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Assessment of carbonic anhydrase IX expression and extracellular pH in B-cell lymphoma cell line models

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Abstract

The expression of carbonic anhydrase (CA IX) and its relation to acidosis in lymphomas has not been widely studied. We investigated the protein expression of CA IX in a human B-cell lymphoma tissue microarray, and in Raji, Ramos, and Granta 519 lymphoma cell lines and tumor models, while also investigating the relation with hypoxia. An imaging method, acidoCEST MRI, was used to estimate lymphoma xenograft extracellular pH (pHe). Our results showed that clinical lymphoma tissues and cell line models *in vitro* and *in vivo* had moderate CA IX expression. Although *in vitro* studies showed that CA IX expression was induced by hypoxia, *in vivo* studies did not show this correlation. Untreated lymphoma xenograft tumor pHe had acidic fractions, and an Acidity Score was qualitatively correlated with CA IX expression. Therefore, CA IX is expressed in B-cell lymphomas and is qualitatively correlated with extracellular acidosis in xenograft tumor models.

Keywords

Carbonic anhydrase IX; lymphoma; acidosis; CEST MRI

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DECLARATION of INTERESTS

The authors declare no conflicts of interest.

INTRODUCTION

Despite significant advances in the diagnosis and treatment of Non-Hodgkin's Lymphoma (NHL) during the last decade, NHL remains a significant cause of morbidity and mortality in the United States with almost 70,000 new cases and 19,000 deaths estimated in 2013 [1]. One common feature of NHL is high cellular metabolic activity necessary to fulfill energy demands and nucleotide biosynthesis that are required for proliferation [2]. A consequence of high cellular metabolic activity is enhanced anaerobic and aerobic glycolysis, also known as the Warburg effect, which results in lactic acid production [3,4]. Poorly vascularized tumors are often poorly oxygenated, and this hypoxia further increases anaerobic glycolysis and increases lactic acid production [5]. Glutaminolysis and oxidative phosphorylation result in CO₂ production that can also be a source of further net H⁺ production and acidosis.

The activity of carbonic anhydrase IX (CA IX) can link the intracellular production of lactic acid and H⁺ ions with extracellular acidosis [11]. A member of a family of zinc metalloenzymes, the CA IX isoform is a transmembrane protein with an extracellular catalytic domain that catalyzes the reversible hydration of CO₂ into bicarbonate [12]. The hydration of extracellular CO₂ provides bicarbonate ions for influx by adjacent transporters, including the chloride/bicarbonate exchanger 1, 2, and 3 and the sodium-bicarbonate co-transporter known as NBCe1, that results in intracellular neutralization [13]. The proton by-products of the CO₂ hydration reaction remain in the extracellular space, acidifying the microenvironment. Thus, CA IX plays a fundamental role in maintaining intracellular pH (pHi) at the expense of decreased extracellular pH (pHe).

In our current study, we investigated the expression of CA IX in human lymphoma tissues, in three B-cell lymphoma cell lines *in vitro*, and in three human B-cell lymphoma tumor xenograft models *in vivo*. We also used a novel imaging method, acidoCEST MRI, to accurately measure tumor pHe with high spatial resolution, using instrumentation and a contrast agent routinely available in radiology clinics [14]. This non-invasive method was used to monitor longitudinal changes in tumor pHe, as well as evaluate the spatial heterogeneity of tumor pHe in each tumor model. To ensure that acidoCEST MRI can measure a range of pHe among different tumors, we also measured the change in pHe with acidoCEST MRI after administering a mitochondrial poison which is known to decrease extracellular pH to a xenograft tumor model.

The high expression of CA IX has previously been correlated to hypoxia. CAIX expression is primarily regulated at the transcriptional level through hypoxia induced transcription factor 1 alpha (HIF-1α) [15], and activating transcription factor 4 (ATF-4), which are activated in response to hypoxia and the unfolded protein response [16]. CA IX is an adverse prognostic factor in multiple tumor types including head and neck, breast, and lung cancers [17]. However, a correlation between CAIX and hypoxia has not been established in NHL. More generally, hypoxia and acidosis have been suggested to be related characteristics of the extracellular tumor microenvironment [18]. CA IX may act as a molecular mechanism that links hypoxia and extracellular acidosis (reviewed by Sedlakova et al, 2014 [19]). Therefore, another primary aim of our study was to investigate the

relationship between CAIX and hypoxia within human lymphoma tissues, and in three B-cell lymphoma cell lines *in vitro* and *in vivo*.

MATERIALS and METHODS

Reagents

All reagents were obtained from SigmaAldrich, Inc., (St. Louis, MO) unless otherwise indicated. The acidoCEST MRI protocol used clinical-grade iopromide at 788 mM (300 mg iodine/mL Ultravist™, Bayer Healthcare, Inc., Leverkusen, Germany).

Immunohistochemistry

Human lymphoma tissue microarrays were obtained from Dr. Lisa Rimsza, Department of Pathology, University of Arizona. Hematoxylin and eosin (H&E) stains were performed with human tissue arrays containing Mantle cell, Diffuse Large B-cell and Follicular lymphoma tissue. H&E stains were also performed with tissues obtained from lymphoma tumor tissues obtained from xenograft mouse tumor models, obtained by cutting 3-micron thick sections from formalin-fixed, paraffin-embedded tissue blocks. Immunohistochemistry (IHC) was performed using anti-pimonidazole mouse monoclonal antibody MAb1, clone 4.3.11.3, diluted 1:50 (Hypoxypore Inc., Raleigh, NC). CA IX staining was performed using anti-CA IX antibody from Novus Biologicals (Littleton, CO) diluted 1:2000. Vascular endothelial growth factor-A (VEGF-A) staining was performed using anti-VEGF-A antibody (Santa Cruz Biotech # sc-7269) diluted 1:70. Tissue sections were stained with a Discovery XT Automated Immunostainer (Ventana Medical Systems, Inc., Tucson, AZ). All steps were performed with VMSI validated reagents, including deparaffinization, cell conditioning (antigen retrieval with a borate-EDTA buffer), primary antibody staining, detection and amplification using a biotinylated-streptavidin-HRP and diaminobenzidine system and hematoxylin counterstaining.

Semi-quantitative scoring of CA IX, VEGF-A, and pimonidazole was performed by a Board Certified Pathologist. Immunoreactivity for VEGF-A was categorized as 0=negative; 1=weak; 2=moderate; 3= strong. The percentages of immunoreactive tumor cells was also evaluated for each intensity level. The overall scores were calculated by multiplying the intensity by the corresponding percentage of immunoreactive cells (scores range between 0–300). For pimonidazole staining, only the percentages of immunoreactive tumor cells were evaluated.

Tissue Culture

Raji, Ramos, and Granta 519 lymphoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were routinely grown in DMEM or RPMI supplemented with 10% HyClone™ fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA) and 100 I.U./ml of penicillin/streptomycin (Cellgro®, Mediatech, Inc., Manassas, MA) in a humidified incubator at 37°C and 5% CO₂. Cells were tested for mycoplasma contamination using the MycoAlert mycoplasma detection assay kit from Lonza (Basel, Switzerland) and found to be negative. Cell line authenticity was verified by extracting genomic DNA. DNA was diluted in TE buffer (10 mM Tris-CL, pH 7.5, 1 mM

EDTA), and submitted for analysis to the University of Arizona Genomics Core (Human Origins Genotyping Lab). Autosomal short tandem repeat (STR) typing was conducted across the 13 core STRs in Combined DNA Index System (CODIS) and referenced against allelic peaks in cell lines of previously confirmed genotype. For hypoxia studies, cells were grown in 0.5% oxygen, in a humidified, 37°C Ruskinn Invivo2 Hypoxia Workstation (Ruskinn Technology Ltd., Pencoe, Bridgend, UK). For pH 6.7 lactic acid media, a dilute stock of lactic acid at 85–90% by volume was added to media until a pH value of 6.7 was achieved, with an estimated concentration of 20 mM of lactic acid. For the lactate control media, a solution of 60% w/w sodium DL-lactate was diluted to achieve 20 mM lactate and then was titrated to pH 7.3.

Quantitative Nuclease Protection Assay (qNPA)

A HTG Molecular Hypoxia & DNA Array (HTG Molecular Diagnostics, Inc., Tucson, AZ) was used to measure CAIX gene expression. Briefly, cells were collected and pelleted following treatment. Cells were lysed in HTG Lysis Buffer and heated to 95°C for 10 minutes and then frozen for shipment to HTG Molecular Diagnostics for analysis as previously described [20]. The gene intensities were normalized to the four housekeeping genes peptidylprolyl isomerase A (PPIA), ribosomal protein L19 (RPL19), ribosomal protein, large PO (RPLPO), ribosomal protein L38 (RPL38). To calculate fold change induced by treatment, intensity values of cells under hypoxia were divided by intensity values of cells grown in normoxia for the same amount of time.

Western Blot Analysis

The cells were washed with PBS and suspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 5 mM Na₃VO₄, 200 μM NaF, 21 μM leupeptin, 230 nM aprotinin, and 1 mM PMSF) (100 μL/mL) and lysed by sonication. Xenograft tumors were homogenized using the same lysis buffer solution. Cell lysate was centrifuged at 10,000 x g for 10 minutes at 4°C. Protein concentration of the supernate was determined using the BioRad Protein Assay (Hercules, CA). Lysates were separated by 10% Bis-Tris gel electrophoresis (Invitrogen, Carlsbad, CA) and then transferred to 0.2 μm PVDF membranes (BioRad) and then blocked for 1 hour in 5% non-fat dry milk/TBST. Membranes were immunoblotted with primary antibodies at concentrations recommended by the manufacture. The CA IX antibody was purchased from Santa Cruz (Dallas, TX) and the α-tubulin antibody was purchased from Calbiochem (San Diego, CA). The membranes were then washed in TBST and then probed with a HRP conjugated anti-rabbit antibody or anti-mouse antibody. Membranes were then visualized with HyGlo Chemiluminescent HRP antibody detection reagent (Denville Scientific Inc., Metuchen, NJ). Blots were stripped in 0.2 M NaOH with shaking for 10 minutes at room temperature. Protein levels were normalized to actin or tubulin levels and expressed in arbitrary units relative to control levels. Densitometer analysis was performed using ImageJ [21].

CA IX ELISA

Cell lines were grown in normoxia or hypoxia conditions as described above. Conditioned media was collected by centrifuging cells at 2000xg and removing the supernatant. Media was immediately frozen at –80°C. Prior to analysis, samples were thawed and gently

vortexed. A quantikine human CA IX immunoassay (R&D Systems, Inc., Minneapolis, MN) was used according to manufacture recommendations.

Xenograft Tumor Studies

Lymphoma cells were mixed 1:1 with Matrigel (BD Biosciences, Inc., Franklin Lakes, NJ), and 10^7 cells were injected s.c. into 4–6 week old SCID mice. Animals were handled according to the guidelines of the Institutional Animal Care and Use Committee at the University of Arizona. Volumes were calculated using T_2 -weighted MR images. Animals were euthanized when tumors reached 2000 mm^3 . For MIBG studies, eight mice with a Raji xenograft tumor were tested when their tumor reached a volume of 800 mm^3 . MIBG (30 mg/kg) and glucose (3 mg/kg) were administered i.p. 4 hours prior to imaging. Immediately before acidoCEST MRI, another dose of glucose (3 mg/kg) was administered i.p. For exogenous hypoxia biomarker studies, mice were injected i.p. with 60 mg/kg pimonidazole and euthanized 1–4 hours after injection. Tumors were excised immediately and the tissues were placed in 10% neutral buffered formalin for 24 hours, transferred to 70% ethanol for dehydration, and then mounted in paraffin blocks.

AcidoCEST MRI

Our acidoCEST MRI protocol has been described in a previous report [14]. Briefly, each mouse was anesthetized with 1.5 – 2.5% isoflurane delivered in 1 L/min oxygen gas ventilation, and then secured to a customized cradle. A 27 g catheter was inserted into the tail vein. The cradle was inserted into a 7T Biospec MRI scanner with a 72 mm-diameter quadrature transceiver coil (Bruker Biospin, Inc., Billerica, MA). The breathing rate was monitored and the core body temperature was regulated at $37.0^\circ\text{C} \pm 0.5^\circ\text{C}$ using an automated feedback loop between the temperature probe and an air heater (SA Instruments, Inc., Stony Brook, NY). A FLASH MRI acquisition sequence was performed to determine the location of the tumor (500 msec TR, 10 msec TE, $625 \times 625 \mu\text{m}$ in-plane resolution, 2.0 mm slice thickness, 64×64 pixels, $4.0 \times 4.0 \text{ cm}$ FOV, one average, 32 sec total acquisition time). A spin-echo MRI acquisition sequence was performed to obtain images with good contrast for determining the tumor volume (2000 msec TR, 35 msec TE, $312.5 \times 312.5 \mu\text{m}$ in-plane resolution, 1.0 mm slice thickness, 128×128 pixels, $4.0 \times 4.0 \text{ cm}$ FOV, one average, 4:20 min total acquisition time). A bolus of 200 μL of iopromide was injected via the catheter within 30 seconds. The catheter was then connected to an infusion pump and iopromide was pumped at a rate of 150 $\mu\text{L/hr}$. Our acidoCEST MRI protocol consisted of 54 FISP MR images acquired at different saturation frequencies, using 2.8 μT saturation power and a saturation period of 5 seconds, which required 4:50 of acquisition time. This process was repeated 6 times, for a total acquisition time of 29 minutes. At the conclusion of the imaging scan, the mouse was removed from the scanner and cradle, and allowed to recover.

The acidoCEST MR images were processed with Matlab[®] R2012B (Mathworks, Inc., Natick, MA). All 6 CEST-FISP images were averaged. To improve signal to noise, groups of 3×3 adjacent pixels were binned. The CEST spectrum was fitted with a sum of three Lorentzian line shapes to account for the direct saturation of water and to measure the CEST effects at 4.2 and 5.6 ppm [22]. The pHe of each binned pixel with two CEST effects greater than $2 \times \text{noise}$ was determined using an empirical calibration based on identical imaging

of chemical solutions. This empirical calibration can determine pH between 6.2 and 7.0 units with a precision of 0.07 pH units [14]. These pixels were represented as colored pixels in the pHe map. Pixels with only a single CEST effect at 4.2 ppm greater than $2 \times$ noise were set to be pH 7.0. These pixels were represented as white pixels in the pHe map. Using a $2 \times$ noise threshold ensured that the CEST contrast was due to the agent with a 95% probability. A histogram showed pixel-wise analysis and the average pHe and standard deviation of pixel distribution of pHe values.

Calculation of the Acidity Score

AcidoCEST MRI can identify tumor regions with no significant uptake of the agent, regions approximated as pH 7.0 or greater, and regions with an acidic pHe. To characterize the tumor fraction that is acidic as well as the degree of acidity, we created an Acidity Score by multiplying the score with the corresponding percentage of pixels (Eq. [1]). An Acidity Score can range between 0 and 500.

$$\begin{aligned} \text{Acidity Score} = & 0 \times (\% \text{ of pixels with pHe} \geq 7) \\ & + 1 \times (\% \text{ of pixels with pHe between 6.90 and 6.99}) \\ & + 2 \times (\% \text{ of pixels with pHe between 6.80 and 6.89}) \\ & + 3 \times (\% \text{ of pixels with pHe between 6.70 and 6.79}) \\ & + 4 \times (\% \text{ of pixels with pHe between 6.60 and 6.69}) \\ & + 5 \times (\% \text{ of pixels with pHe below 6.59}) \end{aligned} \quad (1)$$

Statistical Analysis

A Student's t-test was used to compare pHe, spatial heterogeneity and % agent uptake between the three lymphoma xenografts. A Pearson's coefficient was used to correlate CAIX expression and pHe obtained from acidoCEST MRI.

RESULTS

CAIX is Expressed in Clinical Lymphoma Tissue

CA IX expression was evaluated by using IHC to evaluate clinical lymphoma tissue arrays containing cases of Mantle cell (MCL), Diffuse Large B-cell (DLBCL), and Follicular (FL) lymphomas (Table 1, Figure 1). The median CA IX score was highest in DLBCL and MCL which is consistent with higher proliferation rates in these lymphoma subtypes compared to FL. Strong CA IX expression was observed in a subset of each lymphoma subtype, as shown by high median scores for CA IX IHC. Yet scores of individual tissue samples showed high variability, as exemplified by high interquartile ranges. As another indication of this variability, some of the tissues stained negative (zero score) for CA IX, while others had maximal staining (score of 300). This variability demonstrated heterogeneous expression of this protein, which may reflect differences in the tumor microenvironment for each patient. Overall, the detection of CA IX in 79% (45/57) of the clinical samples demonstrated that CA IX expression is substantial in clinical lymphomas.

CA IX Expression is Induced by Hypoxia *in vitro*

We investigated the induction of CA IX gene expression in response to hypoxia using a qNPA assay in three B-cell lymphoma cell lines (Figure 2A). We tested Raji and Ramos cell lines that represent Burkett's lymphomas, and a Granta 519 cell line that represents a Mantle cell lymphoma. Minimal induction was observed in the Raji and Ramos cell lines, but the Granta 519 cell line demonstrated a time dependent increase in CA IX gene expression with more than a 2-fold induction by 48 hours. We also investigated the secretion of CA IX into cell culture media under normoxia and hypoxia growth conditions (Figure 2B). Similar to message expression, the Granta 519 cells showed the highest induction of secreted CA IX after 48 hours of growth under hypoxic conditions. Western blot analysis of whole cell lysates grown under normoxia or hypoxia for 24 hours demonstrated basal CA IX levels were induced in response to hypoxia in all three cell lines (Figure 2C). Similar to the other results, the Granta 519 cells showed the greatest induction in response to hypoxia, relative to CA IX levels under normoxia. Ramos cells showed minimal induction. The Raji cells also showed induction in response to hypoxia, although this cell line also showed a relatively high basal level of CA IX under normoxia.

CA IX Protein Expression is Not Induced by Lactic Acid *in vitro*

Lactic acid has been shown to activate HIF-1 α expression in tumor cells with oxidative but not Warburg phenotypes [23]. We hypothesized that lactic acid may therefore partially contribute to CA IX expression in lymphomas. Cells were grown in media with 20 mM lactate (pH 7.3), 20 mM lactic acid (pH 6.7), or control media with no lactate or lactic acid added at pH 7.3 (neutral). Western blot analysis of whole cell lysates probed for CA IX, using α -tubulin as a loading control, showed no elevation of CA IX protein in response to either lactate under neutral conditions or to lactic acid in all cell lines investigated (Figure 2D).

CA IX is Highly Expressed in Lymphoma Xenograft Models

Lymphoma xenograft tumor models of the same three B-cell lymphoma cell lines were tested for CA IX expression using IHC (Figure 3A). The three tumor models showed expression of CA IX throughout substantial portions of the tumor areas. The Ramos tumor model had the highest % area that stained positive for CA IX expression, and the Granta 519 tumor model had the most heterogeneous percent of area staining pattern (Figure 3B). We also stained tissues for VEGF-A, to assess if similar staining patterns were observed as with CA IX. Moderate VEGF-A staining was observed in all tumor types (Figure 3C). Similar to CA IX staining results, the Ramos tumor model had the highest VEGF-A score, and the Granta 519 tumor model had the most heterogeneous VEGF-A score.

CA IX is Not Highly Correlated with Hypoxia in Lymphoma Xenograft Models

To evaluate tissue regions of deep hypoxia, we used an exogenous hypoxia probe, pimonidazole, which can be reductively-activated to covalently bind to macromolecules. Following *in vivo* administration, pimonidazole distributes throughout the body where it covalently binds to normal and tumor tissue regions that have ≥ 14 micromolar dissolved

oxygen concentration. This corresponds to a pO_2 of 10 mm Hg at 37°C, consistent with severely hypoxic tissues. Pimonidazole adducts can then be detected by IHC.

Each tumor model showed some staining for the hypoxia probe (Figure 3D). The Raji tumor model had the highest percent area stained positive for pimonidazole. Both the Raji and Granta 519 tumor models showed more heterogeneous staining patterns than the Ramos tumors. The percent area of tumor stained for pimonidazole was significantly less than that stained for CA IX (p -value < 0.001) for each tumor type. Using qualitative assessment by a Board Certified Pathologist, the staining patterns of pimonidazole were not similar to CA IX staining patterns in any of the tumor models.

Lymphoma Xenograft Models Have Extracellular Acidosis

We measured the average tumor pHe in xenograft models of the same three B-cell lymphoma cell lines, using acidoCEST MRI once per week for three weeks. The average tumor volumes for each imaging session showed that the tumor models experienced tumor growth during this time period (Table 2). In particular, the Ramos tumor model was only imaged for two weeks as these tumors exceeded the maximally allowed volume by week 3 and were euthanized. For each group, we calculated the average fraction of tumor that was neutral or acidic, or had no uptake of the contrast agent (Figure 4A). The Granta 519 tumor model had the least agent uptake during the longitudinal study, while the Ramos tumor model had the most agent uptake. These levels of uptake are comparable to uptake levels of MRI contrast agents observed in our other acidoCEST MRI studies [24].

The average tumor pHe across the three models ranged between 6.78 and 6.86 (Figure 4B). These average tumor pHe values were not significantly different between the three tumor models. The pixel-wise pHe values of the Ramos tumor model were less heterogeneous than Raji and Granta 519 tumor models (p -value < 0.002 , Figure 4C). No significant trends were observed for spatial heterogeneities of pHe values relative to growth rates for the three xenograft models. Also, no significant temporal changes in tumor pHe were observed in the Raji or Ramos tumor models. However, the average tumor pHe decreased significantly during the study of the Granta 519 tumor model (pH 6.82 to pH 6.74, $p < 0.02$). We calculated an Acidity Score to provide a better overall assessment of the percent and intensity of pHe across each tumor model (Figure 4D). The Acidity Score had a good dynamic measurement range from 73.1 to 177.5. The Ramos tumor model had a significantly higher Acidity Score than the Raji or Grant 519 xenograft tumors ($p < 0.001$) (Figure 4D).

Xenograft Acidity Scores Correlate with CA IX Protein Expression

Using Pearson's correlation method, we assessed if there was a relationship between the CA IX IHC scores measured in lymphoma xenograft tumor tissue vs. the calculated Acidity Score described above. The correlation coefficient indicated a relationship between extracellular acidity and CA IX expression ($R^2 = 0.80$).

AcidoCEST MRI can Measure a Range of Lymphoma Tumor pHe Values

The three xenograft tumor models of B-cell lymphomas showed a narrow range of average tumor pHe values measured with acidoCEST MRI (Figure 4B). This raised a concern that acidoCEST MRI may not be able to measure a range of tumor pHe values. To validate acidoCEST MRI for assessments of lymphoma xenograft models, we investigated the ability of this imaging method to monitor changes in tumor pHe that are induced by MIBG. This mitochondrial poison causes a cell to shift from intracellular oxidative metabolism to glycolysis metabolism and thereby produce high levels of lactic acid.

To select a lymphoma xenograft model for this study, we excluded the Ramos cell line from consideration because of its rapid growth rate in xenograft models. We then compared the *in vitro* induction of lactate in response to MIBG in the Raji and Granta 519 cell lines. The Raji cell line demonstrated the most consistent dose-response relationship between MIBG treatment and lactate expression and was thus chosen for further study *in vivo* (Figure 5A).

Eight mice bearing Raji xenograft tumors were first scanned with acidoCEST MRI to establish a baseline pHe value two to four days before treatment. Considering that our previous *in vivo* study of the Raji tumor model showed no significant change in tumor pHe over 14 days, the tumor pHe was assumed to be the same as the baseline scan at the time of treatment. The mice were treated with MIBG and acidoCEST MRI was performed 4 hours later (Figure 5B). The mitochondrial poison caused a decrease in average tumor pHe in 6 of 8 mice (Figure 5C, D). The range of changes in the average value of tumor acidosis indicated that the effects of MIBG were variable among the mice. Most importantly, this range of pHe values demonstrated that acidoCEST MRI can accurately measure a range of tumor pHe values *in vivo*.

DISCUSSION

This study is one of the first to report high CA IX expression in clinical samples of B-cell lymphoma and in lymphoma xenograft tissue. We detected CA IX expression in 79% (45/57) of the clinical samples, which represented cases of Mantle cell, Diffuse Large B-cell, and Follicular lymphomas. All three xenograft lymphoma tumor models also showed CA IX expression. For comparison, a previous study reported on the expression of CA IX in only 33% (16/48) of high grade lymphomas [25]. More recently, a preliminary study has indicated a correlation between CA IX expression CA IX lymphoma tissue and plasma in patients with Diffuse Large B-cell Lymphoma, which provides further evidence for the utility of CA IX as a biomarker for lymphoma [26].

HIF-1 α expression, the primary transcription factor that regulates CA IX expression, has been more broadly studied in lymphoma. In a study of 78 DLBCL cases, 67% stained positive for HIF-1 α , which was demonstrated to be a positive prognostic factor for treatment with rituximab [27]. In a small study of 35 cases of MCL, HIF-1 α was expressed in 73.5% of cases and was associated with overall activation of the mammalian target of rapamycin (mTOR) signaling pathway and more aggressive disease [28]. These observations that both CA IX and HIF-1 α are expressed in lymphoma, and yet HIF-1 α and CA IX are not necessarily correlated in lymphoma, support our conclusion that CA IX should be further

investigated as a prognostic biomarker and therapeutic target in lymphoma. Furthermore, our observations of high CA IX expression in lymphoma suggest that future studies that investigate other isoforms of carbonic anhydrase in lymphoma may be warranted.

The three lymphoma xenograft models showed similar average tumor pHe values between 6.78 and 6.86 as measured with acidoCEST MRI. This imaging method can measure a larger range of pHe values, as shown by our acidoCEST MRI studies following mitochondrial poisoning of the Raji tumor model (Figure 5D). We have also observed a greater range of measured tumor pHe values in our other studies [14]. Therefore, this narrow average pHe range in the three lymphoma xenograft models is not an artifact of the imaging method, and instead demonstrates that tumor acidosis is a consistent effect within these xenograft tumor models of B-cell lymphoma.

To further evaluate tumor acidosis in these lymphoma tumor models, we developed an Acidity Score that reflects the pHe values as well as the fraction of the tumor estimated to be acidic. The Ramos tumor model had the highest Acidity Score. Because the Ramos tumor model also had the highest percentage of CA IX staining (Figure 3B), our study provides evidence that extracellular tumor acidosis correlates with tumor CA IX expression. This relationship between tumor acidosis and CA IX expression should be considered when selecting pre-clinical models for studies of small molecule CA IX inhibitors or anti-CA IX antibody therapies [29, 30].

Conversely, our *in vivo* studies did not find a correlation between CA IX expression and hypoxia as measured by pimonidazole staining. This finding may be due to the different level of oxygen required to activate pimonidazole compared to that required to induce CA IX through HIF-1 α [31]. For example, our *in vitro* studies showed that the Granta 519 cell line had greater CA IX expression, relative to other cell lines investigated, when controlled for cell density (Figure 2B) and greater induction of CA IX expression under hypoxia (Figures 2A and C). However, our *in vivo* studies showed the highest CA IX expression and Acidity Score in the Ramos tumor model. This difference between *in vitro* and *in vivo* results suggests that studies involving the tumor microenvironment may need to be studied *in vivo* to answer clinically relevant questions.

The trend in contrast agent uptake observed in our *in vivo* studies of the three lymphoma tumor models paralleled the growth rates of each tumor model. This evidence suggests that the uptake of the contrast agent reflected each tumor models' uptake of similar small molecule nutrients that can promote tumor growth. Furthermore, the Ramos tumor model had the highest Acidity Score and the highest growth rate, suggesting that tumor acidosis may reflect the general metabolic rate and growth of tumors. Similarly, the trend in increasing tumor acidity in the Granta 519 tumor model paralleled the growth of this tumor model, again suggesting that tumor acidosis reflects the general metabolic rate that promotes tumor growth. Future studies with biomarkers of general tumor metabolism should be performed to further investigate this connection between acidoCEST MRI evaluations and tumor growth. As shown by the mitochondrial poisoning study of the Raji tumor model, acidoCEST MRI may also be useful for monitoring the early response to inhibitors of tumor

metabolism and the role pH plays in promoting tumor growth and responsiveness to treatments.

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Figure 1.
Representative images of Mantle cell lymphoma (MCL), Diffuse Large B-cell lymphoma (DLBCL), and Follicular lymphoma (FL) clinical tissue stained for CA IX (200x magnification).

Figure 2.

In vitro evaluations of B-cell lymphoma cell lines. A) A qNPA array was used to measure the fold change in CAIX gene expression in response to hypoxia. B) CA IX expression was measured using an ELISA in conditioned cell culture media from cell lines grown under normoxia or hypoxia. C) Western blot analysis of CAIX protein expression in whole cell lysate from lymphoma cells grown under normoxia or hypoxia for 24 hours. Tubulin expression was used as a loading control. D) Western blot analysis was performed to investigate CA IX protein expression in response to growth in lactic acid (pH 6.7) or lactate (pH 7.3) for 24 hours.

Figure 3.

IHC evaluations of B-cell lymphoma tumor models. A) Representative images of serial xenograft tumor sections stained for CAIX, pimonidazole, and VEGF-A with 10 x magnification. B) The assessment of percent of lymphoma xenograft tumor area stained positive for CA IX (n=4). C) Immunoreactivity scores of lymphoma xenografts stained for VEGF-A (n=4). DD) The assessment of percent of lymphoma xenograft tumor area stained positive for pimonidazole (n=4).

Figure 4.

AcidoCEST MRI evaluations of B-cell lymphoma tumor models. A) The fraction of the tumor estimated as having no significant uptake of agent, acidic pHe < 7.0 , or neutral pHe > 7.0 . B) The average tumor pHe for Raji, Ramos, and Granta 519 tumor xenograft models. The error bars represent the standard deviation of the average of all mice on each day of imaging. C) The average standard deviation of pixelwise pHe values for Raji, Ramos, and Granta 519 tumor xenograft models, which represents the heterogeneity of pHe in a tumor model. The error bars represent the standard deviation of this heterogeneity measurement of all mice on each day of imaging. D) The average Acidity Scores calculated for Raji, Ramos, and Granta 519 xenograft tumor models. The error bars represent the standard deviation of the Acidity Scores of all mice on each day of imaging.

Figure 5.

AcidoCEST MRI evaluations of the mitochondrial poisoning of the Raji tumor model. A) Analysis of secreted lactic acid following 4 hours of MIBG treatment of Raji and Granta 519 lymphoma cell lines *in vitro*. B) The pH map of a mouse bearing a Raji xenograft tumor before and after treatment with MIBG. Colored pixels have acidic pH values ≤ 7.0 that correspond to the color-bar. White pixels represent tumor regions with only a single CEST effect at 4.2 ppm, which were considered to have neutral pH values > 7.0 . C) Cumulative pixel distribution before and after MIBG treatment from the mouse shown in Figure 5B. D) A waterfall plot of the change in average tumor pH in Raji tumors (n=8) following treatment with MIBG. Mice with statistically significant changes are marked with an asterisk ($p < 0.01$).

CA IX expression in clinical lymphoma tissues evaluated with immunohistochemistry.

Table 1

Lymphoma Type	median score	interquartile range	number of tissues		
			total	zero staining	maximal staining
Mantle cell	90	190	19	4	3
Diffuse Large B-cell	125	115	21	1	0
Follicular	50	80	17	7	1

Table 2

Average tumor sizes of xenograft lymphomal models. The number of mice per group is indicated in parantheses.

xenograft model	Week 0 (mm ³)	Week 1 (mm ³)	Week 2 (mm ³)
Raji	217 ± 143 (4)	1005 ± 381(4)	2505 ± 346 (3)
Ramos	321± 141 (5)	1812 ± 384 (5)	N/A
Granta 519	181 ± 108 (6)	556 ± 280 (6)	1585 ± 405 (4)