 Gy combined with bFGF resulted in increases of 0.8 and 0.3 dBr, respectively. Quantified SS and SI results are also presented in Fig. S1 of the supplementary material.\textsuperscript{48}

Figure 2 presents representative B-mode images acquired from a tumor before and after 2 Gy radiation alone, or in combination with Sunitinib treatments, overlaid with MBF parametric maps. Higher levels of treatment response can be observed in parametric maps in tumors treated with 2 Gy radiation and Sunitinib, exhibited as considerable increases in tissue echogenicity as a result of cellular morphological alterations linked to cell death described further below.\textsuperscript{37,38} These parametric maps demonstrate a change in the MBF following treatment in specific regional locations.

Figure 3 exhibits quantified CD31 staining, CA9 staining, and MBF change of control and Sunitinib-treated tumors before and after the 14 days Sunitinib treatment period. From these, a significant MBF decrease ($p < 0.05$) of 2.6 dBr after 14 days of Sunitinib treatment alone was observed, whereas minimal changes occurred in control tumors left to grow in parallel. Similarly, we note a decrease in the vascular density and a
Figure 1. (A) Representative B-mode 2D plane images of tumors following treatments. (B) Normalized power spectrum obtained before (dark blue—solid red line) and after (light blue—broken red line) treatment administration for representative animals treated with 0, 2, or 8 Gy alone or in combination with Sunitinib. These are overlaid with best-fit lines (red). We note a general increase in the MBF value at the 8 Gy dose alone, as well as the 2 and 8 Gy doses when combined with Sunitinib. (C) The average change in the MBF value at 24 h after therapy for all treatment conditions. A significant increase in the MBF value was observed when tumors are treated with 8 Gy radiation only (p < 0.05). However, this effect was nullified when tumors are pretreated with bFGF, likely because of radioprotected endothelial cells. Two weeks treatment with Sunitinib before irradiation significantly enhanced acute tumor response to radiation treatments (2 Gy—p < 0.01; 8 Gy—p = 0.01). Scale bar in (A) is 1 mm. Statistical significance is indicated for treatment condition compared to control condition [α < 0.05 (*)]. Error bars represent the standard error of the mean.

decrease in the CA9 staining (significant—p < 0.05). Associated quantified SS and SI results are presented in Fig. S2 of the supplementary material.\(^{48}\) Qualitative representative CD31 and CA9 histology images are exhibited in Figs. S3 and S4.\(^{48}\)

Representative images of ISEL stained tumor cross sections and cell death area quantification are presented in Fig. 4. A distinctive increase in cell-death staining was observed when Sunitinib was combined with radiation. We also noted some cell death when Sunitinib was delivered alone. Minimal cell-death staining was observed at the 0 and 2 Gy conditions when radiation was administered alone. Quantified ISEL staining in animals pretreated with bFGF was consistent across all radiation conditions indicating minimal tumor cell death. This was found to be in agreement with QUS results. Sunitinib administered alone caused a significant increase in histologically detected cell death (p < 0.05). Cell death due to Sunitinib was enhanced when administered in conjunction with radiation therapy compared to radiation or Sunitinib alone, at both radiation doses (p < 0.01) (Fig. 4). This was also in general agreement with QUS measurements.

Figure 5 presents results of ISEL quantified cell death correlated to the MBF change and analysis indicates a fit within the 95% confidence interval with an \(R^2\) of 0.9. Figures S1 and S2 of the supplementary material present results of two other parameters that have demonstrated some utility tracking response in the past.\(^{48}\) In our results, the 0-MHz
intercept (SI) parameter was relatively invariant other than for radiation treatment groups. SS value changes were observed when Sunitinib was administered in conjunction with 2 and 8 Gy radiation, but not alone. A qualitative decrease in CD31 staining was observed in tumors treated with single doses of 8 Gy (Fig. S3), as previously reported. This was not observed for the 0 or 2 Gy radiation conditions. In Sunitinib treated animals, better-formed tumor vessels were apparent as well as decreased microvascular density.

4. DISCUSSION

Acute (24-h) tumor responses to single dose radiation therapy delivered alone, or in combination with the pharmacological vascular modulators bFGF or Sunitinib, were evaluated using QUS methods and histological analysis. Ultrasound spectroscopy methods have previously been demonstrated to detect microtissue changes associated with cell death following radiation, chemotherapy, and photodynamic therapy and have been demonstrated as useful for monitoring tumor responses to therapy. The aim of the study presented here was to use QUS methods to measure the magnitude of tumor cell death following low (2 Gy) and high (8 Gy) doses and to compare this to radiation treatments combined with Sunitinib as a potential tumor radiosensitizer. In addition, the extent of acute tumor damage resulting from radiation-based vascular damage was investigated by preadministering the endothelial radioprotector bFGF in a subset of animals.

Our results indicate that 8 Gy radiation doses caused rapid tumor responses detectable with QUS by 24 h after therapy administration (MBF increase of 4.4 ± 0.3 dBr and SI increase of 5 ± 0.4 dB). As anticipated, lower 2 Gy doses were not effective in inducing detectable acute cell death with QUS parameters. Minimal changes in QUS parameters were observed when bFGF was used, suggesting that blood vessels may play an important role in regulating tumor response and that direct DNA damage is less relevant when assessing acute radiation effects on tumors with QUS methodologies. These results were generally confirmed with histological ISEL staining assays, further discussed below.

Treatments where Sunitinib was administered in conjunction with radiation therapy caused an increase in tumor responses at both low and high doses of radiation. At 2 Gy, we observed a MBF value increase almost equivalent to a single dose of 8 Gy administered alone (average increase of 3.2 ± 0.3 dBr), demonstrating Sunitinib’s ability to radiosensitize tumors. At the higher 8 Gy dose, Sunitinib resulted in an even greater increase in the MBF value of up to 6 dBr. However, no significant changes were observed in the SS QUS parameter when Sunitinib was used in conjunction with radiation. Similarly, we noted no significant change in the SI QUS parameter. Overall, results suggest that these parameters are not sensitive to radiation-based vascular targeting treatments employed in this study. The combined effects of radiation and Sunitinib hereby may be

![Fig. 2. B-mode images from a tumor cross section before (−) and after (+) 2 Gy radiation alone, or in combination with Sunitinib treatments. Images display typical ROIs with a parametric map of the MBF values selected within the center of the tumor. Yellow color in parametric map indicates regions with increased MBF value, likely responding to radiation (i.e., cell death). The scale bar represents 1 mm.](image)

![Fig. 3. Quantification of CD31 staining (A), CA9 staining (B), and MBF value changes (C) following 14 days of Sunitinib treatment. Animals were imaged at baseline and 14 days after Sunitinib delivery. Data suggest that tumors have decreased vascular densities and fewer hypoxic regions after Sunitinib treatment in comparison to control animals left untreated during the 14-day Sunitinib treatment regimen. The decrease in the MBF value observed may be at least partially linked to changes occurring in the tumor microenvironment. No changes were noted in the MBF of control animals. These changes may be in turn reflected in the normalized power spectrum and the MBF value. Associated CA9 representative images are presented in Fig. S4 Ref. 48. Statistical significance is indicated for treatment condition compared to control condition [α < 0.05 (*)]. Error bars represent the standard error of mean.](image)
inciting tumor and cell morphological heterogeneity related to a decrease in vascular density, distribution, and size (vascular remodeling). This, in turn, may be affecting the averaged spectral parameters in opposite manners. As such, these parameters, when determined as a mean value, may have less sensitivity to such tumor responses (Sunitinib treated tumors). However, these generally reflect well homogeneous responses, as demonstrated in previous work. Considerable levels of heterogeneity were observed in this study within the quantitative ultrasound spectral parametric maps (Fig. 2). This, in particular, suggests that ultrasound-based textural features, capable of quantifying response heterogeneities, may be more sensitive to detect such therapy effects, as demonstrated in other studies.

The MBF results were in agreement with cell death quantification by ISEL staining. A dose-dependent increase in ISEL staining was observed for animals treated with Sunitinib and radiation. Although increased ISEL staining was noted in animals treated with Sunitinib alone at 24-h after the termination of the 14-day drug administration period, a change in MBF was not observed over the 24-h period unless radiation was also delivered. In Fig. 5, correlated quantified ISEL staining with MBF values is presented demonstrating a good fit with an $R^2$ of 0.9, as previously observed.

Sunitinib has been reported to induce a range of vascular effects including vascular remodeling, endothelial radiosensitization, and tumor tissue morphological alterations; histological evidence in this study support this. In Figs. 3, S3, and S4, histological evidence that Sunitinib may be inducing a vascular remodeling process instead of directly radiosensitizing tumor endothelial cells was presented. These include vascular pruning (CD31) and decreased hypoxia (CA9). Sunitinib-based vascular remodeling (normalization)
demonstrated indirectly that tumor perfusion was enhanced in SF188V+ human glioma xenografts 14 days after 20 or 40 mg/kg Sunitinib treatment. Others have further demonstrated using dynamic contrast-enhanced MRI that administration of 20 mg/kg (up to 18 days) Sunitinib to KCl-18 human RCC xenografts in nude mice results in tumor vasculature features similar to those expected when blood vessels are remodeled. Finally, Batra et al. have also suggested increased tumor oxygenation after 6 days of Sunitinib treatment, however, at a Sunitinib dose nearly double that used in this study. Results from the work presented here suggest that QUS parameters may be sensitive to tissue morphology occurring as a result of Sunitinib-based physiological changes. Potential resulting tissue changes include vascular pruning, decreased tumor pressure, decreased cellular density, and/or decreased hypoxia. Specifically, a significant decrease in MBF was noted following the prescribed 14 days of Sunitinib treatment. This occurred in coincidence with a minor decrease in the mean microvascular density (from ~4% to ~3%) and CA9 staining (from ~30% to ~20%). Further investigations will be required to understand the underlying mechanism resulting in the QUS parameter value decrease. Overall, these results suggest that QUS parameters may be sensitive to other changes in the tumor microenvironment separate from cell death. This is the first time that such changes linked to tumor vascular changes are observed with QUS and may present an opportunity to develop QUS-based biomarkers for monitoring vascular targeting cancer therapies and vascular remodeling strategies.

The results presented here have important clinical applications as they suggest that targeting blood vessels may be in instances more important than targeting direct tumor cell DNA damage. This is supported by observations reported in other studies. We further suggest that antiangiogenic agents such as Sunitinib, which target blood vessel growth factors, can be administered in conjunction with radiotherapy or chemotherapy. This may increase their efficacy than when administered alone. It is also possible to obtain better tumor responses using larger radiation fractions (compared to smaller fractions) by using vascular targeting strategies to optimize vascular responses, subsequently leading to increased classic clonogenic cell kill. Our findings are especially important in breast cancer where radiation is used as a primary treatment; this includes recurrent disease and in locally advanced breast tumors, as both are typically characteristically aggressive. In the future, techniques such as QUS could be used to monitor and assess various aspects of the tumor microenvironment and improve treatment regimens to maximize tumor cell death in preclinical studies. This would optimize the process of developing novel cancer therapies or new ways to effectively/strategically administer existing therapies.

Finally, our findings have important implications for the use of higher radiation doses. Such doses (>8 Gy) are often used to rapidly arrest large growing cancers, to induce antivascular effects in order to minimize bleeding, or to help alleviate palliative pain. Doses of 6–10 Gy are commonly used for stereotactic treatments and in rapid response radiation programs. This has been facilitated clinically with the introduction of intensity modulated radiation therapy delivery methods that avoid damage to normal tissues. Overall, there is a pattern indicating significant increases in the number of single radiation doses prescribed in comparison to fractionated radiotherapy. Our studies further demonstrate the potential of single dose radiation delivery when combined with vascular targeting agents.

ACKNOWLEDGMENTS

This work was funded by the Canadian Breast Cancer Foundation (CBCF)—Ontario Region, and also by the Terry Fox Foundation through a program project grant “Ultrasound for Cancer Therapy.” The authors would also like to thank Dr. Kolios for scientific insights. Finally, the authors thank Dr. Kerbel for his generous donation of MDA-MB-231 cells. Dr. Ali Sadeghi-Naini is supported by a Banting Postdoctoral Fellowship. Dr. Omar Falou is a CBCF Postdoctoral Fellow. Dr. Ahmed El Kaffas is the recipient of a Grand Challenges Canada Rising Star award. Dr. Gregory Czarnota is supported by a CCO Research Chair in Experimental Therapeutics and Imaging and a James and Mary Davie Chair in Imaging and Experimental Therapeutics at the University of Toronto.

Medical Physics, Vol. 42, No. 8, August 2015


See supplementary material at http://dx.doi.org/10.1118/1.4926554 for quantified SS and SI and additional representative images of CD31 and CA9 staining. In Figs. S1 and S2, we present quantified SS and SI values linked with MBF values presented in the paper. In Figs. S3 and S4, representative CD31 and CA9 qualitative histologic images are exhibited.