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Transcriptomic and Metabolomic Differentiation of Breast Cancers: Progression and Therapy Response via Magnetic Resonance Spectroscopy and Quantitative Expression Profiling in the Choline pathway

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Abstract

Tumor choline metabolites have potential for use as diagnostic indicators of breast cancer progression and can be non-invasively monitored *in vivo* by magnetic resonance spectroscopy. Both a ³¹P MR visible phosphomonoester peak (~3.9 ppm) and a total choline metabolite peak (~3.2 ppm) detected by ¹H MRS are elevated in human breast tumors. As determined by tumor extract studies, the principle diagnostic component of these peaks is phosphocholine (PCho), the biosynthetic precursor to the membrane phospholipid, phosphatidylcholine (PtdCho). In extracts of human breast cells, PCho and the PtdCho breakdown product, glycerophosphocholine, are incrementally increased in cell lines of increasing metastatic potential. The ability to resolve and quantify PCho *in vivo* would improve the precision of this putative diagnostic tool. Additionally, determining the biochemical mechanisms underlying these metabolic perturbations will improve the understanding of metastatic cancer and could suggest potential molecular targets for drug development. Reported herein is the *in vivo* resolution and quantification of PCho in a breast cancer xenograft model in SCID mice via image-guided ³¹P MR spectroscopy, localized to small voxels. Also reported is the quantification of cytosolic and lipid metabolites in breast cancer cells of increasing cancer progression, and the identification of metabolites that differ amongst cell lines by degree of cancer aggressiveness. These metabolic differences are correlated with differences in expression of genes of the choline metabolic pathway. Gene expression changes following taxane therapy are also correlated with previously reported changes in choline metabolites following the same therapy in the same tumor model. Biochemical models explaining the metabolic changes are discussed.

Keywords

Choline; phosphatidylcholine; metabolism; gene expression; breast; cancer

Introduction

Breast cancer is a significant factor in women's health worldwide. In the US, approximately 212,920 new cases of invasive breast cancer, 61,980 in situ cases and 40,970 deaths were predicted for 2006 (1). Diagnosis and choice of appropriate therapy is complicated by the heterogeneous nature of the disease. Breast cancer is presented in a range of histological types and molecular profiles (2). Seventy percent of cases are classified as infiltrating ductal carcinoma, but there is considerable heterogeneity of gene expression and a range of therapeutic outcomes in these cases. A number of additional classifications exist, each type with a general prognosis but with considerable heterogeneity of therapeutic response.

The most established prognostic markers are involvement of axillary lymph nodes, tumor size and histologic grade. A number of molecular markers are also being investigated for proliferation (Ki-67, PCNA); expression of oncogenes (HER-2, EGF receptor); tumor suppressor activity (p53, nm-23); invasiveness (cathepsin D, plasminogen activator inhibitor and urokinase-plasminogen activator receptor); and angiogenesis (VEGF) (3). DNA arrays have established expression patterns of 70 genes involved in cell cycle regulation, invasion and metastasis, angiogenesis and signal transduction that are highly predictive of poor prognosis in breast cancer (4).

Non-invasive magnetic resonance spectroscopy (MRS) localized to the tumor can also provide useful information about tumor progression and metabolism. MR-visible choline metabolites are elevated in breast cancer (5-8). The ^{31}P MRS visible phosphomonoester (PME) resonance (~3.9 ppm) of breast tumors is elevated relative to normal tissue, yet does not yet accurately differentiate benign from metastatic cancerous lesions (9-13). MRS of tumor extracts have established that the PME signal is a composite resonance, primarily of phosphocholine (PCho), phosphoethanolamine (PEtn) and sugar phosphates (14-16). PCho is implicated as the elevated component of the PME resonance in breast tumors (17,18).

In more recent years, the identification of elevated choline metabolites *in vivo* by ^1H MRS was explored in breast tumors and axillary lymph node metastases (19-26). At lower field strengths, detection of the total choline (tCho) resonance (~3.2 ppm) has distinguished malignant from benign tumors with high specificity and sensitivity (27-29). However, as higher field strengths were used clinically, tCho peak detection limits were lowered, requiring the quantification of relative tCho levels (30). Quantification at low field strength (1.5 T) was subsequently achieved (31). The same group performed a clinical blind study at 4 Tesla demonstrating that when MRS and MRI readings are combined the sensitivity and accuracy of radiological assessments were significantly improved (32).

Studies of tumor extracts have identified individual components of the tCho resonance and PCho is determined to be the most diagnostic component (33). Use of the PME and tCho resonance to stage breast cancer progression is limited by the inability to resolve PCho *in*

vivo. Since PCho appears to be the most diagnostic component, differences in other components of these resonances may dampen the effect. By image localized ^{31}P MR spectroscopy, the resolution and quantification of PCho in small voxels of human breast cancer cell xenografts in SCID mice was recently reported (34).

High resolution NMR studies of breast cancer cell extracts have determined that PCho increases with cancer progression (35-37). PCho is a cytosolic intermediate for the synthesis of the lipid membrane component, phosphatidylcholine (PtdCho). NMR of breast cells is used to study the underlying biochemistry behind these cancer-related differences in choline metabolite levels. Transport of choline into MCF-7 breast cancer cells is 2-fold higher relative to human mammary epithelial cells (HMECs), and conversion of choline (Cho) into PCho by choline kinase is 10-fold higher, but conversion of PCho into PtdCho occurred at a similar rate (38). Glycerophosphocholine (GPC) is a degradation product of PtdCho. GPC is also elevated with breast cancer progression and relative PCho/GPC ratios are reported to increase dramatically in cells during log phase growth (37). Determining the biochemical mechanisms behind these metabolic perturbations will improve the understanding of metastatic breast cancer and may suggest novel molecular targets for drug development.

This communication is a study of the breast cancer metabolome. In particular, choline metabolites are quantified and compared with the choline pathway transcriptome for the same tumors and cells. Quantification of PCho and GPC *in vivo* in tumorigenic (MCF-7/S), doxorubicin resistant (MCF-7/D40) (39) and metastatic (MDA-mb-231) breast tumor xenografts is reported. Other choline compounds are quantified from tumor extracts. Also reported are the relative concentrations of lipid and cytosolic metabolites in breast cells of increasing cancer progression (normal MCF-10A, tumorigenic MCF-7 and metastatic MDA-mb-231) (40) and differences identified in choline metabolites and other metabolites varying amongst these cells. These results are correlated with quantification of mRNA expression of genes in the choline pathway. Alterations in gene expression are also correlated with changes in choline metabolites in response to therapy (34). These data suggest that a combination of anabolic and catabolic perturbations in PtdCho metabolism contribute to the alterations in choline metabolism observed with cancer progression and response to therapy.

Materials and Methods

Tumor Model

As a model for tumor therapeutic response, human tumor xenografts have been grown in SCID mice and effectively treated (41-43). In the context of therapeutic response, diffusion MRI and spectroscopy results for the animals discussed in this communication have been previously reported (34,44). Therefore methods will be presented in brief. MCF-7/S (Arizona Cancer Center Cell Culture Shared Services, Tucson, AZ), MCF-7/D40 (Arizona Cancer Center Experimental Mouse Shared Services) and MDA-mb-231 (American Type Culture Collection, Rockville, MD) human breast cancer cells were grown in DMEM-F12 media (Sigma, St. Louis, MO) supplemented with 10% FBS (Omega Scientific, Inc., Tarzana, CA), detached using trypsin (GIBCO/Invitrogen life technologies, San Diego, CA) or scraping, and placed in a 1:1 suspension of Hank's buffered saline (Sigma) and matrigel (Becton Dickinson Labware, Bedford, MA). Cells (1 to 2×10^7) were injected into the

mammary fat pad of 5 to 7 week old female SCID mice (Arizona Cancer Center Experimental Mouse Shared Services). Xenografts were allowed to grow into tumors.

In vivo localized ^{31}P MR spectroscopy

Our method for *in vivo* image-localized spectroscopy has previously been reported in detail (34). Briefly, image-guided ISIS (45) or OSIRIS (46) localized spectroscopy was performed using quantitative acquisition parameters, at a field-strength of 81.15 MHz, spectral width of 8013 Hz, acquisition size of 4096 data points, T_R of 10 s, either 23 (184/8) averages for an acquisition time of 1/2 h or 46 (368/8) averages for an acquisition time of 1 h, a secant hyperbolic inversion pulse, an adiabatic fast passage excitation pulse, a Hermitian refocusing pulse, and a dwell time of 62.4 μsec were used. Localized autoshimming on the water resonance was accomplished using a PRESS sequence. Voxel size was variable (0.245 to 1.450 mL) and set within the boundaries of the tumor. Mean voxel volume was 831 ± 62 μL for MCF-7 tumors, and 436 ± 7 μL for MDA-mb-231 tumors. Spectral processing included an exponential line broadening factor of -30 and a Gaussian factor of 0.06, manual phasing and baseline correction, and calibration of the α -NTP peak was (-10.05 ppm). Peak areas were determined using a Levenberg-Marquardt deconvolution routine. Individual peak areas were normalized to the total phosphate signal.

Cell Culture

MCF-10A cells were obtained from the Michigan Cancer Foundation (now Karmanos Cancer Institute, Detroit, MI), MCF-7 cells were obtained from the Arizona Cancer Center cell culture shared service (Tucson, AZ) and MDA-mb-231 cells were obtained from the American Type Culture Collection (Rockville, MD). All cell lines have been maintained at low passage number. Commercial sources of cell growth medium are as follows: EGF (Upstate Biotechnology, Lake Placid, NY), Fetal bovine serum (FBS) (Omega Scientific, Inc., Tarzana, CA), Trypsin (Invitrogen life technologies, San Diego, CA). All other media, chemicals and reagents were purchased from Sigma, St. Louis, MO) unless otherwise stated. Cells were grown in DMEM-F12 media with 5 mg/mL insulin. Due to EGF dependence for growth (47), 20 ng/mL EGF was added to MCF-10A media. Media contained 10% FBS of the same lot.

Dual-Phase Tumor and Cell Extract

Tumor metabolites were extracted via a dual-phase extraction (DPE) method previously described by Katz-Brull, *et al.* (48). Frozen tumors were placed in 10 mL ice-cold 0.4 mM phenylphosphonic acid in methanol for 1 h, after which the tumor tissue was homogenized to an even consistency using a Dremel hand-held homogenizer [Racine, WI] while maintaining on ice. Equal volumes (10 mL) of ice-cold chloroform and distilled and deionized water were sequentially added to the homogenate followed by vigorous vortex mixing after each addition. The mixture was left overnight in the -20°C freezer allowing phase separation.

Human breast cancer cells representing a range of cancer progression were used (40). Prior to treatment, MCF-10A (normal), MCF-7 (tumorigenic) and MDA-mb-231 (tumorigenic and metastatic) cells were grown to steady-state (confluence + 1 d) in order to best model a

tumor. For metabolite comparison amongst cell lines, media was replaced and cells were allowed to grow for an additional 24 h. Replaced media contained a dilute concentration of drug carrier which we have previously shown to have no effect on cell viability or growth (40). Dual-phase cell extracts were performed as described by Tyagi, *et al.* (49). Four extracts per treatment group. Immediately following three initial 0.9% saline washes at room temperature, plates were placed on ice and covered during the 10 to 20 minute incubation in ice-cold methanol. Cells in methanol were scraped from each plate and ice-cold water and chloroform were added in succession with vortexing between additions. Equal volumes of methanol, water and chloroform were used (1:1:1). Extracts were kept overnight at 4°C for phase separation.

Following phase separation of both tumor and cell extracts, the upper methanol-water and the lower chloroform phases were transferred into separate containers. Methanol was reduced from the aqueous phase by blowing nitrogen gas over the mixture while on ice and samples were frozen in liquid nitrogen and lyophilized at -80°C. After lyophilization, water-soluble metabolites were resuspended in 1 mL of $^2\text{H}_2\text{O}$. Chelex (~5 mg) was added to the resuspendate and removed by centrifuge. For ^1H spectroscopy, 400 μL of resuspendate were added to a 5 mm NMR tube (Wilmad, Buena, NJ). For ^{31}P spectroscopy, EDTA (pH 8) was added to reach a final concentration of 10 mM. Tumor samples were then diluted 1:1 in tricine buffer (pH 8.1) bringing the sample pH to within the range of pH 7.8 to 8.0. Cell extracts were already in the appropriate pH range for resolution of peaks (~8.5) so adjustment was not necessary. Protein at the interface between the aqueous and lipid phases was air dried, resuspended in 0.1 N NaOH, let stand for 30 minutes, boiled 5 minutes and neutralized in HCl. Protein content was determined via Bradford assay using BSA standards (50). Before resuspending, samples were maintained at -20°C. Resuspended samples were maintained on ice or at 4°C.

High-Resolution NMR Spectroscopy

High-resolution spectroscopy of extracts was performed on a Bruker-Biospin DRX-500 NMR spectrometer [Billerica, MA]. Phosphorous spectroscopy of the aqueous and lipid phases was performed using a Bruker-Biospin 5 mm multinuclear probe at a field strength of 202 MHz with an acquisition size of 65,000 data points, a sweep width of 50 ppm, T_R of 12 s, with 5000 scans for aqueous extracts and 1200 scans for lipid extracts. Linewidths were < 1 Hz for aqueous extracts and < 2 Hz for lipid extracts. Processing included zero filling and a line broadening factor of 1 Hz was applied. As a standard, a 10 mM 1-aminopropylphosphonic acid (1-APP) in $^2\text{H}_2\text{O}$ was used. Proton spectroscopy was performed using a Bruker-Biospin 5 mm triple-resonance triple-gradient probe at a field strength of 500 MHz with an acquisition size of 32,000 data points, a sweep width of 20 ppm, T_R of 3.6 s, with 256 scans. Linewidths were ~ 3 Hz. Processing included zero filling and a line broadening factor of 0.3 Hz was applied. As a standard, 0.5 mM 3-(trimethylsilyl) tetra-deutero sodium propionate (TSP) in $^2\text{H}_2\text{O}$ was used. 90° pulse angles were applied for quantitative results. T_1 tests determined that acquisition conditions were quantitative ($5 \times T_1$) for all resonances analyzed except for inorganic phosphate (Pi) during ^{31}P spectroscopy. Pi was determined to be 81.9% of the fully relaxed signal, and the appropriate correction was applied.

Tumor extract and lipid phase cell extract standards were placed into a Wilmad 5 mm O.D. stem coaxial insert [Buena, NJ] and inserted into the sample NMR tube. For aqueous phase of cell extracts, standards were sealed in a capillary tube and placed in the NMR tube. The ratio of sample to standard was determined from manufacturer's specifications of the NMR tube and insert, or from a cross-section image of the capillary tube by which the inside and outside diameters were determined. For cell extracts, initial spectra were acquired without the standard. A second spectrum was acquired with the standard without spinning. Since the initial spectra were of higher resolution due to the spinning, a ratio of the standard peak and the largest metabolite peak was determined from the standard spectra and applied to all peaks of the higher resolution spectra.

Peak assignments were made by addition of known metabolites and from the literature (51). Peak areas were determined using a deconvolution routine based on the Levenberg-Marquardt algorithm. The signal to noise ratio was used to determine values for undetectable peaks with the ratio being defined as: largest peak intensity/(2*noise intensity). Undetectable peaks were given the value of 2X noise, which is the largest peak area divided by the signal to noise ratio. The deconvolution linefit was assessed for each peak. Peaks with poor linefits due to very low signal-to-noise ratio were removed from the analysis. All peak areas were normalized to the total aqueous phosphorous signal for a given extract. Cho peak areas were normalized by applying the Cho/PCho ratio from the ^1H spectra to the PCho/total aqueous phosphorous ratio from the ^{31}P spectra of the same extract. Lipid peak areas were normalized by applying the ratio of lipid peak concentration ($\text{nmol} \cdot \text{mg protein}^{-1}$) / PCho concentration ($\text{nmol} \cdot \text{mg protein}^{-1}$) to the PCho/total aqueous phosphorous ratio from the ^{31}P spectra of the same extract. Excel spreadsheet (Microsoft, Seattle, WA) was used to manually align acceptable signals within spectra from each cell line ($n = 4$). Alignments were then made of signals amongst all cell lines. Mean peak area and standard error of the mean (sem) were calculated for each peak by cell line. Significant differences in mean peak areas amongst cell lines were determined by t-test.

RNA extraction

For comparison of gene expression amongst cell lines, cells were grown one day past confluence, media was then replaced with media containing dilute drug carrier and incubated an additional 24 h before RNA extraction. For gene expression response to therapy, replacement media contained 10 nM docetaxel or drug-carrier only as a no drug control, and cells were incubated an additional 48 h before RNA extraction. RNA was extracted using the GenElute™ Mammalian Total RNA Miniprep kit (Sigma, St. Louis, MO). To remove genomic DNA contamination, RNA extracts were treated with DNase using the DNA-free™ kit (Ambion, Austin, TX). RNA purity and concentration were determined by A260/A280 ratio.

Real-time RT-PCR

Primer sets were designed by us to generate cDNA and perform RT-PCR from β -actin, CTL1, CK1, CHKL, CTP1 α , CTP1 β , CPT, PLD1 α , PLD2 α and PLD2 β mRNA. Primers were synthesized by Invitrogen life technologies (San Diego, CA). PCR conditions were optimized for each primer set by varying annealing temperature and, if necessary, Mg^{++}

concentration so that maximum yield without spurious priming was achieved. RNA extracts from multiple cell lines were used as template RNA during optimization (Table 1).

Real-time RT-PCR was conducted using a Smart Cycler® (Cepheid, Sunnyvale, CA) and, for comparison of gene expression between cell lines, the SuperScript™ One-Step RT-PCR system with Platinum® Taq (Invitrogen life technologies, San Diego, CA) was used. For detection of gene expression response to therapy, the HotStarTaq DNA polymerase kit was used (Qiagen, Valencia, CA). Real-time detection was acquired using a SYBR-green detection format (Molecular Probes, Eugene, OR). Reverse transcriptase (RT) conversion of RNA into cDNA was performed during a 15 min (SuperScript™) or 20 min (HotStarTaq) incubation at 52°C (SuperScript™) or 50°C (HotStarTaq), which was immediately followed by a 15 min incubation at 94°C (SuperScript™) or 95°C (HotStarTaq) to stop the reaction and denature the cDNA. Next, 35 cycles were run with a 94°C melting temperature for 15 s, and a primer-set specific temperature for 60 s (annealing/extension, SuperScript™) or 30 s (annealing, HotStarTaq). HotStarTaq runs had an additional 20 s extension step at 72°C. To control for DNA contamination of the RNA extracts, a no-RT reaction was run for each extract. A no-template reaction was included during each experiment to control for DNA contamination in the reagents. In some cases, amplification of primer-dimers was observed in the no-template control at later cycles. In all cases, amplification of template occurred prior to primer-dimer amplification and primer-dimer amplification was not detected before template fluorescence had reached a maximum limit. Melt curves ranging from 60 to 90°C yielded a single melt-peak for all template reactions and a minimal melt peaks for the no-template control reaction.

Raw mRNA expression values were determined as being 2^{-C_T} , where C_T is the number of cycles required for the SYBR green I fluorescence to cross the threshold of 30 arbitrary fluorescence units (SuperScript™), or the second derivative of the fluorescence curve (HotStarTaq). Expression of individual genes in the choline metabolism pathway was normalized to β -actin expression. We have previously shown that β -actin expression is reasonably invariable between these breast cancer cell lines (52). Three extracts per cell line were analyzed ($n = 3$). Repeatability of measurements by this method is high (Cronbach's alpha of 0.93) (52), so only one run per primer set per extract was performed. Results are reported as the mean and error is reported as standard error of the mean (sem). In some cases when using SuperScript™, primer-dimer formation was observed in conjunction with mRNAs expressed at very low levels. These transcripts are reported as being expressed at low, unquantifiable levels.

Results

In Vivo and Ex Vivo Quantification of Tumor Choline

Image-guided, localized ^{31}P MR spectroscopy was performed on MCF-7/S ($n = 16$), MCF-7/D40 ($n = 17$) and MDA-mb-231 ($n = 23$) mammary fat pad xenografts in SCID mice. PCho and GPC peaks were resolved and quantified (Figure 1A). Peak areas normalized to the total tumor phosphate signal were determined for PCho and GPC (Table 2). *Ex vivo* spectra from corresponding tumors and phantom studies are used to demonstrate that this method is quantitative (34). Following spectroscopy, tumors were excised and both

aqueous and lipid metabolites were extracted using a dual-phase method (48). High resolution NMR spectroscopy was performed on the aqueous and lipid phases of the tumor extracts for peak identification. Peaks representing choline metabolites (PCho, GPC and Cho) were resolved and identified in ^{31}P spectra (Figure 1B) and ^1H spectra (1C) of aqueous phase samples. Choline phospholipids and metabolites (PtdCho, PtdCho plasmalogen and Lyso-PtdCho) were identified in ^{31}P spectra of non-aqueous extracts (1D). All tumor choline metabolite peaks were normalized to the total aqueous phosphorous signal and therefore concentrations can be directly compared (Table 1). *In vivo* PCho was significantly higher in doxorubicin-resistant MCF-7/D40 tumors ($p < 0.01$ relative to MCF-7/S and $p < 0.001$ relative to MDA-mb-231 tumors). GPC and the PCho/GPC ratio did not vary significantly between tumor types. Extracts were primarily used for peak identification and hence, the numbers were too low to determine statistical significance *ex vivo*. However, *ex vivo* quantifications generally agreed with *in vivo*. Cho and phospholipid levels were only identifiable *ex vivo*.

In Vitro Quantification of Choline Metabolites

Quantitative extracts were obtained from cultured cells which not only allowed careful control over conditions but also allowed comparison to non-tumorigenic cells, *viz.* MCF-10A. Both ^1H and ^{31}P high resolution MR spectra were acquired from the aqueous phase of MCF-10A, MCF-7 and MDA-mb-231 cell extracts. Cells were grown to steady-state before extraction in order to mimic tumor conditions. Figure 2 shows the choline region of ^1H spectra (left column) and the PME and PDE region of ^{31}P spectra (right column). Choline phospholipids were identified in ^{31}P spectra of the lipid phase (Figure 3). Cellular metabolites were normalized as both nmols per mg protein, or as a ratio of metabolite peak area to total aqueous phosphorous peak area. Similar peak profiles were observed by either method of normalization (Figure 4), but the peak area ratios yielded considerably lower error estimates (4A versus 4B), implying a higher degree of precision when quantifying as a percentage of total aqueous phosphorous. Both cytosolic and lipid choline metabolites were quantified as a percentage of total aqueous phosphorous and are reported in table 3.

In vitro, PCho increased significantly by degree of cancer progression. The lowest PCho concentrations were observed in normal MCF-10A cells. Tumorigenic MCF-7 cells had significantly higher PCho, and metastatic MDA-mb-231 cells had yet a significantly higher concentration. This same profile is seen for the phosphatidylcholine (PtdCho) degradation product, Lyso-PtdCho. GPC also appears to increase by degree of cancer progression, with MCF-7 and MDA-mb-231 concentrations being significantly higher than in MCF-10A cells, although the differences between the tumorigenic cell lines were not significant. This same effect is seen with the PCho/GPC ratio, where the cancer cell lines do not have significantly different ratios, but both ratios are significantly lower than that of the normal cell line. While PCho levels are higher in cancer cells, the inverse is seen for Cho concentrations. Cho is significantly lower in MCF-7 and MDA-mb-231 cells compared to MCF-10A cells. However, Cho is lowest in MCF-7 cells. There were no significant differences observed in the phospholipid PtdCho or the etherlipid PtdCho plasmalogen concentrations.

In Vitro Quantification of All Metabolites Relative to Cancer Progression

In addition to the choline metabolites, quantifications of all metabolites were compared amongst cell lines to determine significant differences correlated with cancer progression. Four regions of high peak density were identified in ^{31}P MR spectra of the extract aqueous phase (Figure 5): the phosphomonoester (PME)/nucleotide monophosphate (NMP)/sugar phosphate region (5A), the nucleotide triphosphate- γ (NTP γ) region (5B) which also includes nucleotide diphosphate- β resonances (NDP β), the NTP α region (5C) which also includes NDP α 's, nicotinamide adenine dinucleotides (NADs), and UDPG's (uridine diphosphate-glucourinate, -glucose, -galactose, -*N*-acetylglucosamine, -*N*-acetylgalactosamine), and the NTP β region (5D). Table 4 is a listing of metabolites which increase with cancer progression. These include the cytosolic phospholipid synthesis and degradation intermediates PCho, Lyso-PtdCho, GPC and GPE, and the phospholipid cardiolipin (diphosphatidylglycerol). Also, some sugar phosphates were elevated including glycerol-3-phosphate and fructose-1,6-diphosphate. A single uridine diphosphate-glucose- β (UDPGlc β) resonance increased.

Table 5 includes metabolites which are significantly higher or lower only in the metastatic MDA-mb-231 cell line, although a number of them appear to increase incrementally with cancer progression but are below detection limits needed to verify this statistically. These include a number of unidentified resonances in the sugar phosphate range. Both table 4 and 5 show NTPs which increase or decrease, with the majority being UTP/CTP coresonances. Table 6 is a listing of metabolites that are higher or lower only in MCF-7 cells. Generally, metabolites increased in MCF-7 cells were NDP, NAD, and UDPG resonances. Two lipid resonances were lower in MCF-7 cells, phosphatidylethanolamine (PtdEtn) plasmalogen and an unidentified lipid at -0.225 ppm. A number of metabolites decreased with cancer progression (Table 7). These were Cho, phosphoethanolamine (PEtn), phosphocreatine (PCr), adenosine monophosphate (AMP), NDP α , and various NTPs. Metabolites remaining unchanged with cancer progression (Table 8) included two unidentified sugar-phosphates, inorganic phosphate (Pi), NDP β , a number of UDPG and NAD resonances, and the majority of phospholipids.

Choline Pathway Gene (mRNA) Expression

The choline metabolic pathway is well characterized and gene sequences of enzymes involved in the biosynthesis of phosphatidylcholine (PtdCho) are known (Figure 6) (7,8,53). PtdCho catabolism is mediated by three classes of PtdCho specific phospholipases (PLA $_2$, PLC and PLD). Each class mediates PtdCho degradation by a separate pathway (Figure 6), but all can mediate the conversion of PtdCho back into cytosolic Cho or PCho. Using real-time RT-PCR, the relative mRNA expression of choline metabolism genes in normal and cancerous breast cells is quantified (Table 9). Expression is normalized to β -actin mRNA, which is known to be relatively unchanged amongst these cell lines (52) and is determined from multiple RNA extracts ($n = 3$).

Choline kinase activity mediates the conversion of intracellular choline (Cho $_i$) into PCho. Expression of the choline kinase gene (CK) appears to be higher in cancer lines but the difference is not statistically significant. Choline-kinase like gene (CHKL) is the result of a

recent gene duplication event of CK and is highly homologous (54), however CHKL's biological activity has not been determined. These analyses detected CHKL transcripts at levels comparable to those of CK. CHKL mRNA is expressed at significantly higher levels in MDA-mb-231 cells but at significantly lower levels in MCF-7 cells. This is the first record of CHKL being expressed in breast cancer. The CTP:PCho cytidylyltransferase (CTP1) gene product converts PCho and CTP into CDP-choline. CTP1 has undergone a gene duplication event and is known to be expressed as both α and β forms. CTP1 α was expressed in all of the breast cancer cell lines, but at significantly lower levels in MDA-mb-231 cells. Expression of CTP1 β was also detected but at levels too low to accurately quantify. Choline phosphotransferase (CPT) activity converts CDP-Choline into PtdCho. CPT transcript was expressed at significantly higher levels in MDA-mb-231 cells. PLD mediates the conversion of PtdCho into Cho and phosphatidate (PA). PLD1 was found to be expressed at low unquantifiable levels. PLD2 is known to be expressed as two variants (α and β). Both variants were expressed in the breast cancer lines, however, PLD2 α expression was significantly higher in MCF-10A cells and PLD2 β expression was significantly lower in MCF-7 cells. Gene expression of PtdCho -PLC and PLA₂ were not determined.

In addition to genes involved in PtdCho biosynthesis and catabolism, expression was determined for a number of putative choline transporters and their splice variants, including CHT1, OCT1, OCT2 and CTL1 (55). High expression was observed in all cell lines for CTL1 splice variant A (Table 9) and no expression was observed for variant B. Although expression appears to decrease with cancer progression, the values were not significantly different. Very low expression of OCT1 was detected and expression of CHT1 and OCT2 was undetectable (data not shown).

Changes in Expression of Choline Metabolism Genes in Response to Docetaxel

Significant changes in choline metabolite concentrations in these same breast cancer cell lines, 48 h after treatment with 10 nM docetaxel (34). In MDA-mb-231 cells, expression of the putative choline transporter, CTL1A (55), increased significantly 48 h post treatment with 10 nM docetaxel (Figure 7A). CTL1A also appears to increase in the cancer line MCF-7 but not with significance. The normal MCF-10A cell line did not increase CTL1A expression post-treatment. A similar profile was seen for CK1 expression response (7B), with significant increases in MDA-mb-231. CHKL, CTP1 α and PLD2 β all had similar expression response profiles with small post-treatment decreases in expression. Of the three, only PLD2 β expression decreased significantly in the metastatic MDA-mb-231 cell line.

Discussion

Tumor choline metabolites are being studied as potential diagnostic markers for breast cancer which can be noninvasively detected and quantified via magnetic resonance spectroscopy (7,8). Herein, the *in vivo* detection and quantification of PCho and GPC in three different breast cancer xenograft tumor types in SCID mice (MCF-7/S, MCF-7/D40 and MDA-mb-231) is reported. Also reported is the *ex vivo* detection of tumor cytosolic and lipid choline metabolites. By MR spectroscopy of *in vitro* cell extracts, including two of the cancer cell lines and a normal breast line, an incremental increase in PCho, GPC and Lyso-

PtdCho in cell lines of increasing cancer progression, and a general decrease in Cho are observed. Additionally, differences in other metabolites parsed by cancer progression are quantified.

In vivo results are not always recapitulated *in vitro*. Although generally elevated, PCho was quantifiably higher in the metastatic MDA-mb-231 cells *in vitro*, but not significantly different from MCF-7 tumors *in vivo*. We have previously shown that MDA-mb-231 tumor xenografts are more necrotic *cf.* MCF-7 tumors (44). Hence, this discrepancy might be explained by the partial volume effects of having a lower proportion of viable cancer cells in the whole tumor volume when compared to MCF-7 tumors. How this partial volume issue might influence clinical measurements is not entirely clear since the xenograft model is not necessarily an ideal model of human breast tumors. Differences in tumor perfusion and host interactions could have an effect on the proportion of viable cells to necrotic cells within a tumor.

Lowered Cho concentration in the cancerous cells implies lower choline transport and/or increased CK activity. A 2-fold increase in MCF-7 choline transport relative to HMECs was reported (38). Additionally, lower transport would be inconsistent with the observed elevation of PCho in cancer lines. An elevation in transcription of the kinases, CK1 and CHKL is observed in the cancer cells. This observation is consistent with reports of increased CK activity in breast cancer cells of increasing tumor grade (38,56), increased CK mRNA expression by micro array (57), and decreased PCho following treatment with CK inhibitors or RNAi (58,59). Increased CK activity would at least in part explain the observed lower levels of cytosolic Cho and elevated levels of PCho.

Significantly decreased CTP1 α mRNA is observed in metastatic cancer cells, which codes for CTP:PCho cytidyltransferase (CCT). This enzymatic step is rate-limiting for the generation of PtdCho from PCho, and CCT activity is subject to regulation at the level of transcription (60) and by higher-order, post translational regulatory mechanisms (61,62). CCT tightly regulates membrane lipid homeostasis and it is proposed that depletion of PtdCho in the membrane is followed by CCT activation. Regardless of higher order regulation, decreased transcript levels could lead to decreased CCT levels, translating to lower enzyme activity, and contributing to the elevation of PCho. Decreased CCT activity in NIH 3T3 fibroblasts was observed following Ha-ras transformation (63), which would be consistent with a role for CCT in the elevation of PCho in malignant cells.

Increased PtdCho degradation products in the phospholipase A₂ (PLA₂) pathway are detected (Figure 6). Both Lyso-PtdCho and GPC are products of PLA₂ degradation of PtdCho and are significantly elevated by degree of cancer progression. GPC is converted to Cho and glycerol-3-phosphate (Gro3P) by GPC phosphodiesterase (64). Elevated Gro3P is observed incrementally by cancer progression. Based on this metabolic evidence, PLA₂ activity appears to be elevated by degree of cancer progression. This increased activity would add to the PCho pool by providing Cho substrate for CK (65). These observations are possibly incongruous with the recent reporting of decreased PLA mRNA by micro array (57), however decreased mRNA expression does not necessarily lead to decreased levels of gene product or enzyme activity if other levels of regulation exist. Our result of decreased

PLD mRNA in cancer is in agreement with the micro array data (57). Increased PLC activity was also reported by micro array (57), however we did not include PLC in our study. A number of PLC genes and activities have been identified, but we were not able to identify a PLC clone with activity specific for PtdCho.

Elevated transcription of genes does not necessarily translate to elevated gene product within the cell or elevated enzyme activity, but in many cases increased transcription does lead to increased activity. The combination of elevated choline metabolites and elevated expression of genes in the choline pathway suggests that multiple mechanisms are involved in generating increased tumor PCho (Table 10). Increased CK and PLA activity generating more PCho, coupled with tight regulation of CCT activity would lead to a buildup of PCho.

Changes in expression of genes in the choline pathway were observed following docetaxel therapy. However, it is not simple to explain the coincident PCho decrease and GPC increase from the observed changes. Expression of both the choline transporter and kinase are increased, either of which would intuitively suggest an increase in PCho. Note that the observed decrease in PCho is coupled to an increase in GPC, which suggests increased PtdCho turnover. Since the homeostasis of membrane lipid content is tightly regulated by CCT, increased PtdCho turnover would suggest CCT activation and utilization of the PCho substrate pool. CTP1 mRNA did not increase, but, as discussed above, this gene's product, CCT, is regulated post-translationally by a number of mechanisms (61,62). CCT activity increases following treatment of MCF-7 breast cancer cells with tumor necrosis factor (TNF) (66), which is consistent with a role for CCT in depletion of PCho following therapy. A significant therapy-induced decrease in PLD2 β expression was also observed, which could contribute to the decrease in PCho levels by reducing the amount of choline substrate available to choline kinase. Further elevation of GPC post treatment suggests an additional increase in PLA₂ activity. However, additional Gro3P was not observed post treatment (data not shown), suggesting that GPC phosphodiesterase activity is decreased. In this case, the degradation of GPC into choline would decrease and could contribute to the decrease in PCho by reducing the amount of choline substrate (Table 10).

In general terms, the evidence points toward the combination of elevated choline transport and kinase activity, decreased CCT activity and increased phospholipase activity as the factors leading to increased PCho in breast cancer. In contrast, the mechanism behind post therapy decreases in PCho appears to be a combination of increased CCT activity and decreased phospholipase and GPC phosphodiesterase activities. These conclusions are based on metabolite differences, changes in gene expression and reports from other groups. Absolute differences in enzyme activities have yet to be determined.

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Figure 1.

MR Spectra of MCF-7 tumor metabolites: localized ^{31}P MRS in vivo (A), ^{31}P MRS ex vivo, PME and PDE region (B), ^1H MRS ex vivo, choline region (C), and ^{31}P MRS ex vivo, lipid phase. Peak abbreviations are: composite phosphoethanolamine / nucleotide monophosphate / sugar phosphate peak (PEtn'), phosphocholine (PCho), inorganic phosphate (Pi), glycerophosphoethanolamine (GPE), glycerophosphocholine (GPC), phosphocreatine (PCr), nucleotide triphosphate ($\text{NTP}\gamma$), cardiolipin (CL), phosphatidylethanolamine plasmalogen (PtdEtn plas), phosphatidylethanolamine (PtdEtn), lyso-phosphatidylcholine (Lyso-PtdCho), phosphatidylinositol (PI), phosphatidylcholine plasmalogen (PtdCho plas) and phosphatidylcholine (PtdCho).

Figure 2.

Cell extract MR spectra of human breast cancer cell lines of increasing cancer progression: normal MCF-10A cells (top row), tumorigenic MCF-10A cells (middle row), and tumorigenic/metastatic MDA-mb-231 cells (bottom row). ^1H spectra of the choline region are in the left column, and ^{31}P spectra of the PME and PDE regions are shown in the right column. Peak abbreviations are: glycerophosphocholine (GPC), phosphocholine (PCho), choline (Cho), phosphoethanolamine (PEtn), inorganic phosphate (Pi), phosphocreatine (PCr), nucleotide triphosphate ($\text{NTP}\gamma$) and glycerophosphoethanolamine (GPE).

Figure 3.

^{31}P MR spectra of the cell extract lipid phase: MCF-10A (top), MCF-7 (middle) and MDA-mb-231 (bottom). Peak abbreviations are: phosphatidylethanolamine plasmalogen (PtdEtn plas), phosphatidylethanolamine (PtdEtn), phosphatidylinositol (PI), phosphatidylcholine plasmalogen (PtdCho plas), phosphatidylcholine (PtdCho), cardiolipin (CL), phosphatidyl serine/sphingomyelin composite (PS/SPH) and lyso phosphatidylcholine (Lyso-PtdCho).

Figure 4.

A Comparison of normalization methods: Areas of PCCho peaks from cell extracts (n = 4) normalized as a percentage of total aqueous phosphate signal (A), and normalized as nmol per mg protein (B).

Figure 5.

Regions of high signal density from ^{31}P MR spectra of cell extracts (MCF-7 spectrum shown): PME/sugar phosphate region (A), NTP γ region (B), NTP α region (C) and NTP β region (D). Peak assignments are: glycerol-3-phosphate (Gro3P), fructose-1,6-diphosphate (Fru-1,6-DP), phosphoethanolamine (PEtn), adenosine monophosphate (AMP), uridine monophosphate (UMP), phosphocholine (PCho), nucleotide triphosphate (NTP γ , - α , - β), nucleotide diphosphate (NDP β , - α), uridine diphosphate-glucourinate, -glucose, -galactose, -N-acetylglucosamine, -N-acetylgalactosamine (UDPG α , - β), and nicotinamide adenine dinucleotide (NAD α , - β).

Figure 6.

Phosphatidylcholine (PtdCho) metabolic pathway. The choline transporter (CT), mediates the uptake of extracellular choline (Cho_e) across the plasma membrane (PM). The Kennedy pathway enzymes: choline kinase (CK), converting intracellular choline (Cho_i) into phosphocholine (PCho); CTP:phosphocholine cytidylyltransferase (CCT), converting PCho and CTP into CDP-Cho; and CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT), converting CDP-Cho and diacylglycerol (dAG) into PtdCho and CMP. PtdCho degradation is mediated by phospholipases A, C and D: PL-A₂ converts PtdCho into lyso-PtdCho and free fatty acid (FFA); PL-A₁ converts Lyso-PtdCho into GPC and FFA; PL-C converts PtdCho into PCho and dAG; and PL-D converts PtdCho into Cho and phosphatidate (PA).

Figure 7.

Changes in mRNA expression of choline metabolism genes in human breast cancer cells 48 h post treatment with 10 nM docetaxel. Data are shown for a choline transporter (CTL1 α), choline kinase (CK1), choline ethanolamine kinase (CHKL), CTP:phosphocholine cytidyltransferase (CTP1 α) and phospholipase 2 (PLD2 β).

Optimal PCR conditions per primer set. For the Invitrogen kit, [Mg⁺⁺] was 1.8 mM in all cases and for the Qiagen Hot Start kit, stock [Mg⁺⁺] was used.

Table 1

Gene	Accession #	Primer	Sequence (5' → 3')	Product Length	Annealing T (°C)
CTL1, var. A	HSA420812	forward	gtg ctg atg gag ttg gtc g	107	57.8**
		reverse	aga act tgc tcc cga agc		
CK1	D10704	forward	aga ggc ggc ctt agc aac a	76	60
		reverse	ttc cga ggc tca tca cca a		
CHKL	NM_005198	forward	gct ctc aga gcc aga aaa tgc t	85	60
		reverse	tcc caa tgt caa agc ccc tat a		
CTP1α	NM_005017	forward	gtt tat gcc gat gga ata ttg gac t	71	64.8, * 58.8**
		reverse	aag gtt ctt cgc tgc cat cag		
CTP1β	NM_004845	forward	gcc cac agc ctc gac tga *	69*, 104**	60.8, * 59**
		reverse	cct ttc caa tga gcc tcc ** ctt gac act ggc agt tgg tt c * tgg tt cat cag caa atg g **		
CPT	AF195623	forward	cgc tgc tgc tca tct cct act	76	64.8, * 61.8**
		reverse	ccc agt gca cat aaa agg tat gtc		
PLD1α	NM_002662	reverse	caa gat agc caa gga caa cc	94	59.2 *
		forward	tgt tcc ttc agt aat gga tgg		
PLD2α	NM_002663	forward	tct gcg gaa gca ctg c	91	64.8, * 61.8**
		reverse	act gga aga agt cat cac aga tgg		
PLD2β	AF038441	forward	agg cgg gca gtc tga ttc	123, * 80**	60.8, * 58.8**
		reverse	gct cat aga tat tgg cgt tgc tc * act gga aga agt cat cac aga tgg **		
β-actin	BC016045	forward	cgg cat cgt cac caa ctg	71	69.9, * 61.8**

Gene	Accession #	Primer	Sequence (5' → 3')	Product Length	Annealing T (°C)
		reverse	ggc aca cgc age tca ttg		

* Parameters for Invitrogen RT-PCR kit.
** Optimal conditions for Qiagen Hot-Start Kit.

Table 2

Quantifications of tumor choline, in vivo and ex vivo.

Tumor Type	Number of Spectra (n)		Tumor Choline Metabolites as % total aqueous phosphate signal (± sem)									
			PCho, 3.93		GPC, 0.49		PCho/GPC		Cho, *	PtdCho, **	PtdCho plas, **	Lyso-PtdCho, **
	<i>in vivo</i>	<i>ex vivo</i>	<i>in vivo</i>	<i>ex vivo</i>	<i>in vivo</i>	<i>ex vivo</i>	<i>ex vivo</i>	<i>ex vivo</i>	<i>ex vivo</i>	<i>ex vivo</i>	<i>ex vivo</i>	
MCF-7/S	16	1	8.4 (1.0)	15.0	3.6 (0.8)	6.5	2.31 (1.21)	2.32	0.064	298	2.8	7.0
MCF-7/D40	17	2	15.1 (2.0)	16.9	3.1 (0.5)	3.3	10.1 (4.30)	5.14	0.088	321	13.8	13.4
MDA-mb-231	23	7 (6 lipid)	7.1 (0.9)	7.2 (0.7)	4.6 (1.1)	4.8 (0.8)	6.88 (4.39)	1.84 (0.42)	0.035 (0.023)	87.2 (23.0)	4.0 (0.73)	2.3 (0.66)

* (Cho/PCho from ¹H spectra) * (PCho/tot aqueous PO₄ signal from ³¹P spectra) * 100.
** (PtdCho nmol/mg protein from lipid ³¹P spectra) / (PCho nmol/mg protein from aqueous ³¹P spectra) * (PCho/total aqueous PO₄ signal from ³¹P spectra) * 100.

Table 3

Quantifications of choline metabolites in vitro.

Cell Line	Metabolites, ppm, as % total aqueous phosphate signal, n = 4 (± sem)						
	PCho, 3.93	GPC, 0.49	PCho/GPC	Cho, * 3.2	PtdCho, ** -0.84	PtdCho plas, ** -0.77	Lyso-PtdCho, ** -0.33
MCF-10A (normal)	11.0 ^{c,c} (0.14)	n.d. ^{c,c}	80.7 ^{c,c} (2.2)	2.9 ^{c,a} (0.27)	38.7 ^{0,0} (3.2)	5.3 ^{0,0} (2.0)	n.d. ^{0,b}
MCF-7 (tumorigenic)	19.4 ^{c,c} (0.38)	2.6 ^{c,0} (0.020)	7.3 ^{c,0} (0.1)	0.20 ^{c,0} (0.15)	32.5 ^{0,0} (9.0)	2.6 ^{0,0} (1.2)	0.18 ^{0,0} (0.048)
MDA-mb-231 (metastatic)	28.6 ^{c,c} (0.37)	3.2 ^{c,0} (0.39)	9.2 ^{c,0} (1.0)	1.3 ^{a,a} (0.53)	50.0 ^{0,0} (17.0)	3.9 ^{0,0} (1.1)	1.4 ^{b,0} (0.15)

n.d. = not detected at quantifiable levels, significance calculated from 2X noise.

* (Cho/PCho from ¹H spectra) * (PCho/tot aqueous PO₄ signal from ³¹P spectra) * 100.

** (PtdCho nmol/mg protein from lipid ³¹P spectra) / (PCho nmol/mg protein from aqueous ³¹P spectra) * (PCho/total aqueous PO₄ signal from ³¹P spectra) * 100.

⁰ not significant,

^a p < 0.05,

^b p < 0.01,

^c p < 0.001 vs. other cells in order from top to bottom.

Table 4

Metabolites increasing with cancer progression.

Cell Line	Metabolites, ppm, as % total aqueous phosphate signal, n = 4 (± sem)										
	incremental increase					generally elevated in cancers					
	Gro3P, 4.91	F1,6BP, 4.53	4.46*	PCho, 3.93	Lyso-PtdCho, **	F1,6BP, 4.75	GPE, 1.03	GPC, 0.49	UTP/CTPy, -4.93	UDPGlcβ, -12.09	CL, **, 0.165
MCF-10A (normal)	n.d. ^{0,b}	n.d. ^{c,c}	n.d. ^{0,c}	11.0 ^{c,c} (0.14)	n.d. ^{c,b}	n.d. ^{c,a}	0.19 ^{c,c} (0.051)	n.d. ^{c,c}	1.7 ^{b,0} (0.078)	n.d. ^{a,0}	n.d. ^{a,a}
MCF-7 (tumorigenic)	0.39 ^{0,a} (0.11)	0.62 ^{c,c} (0.072)	0.18 ^{0,a} (0.084)	19.4 ^{c,c} (0.38)	0.18 ^{c,c} (0.048)	0.39 ^{c,c} (0.020)	2.1 ^{c,a} (0.031)	2.6 ^{c,0} (0.020)	2.0 ^{b,0} (0.057)	0.23 ^{a,0} (0.027)	3.2 ^{a,0} (0.88)
MDA-mb-231 (metastatic)	0.85 ^{b,a} (0.14)	2.0 ^{c,c} (0.21)	0.49 ^{c,a} (0.043)	28.6 ^{c,c} (0.37)	1.4 ^{b,c} (0.15)	0.18 ^{a,c} (0.014)	1.7 ^{c,a} (0.11)	3.2 ^{c,0} (0.39)	2.3 ^{0,0} (0.90)	0.20 ^{0,0} (0.076)	3.7 ^{a,0} (1.3)

n.d. = not detected at quantifiable levels, significance calculated from 2X noise.

* Unidentified sugar phosphate.

** (PtdCho nmol/mg protein from lipid ³¹P spectra) / (PCho nmol/mg protein from aqueous ³¹P spectra) * (PCho/total aqueous PO₄ signal from ³¹P spectra) * 100.

0 not significant,

a p < 0.05,

b p < 0.01,

c p < 0.001 vs. other cells in order from top to bottom.

Table 5

Metabolites differing only in metastatic MDA-mb-231 cells.

Cell Line	Metabolites, ppm, as % total aqueous phosphate signal, n = 4 (± sem)					
	higher in MDA-mb-231 (peaks ranging from 5.29 to 4.42 are sugar phosphates)			lower in MDA-mb-231		
	5.29	4.58	4.42	1.56*	UTP/CTPγ, -4.84	NTPα, -10.22
MCF-10A (normal)	n.d. <i>0,a</i>	n.d. <i>0,b</i>	n.d. <i>0,b</i>	n.d. <i>0,c</i>	1.5 <i>c,a</i> (0.024)	1.1 <i>0,a</i> (0.07)
MCF-7 (tumorigenic)	n.d. <i>0,a</i>	0.12 <i>0,b</i> (0.073)	0.066 <i>0,b</i> (0.014)	n.d. <i>0,c</i>	1.8 <i>c,a</i> (0.00007)	1.3 <i>0,a</i> (0.04)
MDA-mb-231 (metastatic)	0.48 <i>a,a</i> (0.12)	1.4 <i>b,b</i> (0.22)	0.98 <i>b,b</i> (0.17)	0.46 <i>c,c</i> (0.036)	0.81 <i>a,a</i> (0.28)	0.80 <i>a,a</i> (0.18)

n.d. = not detected at quantifiable levels, significance calculated from 2X noise.

* Unidentified.

** (PtdCho nmol/mg protein from lipid ³¹P spectra) / (PCho nmol/mg protein from aqueous ³¹P spectra) * (PCho/total aqueous PO₄ signal from ³¹P spectra) * 100.

0 not significant,

a p < 0.05,

b p < 0.01,

c p < 0.001 vs. other cells in order from top to bottom.

Table 6

Metabolites differing only in tumorigenic MCF-7 cells.

Cell Line	Metabolites, ppm, as % total aqueous phosphate signal, n = 4 (± sem)																
	higher (peaks ranging from -10.32 to -10.74 are UDPG's and NAD's, peaks ranging from -12.02 to -12.33 are UDPG's)											lower					
	-4.63 *	-4.73 *	ADP/GDP's, -9.70	-10.32	-10.39	-10.57	-10.67	-10.74	NAD's, -10.81	NAD's, -10.91	-11.93	-12.02	-12.13	-12.24	-12.33	**	protein plus, **
MCF-10A (normal)	0.14 <i>bO</i> (0.00004)	n.d. <i>cO</i>	0.41 <i>aO</i> (0.10)	1.7 <i>bO</i> (0.05)	n.d. <i>bO</i>	1.6 <i>aO</i> (0.26)	n.d. <i>cO</i>	0.38 <i>bO</i> (0.12)	1.1 <i>cO</i> (0.07)	0.72 <i>bO</i> (0.17)	n.d. <i>cO</i>	0.43 <i>aO</i> (0.070)	n.d. <i>aO</i>	0.25 <i>cO</i> (0.11)	n.d. <i>cO</i>	7.8 <i>aO</i> (0.70)	7.9 <i>aO</i> (2.6)
MCF-7 (tumorigenic)	0.24 <i>bO</i> (0.11)	0.28 <i>c.a</i> (0.015)	0.70 <i>a.a</i> (0.041)	2.0 <i>b.b</i> (0.06)	0.77 <i>b.b</i> (0.16)	2.2 <i>a.a</i> (0.15)	0.44 <i>c.a</i> (0.047)	0.92 <i>b.a</i> (0.051)	1.8 <i>c.c</i> (0.01)	1.2 <i>b.c</i> (0.06)	0.34 <i>c.a</i> (0.033)	0.81 <i>a.b</i> (0.083)	0.31 <i>a.b</i> (0.051)	0.75 <i>c.b</i> (0.050)	0.61 <i>c.b</i> (0.023)	3.8 <i>aO</i> (1.1)	1.2 <i>a.a</i> (1.0)
MDA-mb-231 (metastatic)	0.16 <i>OO</i> (0.019)	0.12 <i>OO</i> (0.054)	0.52 <i>OO</i> (0.030)	1.4 <i>Ob</i> (0.12)	0.38 <i>Ob</i> (0.11)	1.6 <i>OO</i> (0.13)	0.16 <i>OO</i> (0.082)	0.65 <i>OO</i> (0.066)	1.2 <i>OO</i> (0.03)	0.76 <i>Ob</i> (0.066)	0.16 <i>OO</i> (0.048)	0.25 <i>Ob</i> (0.10)	0.082 <i>Ob</i> (0.00088)	0.40 <i>Ob</i> (0.065)	0.21 <i>Ob</i> (0.085)	6.2 <i>OO</i> (2.3)	12.6 <i>OO</i> (5.7)

n.d. = not detected at quantifiable levels, significance calculated from 2X noise.

* Unidentified.

** (PdCho nmol/mg protein from lipid ³¹P spectra) / (PCho nmol/mg protein from aqueous ³¹P spectra) * (PCho/total aqueous PO₄ signal from ³¹P spectra) * 100.

O not significant,

a p < 0.05,

b p < 0.01,

c p < 0.001 vs. other cells in order from top to bottom.

Table 7

Metabolites decreasing with cancer progression.

Cell Line	Metabolites, ppm, as % total aqueous phosphate signal, n = 4 (± sem)											
	PEtn, 4.40	AMP, 4.33	PCr, -2.54	ATP/GTPα, -4.78	ATP/GTPα, -4.87	NDPα, -9.81	ATP/GTPα, -10.09	NTPα, -10.18	ATP/GTPβ, -20.24	NTPβ, -20.34	NTPβ, -20.44	Cho,* 3.2
MCF-10A (normal)	7.5 <i>c,b</i> (0.26)	0.74 <i>0,c</i> (0.042)	8.7 <i>c,c</i> (0.20)	6.8 <i>c,c</i> (0.06)	7.0 <i>c,c</i> (0.04)	0.75 <i>0,a</i> (0.085)	6.8 <i>c,c</i> (0.10)	7.2 <i>c,c</i> (0.07)	3.3 <i>c,c</i> (0.10)	7.8 <i>c,c</i> (0.17)	2.9 <i>c,c</i> (0.07)	2.9 <i>c,a</i> (0.27)
MCF-7 (tumorigenic)	1.3 <i>c,c</i> (0.054)	0.43 <i>0,0</i> (0.14)	1.9 <i>c,c</i> (0.00005)	5.0 <i>c,a</i> (0.02)	5.1 <i>c,c</i> (0.05)	0.64 <i>0,b</i> (0.027)	4.9 <i>c,a</i> (0.03)	5.3 <i>c,c</i> (0.03)	2.5 <i>c,0</i> (0.04)	6.2 <i>c,b</i> (0.03)	2.2 <i>c,a</i> (0.02)	0.20 <i>c,0</i> (0.15)
MDA-mb-231 (metastatic)	0.45 <i>b,c</i> (0.13)	0.13 <i>c,0</i> (0.031)	0.73 <i>c,c</i> (0.060)	4.7 <i>c,a</i> (0.084)	4.6 <i>c,c</i> (0.28)	0.45 <i>a,b</i> (0.042)	4.6 <i>c,a</i> (0.01)	5.0 <i>c,c</i> (0.26)	2.4 <i>c,0</i> (0.14)	5.4 <i>c,b</i> (0.17)	2.0 <i>c,a</i> (0.05)	1.3 <i>a,0</i> (0.53)

n.d. = not detected at quantifiable levels, significance calculated from 2X noise.

* (Cho/PCCho from ¹H spectra) * (PCho/tot aqueous PO4 signal from ³¹P spectra) * 100, from ³¹P spectra) * 100.

0 not significant,

a p < 0.05,

b p < 0.01,

c p < 0.001 vs. other cells in order from top to bottom.

Table 8

Metabolites remaining unchanged regardless of cancer phenotype.

Cell Line	Metabolites, ppm, as % total aqueous phosphate signal, n = 4 (± sem) (peaks at 5.38 and 5.07 are sugar phosphates, and ranging from -10.26 to -10.64 are UDPGα's and NADα's)																
	5.38	5.07	PI, 3.15	NDPβ, -5.26	NDPβ, -5.36	-10.26	-10.44	-10.47	-10.54	-10.64	UDPGβ, -11.99	PtdEtn, *, -0.083	PS/SPH, *, -0.157	PI, *, -0.400	PtdCho *, plas, -0.772	PtdCho, *, -0.840	-13.1 *
MCF-10A (normal)	n.d.	n.d.	10.5 (0.66)	0.62 (0.016)	0.73 (0.038)	0.52 (0.054)	0.94 (0.50)	0.69 (0.41)	0.55 (0.16)	0.44 (0.18)	n.d.	14.3 (1.5)	0.68 (0.44)	4.9 (1.3)	5.3 (2.0)	38.7 (3.2)	8.2 (0.96)
MCF-7 (tumorigenic)	0.13 (0.031)	0.16 (0.04)	10.4 (0.10)	0.61 (0.029)	0.83 (0.041)	0.53 (0.046)	0.53 (0.18)	1.4 (0.42)	0.38 (0.11)	0.73 (0.040)	0.23 (0.042)	23.8 (6.6)	0.62 (0.44)	4.4 (1.3)	2.6 (1.2)	32.5 (9.0)	1.3 (0.39)
MDA-mb-231 (metastatic)	0.14 (0.021)	0.19 (0.071)	10.2 (1.0)	0.50 (0.079)	0.62 (0.033)	0.36 (0.11)	0.63 (0.19)	0.53 (0.085)	0.14 (0.062)	0.57 (0.22)	0.13 (0.033)	22.3 (7.7)	0.36 (0.13)	4.8 (1.9)	3.9 (1.1)	50.0 (17.0)	2.6 (0.87)

n.d. = not detected at quantifiable levels, significance calculated from 2X noise.

* (PtdCho nmol/mg protein from lipid ³¹P spectra) / (PCho nmol/mg protein from aqueous ³¹P spectra) * (PCho/total aqueous PO₄ signal from ³¹P spectra) * 100.

Table 9

Expression (mRNA) of genes in the choline pathway.

Cell Line	Choline Metabolic Enzyme/ β -actin ratio ($2^{-C_T}/2^{-C_T}$) * 1000						
	CTL1A	CK1	CHKL	CTP1a	CPT	PLD2a	PLD2b
MCF-10A (normal)	13.4 (6.2)	2.8 (0.31)	3.8 ^a (0.30)	50.0 (2.7)	1.3 (0.79)	16.0 ^d (4.7)	3.0 (0.38)
MCF-7 (tumorigenic)	8.9 (2.6)	7.4 (3.1)	4.8 (0.20)	41.0 (4.0)	1.6 (0.23)	2.9 (0.23)	1.0 ^e (0.13)
MDA-mb-231 (metastatic)	3.5 (0.39)	4.6 (1.4)	8.4 (0.27)	15.0 ^b (1.0)	6.2 ^c (0.52)	2.5 (0.12)	2.2 (0.20)

^a p < 0.002,
^b p < 0.004,
^c p < 0.007,
^d p < 0.05,
^e p < 0.01.

Table 10

Proposed mechanisms for increased PCho by cancer progression and decreased PCho in response to docetaxel therapy.

Condition	Evidence and Proposed Model	Net Metabolic Effect
Cancer Progression	↑ choline transport (provides substrate for CK), ↑ CK activity and expression (converts Cho to PCho), ↓ CTP1α (CCT) expression and activity (inhibits conversion of PCho to PtdCho, activity decrease shown by others in MCF-7 cells), ↑ PLA activity (proposed because activity converts PtdCho to Lyso-PtdCho and GPC which are elevated), ↑ GPC phosphodiesterase activity (proposed because activity converts GPC to Cho and Gro3P (elevated), providing substrate for CK further elevating PCho).	↓ Cho, ↑ PCho, ↑ Lyso-PtdCho, ↑ GPC, ↑ Gro3P.
Docetaxel Therapy	↑ CTL1A* (suggesting increased transport), ↑ CK expression* (suggesting increased phosphorylation of Cho to PCho), ↑ CCT activity (proposed because ↑ GPC suggests PtdCho turnover which signal CCT activity, and is observed to increase following treatment with TNF), ↓ PLD expression, ↓ GPC phosphodiesterase activity (Proposed because activity converts GPC to Cho and Gro3P (not elevated), providing substrate for CK and elevating PCho. The opposite is observed).	↓ PCho, ↑ GPC.

* These results are counterintuitive since a decrease in PCho is observed. However, GPC is further increased post treatment suggesting increased PtdCho turnover post treatment, potentially signaling an increase in CCT activity, utilizing PCho substrate pool.