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Breast Cancer Redox Heterogeneity Detectable with Chemical Exchange Saturation Transfer (CEST) MRI

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Abstract

Purpose—Tissue redox state is an important mediator of various biological processes in health and diseases such as cancer. Previously, we discovered that the mitochondrial redox state of *ex vivo* tissues detected by redox scanning (an optical imaging method) revealed interesting tumor redox state heterogeneity that could differentiate tumor aggressiveness. Because the noninvasive chemical exchange saturation transfer (CEST) MRI can probe the proton transfer and generate contrasts from endogenous metabolites, we aim to investigate if the *in vivo* CEST contrast is sensitive to proton transfer of the redox reactions so as to reveal the tissue redox states in breast cancer animal models.

Procedures—CEST MRI has been employed to characterize tumor metabolic heterogeneity and correlated with the redox states measured by the redox scanning in two human breast cancer mouse xenograft models, MDA-MB-231 and MCF-7. The possible biological mechanism on the correlation between the two imaging modalities was further investigated by phantom studies where the reductants and the oxidants of the representative redox reactions were measured.

Results—The CEST contrast is found linearly correlated with NADH concentration and the NADH redox ratio with high statistical significance, where NADH is the reduced form of nicotinamide adenine dinucleotide. The phantom studies showed that the reductants of the redox reactions have more CEST contrast than the corresponding oxidants, indicating that higher CEST effect corresponds to the more reduced redox state.

Conclusions—This preliminary study suggests that CEST MRI, once calibrated, might provide a novel noninvasive imaging surrogate for the tissue redox state and a possible diagnostic biomarker for breast cancer in the clinic.

Keywords

Cancer metabolism; Redox state; Intratumor heterogeneity; CEST; NADH

Introduction

Tumors are known to be heterogeneous in their antigenic properties, immunogenicity, hormone receptors, pigment production, metabolic rate, susceptibility to cytotoxic drugs, as well as invasiveness and metastatic potentials [1]. Emerging evidence reveals that tumors are biologically complex structures exhibiting marked spatial variation in angiogenesis [2, 3], hypoxia [4], cell death [5], glucose metabolism [6], redox state [7–10], genetic mutations [11], and others. The presence and degree of tumor heterogeneity may be an important determinant of tumor metastatic potential [7, 10, 12] and response to therapy [13]. Intratumor heterogeneity may foster tumor evolution and adaptation, contributing to treatment failure and drug resistance [11].

However, measurement of tumor heterogeneity is largely overlooked in clinical practice, and the gold standard for diagnosis is based on histopathological analysis of localized biopsy specimens, which is subject to potential sampling error [11]. Genomic analyses from localized biopsy specimens may underestimate the mutations of heterogeneous tumors. Recently, multiregion genetic analysis of primary and metastatic tumors from clinical renal cell carcinomas provided evidence of intratumor heterogeneity in gene mutations, expressions, chromosome aberrations, and ploidy profile, and gene expression signatures of good and poor prognosis were shown to exist in different regions of the same tumor [11]. In addition, optical imaging of clinical biopsy specimens has revealed a high degree of intratumor metabolic heterogeneity and difference between cancerous and normal tissues [8, 9]. Thus, there is an increasing clinical need of imaging tumor heterogeneity *in vivo* that can be useful for the development of imaging biomarkers for cancer management.

Cellular redox state has been demonstrated, mostly by *in vitro* studies, as a key player in cellular biology, important for many cellular events including metabolism, signaling, reactive oxygen species generation, growth, differentiation, survival, apoptosis, and motility [14–16]. We have employed the optical redox scanning technique [17] to show the heterogeneous characteristics of the mitochondrial redox state of tumor tissues. This technique probes the tissue mitochondrial metabolic state and its spatial heterogeneity *ex vivo* by imaging the intrinsic fluorescence of NADH and oxidized flavoproteins (Fp) to obtain the redox ratios (*e.g.*, NADH/Fp, NADH/(Fp+NADH)) with a submillimeter spatial resolution. The results of the redox scanning from various cancer/precancer mouse models showed that the heterogeneity of the redox indices correlated to cancer transformation and progression, and these intratumor heterogeneity-associated redox indices were able to predict the metastatic risk of the tumor xenografts of human melanoma and breast cancer lines [7, 8, 10, 12, 18] and discriminate the PTEN-null premalignant pancreases from the

controls [19] and were sensitive to p53 status in colon cancer [20], whereas the global averaged redox indices minimizing the tissue heterogeneity failed to do so or were less effective. In addition, multiphoton NADH/Fp fluorescence imaging has also been used to probe cancer cell metabolism, genetic types, and the response to chemotherapy [21, 22].

However, redox scanning is a highly invasive technique and multiphoton NADH/Fp fluorescence imaging is limited by its tissue penetration depth. Noninvasive imaging, such as MRI of the redox state and its heterogeneity in deep tissues, is therefore desired for tumor characterization, monitoring treatment response, and developing personalized treatment strategies.

In this project, we set out to investigate the potential of using the CEST MRI to probe the tumor redox state. CEST MRI, on the basis of the chemical exchange saturation transfer effect from amine, amide, and hydroxyl protons, has been recently employed to noninvasively map various metabolites in tissue, for example, amide protons [23], liver glycogen [24], cartilage glycosaminoglycans [25], brain myo-inositol [26], and glutamate [27] with a spatial resolution down to submillimeter. It was also used to characterize the metabolic heterogeneity of the prostate cancer mouse xenografts [28]. In this study, the CEST MRI was followed by the optical redox scanning for mapping redox heterogeneity in two breast cancer mouse xenograft models (MDA-MB-231 and MCF-7). Conventional structure MRI methods including T₁- and T₂-weighted MRI and magnetization transfer (MT) MRI were used for comparison. The phantom studies were conducted to investigate the possible biological connections between the CEST contrast and the redox indices, NADH and the NADH redox ratio (NADH/(Fp+NADH)).

Rationale and Methods

Rationale

In the mitochondria, the electron transport chain couples electron transfer between an electron donor/reductant (such as NADH) and an electron acceptor/oxidant (R) with the transfer of H⁺ ions across the mitochondrial membrane. In general, as shown in the following redox reaction,

two hydrogen atoms (in the form of a hydride ion (H⁻) and a proton (H⁺)) were transferred to RH₂ while NAD⁺ is reduced to NADH. The two exchangeable protons in the reductant RH₂ are more likely to dynamically exchange with bulk water. Frequency selective saturation of those exchangeable protons can indirectly reduce bulk water signal and create the CEST contrast. In other words, the reductant RH₂ is more likely to be a CEST agent than the oxidant R. At (near) equilibrium, RH₂/R is linked to NADH/NAD⁺ by the equilibrium constant of the reaction. The overall CEST contrast, hence, is expected to be coupled to NADH and NADH/NAD⁺ redox potential. Although the co enzymes in the redox reactions, such as NAD⁺ and NADH, have the -NH₂ or -OH groups that may also contribute to the CEST contrasts, these moieties are not modified in the redox reactions. In addition, the concentrations of NAD⁺ and NADH are less than 1 mM *in vivo*. This indicates that these co

enzymes themselves should not contribute significantly to the redox correlated CEST contrast.

Thus, CEST MRI of tumor metabolites could be related to the tumor cellular redox state through the redox reactions in which tumor metabolites are involved. Among the major metabolites [29, 30] in breast cancer, alanine, glutamate, and creatine are known to exhibit notable CEST effect *in vivo*. They are directly or indirectly coupled with the redox reactions. For instance, glutamate is known to participate in the following mitochondrial redox reaction catalyzed by glutamate dehydrogenase:

Alanine is involved in the redox reaction catalyzed by the alanine dehydrogenase.

Therefore, CEST contrasts from glutamate and alanine are expected to be coupled with $NAD^+/NADH$. For creatine, the creatine/phosphocreatine correlates with the ADP/ATP ratio by the creatine kinase reaction *in vivo*.

The cellular ratio ADP/ATP is proportional to or tightly coupled to $NADH/NAD^+$ due to the high activity of the glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase reactions [31]. Since the CEST effects of phosphocreatine, ATP, and ADP are negligible compared to the CEST contrast of creatine as we previously demonstrated [32], the creatine CEST contrast is expected to be proportional to $NADH$ or $NADH/NAD^+$. Therefore, the overall *in vivo* CEST MRI contrasts from metabolites, such as glutamate, alanine, and creatine, may correlate with $NADH$ and $NADH/NAD^+$ in breast cancer.

Animal Studies

In this study, all animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania, USA. Human breast cancer cell line MDA-MB-231 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and tested by RADIL (MU Research Animal Diagnostic Laboratory, Columbia, MO, USA) in August 2006 for IMPACT III PCR Profile, and human breast cancer line MCF-7 was a gift from Dr. Jerry Glickson's lab (Department of Radiology, University of Pennsylvania, USA). The two lines were subcutaneously inoculated to the upper thighs of female athymic nude mice with 10 million cells per site to grow tumors [10]. An estrogen pellet (17 β -estradiol, 0.72 mg/pellet) was inserted subcutaneously to the chest areas of the mice that were inoculated with MCF-7 cells. The volume of the grown tumors for the present study ranges from less than 0.1 to over 1 cm³ as measured by a caliper ($n=8$ and 6 for MDA-MB-231 and MCF-7 tumors, respectively). Tumor volume was estimated using an ellipsoid volume formula $1/2 a \times b^2$,

where parameter a is the long axis and b is the short axis. The xenografts were scanned at a Varian 9.4-T horizontal MRI scanner using a 35-mm ^1H volume coil. CEST Z spectra images from -5 to 5 ppm with a step size of 0.25 ppm were collected from tumor central cross section using a custom programmed sequence, with a frequency selective rectangular saturation pulse ($\mathbf{B}_1=250$ Hz, 1 s) followed by a segmented Fast Low-Angle SHot imaging (FLASH) readout. Sequence parameters were as follows: field of view 35×35 mm², slice thickness 1 to 2 mm, flip angle 15° , readout TR 6.3 ms, TE 3.0 ms, matrix size 128×128 , and number of averages 2 . One saturation pulse followed with 128 segment acquisition was repeated every 9 s. $\mathbf{B}_0$ and $\mathbf{B}_1$ field inhomogeneity maps were acquired for the correction of the CEST contrast maps from $+2$ to $+4$ ppm (step size 0.25 ppm), normalized to the corresponding negative saturation frequencies as previously reported [27]. In addition, MT “on” and “off” images with $+25$ and $+100$ ppm saturation offsets were acquired. T_1 - and T_2 -weighted images of the same slice were also acquired with TR/TE= 100 ms/ 3 ms and 2 s/ 80 ms, respectively.

After the MRI tests, the mice under anesthesia underwent snap-freezing procedures for tumor harvest. The excised frozen tumors were embedded for multislice fluorescence imaging of NADH and Fp using the Chance redox scanner [10]. Two-dimensional images of the tissue sections at various tissue depths were obtained by repeated tissue grinding and raster scanning using a fiberoptic probe coupled to two multichannel filter wheels. For the NADH channel, the excitation bandpass filter was $365\text{BP}26$ nm and the emission filter $455\text{DF}70$ nm. For the Fp channel, the excitation and emission filters were $440\text{DF}20$ nm and $515\text{DF}30$ nm, respectively. The in-plane image resolution was 200 μm . Based on the signals of NADH and Fp and the signals of the reference standards embedded adjacent to the tissue, the nominal concentration of NADH and Fp and the concentration-based NADH redox ratio $\text{NADH}/(\text{Fp} + \text{NADH})$ were quantified. The redox images of the sections closest to the CEST MRI imaging slices were chosen for the quantitative analysis and correlation with the CEST contrast.

Phantom Studies

CEST MRI were conducted for the major metabolites of three representative redox reactions, including 10 mM glutamate, 34 to 125 mM NH_4OH , 50 mM 2-oxoglutarate , 10 mM alanine, 10 mM pyruvate, 20 mM reduced glutathione (GSH), and 10 mM oxidized glutathione (GSSG). Because two GSH molecules are converted into one GSSG molecule in the redox reaction (see “Discussion”), the comparison of CEST effect of GSH and GSSG was conducted with a $2:1$ concentration ratio. All solutions were freshly prepared in phosphate buffered saline (PBS) at pH 7.0 , stored in 10 mm NMR tubes, and maintained at 37 ± 1 $^\circ\text{C}$ during the measurement period. For easy setting up of multisolution phantoms, a whole body Siemens 7 T scanner with a vendor supplied 32 -channel volume radiofrequency coil was used. The imaging protocol is similar to the animal study: number of averages= 2 , slice thickness= 10 mm, field of view= 120×120 mm², matrix size= 128×128 , Hanning-windowed saturation pulses with $\mathbf{B}_{1\text{ rms}}$ of 155 Hz (3.6 μT) for 2 s duration, and a single shot FLASH readout every 15 s. Raw CEST images were acquired at varying saturation offset frequencies ranging from -5 to $+5$ ppm (relative to water resonance) with a 0.2 -ppm increment. $\mathbf{B}_1$ and $\mathbf{B}_0$ field inhomogeneity were mapped and used for removing field

inhomogeneity-induced artifacts during the reconstruction of the Z spectra as previously described [27]. CEST asymmetric curves were then calculated from the normalized Z spectra by subtracting the signal of positive frequency offsets from that of the corresponding negative frequency. CEST asymmetric contrast is generally localized within a small saturation offset range in the Z spectrum acquired with the typical low saturation amplitude (~50 Hz or ~1.2 μ T). While using a high saturation amplitude of 250 Hz or 5.9 μ T in this study, CEST asymmetry broadens and covers across a few parts per million similar to previous studies [27, 28].

Image Processing and Data Analysis

All image processing and data analysis were performed using customized Matlab® (Mathworks, Natick, MA, USA) programs. The CEST contrast in percentage at any saturation offset was calculated according to the following equation:

$$CEST \text{ contrast} = \frac{S_{neg} - S_{pos}}{S_{neg}} \times 100\% \quad (5)$$

where S_{neg} and S_{pos} are B_0 inhomogeneity-corrected images with saturation offset of negative or positive chemical shifts (in ppm) relative to water resonance at 0 ppm. The MT contrast was quantified with the MT ratio (MTR), which was computed using the following equation:

$$MTR = \left(1 - \frac{MT_{\text{“on”}}}{MT_{\text{“off”}}} \right) \times 100\% \quad (6)$$

where $MT_{\text{“on”}}$ and $MT_{\text{“off”}}$ referred to images acquired with saturation “on” at 25 ppm and “off” at 100 ppm, respectively. Tumor CEST and MTR contrasts were then false colored and overlaid onto the MR structural images in gray scale.

As our preliminary observation of CEST MRI images showed two distinct regions with higher and lower CEST contrasts within a tumor, we denote these two regions as CEST-rim and CEST-core, respectively. We call the region of lower CEST contrast as CEST-core because it tends to be located at or near the geometrical center (but not necessarily true always). We found that the CEST-core and CEST-rim contrasts appeared to be correlated, respectively, with the redox-core (regions of lower NADH redox-ratio) and the redox rim (regions of higher NADH redox ratio) images of the same individual tumor. Such bimodal distributions were not distinctly visible on T_1 - and T_2 -weighted and MTR images. For a more quantitative analysis, an initial survey of CEST-rim to CEST-core ratio of multiple MRI contrasts (T_1 - and T_2 -weighted, MTR, and CEST, Fig. 2) were performed from regions of interest (ROIs) of CEST-rim and CEST-core that were manually drawn. If core-rim separation was not visible on the images, approximately an inner circle covering half of the tumor diameter was treated as the tumor core and the rest of the tumor (outer segment) as the tumor rim. A similar approach of drawing ROI for defining tumor core and rim has been reported in a previous research [7].

To further confirm the correlation of the CEST contrast with the redox signals among individual tumors, histogram analysis was performed for each tumor to quantify the core and rim CEST contrasts (Figs. 4 and 5). Basically, the histogram distribution of all the pixels within each tumor was fitted using two Gaussian functions as described previously [19] to obtain CEST-rim or CEST-core values and confirmed with visual readings from both the images and their corresponding histograms (see Fig. 4). Similarly, the NADH and NADH redox ratios of the redox-core and redox-rim were obtained from the histograms of the corresponding redox images. The NADH redox ratio as defined by $\text{NADH}/(\text{Fp}+\text{NADH})$ was used to represent tissue mitochondrial redox state in this study.

Statistics Analysis

Two tailed paired Student's *t* test was performed for comparing the redox or CEST indices between rim and core within a tumor line and two-tailed unpaired Student's *t* test for the comparison between two tumor lines. Significant difference is reached if $p < 0.05$. The mean CEST contrasts in the core and rim were pooled for a linear correlation with the corresponding redox indices. The correlation coefficients R^2 and the *p* values were obtained for all linear regressions. The results are reported as mean $\pm$ standard error (SE) with $n=8$ and 6 for MDA-MB-231 and MCF-7 tumors, respectively.

Results

Representative T_1 - and T_2 -weighted structural images, MTR, and the CEST contrast maps of MDA-MB-231 and MCF-7 breast tumors are shown in Fig. 1. Although spatial signal variation is generally visible for each imaging method, the T_1 - and T_2 -weighted and MTR images did not exhibit a clear bimodal imaging pattern such as the rim and core in either of the tumor models. Only CEST MRI gave clear core-rim separated patterns in the MDA-MB-231 tumors as shown in Fig. 1d (2 ppm) and Fig. 1e (3 ppm). The CEST contrast of the MCF-7 tumors showed an obscure core-rim separation. In general, tumor CEST contrast at 2 ppm is higher than that at 3 ppm, while the pattern of the core-rim difference appears similar at both chemical shifts. Quantitatively, Fig. 2 shows that the CEST contrast at 3 ppm of the MDA-MB-231 tumors has significant higher rim-to-core ratio than the MCF-7 tumors at the same chemical shift (1.54 ± 0.17 versus 1.21 ± 0.19 , $p < 0.01$, Fig. 2d). The rim-to-core ratios of the other MRI methods using the same ROIs for CEST are all close to 1 for both tumor lines without statistically significant difference between them (Fig. 2a-c).

Further investigation of the CEST contrast at various frequency offsets from 2 to 4 ppm revealed that rim-to-core CEST contrast ratio remains unchanged and insensitive to chemical shifts for both tumor models (Fig. 2e). Quantification beyond this offset range may be subject to errors. The CEST contrast at larger offset (>4 ppm) may not be fully corrected from B_0 inhomogeneity given that the Z spectra were collected only until 5 ppm. The CEST contrast near water resonance (0 ppm), on the other hand, is highly affected by direct saturation effect and vulnerable to small inaccuracy of B_0 mapping and correction.

The optical redox images from the MDA-MB-231 tumors demonstrate a clear redox-core-rim bimodal pattern of NADH and the NADH redox ratio, whereas those of the MCF-7 tumors appear to be relatively less distinct (Fig. 3a-c), similar to that observed by the CEST

MRI (Figs. 1 and 2). The mean rim-to-core ratio of the NADH is significantly higher in MDA-MB-231. However, the mean rim-to-core ratio of the NADH redox ratio is not statistically higher in the MDA-MB-231 tumors compared to the MCF-7 tumors (Fig. 3d–f). The redox state heterogeneity is evident in both models.

We have further performed Gaussian-fitting histogram analysis of tumor CEST contrasts to quantify the core–rim difference with typical examples demonstrated in Fig. 4a, b. In Fig. 4c, we demonstrate that the histogram analysis result for the core and rim CEST contrasts is linearly correlated with that obtained with the initial manually drawn ROI analysis. Table 1 summarizes the mean values of the CEST contrast, NADH, and the NADH redox ratios for the tumor cores and rims. Comparing the CEST contrast and the redox indices for the core and rim of individual tumors (when pooling data from both tumor lines), we found a highly significant linear correlation between the CEST contrast and the nominal concentration of NADH ($R^2=0.55$, $p<0.00005$) or between the CEST contrast and the NADH redox ratio ($R^2=0.58$, $p<0.00005$) (Fig. 5), indicating that higher CEST contrast corresponds to the more reduced redox state. For the MCF-7 line only, the linear regression of the core and rim values of CEST contrast *versus* the core and rim values of NADH redox ratios is also highly significant ($p<0.01$, $R^2=0.57$). The core and rim CEST contrasts correlate with the core and rim NADH with a border line significance ($p=0.076$, $R^2=0.28$) for MCF-7. For MDA-MB-231 tumors, the linear regression of core and rim CEST contrasts *versus* NADH or NADH redox ratios is highly significant ($p<0.0001$, $R^2>0.8$).

To explore the possible mechanism that correlates the CEST contrast with tissue redox state, phantom studies were performed on metabolites such as glutamate, alanine, and glutathione. As shown in Fig. 6a, glutamate, the reductant of glutamate dehydrogenase reaction, is significantly more effective in generating CEST contrast than 2-oxoglutarate and ammonia (NH_3) (in the form of ammonium hydroxide (NH_4OH) in the solution) combined. Alanine is also more CEST-effective than its oxidative counterparts pyruvate and ammonia in the reaction of alanine dehydrogenase (Fig. 6b). Similarly, for the redox reaction of glutathione reductase coupled with $\text{NADP}^+/\text{NADPH}$ (oxidized and reduced nicotinamide adenine dinucleotide phosphate), the reductant GSH has more significant CEST effect than the oxidant GSSG (Fig. 6c).

Overall, the reductants of these redox reactions dominate the CEST contrast, while the other species in these reactions have no or significantly less CEST contrast at comparable concentrations. Since these reductants are coupled with $\text{NAD(H)}/\text{NAD(P)H}$ in the reactions, the *in vivo* CEST contrast mainly due to the reductants should be proportional to the sum of NADH and NADPH (denoted as NAD(P)H) levels and/or the ratio of $\text{NAD(P)H}/\text{NAD(P)}^+$.

Discussion

In this study, we have employed CEST MRI to characterize the metabolic heterogeneity in the mouse xenografts of two breast tumor lines and compared the results with redox scanning. As we know that proteins and peptides also express CEST contrast due to their slow exchangeable amide protons, an effect called amide proton transfer (APT) is optimally generated under low amplitude and prolonged saturation RF pulses (typically 50 Hz for a

few seconds) [33]. However, using relatively high saturation amplitude (250 Hz) in this study, the free metabolites with small molecular weight should be the ones that majorly contribute to the overall *in vivo* CEST contrast [27].

Our results showed a significant correlation between the CEST contrast and the NADH redox state. Core–rim heterogeneity in the two breast tumor lines was visible in both the CEST MRI and the redox scanning images, with the rim–core bimodal pattern more distinct in the MDA-MB-231 than in the MCF-7 xenografts (Figs. 2, 3, and 5). We demonstrated that the conventional T₁- and T₂-weighted MRI and MT contrasts have less distinct delineation of the tumor core–rim heterogeneity than CEST MRI.

Although we could not rule out that there may be sporadic core regions with necrosis, the lack of reduced MTR contrasts and signal increment in T₂-weighted images indicates that the tumor cores are not necrotic in a large scope probably due to the majority of tumors under study are relatively small. For instance, the MDM-MB-231 tumors under study have an average tumor volume of 0.32 cm³. In case that tumors further grow to up to 1.5 cm³, large-scale necrosis in tumor cores is expected as shown in the previous literature [34]. In the clinic, tumor tissues are commonly classified into viable and necrotic regions based on hematoxylin and eosin (H&E) staining, and there is so much interest in the necrotic regions from the pathologists in general. However, our redox imaging along with various MRI and optical imaging methods has repeatedly indicated that this binary tissue classification could be an oversimplified picture of tumor progression because cancer cells may survive in necrotic regions. As shown in both H&E and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining images in our previous paper [10], there are numerous viable cells in the tumor core region, which contributed hypointense but significant NADH signals. The existence of viable cancer cells in apparently necrotic tumor cores was also shown in our previous publications [8, 35]. The redox properties of these necrotic regions may contain useful information related to clinical prognosis and tumor metastatic risks [8, 10, 35].

To understand the molecular mechanism for the redox-sensitive CEST contrast, we have also performed CEST phantom experiments on the metabolites that are expected to make major contributions to the CEST contrast in breast cancer. Our results indicated that the reductants of the typical cellular NAD⁺/NADH-coupled redox reactions in animal tissues (Eqs. 2 and 3), *i.e.*, glutamate and alanine, are expected to generate higher CEST contrasts than the oxidants, *i.e.*, glutamine and pyruvate. It is important to note that both breast tumor lines under study are glutamine dependent. Glutamine plays a critical role in energy formation, redox homeostasis, macromolecular synthesis, and signaling in cancer cells [36]. Cell culture studies showed that millimolars of glutamine was necessary for MDA-MB-231 cells to maintain their normal growth and MCF-7 cells were less affected [37]. Abundant glutamine, glutamate, and alanine in millimolar levels were detected from representative breast cancer cell lines including MCF-7 and MDA-MB-231 [29, 30, 38]. Creatine, another major metabolite contributing to CEST contrast, is indirectly coupled with NAD⁺/NADH redox state (Eq. 4). Thus, the overall CEST contrast is expected to correlate with the NADH level and the cellular redox state. In order to quantitatively understand how the tumor redox state and the overall tumor CEST effect are affected by those key metabolites, additional ¹H-

MRS studies on the corresponding PCA-extracted tissues are necessary, which could be pursued in the future.

An additional example of the generic redox reactions is the glutathione reductase-catalyzed conversion of the oxidized glutathione (glutathione disulfide, GSSG) to its reduced form glutathione (GSH) with NADPH as a coenzyme, *i.e.*,

Our data indicate that glutathione has higher CEST effect in its reduced form (GSH) than its oxidized form (GSSG) in the equivalent concentration. The overall CEST contrast of this reaction is also expected to correlate with NADPH and NADPH/NADP⁺. Thus, both NAD- and NADP-coupled reactions can contribute to the CEST contrast, while the redox signals imaged by the optical redox scanning are mainly from the bound forms of NADH and Fp in the mitochondria [39]. However, the mitochondrial NADH may be coupled to NADPH in the cytosol *via* NADH transhydrogenase [40]. Thus, the mitochondrial NADH level may be proportional to the total level of NAD(P)H in the cell. To some degree, this may explain the linear correlation between the CEST contrast and the mitochondrial NADH or NADH redox ratio from the redox scanning. Although more validations *in vivo* are needed, these results suggest a plausible mechanism underlying the CEST contrast that is sensitive to NAD(P)H redox state.

It is worth noting that in the redox reactions we studied, the reductants and their corresponding oxidants, if they show any CEST contrast, have similar resonant frequency offsets for CEST. It is possible that the CEST effects of reductants may resonate at different frequency offsets than the oxidants. If this is the case, the overall CEST asymmetry summed over a wide range (*e.g.*, 0–5 ppm) in the Z spectrum should still be higher for reductants than oxidants and may be used as an indicator of redox state.

There are a few limitations associated with this study. First, due to significant variations within and among tumors, it is impossible to have a simple and objective standard for defining the core and rim. In the literature, core and rim heterogeneity has been defined in different ways on the basis of different criteria including histology, hypoxia level, contrast enhancement ratio, or physically drawing a thin layer of certain thickness for the rim [41–43]. In this study, both ROI and histogram analyses were employed to define the core and rim and consistent contrast results were obtained (Fig. 4c).

Second, only a single slice CEST map of each tumor was acquired due to imaging time constraints. The image co-registration between the MRI and the optical scanning becomes difficult due to the possible tissue distortion occurring during the preparation for the *ex vivo* cryogenic optical scanning. However, for the tumors used in this study, the redox state does not vary much with the slice depth in the tumor central region which occupies a large portion of tumor volume [10, 28]. This should make the precise image co-registration less critical.

Thirdly, the CEST effect *in vivo* may also be affected by other factors, such as the exchange rate of exchangeable protons (pH dependent), T_1 and T_2 of tissue, and the metabolites that are not directly involved in the redox reactions. The similarities in the T_1 and T_2 MR contrasts between tumor core and rim and between the two tumor lines (Figs. 1 and 2) indicate that T_1 and T_2 should not contribute much to the CEST core–rim difference. There were no apparent core–rim patterns observed in the typical extracellular pH maps of MCF-7 [44] and MDA-MB-231 [45] tumor xenografts. Even if there is a slight pH difference between the tumor core and rim, lower pH is expected in the core compared to the rim. Lower pH most likely leads to higher CEST effect. This is opposite to what we observed that lower CEST contrast was seen from the core compared to the rim. Therefore, pH contribution to the CEST core–rim heterogeneity should not be a major factor. Nevertheless, using the overall CEST effect *in vivo* as an indicator of the redox state requires more thorough calibrations and a good control of the other potential contributing factors independent of tissue redox state. More investigations are also needed to see if this correlation can be extended to other tissues or pathological conditions.

More controlled studies with metabolic perturbations can be done in the future to strengthen the correlation between CEST and redox imaging. Varying the oxygen supply to tissues may be used to modify tissue redox. However, it is shown that the required pO_2 level is very low (<1 mmHg) to affect directly the mitochondrial oxidative phosphorylation and redox state [10]. On the other hand, more effective means are to employ biochemical agents such as inhibitors of electron transport or uncouplers to modify the tissue mitochondrial redox state.

Moreover, we compared CEST MRI with a few conventional MRI methods for probing the rim to core heterogeneity. These methods such as T_1 - and T_2 -weighted MRI and MTR contrast are not expected to be sensitive to tissue function and metabolism. In the future, it may be worth comparing CEST MRI with diffusion-weighted and dynamic contrast-enhanced (DCE) MRI. We notice that T_1 -weighted images enhanced by T_1 -shortening agent such as Gd-DTPA can greatly increase the signal from tumor vasculature (characterized with angiogenesis) that is mainly distributed in the tumor rim. However, based on previous studies [46–48], the tumor angiogenesis-related contrast enhancement is located in a much thinner outer layer than the rim that is observed by the CEST MRI and redox scanning in this study. The CEST MRI should be further investigated for its utility on noninvasive mapping of the metabolic heterogeneities in tissue.

Conclusion

CEST contrast was found to correlate to the tissue mitochondrial NADH and the NADH redox ratio measured by the redox scanning. This correlation indicates that CEST MRI is sensitive to the reductive reactants in NAD(P)-coupled cellular redox reactions as supported by the phantom studies. More studies are needed in the future to validate this correlation. Due to the increasingly revealed importance of the redox state in many biological processes including various diseases, noninvasive CEST MRI sensitive to tissue redox state, once further confirmed, might have a great potential for clinical applications.

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Fig. 1.

Imaging of the two breast cancer models: MDA-MB-231 and MCF-7 with multimodality MRI: T₁-weighted (T₁-w; **a, f**), T₂-weighted (T₂-w; **b, g**), MTR (**c, h**), and CEST maps at 2 ppm (**d, i**) and 3 ppm (**e, j**).

Fig. 2.

Averaged rim-to-core signal or contrast ratios obtained with multimodality MRI: **a** T₁-weighted (T₁-w), **b** T₂-weighted (T₂-w), **c** MTR, and **d** CEST (at 3 ppm). Only the CEST contrast exhibits a significant difference between the two tumor lines. **e** Saturation offset dependence of the rim-to-core ratios of the CEST contrast.

Fig. 3.

Redox scanning provides quantitative NADH (a, b) and NADH redox ratio (d, e) from representative MDA-MB-231 and MCF-7 tumors. Significantly higher rim–core ratio of NADH concentration (e) was seen from MDA-MB-231 than MCF-7 tumors ($p < 0.05$), although their rim–core ratio of NADH redox ratio (f) was not significantly different.

Fig. 4.

Demonstration of the Gaussian-fitting histogram analysis of the CEST contrasts (at 3 ppm) of representative **a** MDA-MB-231 and **b** MCF-7 tumors. The histogram analysis result linearly correlates with the manual ROI analysis result (**c**). Each point shown in **c** represents the mean CEST contrast of individual tumor rim or core. The p values indicate the significance of the linear regressions.

Fig. 5.

The significant correlation between the CEST contrast and NADH concentration (**a**) and between the CEST contrast and NADH redox ratio (**b**). The CEST contrasts and the redox indices from the core or rim of individual tumors were obtained by histogram analysis using Gaussian fitting. In the figure, “231R” and “231C” indicate the data points from the rim and core of MDA-MB-231 tumors, respectively. The similar indications apply to “MCF7R” and “MCF7C”. “Linear (all)” indicates the linear regressions performed using all data points (denoted by *circles*).

Fig. 6.

Z spectra (*solid lines*) and the rescaled CEST asymmetrical curves (*dots*) of the key metabolites in the representative redox reactions of glutamate dehydrogenase (**a**), alanine dehydrogenase (**b**), and glutathione reductase (**c**). **a** 10 mM glutamate (*red*), 34 to 125 mM NH₄OH (*blue*), 50 mM 2-oxoglutarate (*green*); **b** 10 mM alanine (*red*), 34 to 125 mM NH₄OH (*blue*), 10 mM pyruvate (*green*); **c** glutathione (GSH, 20 mM) and its oxidized form glutathione disulfide (GSSG, 10 mM). All solutions are freshly prepared in PBS at pH 7.0 and maintained at 37 °C.

Table 1

Summary of the core and rim values (mean $\pm$ SD) of the CEST contrast and NADH and NADH redox ratios (NADH are in nominal concentrations, between the core and the rim)

Tumor	MCF-7			MDA-MB-231		
	CEST (%)	NADH (μ M)	NADH redox ratio	CEST (%)	NADH (μ M)	NADH redox ratio
Core	15 $\pm$ 2 [*]	92 $\pm$ 50 [*]	0.10 $\pm$ 0.06 [*]	14 $\pm$ 1 [*]	64 $\pm$ 24 [*]	0.10 $\pm$ 0.07 [*]
Rim	20 $\pm$ 1	196 $\pm$ 86	0.44 $\pm$ 0.12	24 $\pm$ 3	210 $\pm$ 50	0.40 $\pm$ 0.05

^{*}
 $p < 0.05$