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A Study of the Relationship of Metabolic MR Parameters to Estrogen Dependence in Breast Cancer Xenografts

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Abstract

¹H Magnetic Resonance Spectroscopy (MRS), ³¹P MRS and Diffusion Weighted MRI (DW-MRI) were applied to study the metabolic changes associated with estrogen dependence in estrogen receptor (ER) positive BT-474 and triple negative HCC1806 breast cancer xenografts supplemented with or without 17 β -estradiol (E₂) at a dose of 0.18 or 0.72 mg/pellet. Furthermore, the effect of estrogen withdrawal on metabolism of BT-474 and HCC1806 breast cancer xenografts were studied on day 0, day 2 and day 10. Increasing the dose of E₂ resulted in a rapid growth and increase in lactate level, PME/ β NTP, PCr/Pi and β NTP/Pi ratios in BT-474; however no significant changes were found in HCC1806 breast cancer xenografts. Estrogen withdrawal resulted in a marked decrease in lactate level and PME/ β NTP and an observed increase in β NTP/Pi, PCr/Pi and apparent diffusion coefficient (ADC) values of BT-474 xenografts on day 10. These data suggest that lactate, PME/ β NTP, PCr/Pi and β NTP/Pi ratios of ER-positive tumors were closely related to ER dependence.

Graphical Abstract

Increasing the dose of E₂ (0.72 mg) resulted in a rapid growth and increases in lactate, PME/ β NTP, PCr/Pi, and β NTP/Pi ratios in BT-474, whereas no significant differences occurred in HCC1806 tumors. Estrogen withdrawal resulted in marked decreases in lactate and PME/ β NTP and observed increases in β NTP/Pi, PCr/Pi, and ADC values of BT-474 tumors on day 10. These data suggest that lactate, PME/ β NTP, PCr/Pi, and β NTP/Pi ratios of ER-positive tumors were closely related to ER dependence. Lactate was the most important parameter for predicting the response to endocrine therapy in breast cancer.

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Keywords

¹H MRS; ³¹P MRS; DW-MRI; HadSelMQC; lactate; ER– Positive Breast Cancer; Estrogen Dependence; Endocrine therapy

Introduction

Breast cancer is the most common cancer in women worldwide. About 75% of all breast cancers are estrogen receptor (ER) positive, and their dependence on estrogen stimulation for growth makes endocrine therapy an attractive choice (ET) (1–3). Lack of ER expression predicts endocrine therapy failure in breast cancer; however, not all ER positive tumors respond to endocrine treatment (4). Only 50%–75% of untreated patients and even fewer (25%) of previously treated patients with ER positive tumors respond. Therefore, it is equally important to know the presence of ER as well as response to endocrine therapy.

A “clinical flare reaction” after initiation of endocrine therapy, which is often characterized by increased pain in areas of osseous metastasis, enlargement of soft tissue tumor foci and increase in serum marker (5–9), may predict the response more accurately than tumor ER status. The majority of patients (65–90%) who experience a clinical flare reaction will subsequently experience the benefit of endocrine therapy (6,7,10). However, the clinical flare reaction is relatively infrequent and is difficult to distinguish from actual disease progression. The development of a “metabolic flare” reaction may have potential to predict the response to endocrine therapy (11,12). One approach based on ER-stimulated growth in breast cancer has been studied as a measurement of response; it included serial tumor biopsy and *in vitro* assay of the Ki-67 index of cellular proliferation, (13,14). However, this approach is invasive and subject to sampling error and is unlikely to be implemented in the clinic. Tumor response to endocrine therapy can be visualized by using fluoro-deoxyglucose (FDG)-PET which is capable of detecting focused areas with high glucose uptake (11,12,15). Given the high cost of the procedure and low resolution images, it would be challenging to adopt it as a standard of care for endocrine therapy.

Estrogen and ER have been shown to play an important role in cellular proliferation and metabolism (16,17), and to increase glucose transport and lactate production in breast cancer

cells (18–21). The antiproliferative effect of estrogen withdrawal or antiestrogen therapy has been used clinically for decades, but only little is known about the influence of this treatment on cellular metabolism. The noninvasive and efficient detection of ER-dependent tumor metabolism may have great potential predicting response to endocrine therapy. Magnetic resonance spectroscopy (MRS) has been used as a noninvasive tool for detecting tumor metabolism *in vivo* (22–25). Lactate MR spectroscopy has been used for the measurement of changes in lactate levels in lymphoma, breast and prostate cancer xenografts as an early biomarker of response to chemotherapy (26–28). Furthermore, ³¹P phosphorus magnetic resonance spectroscopy (³¹P MRS) can precisely assess the pH and bioenergetic status of the tumor (29,30). Diffusion-weighted MRI (DW-MRI) holds promise to serve as a biomarker of treatment response. DW-MRI is sensitive to microstructural changes occurring at cellular level during treatment rather than to anatomical changes (31,32). Therefore, MRS and DW-MRI can be used for predicting the response to endocrine therapy. The purpose of this study was to investigate the effect of estrogen withdrawal on the metabolic MR parameters and the relationship between metabolic MR parameters and estrogen dependence in breast cancer xenografts using MRS and DW-MRI techniques.

Materials and Methods

1. Cell Lines

BT-474 and HCC1806 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). BT-474 is a breast cancer cell line that expresses the estrogen receptor (ER). HCC1806 cell is a triple-negative breast cancer (TNBC) that lacks receptors for estrogen (ER), progesterone (PR), and human epidermal growth factor receptor 2 (HER2) (33).

2. Animals

To establish the BT-474 breast tumor model, 10⁷ cells were subcutaneously inoculated into the flank of 01b74 athymic female nude mice with a mixture of 75 µl matrigel (BD Biosciences, California, USA) and 75 µl DMEM medium. To establish the HCC1806 breast tumor model, a total of 10⁶ HCC1806 cells were subcutaneously injected into the flank of 01b74 athymic female nude mice. Animals were anesthetized during the experiment with 1 to 1.5% isoflurane. 01b74 athymic female nude mice were purchased from NCI Production. The experimental design was approved by the University of Pennsylvania Committee on Use and Care of Animals.

3. Hormone Treatments

Animals were supplemented with or without 17β-estradiol with dose of 0.18 or 0.72 mg/pellet (60-day release, Innovative Research of America) 7 days before inoculation with BT-474 tumor cells. As a control, animals were inoculated with HCC1806 tumor cells with or without estrogen supplement (0.72 mg/pellet). The pellet was placed with the aid of a 10-gauge trocar into the neck 7 days before tumor cell inoculation. The tumor dimensions were measured once a week with calipers in three orthogonal directions; tumor volumes were calculated using a hemiellipsoid formula: $V = (\pi/6) \times x \times y \times z$, where x, y and z are the length,

width and height of the tumor, respectively (34). MRI and MRS were performed when the tumor volumes reached approximately 200 mm³ and 500 mm³. Five mice were used in each group (Fig. 1).

In a separate experiment, the mice carrying BT-474 and HCC1806 tumors received a 17 β -estradiol pellet (60-day release, 0.72 mg, Innovative Research of America) subcutaneously (s.c.) 7 days before tumor cell inoculation. When tumors reached 200 mm³, 17 β -estradiol pellets were removed from half of the animals after the MRS and MRI study by puncturing the skin with a No. 11 scalpel blade and retrieving the pellet with forceps (day 0). Lactate MR spectra, ³¹P MRS and DWI were performed on day 0, day 2 and day 10 (Fig. 1). Five mice were used in each group at each time point.

4. *In vivo* MRI and MRS

The experiment were performed on a 9.4-T/31 cm horizontal bore magnet with a 21 cm ID gauss/cm and a 12 cm ID gauss/cm gradient tube and interfaced to a Varian Direct Drive console (Varian, Palo Alto, CA, USA). A 35 mm ID volume coil was used for MR imaging. During imaging, mice were anaesthetized by 1–1.5% isoflurane mixed with oxygen, and the core body temperature, electrocardiogram (ECG) and respiration were monitored (SA Inc, Stony Brook, NY, USA). The core body temperature was maintained at 37°C by blowing warm air into the bore of the magnet, and the respiration signals were used for gating during the data acquisition to minimize the motion interference. DW-MRI was used with following parameters: [500/35.85 (repetition time msec/echo time msec)], matrix of 128×96 with a multi-slice spin-echo sequence with 12 bipolar gradients (field of view of 30×30 mm at b = 0 and 939.48 s/mm²). A separate scan using b=0 and 66.8 s/mm² was performed for calculation of apparent diffusion coefficient (ADC_(low)) values. The gradients were applied in six orthogonal directions and were combined to produce a trace data set to minimize the effects of diffusion anisotropy.

A Hadamard-selective multiple quantum coherence transfer pulse sequence (HadSelMQC) was used to detect lactate and to filter out overlapping lipid signals (35). To maximize the signal-to-noise ratio, we used only one slice covering the entire tumor, based on scout imaging, which excluded muscle. The slice thicknesses for all ¹H MRS experiments were 10 mm. Gaussian pulses of 8192 μ s were used as frequency selective pulses. For selection of coherence pathway, shaped gradients with amplitude ratios of 0:–1.5:3 Gauss/cm and duration of 3 ms were applied. Other acquisition parameters were as follows: sweep width, 4 kHz; 2048 data points; TR=8 s; 128 scans. Receiver gain for HadSelMQC experiments was set to 60 dB. For quantitation, a Hadamard-encoded spin echo sequence was used to detect water in the same slice (TE= 140 ms, TR= 8 s, NA=4)(36). Lactate/water ratios were calculated for each *in vivo* experiment.

In vivo ³¹P MRS spectra were acquired with a homebuilt dual-frequency (¹H/³¹P) slotted-tube resonator (inner diameter, 10 mm), which is capable of producing a very homogenous B1 field. The mice were in the lateral position, and the tumor were put in the coil center. The legs of mice were fixed to prevent motion. Respiration was monitored. Shimming was performed on the flat top of the respiration. The magnet was shimmed until the water line-width of the tumor monitored via the ¹H channel reached 60–70 Hz. Localized ³¹P MRS

was performed using the image-selected *in vivo* spectroscopy (ISIS) technique with the following parameters: 256 scans with a radiofrequency pulse width of 60 μ s, corresponding approximately to a 90° flip angle; sweep with, 12 kHz; 512 data points; TR=4 s. A point-resolved spectroscopy sequence was used to manually shim the B0 field within the ISIS tumor voxel(37). Five mice were used in each group.

5. Data analyses

NUTS (Acorn NMR Inc., Livermore, CA, USA) and MestRec (Mestrelab Research, Santiago de Compostela, Spain) were used to process all spectroscopic data. A 20-Hz exponential filter was used to improve the apparent signal-to-noise ratio of ^{31}P MRS data, and baseline correction was applied before plotting and calculation of the peak areas. Peak integration was performed to calculate metabolites. pH_i was determined from the chemical shifts of inorganic phosphate (Pi) referenced to the α -nucleoside triphosphate (α -NTP) resonance. For pH_i , a pK_a value of 6.57 ± 0.03 , limiting acid chemical shift of 13.52 ± 0.03 ppm and base chemical shift of 11.24 ± 0.02 ppm were used.

ADC maps were generated using IDL software (Research Systems, Boulder, Colorado) program. The region of interest (ROI) of the tumor was drawn on the ADC map with necrotic areas excluded by visual comparison with T_2 weighted images from the same level. ADC values from the ROI of the tumor were quantified in square millimeters per second. These values were pooled to construct the ADC histogram for the entire tumor using 256 bins and bin width of $9.57 \times 10^{-6} \text{ mm}^2/\text{sec}$. To validate the MRI-based estimation of ADC, we first measured the ADC of a water phantom at 37°C using a diffusion-weighted imaging pulse sequence.

6. High-resolution NMR spectroscopy

Animals were anesthetized by i.p. injection of ketamine after completing the *in vivo* experiment to analyze the tumor metabolites by high-resolution ^1H and ^{31}P NMR spectroscopy using the perchloric acid (PCA) extraction method as described previously(36). A skin incision was made and the tumor tissue was carefully excised from the body and freeze clamped with a pair of tongs, dipped in liquid nitrogen and weighed. After excision, animals were euthanized under CO_2 inhalation. Frozen tissues were ground and 6% perchloric acid (3.25 ml per 1 g of tissue) was slowly added while grinding. The ground tumor tissue powder was homogenized and the homogenate was centrifuged at 13000 rpm for 30 min at 4°C. The supernatant was neutralized to $\text{pH } 7.0 \pm 0.2$ using 100 mM KOH. The precipitated salt was removed following centrifugation at 1000 rpm for 10 min and the supernatant was stored at -20°C until further processing.

The tumor tissue extract was concentrated by lyophilization. The resulting powder was re-dissolved in 0.6 ml D_2O and the pH was adjusted to 7.0 ± 0.1 using 1mM KOH. The solution was transferred to a 5-mm NMR tube, and a capillary tube containing 0.56 mM sodium 3-(trimethylsilyl)-[2,2,3,3,-2H4]-1-propionate (TSP) or an 8.7 mM solution of 1-APP (1 aminopropylphosphonate) was inserted as an external reference standard. High resolution ^1H NMR spectra were acquired with a 9.4T superconducting magnet interfaced to a Bruker DMX400 spectrometer (Bruker BioSpin, Billerica, MA, USA). The following parameters

were used: 45° flip angle, TR 8.8 s and 128 averages. A pre-saturation pulse was used to suppress the water signal. High resolution ^{31}P NMR spectra were recorded on a DMX-400 spectrometer (Bruker) at 162 MHz by applying 45° pulses, a repetition time of 5 s, and continuous composite pulse proton decoupling. The Fourier transformed spectra were analyzed using the XWIN NMR program (Bruker). Five mice were used in each group at each time point.

7. Histological analyses

Animals were supplemented with 17 β -estradiol at a total dose of 0.72 mg/pellet 7 days before inoculation with BT-474 tumor cells. When tumor volume reached 200 mm³ and 500 mm³, tumors were excised from the body and embedded in OCT medium and snap frozen after MR imaging. Animals were euthanized using CO₂ inhalation after tumor excision. Twenty micrometer sections were cut and fixed in 10% paraformaldehyde solution followed by either hematoxylin or eosin (H&E) for detection of tumor necrosis or immunostaining for endothelial cell density. For immunostaining of endothelial cell density, CD 31 antibody (rat-anti-mouse, AbD Serotec, USA) was used as the primary antibody. Primary antibody was detected by sequential incubation with secondary antibody (goat anti-rat IgG conjugated to Cy3, Life Technologies, NY, USA). Images were captured and processed with an epifluorescence microscope (Nikon E600 Upright Microscope, NY, USA) and ImageJ software (NIH, USA). Five mice were used in each group.

The fractional area of tumor necrosis was determined from digital images of H&E stained whole tumor sections using Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA, USA). The whole tumor and regions of necrosis were manually delineated, and the necrotic fraction was calculated as the ratio of the necrotic area to the whole tumor area.

8. Statistical analyses

Numeric data were reported as the mean $\pm$ standard deviation. One-way ANOVA with Bonferroni correction was carried out using SPSS (version 17.0J for windows; SPSS, Chicago, IL, USA) software to compare values obtained from groups. A p value less than 0.05 was considered as statistically significant.

Results

Absence of estrogen supplementation resulted in no development of BT-474 tumors. Supplementation of 0.72 mg E₂ resulted in a rapid outgrowth of BT-474 tumors in comparison to the 0.18 mg group, however no significant differences were observed in HCC1806 tumors. The BT-474 tumors in the 0.18 mg group grew slowly and took 120 days after inoculation to reach volumes of approximately 300 mm³ (Fig. 2a). Thus, we failed to acquire the 500 mm³ volume data for the 0.18 mg group of BT-474 tumors. HCC1806 tumors grew more rapidly than BT-474 tumors.

BT-474 tumors supplemented with 0.72 mg/pellet E₂ showed higher lactate levels, PME/ β NTP, β NTP/Pi and PCr/Pi compared to the 0.18 mg group, however no significant difference was observed in HCC1806 tumors (Fig. 2b). In all tumor types, a higher lactate level, a lower PME/ β NTP, a higher β NTP/Pi, and a higher PCr/Pi were found in the small

tumors ($\sim 200 \text{ mm}^3$) in comparison to the large tumors ($\sim 500 \text{ mm}^3$) (Fig. 2b). Concentrations of lactate, PC, PE, β NTP, PCr and Pi in tumor extracts were quantified by high-resolution NMR spectroscopy (Table 1). There was no significant difference in pH of tumors in different groups, which was around 6.8 ± 0.2 .

In both tumor types, lower ADC values were found in large tumors, but the difference was not significant ($P > 0.05$). No significant difference was found in ADC values of tumors supplemented with different doses of E_2 ($P > 0.05$). There was no significant difference in ADC values between two tumor types ($P > 0.05$). The ADC value of the water phantom was identical to that reported in the literature (32). The water phantom appeared homogeneous on the ADC map, and its mean ADC value was $(3.0 \pm 0.1) \times 10^{-3} \text{ mm}^2/\text{s}$ at 37°C .

To determine and compare the ischemia in ER– positive small ($\sim 200 \text{ mm}^3$) and large ($\sim 500 \text{ mm}^3$) tumors, H&E staining and CD31 immunostaining were performed for tumor necrosis and endothelial cell density, respectively. Small tumors showed no necrosis and a uniform endothelial cell density (Figs. 3b, 3c and 3d); however, larger tumors showed some central necrosis and a decrease in endothelial cell density (Figs. 3f, 3g and 3h). The fractional area of tumor necrosis in small tumors and larger tumors were $1.24 \pm 0.07\%$ and $13.95 \pm 1.00\%$, respectively. When a low b-value range ($0\text{--}100 \text{ s/mm}^2$) was used to calculate the ADC map, most of the diffusion signal was contributed by the blood flow (31,38). Recognizing that low b-value is sensitive for perfusion effects, we also compared the $\text{ADC}_{(\text{low})}$ values of small BT-474 tumors to large BT-474 tumors to investigate the perfusion in ER– positive tumors. Higher $\text{ADC}_{(\text{low})}$ values were found in small tumors ($3.94 \pm 0.05 \times 10^{-3} \text{ mm}^2/\text{sec}$) in comparison to large tumors ($1.63 \pm 0.03 \times 10^{-3} \text{ mm}^2/\text{sec}$) ($P < 0.05$) (Figs. 3a and 3e).

To investigate the effect of estrogen ablation on tumor metabolism, 17β -estradiol pellets were removed, when tumors reached 200 mm^3 . Lactate MRS, ^{31}P MRS and DWI were performed on day 0, day 2 and day 10. After estrogen withdrawal, a prolonged plateau phase was shown in the growth curve of BT-474 tumors; however, HCC1806 tumors continued to grow (Fig. 2a). Estrogen withdrawal resulted in a marked decrease in lactate level in BT-474 tumors on day 2 ($41.18 \pm 2.14\%$) and day 10 ($82.35 \pm 3.22\%$) ($P < 0.05$); however, no significant decrease in lactate level was found in the continuously estrogen-stimulated BT-474 tumors ($P > 0.05$, Fig. 4e). A typical lactate MR spectrum of a BT-474 tumor on day 0 and day 10 (Figure 4a), shows a sharp decrease in lactate signal on day 10 after estrogen withdrawal. Although a decrease in lactate level in HCC1806 tumors was observed on day 10 after estrogen withdrawal ($P < 0.05$), no significant difference between the estrogen-stimulated and the estrogen-deprived HCC186 tumors was found ($P > 0.05$, Fig. 4e).

Estrogen withdrawal resulted in significant decrease in the ^{31}P MRS PME/ β NTP ratio of BT-474 tumors on day 2 ($2.79 \pm 0.76 \%$) and day 10 ($44.43 \pm 8.31\%$) ($P < 0.05$); however, no significant decrease in PME/ β NTP was found in the continuously estrogen-stimulated BT-474 tumors ($P > 0.05$, Fig. 4e). An increase in PME/ β NTP in HCC1806 tumors was found on day 10, but no significant difference between the estrogen-stimulated and estrogen-deprived HCC1806 tumors was observed ($P > 0.05$, Fig. 4e).

The decreases in β NTP/Pi and PCr/Pi ratios in BT-474 tumors on day 2 after estrogen withdrawal were found to be statistically insignificant ($P>0.05$) (Fig. 4e). On day 10, significant increases in β NTP/Pi ($81.25 \pm 7.25\%$) and PCr/Pi ($128.57 \pm 7.36\%$) were observed (Fig. 4e). Typical ^{31}P MR spectra of BT-474 tumors on day 0 and day 10 are shown in Figure 4c, where a sharp decrease in PME/ β NTP and the marked increases in β NTP/Pi and PCr/Pi on day 10 after estrogen withdrawal are visible. HCC1806 showed decreases in β NTP/Pi and PCr/Pi on day 10, but no significant difference between the estrogen-stimulated and estrogen-deprived HCC1806 tumors was observed ($P>0.05$, Fig. 4e). Concentrations of lactate, PC, PE, β NTP, Pi and PCr in tumor extracts were quantified by high-resolution NMR spectroscopy (Table 2)

Estrogen withdrawal resulted in an increase in ADC values of BT-474 tumors on day 2 ($12.72 \pm 7.34\%$) and day 10 ($31.92 \pm 7.21\%$) (Figs. 5a, 5c and 5e). Decreases in ADC values were found in the continuously estrogen-stimulated BT-474 tumors and HCC1806 tumors (Fig. 5e). A right shift of ADC mean values in the ADC histogram of BT-474 tumors was found on day 10 (Figs. 5a and 5c); however, a left shift was found in HCC1806 tumors (Figs. 5b and 5d).

Discussion

1. Lactate

Our study showed that increasing the dose of E_2 resulted in a significant increase in lactate in ER positive BT-474 tumors; however, no lactate increase was found in triple negative HCC1806 tumors. The dose-dependent effect of estrogen stimulation on lactate in ER positive BT-474 tumors suggested that lactate metabolism was closely related to estrogen dependence. The marked decrease in lactate in BT-474 tumor after estrogen withdrawal further validated the hypothesis that this phenomenon was in fact related to estrogen dependence. These *in vivo* data are consistent with a metabolic study of estrogen stimulation by ^{13}C NMR; estrogen increased the rate of glucose consumption and lactate production in MCF-7 xenografts (19). ER has been shown to stimulate glycolysis not only under hypoxia but also under normoxia, acting as an important transcriptional activator of the glycolytic pathway and contributing to the Warburg effect in breast cancer cells(39). Acceleration of glycolysis by estrogen stimulation may account for the increase in lactate in ER positive tumors. In a clinical study, higher levels of lactate were found to be associated with lower survival rates for ER-positive patients; however, similar metabolic differences were not observed in ER-negative patients, where survivors could not be separated from nonsurvivors (40). The results presented here further indicate that lactate was closely related to ER dependence. MRS using the HadSelMQC sequence has been used for noninvasive detection of lactate levels *in vivo*(26,35,36). In our study, the water signal was detected as reported before (36) at an echo time of 140 ms, which may lead to T_2 weighted effects on lactate/water signals. However, well-defined lactate peaks were observed in BT-474 tumors, which was sensitive enough to detect the decrease in lactate levels of tumors on day 2 and day 10 after estrogen withdrawal. And the errors in lactate/water due to T_2 of water were $<10\%$ at an echo time of 140 ms.

2. PME/ β NTP

The phosphomonoesters (PMEs) phosphocholine (PC) and phosphoethanolamine (PE) are precursors of the phosphatidylcholine and phosphatidylethanolamine in biological membranes (29,30). Increased PME is observed in a wide range of human tumors in comparison to normal tissue (41,42). The ratio of PME/ β NTP in ^{31}P MRS generally decreases in response to therapy (43). In the current study, a dose-dependent effect of estrogen stimulation on the PME/ β NTP ratio in ER positive tumors was found; increasing the dose of estrogen resulted in an increase in PME/ β NTP in BT-474 tumors. On day 10 after estrogen withdrawal, PME/ β NTP was significantly decreased in BT-474 tumors, whereas this ratio was increased in HCC1806 tumors. A difference in tumor size between BT-474 tumors and HCC1806 tumors on day 10 after estrogen withdrawal would not account for the difference in PME/ β NTP changes between BT-474 tumors and HCC1806 tumors after estrogen withdrawal, because the changes of PME/ β NTP in HCC-1806 tumors with or without estrogen supplementation after estrogen withdrawal were similar. These *in vivo* data indicate that PME/ β NTP in ER-positive tumors was closely related to estrogen dependence. These data were in agreement with a previous study of the effect of estrogen withdrawal on phosphate metabolism in breast cancer (44); PME relative ratio was found to be the most important single contributor to the predictability of estrogen dependent tumors. A decrease in PME concentration in ER positive tumors may account for the observed decrease in PME/ β NTP in BT-474 on day 10 after estrogen withdrawal because β NTP was unchanged. A receptor-mediated regulatory effect of estrogen on phospholipid metabolism might explain the observed decrease in PME after estrogen withdrawal. Upregulation of choline kinase and ethanolamine kinase by estrogen stimulation of the CDP-choline and ethanolamine pathways may result in increased production of phosphocholine and phosphoethanolamine. Furthermore, a strong relationship between PME and tumor proliferation has been found, and the presence of increased PME was considered to be a consequence of a shift in the balance of phospholipid metabolism with more rapid synthesis in the faster growing tumors (45,46). In our study, estrogen stimulation resulted in a rapid growth of estrogen dependent tumors; however, no tumor developed without estrogen supplementation.

3. β NTP/Pi and PCr/Pi

Estrogen withdrawal induced a significant increase in β NTP/Pi and PCr/Pi ratios in BT-474 tumors on day 10. Higher levels of PCr in BT-474 tumors on day 10 after estrogen withdrawal were found; however, there were no differences in β NTP and Pi concentrations between estrogen-stimulated and estrogen-deprived BT-474 tumors. The observed increases in β NTP/Pi and PCr/Pi after estrogen withdrawal were consistent with the finding in a previous study of the effect of estrogen withdrawal on energy-rich phosphates in ^{31}P MRS of four human breast cancer xenografts (19,44). That study suggested that a decrease in the Pi was probably the explanation of the increase in β NTP/Pi and PCr/Pi; a switch from a glycolytic state to oxidative phosphorylation was followed by a decrease in NMR-detectable Pi and a corresponding increase in intramitochondrial Pi concentration. Estrogen may inhibit creatine kinase activity in ER positive tumors, which may lead to an increase in PCr concentration in estrogen-depleted BT-474 tumors. β NTP/Pi and PCr/Pi have been found to

be sensitive indicators of energy metabolism (44,47). The decrease in β NTP/Pi and PCr/Pi caused by decreasing the estrogen dose and the initial decrease in β NTP/Pi and PCr/Pi after estrogen withdrawal (day 2) may represent a decrease in the energy status of ER positive tumors due to the reduction of glycolysis that occurs in the absence of estrogen stimulation. These data are consistent with the observation of the early responses of ER positive tumor cells to tamoxifen (48); one of the early responses to estrogen is concerned with fast stimulation of energy production via glycolysis. Additionally, the energy of tumors also can be compromised by hypoxia and nutrient deficiency. We found increased ischemia in the large BT-474 tumors, which had a low ATP, a high Pi and a low PCr.

4. Study limitations

The use of ER+ breast cancer cell lines as xenograft models has enabled a wide variety of *in vivo* studies examining response to therapy, including anti-hormonal approaches (49). Experiments are usually performed in immunocompromised mice which may impact on tumor formation and progression. Although breast cancer cell line xenografts are imperfect, the choice of these tumor models for investigation of estrogen dependent MR parameters is reasonable. Further studies with genetically modified models and patient derived xenografts are feasible.

Conclusion

Lactate, PME/ β NTP, PCr/Pi and β NTP/Pi in the MR spectra of ER-positive tumors were closely related to ER-dependence.

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Abbreviations

ER	estrogen receptor
ET	endocrine therapy
TNBC	triple-negative breast cancer
PR	progesterone
HER2	human epidermal growth factor receptor 2
E₂	17 β -estradiol
HadSelMQC	hadamard-selective multiple quantum coherence

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Fig. 1.

The scheme for hormone treatments. (n=5 in each group at each time point) **a.** Animals were supplemented with or without 17 β -estradiol with dose of 0.18 or 0.72 mg/pellet (60-day release, Innovative Research of America) 7 days before inoculation with BT-474 tumor cells. As a control, animals were inoculated with HCC1806 tumor cells with or without estrogen supplement (0.72 mg/pellet). MRI and MRS were performed when the tumor volumes reached approximately 200 mm³ and 500 mm³. **b.** the mice carrying BT-474 and HCC1806 tumors received a 17 β -estradiol pellet (60-day release, 0.72 mg, Innovative Research of America) s.c. 7 days before tumor cell inoculation. When tumors reached 200 mm³, 17 β -estradiol pellets were removed from half of the animals (day 0). Lactate MR spectra, ³¹P MRS and DWI were performed on day 0, day 2 and day 10.

Fig. 2.

a. Effect of estrogen dose on growth of BT-474 and HCC1806 tumors. Tumor volume was measured on the days as shown. 17 β -estradiol (E₂) pellets were removed when tumors reached 200 mm³ (arrows). The values are presented as the mean (n=5) $\pm$ standard deviation (SD). **b.** Effect of estrogen dose on magnetic resonance (MR) signal intensity ratios of lactate/water, PME/ β NTP, β NTP/Pi and PCr/Pi. (* p<0.05; ** p<0.005)

Fig. 3.

Immunohistochemical staining and apparent diffusion coefficient ($ADC_{(low)}$) map of BT-474 tumors. $ADC_{(low)}$ value of small tumors (200 mm^3) (**a**) was more uniform and much higher in comparison to large tumors (500 mm^3) (**e**). Histology (H&E) and endothelial cell density (CD31, red) were compared. Small tumors (200 mm^3) showed no necrosis (**b** 1x magnification, **c** 4x magnification) and a uniform endothelial cell density (**d** 4x magnification). Large tumors (500 mm^3) showed some central necrosis (**f** 1x magnification, **g** 4x magnification) and a decrease in endothelial cell density (**h** 4x magnification).

Fig. 4. Typical ^1H MR Spectra with lactate editing and ^{31}P MR spectra of BT-474 (**a, c**) and HCC1806 tumors (**b, d**) on days 0 and 10 after estrogen withdrawal. **e** The changes in MR signal intensity ratio of lactate/water, PME/ β NTP, PCr/Pi and β NTP/Pi on days 2 and 10 after estrogen withdrawal.

Fig. 5.

The apparent diffusion coefficient (ADC) map and the ADC histogram of BT-474 and HCC1806 tumors on days 0 (**a** BT-474, **b** HCC1806) and 10 (**c** BT-474, **d** HCC1806). A right shift of ADC mean values was found on day 10 in the ADC histogram of BT-474 tumors; however, a left shift was found in HCC1806. **e.** The changes in ADC values of tumors on days 2 and 10 after estrogen withdrawal.

Table 1
The concentrations of metabolites in tumor extracts supplemented with or without 17β-estradiol (E₂)

	Lactate (μmol/g)	PC (μmol/g)	PE (μmol/g)	βNTP (μmol/g)	PCr (μmol/g)	Pi (μmol/g)
BT-474 0.72 mg E ₂ (small)	29.89±8.9	9.21±0.3	3.02±0.02	2.04±0.04	0.83±0.07	5.02±0.34
BT-474 0.72 mg E ₂ (large)	27.38±0.56	9.12±0.12	2.98±0.09	1.76±0.06	0.76±0.07	5.32±0.23
BT-474 0.18mg E ₂ (small)	6.18±0.45	5.04±0.34	2.08±0.12	1.73±0.09	0.94±0.05	6.22±0.33
HCC1806 0.72 mg E ₂ (small)	9.17±0.23	4.28±0.22	0.58±0.04	1.54±0.09	0.65±0.03	6.22±0.21
HCC1806 0.72 mg E ₂ (large)	5.02±0.44	4.09±0.12	0.52±0.04	0.84±0.11	0.56±0.03	7.36±0.56
HCC1806 0 mg E ₂ (small)	9.13±0.22	4.3±0.21	0.59±0.03	1.53±0.09	0.63±0.04	6.23±0.23
HCC1806 0 mg E ₂ (large)	4.98±0.33	4.1±0.23	0.52±0.04	0.84±0.07	0.56±0.04	7.32±0.56

Data are expressed as the mean ± SD. PC, phosphocholine; PE, phosphoethanolamine; PME, phosphomonoester; PCr, phosphocreatine; βNTP, β-nucleoside triphosphate; Pi, inorganic phosphate. PME=PC+PE (n=5 in each group)

Table 2

The concentrations of metabolites in tumor extracts on day 10 after 17β-estradiol (E₂) withdrawal

	Lactate (μmol/g)	PC (μmol/g)	PE (μmol/g)	βNTP (μmol/g)	PCr (μmol/g)	Pi (μmol/g)
BT-474 E ₂ -	4.18±0.33	5.19±0.45	1.70±0.09	2.03±0.11	1.03±0.05	5.03±0.34
BT-474 E ₂ +	28.89±0.93	9.18±0.54	3.00±0.74	2.03±0.56	0.58±0.04	5.0±0.78
HCC1806 E ₂ -	8.56±0.34	4.30±0.56	0.58±0.07	1.32±0.07	0.61±0.07	6.87±0.76
HCC1806 E ₂ +	8.33±0.42	4.01±0.35	0.51±0.07	1.40±0.07	0.69±0.06	6.80±0.66

Data are expressed as the mean ± SD. PC, phosphocholine; PE, phosphoethanolamine; PME, phosphomonoester; PCr, phosphocreatine; βNTP, β-nucleoside triphosphate; Pi, inorganic phosphate. PME=PC+PE (n=5 in each group)