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Alterations of Choline Phospholipid Metabolism in Endometrial Cancer Are Caused by Choline Kinase Alpha Overexpression and a Hyperactivated Deacylation Pathway

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Abstract

Metabolic rearrangements subsequent to malignant transformation are not well characterized in endometrial cancer. Identification of altered metabolites could facilitate imaging-guided diagnosis, treatment surveillance, and help to identify new therapeutic options. Here, we used high-resolution magic angle spinning magnetic resonance mass spectroscopy on endometrial cancer surgical specimens and normal endometrial tissue to investigate the key modulators that might explain metabolic changes, incorporating additional investigations using qRT-PCR, Western blotting, tissue microarrays (TMA), and uptake assays of [3H]-labeled choline. Lipid metabolism was severely dysregulated in endometrial cancer with various amino acids, inositols, nucleobases, and glutathione also altered. Among the most important lipid-related alterations were increased phosphocholine levels (increased 70% in endometrial cancer). Mechanistic investigations revealed that changes were not due to altered choline transporter expression, but rather due to increased expression of choline kinase α (CHKA) and an activated deacylation pathway, as indicated by upregulated expression of the catabolic enzymes LYPLA1, LYPLA2, and GPCPD1. We confirmed the significance of CHKA overexpression on a TMA, including a large series of endometrial hyperplasia, atypical hyperplasia, and adenocarcinoma tissues, supporting a role for CHKA in malignant transformation. Finally, we documented several-fold increases in the uptake of [3H]choline in endometrial cancer cell lines compared with normal endometrial stromal cells. Our results validate deregulated choline biochemistry as an important source of noninvasive imaging biomarkers for endometrial cancer. Cancer Res; 74(23): 6867–77. ©2014 AACR.

Introduction

Endometrial cancer (EC) is the most common gynecologic malignancy in North America and Europe, and despite research efforts, the 5-year overall survival in patients with metastatic disease remains less than 20%. The main risk factor in the development of endometrial cancer is unopposed estrogen, a complication of obesity, hence the incidence of endometrial cancer is projected to dramatically increase in the next decade (1).

Early-stage endometrial cancer can be effectively managed with surgery with a 5-year survival of >80%. Although surgery is curative in patients with disease confined to the uterus, lymphovascular invasion and spread to the regional lymph nodes are most important prognostic factors in endometrial cancer, and will dictate the choice of adjuvant therapy (2). Currently, patients deemed to be at "high risk" of extraterine spread undergo staging pelvic lymphadenectomy such that a certain number of patients will undergo an unnecessary surgery, whereas others will be undertreated. Reliable staging, therefore, is critical to both the management and prognostic stratification of patients. Conventional imaging techniques have limited sensitivity in the assessment of lymph node status and more effective imaging modalities are urgently needed (3). Understanding the metabolic and biochemical changes that underlie the malignant progression of the disease would allow utilization of noninvasive molecular imaging techniques for clinical staging and follow-up. Furthermore, as an increasing number of novel targeted therapies are entering clinical trial, more effective imaging can facilitate drug development efforts in this tumor type, aid patient stratification, and thereby improving clinical outcome.

Choline (Cho) is an essential nutrient that is phosphorylated to phosphocholine (PCho) by choline kinase (CHK) and then

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incorporated into the cell membrane via the Kennedy pathway (4). CHK exists in at least three isoforms, α1, α2 (CHKA), and β (CHKB), of which the alpha but not beta isoforms have been associated with malignancy (5). CHKA overexpression, elevated Cho uptake, and PCho formation have been shown in a wide array of human cancers, including breast, ovarian, lung, and prostate cancer (6–10). These changes in choline handling have most commonly been studied using magnetic resonance spectroscopy (MRS). 1H MRS studies report alterations in the spectral profile in the region of 3.20 to 3.24 ppm that are indicative of PCho, glycerophosphocholine (GPC), and free Cho. In the majority of tumor models, an increased PCho/GPC ratio has been reported compared with normal tissues (11). More recently, the enzyme endometrial differential 3 has been identified as cleaving GPC to form glycerol-3-phosphate and choline, which may also contribute to the increased PCho/GPC ratio (12). Mechanisms of PCho accumulation within tumor cells can occur via a number of mechanisms including enhanced choline transport, CHKA dedicated phosphorylation, and activation of phosphatidylcholine-specific phospholipases. Furthermore, overexpression of CHK (6–8) and abundance of choline metabolites (13–15) have been shown to be prognostic in a number of cancers including prostate, breast, lung, and ovarian cancer. More recently inhibition of CHKA, both by shRNA and small-molecule inhibitors, has been shown to have both an antimitogenic and antiproliferative effects in vitro and in vivo (16–21).

Celik and colleagues identified an abnormal choline signal in proliferating endometrium compared with endometrial cancer by spectroscopy (7). However, neither alterations in choline phospholipid metabolism in the development of endometrial cancer nor alterations in CHKA expression and activity have been considered in endometrial cancer, and may represent both a novel therapeutic target and imaging strategy. The aim of this study was therefore to confirm an altered choline profile in endometrial cancer compared with normal endometrial tissue from patients using high-resolution magic angle spinning (HR-MAS) 1H nuclear magnetic resonance (NMR) technique. We then further investigated alterations in the enzymes responsible for choline homeostasis and choline uptake in endometrial cancer using comparative mRNA analysis of relevant genes. We then considered whether the expression of CHKA correlates with progressive histologic changes from a normal endometrium, hyperplasia, and endometrial cancer using a tissue microarray (TMA) approach. Furthermore, we investigated differential activity of CHKA using radioactivity uptake studies.

Materials and Methods

Clinical specimens

Fresh-frozen endometrial tissue (∼5 mm3) was obtained following approval from the Hammersmith Hospital tissue bank (London, United Kingdom). Informed consent to use leftover biologic material for investigative purposes was obtained from all patients. Samples were taken at the time of initial surgery from 20 patients, 10 with histologically confirmed grade III endometrioid endometrial cancer and 10 with normal endometrium. The median age of the sample set with normal tissue was 47.8 years, whereas with the cancer set was 65.8 years. Samples were immediately frozen in liquid nitrogen and stored within the tissue bank at −80°C until use. The natural morphology in this tumor type is that of discrete lesions rather than diffuse infiltrate; contamination by non-tumor tissue is deemed negligible.

1H NMR spectral acquisition and analysis

Tissue preparation and NMR acquisition were undertaken as per previously published (22, 23). Briefly, for HR-MAS, approximately 5 mm3 (5–18.5 mg) endometrial tissue was thawed and rinsed with a small volume of 0.9% saline (90 mg NaCl in 10 mL D2O) before being placed into a zirconia rotor (Bruker Biospin). Before spectral acquisition using the Bruker DRX600 spectrometer operating at 600.13-MHz 1H NMR, sufficient time was allowed for the rotor to reach a steady spin rate of 5 kHz and a temperature of 283 K. Standard 1D Nuclear Overhauser Effect Spectroscopy (NOESY)-presaturation pulse sequence (RD-90°-τ-90°-τm-90°-AQ) was used to manually determine the receiver gain, water saturation power, 90° pulse length, and shimming for each sample. Each spectrum was acquired with eight dummy scans (to allow sufficient time for the nuclei spin to reach steady state) and 32 scans (2.73 seconds per scan). Field homogeneity was optimized by measuring the v1/2 (∼1.5–2.2 Hz) for L-lactate (δ1.33) before the final acquisition and manual adjustment of the shim coils (24). To suppress signals arising from macro-molecules, 1D Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse sequence [RD-90°-τ-180°-τm-AQ] was used with τ = 40 μs and n = 300. For 1D CPMG experiments, 16 dummy scans followed by 256 transients were collected. A line-broadening factor of 0.3 Hz was multiplied to all free induction decays before Fourier transformation. For peak assignment, 2D J-Resolved and correlation spectroscopy experiments were also performed on a selected sample. Spectra were calibrated to the L-alanine resonance (δ1.48), phasing and baseline correction were done manually using TopSpin software (v.3.1, Bruker Biospin). Subsequent data processing was carried out using in-house scripts written in MATLAB (R2011a, Mathworks) by Drs. T.M.D. Ebbels, H.C. Keun, J.T. Pearce, K. Veselkov, and O. Cloarec (Department of Surgery and Cancer, Imperial College London, London, United Kingdom). Ethanol resonances (δ1.06–1.31, δ3.56–3.75), introduced during sample collection, and the residual water resonance (δ4.68–5.16) were removed. Regions at δ < 0.6 and δ > 9.0 were also removed to reduce effects of noise at baseline. Median fold-change normalization was performed on all reduced spectra to compensate for effects arising from variation in tissue weight or any systematic preparation error (25). Multivariate analysis (Principal Component Analysis and Partial Least Squares Discriminant Analysis; PLS-DA) was applied to the reduced and normalized data using SIMCA P+ software (v.12.0.1, Umetrics). Seven-fold cross-validation and permutation tests were carried out to validate the robustness of our models and to assess their predictability (26). ROC curve analysis was used to indicate the model’s ability to accurately discriminate the presence of
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Nonparametric Mann–Whitney U test and Wilcoxon signed-rank tests were performed using Excel (Microsoft). The Benjamini–Hochberg multiple testing correction (27) was used to assess FDR and counteract multiple hypothesis testing. Specifically for more accurate estimation of ratios between PCho, GPC, and choline, the relative abundance of each was estimated using Chenomx software. The in vitro cell samples were collected and the aqueous metabolites extracted as previously described (28). Briefly, cold methanol was used to quench metabolism and lysates extracted in a dual-phase process. Chloroform/methanol (300 μL, 2:1, v/v) was added to the dried methanol quenched samples, vortexed (30 seconds) and centrifuged (16,000 g, 10 minutes). Ultrapure water (300 μL) was added and samples vortexed and centrifuged as before, the aqueous and organic phases were then aspirated to fresh sample tubes. To maximize metabolite recovery, the extraction was repeated and the samples pooled and dried. Blank samples were also generated to account for any contamination from the extraction solvents. All ID CPMG experiments were conducted in solution state at 600 MHz 1H and 300K using a Bruker AVANCE DRX600C spectrometer fitted with a 5-mm broadband-inverse probehead (Bruker Biospin). The spectral data were processed, digitized, and imported into MATLAB using in-house scripts (see above). The area under the peak was measured for the PCho and Cho resonances at approximately 3.2 ppm and the ratio calculated for each of the three endometrial cancer cell lines.

**Tissue microarray and IHC**

Three types of TMAs were constructed comprising normal endometrium (n = 17), endometrial hyperplasia (n = 15), and endometrial cancer (n = 28). Briefly, formalin-fixed, paraffin-embedded specimen and corresponding hematoxylin and eosin (H&E) slides were retrieved from the local pathology archive. After review of H&E-stained sections, three 1-mm cores were identified from the most representative areas of the tumor tissue, then re-embedded into recipient TMA blocks using an MTA-1 Manual Tissue Microarrayer (Beecher Instruments). Consecutive 4-μm thick sections of the TMA blocks were cut and stained for CHKA using an automated protocol on a Bond Max Autostainer (Leica Microsystems) using the Polymer-HRP system (BioGenex) with subsequent development in diaminobenzidine and Mayer’s Hematoxylin counterstaining. CHKA rabbit polyclonal antibody (Sigma-Aldrich, HPA024153) was used at a 1:25 dilution in Dako EnVision Peroxidase/DAB+ Rabbit kit (Dako) secondary antibody was used to visualize antibody binding. Slides were counterstained with hematoxylin. Normal bronchial tissue was used as positive control to confirm reaction specificity. Omission of the primary antibody was used as negative control reaction. Tissue samples were scored manually using the immunohistochemical score method (IHS), as described previously (29). Briefly, each sample was assigned an IHS ranging between 0 and 300, based on the product of the percentage of cells showing IHC expression (0–100) and the intensity of the signal (graded 1–3). Every core was assessed individually. A composite score of 0 to 300 was generated from the product of intensity and percentage. A final score on a scale of 0 to 3 was obtained; 0, no expression; 1, weak expression (composite score <100); 2, moderate expression (composite score 101–200), and 3, strong expression (composite score 201–300). For statistical purposes, immunoexpression patterns were divided into two groups: reduced phenotype with a staining scored 0 or 1 and preserved phenotype with a staining scored 2 or 3. The pattern of staining (cytoplasmic, membranous, nuclear, or diffuse) was also described in each case. Two observers (R. Dina and R. Sharma) scored all the cases independently, blindly from clinical data and results were found to be consistent.

**Real-time PCR**

RNA was extracted from cells using RNasy Mini Kit (Qiagen) according to manufacturer’s instructions and 1 μg total RNA reverse-transcribed using QuantiTect Reverse Transcription Kit (Qiagen). Gene expression was analyzed by qRT-PCR using SYBR green method and Platinum SYBR Green qPCR SuperMixUDG (Invitrogen) master mix according to manufacturer’s instructions. The reaction was run in a total volume of 20 μL using 25 ng cDNA at a final primer concentration of 400 nmol/L. The following custom designed primers were supplied by Invitrogen: CHKA (Fwd. CCGGAGTAGGAGAACTGATAC, Rev. TCCCCAGAGGAATGAGATGT), CHKB (Fwd. TGGTGCTAGAAAGGTGTAGT; Rev. GCCGACTTGGAGATGACTG), PCYT1A (Fwd. GCAACCAGCTCTTTTTCTG; Rev. GCACACTCCCCAAATGGAGT), SLC22A3 (Fwd. TAGGACTCTCCTGGCTCTCG; Rev. CATGCTTTGTTCATCCCA), GPCPD1 (Fwd. CCGTGGACTCTCTCTCTG; Rev. GAAGTTTCCACAAGGGTTCAG), LYPLA1 (Fwd. AGAAAGTGCGAGTGTCAC; Rev. GTGCGACTTGGGAATAGAAATATC), LYPLA2 (Fwd. AAGAAGGGCAGCAGAACAT; Rev. CTCCCCAGAGGATCTGTTG), SLC5A7 (Fwd. CTTGCCATCTAATTTCA; Rev. CATTCTGAGACCTTTGCTG), SLC5A8 (Fwd. CTTGCCATCTAATTTCA; Rev. CATTCTGAGACCTTTGCTG).

Gene expression assays were performed on an ABI 7900HT Fast Real-Time PCR machine (Applied Biosystems) under the following conditions: 50°C for 2 minutes, 95°C for 2 minutes, 40 cycles of 95°C for 3 seconds, and 60°C for 30 seconds. Data were analyzed using comparative CΔ method as previously described with PPIA (Fwd. CTGCAGTCTGAGACATG; Rev. CATTCTGAGACCTTTG). Tissue samples were homogenized in RIPA buffer containing protease and phosphatase inhibitors (all Sigma-Aldrich) using a Precellys 24 homogenizer with CK14 beads. Homogenates were cleared of debris by centrifugation at 5,000 × g at 4°C for 5 minutes. Supernatants were recovered and 20 μg protein was resolved on a 4% to 15% Mini-PROTEAN TGX gel.
Membranes were blocked for 1 hour in 5% milk in TBS for one hour at room temperature. Signals were visualized using Amersham ECL Western Blotting Detection Reagent (GE Healthcare) and Amersham Hyperfilm (GE Healthcare).

Cell culture

Ishikawa (Health Protection Agency Culture Collections) and HEC-1B (ATCC) were maintained in DMEM (Invitrogen). Endometrial stromal cells, St-T1B, were a kind gift of Professor Jan Brosens (University of Warwick, Coventry, United Kingdom) and cultured in DMEM/F12. All media were supplemented with 10% FCS, glutamine, penicillin, and streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2. Commercially available cell lines were authenticated by provider.

By short-tandem repeat analysis and immediately expanded. Commercially available cell lines were authenticated by provider.

St-T1B, HEC-1B and Ishikawa cells were plated into 6-well plates at a density of 5 x 10⁵ cells per well. The following day, cells were pulsed with 0.1 μCi/mL [3H]choline (PerkinElmer) for one hour then washed once with PBS, trypsinized, and centrifuged. The cell pellet was washed three times with PBS and lysed in RIPA buffer (Sigma-Aldrich). Radioactivity was measured on a scintillation counter after addition Ultima Gold scintillation cocktail (PerkinElmer). Counts were normalized to protein content as determined by BCA assay (Pierce).

**Statistical analysis**

Unless stated otherwise, data were analyzed using either GraphPad Prism software version 5.01 or SPSS version 5.01. Statistical significance of differences was determined as specified and a P value of <0.05 deemed significant.

**Results**

MAS analysis illustrates unique endometrial cancer spectra compared with normal endometrial tissue and increased levels of choline-containing metabolites in endometrial cancer tissue

CPMG-derived ¹H NMR spectra analysis of the tissue samples revealed prominent differences in concentration of multiple metabolites (Fig. 1A). In total 18 samples (10 normal endometrial tissue and 8 endometrial cancer) were analyzed. Two samples were excluded from further analysis due to poor spectral quality. To model all these differences, a total of 68 spectral regions covering most distinguishable resonances across the spectra were integrated for multivariate analysis.

**Figure 1.** ¹H HR MAS NMR spectral analysis of endometrial tissue reveals metabolic perturbations in endometrial cancer. A, mean spectra (1D-CPMG experiments) for tumor (n = 8) and normal tissue (n = 10); BCAA, branched chain amino acids. B–D, multivariate analysis of endometrial tissue based on integrals of metabolite data extracted from ¹H HR MAS NMR CPMG spectra. B, unsupervised PCA scores plot (UV scaled) of the first two principal components reveal discrimination between control (black squares) and cancer (red diamonds) groups, with each point representing a patient’s metabolic profile. C, validation of the two-component PLS-DA model by permuting 999 random combinations of the class matrix, giving P = 0.001. R²Y and Q²Y represent cumulative values for the two-component model, measuring “goodness of fit” and “goodness of prediction,” respectively. D, the ROC curve summarizes the diagnostic accuracy in classifying endometrial cancer and controls.
Unsupervised principal component analysis (PCA) with unit variance scaling was initially used to look for visible separation between the metabolic profiles of different classes. This revealed a clear division between the normal endometrial tissue and cancer groups in model scores (Fig. 1B). To examine further the relationships between cancer and normal endometrial tissue, PLS-DA was exploited to model specifically the covariance between the class membership of the samples and spectral variables. The resulting two-component model explained 95% of the class variance (R²Y), which fell to 75.7% with cross-validation (Q²Y). A permutation test where models were generated on randomly classified data indicated that these model statistics were significant (Fig. 1C). The ROC curve, based on fitted Y values, gave an AUC of 0.9875 indicative of the ability of the model to positively identify endometrial cancer from normal endometrial tissue (Fig. 1D).

Following the establishment of a valid and significant model, we began to deconvolute the metabolic fingerprint of endometrial cancer to identify the metabolites contributing to the classification of normal endometrial tissue and endometrial cancer observed in the PLS-DA model. Figure 2 illustrates a variable importance for the projection (VIP) plot, which summarizes in order of the overall importance individual metabolite/regions that correlates to our model. The metabolites observed to be contributing most to the classification model were PCho, myo-inositol, nucleosides, glutathione, several amino acids, lipids, and glucose, suggesting generally deregulated metabolism in endometrial cancer. To confirm which metabolite changes were statistically significant, we compared the integrals of individual metabolites of cancer against control using Mann–Whitney U test, which provided a semiquantitative analysis in terms of relative metabolite concentration change. In the case where one metabolite has more than one signal across the spectrum, the one with the least signal overlapping was selected; for metabolites of low abundance (e.g., phenylalanine), the signal with the highest contrast from the baseline was selected on the basis of visual inspection, otherwise the metabolites with negative integrals were excluded (e.g., uracil), as signal-to-noise ratio tends to be relatively poor upfield of the NMR spectrum. Out of the 44 metabolites tested, metabolites showing statistical significance were similar to those observed from our multivariate model (Table 1; a list of all metabolites is summarized in Supplementary Table S1). In endometrial cancer, significantly elevated metabolites included a number of amino acids, leucine, valine, alanine, proline, tyrosine, in addition to PCho glutathione, inositols, inosine/adenosine, and unsaturated lipids and triglycerides. Importantly, on superimposing spectra from normal endometrial tissue and endometrial cancer, a significantly increased PCho:Cho ratio (1.59-fold) was detected in endometrial cancer compared with normal endometrial tissue, indicative of activated CHK (Fig. 3). No significant differences were observed in GPC and Cho content, whereas an increase in the PCho:GPC ratio (1.29-fold) approached but did not reach significance (P = 0.08).

Expression of enzymes responsible for phosphocholine synthesis is increased in endometrial cancer

Increased levels of PCho can be attributed to increased Cho uptake and CHKA activity or increased activity of phospholipid catabolic pathways. To establish which aspects of the choline biochemistry are deregulated in endometrial cancer, we investigated the mRNA expression of enzymes involved in differing aspects of choline metabolism using tissues from normal endometrial tissue (n = 10) and grade III endometrioid endometrial cancer (n = 10, Fig. 4A). We observed a significant increase in the expression of CHKA (P < 0.01), GPCPD1 (P = 0.01), LYPLA1 (P < 0.01), and LYPLA2 (P = 0.01) in endometrial cancer compared with normal endometrial tissue, but found no alterations in expression of the organic cation transporters or choline transporter-like proteins. These results suggest that the deacylation pathway may be activated in clinical specimens.
of endometrial cancer and the generated Cho is consequently converted by CHKA to PCho, which results in the high PCho accumulation in the metabolic profile.

Choline kinase is overexpressed in endometrial cancer compared with normal endometrial tissue and endometrial hyperplasia

Because of the high intracellular accumulation of PCho, we hypothesized that CHKA is a key driver for deregulated choline biochemistry and further investigated the role of CHKA in endometrial cancer. In an initial study, we performed Western blot analysis on the same sample set we used for mRNA analysis and found an increased expression of CHKA in endometrial cancer compared with normal endometrial tissue ($P < 0.001$, Fig. 4B and C). To confirm this, we used a TMA approach to assess whether the IHC expression of CHKA changes between normal endometrial tissue, typical and atypical hyperplasia of endometrium and endometrial cancer. The expression of CHKA was cytoplasmic in all samples, and a significantly increased CHKA expression was observed in endometrial cancer and atypical hyperplasia compared with normal endometrial tissue ($\chi^2$ test, $P < 0.02$). However, no difference was observed between atypical endometrial hyperplasia and endometrial cancer (Fig. 5A).

Choline kinase activity in endometrial cell lines

Finally, to investigate whether upregulated CHKA expression and activity would allow utilization of choline-based PET examinations, we measured $[^3]H$choline uptake in HEC-1B and Ishikawa endometrial cancer cell lines compared with that of St-T1b endometrial stromal cells. Incorporated radioactivity was increased 7- and 12-fold in the two endometrial cancer cell lines, respectively, which indicates good differential uptake to stromal tissue ($P < 0.001$, Fig. 4B and C). Increased CHKA activity was further confirmed by MRS as denoted by increased PCho/Cho ratios in cancer cell lines versus normal control (Fig. 5C). Elevated flux was caused by higher baseline expression of choline transporters SLC44A, SLC22A3, and CHKA. In addition, we found that, comparably to the patient-derived samples,
PCYT1B and LYPLA1 and 2 were significantly higher expressed in cancer-derived cell lines compared with St-T1b (Fig. 5D).

**Discussion**

Abnormal NMR-detectable metabolic profiles have been reported in a number of tumor types, including breast, cervical, and colorectal cancers (9, 31–34). However, this has not been studied in endometrial cancer. In this study, we investigated metabolic alterations in endometrial cancer using complementary approaches with the aim of identifying novel potential therapeutic and molecular imaging targets. We reveal an increase in the PCho pool in endometrial cancer compared with normal endometrial tissue that can be attributed to an increase in CHKA activity and upregulation of enzymes involved in the deacylation pathway. Alterations in choline handling directly impacts radiolabeled choline uptake allowing PET imaging to be used to assess tumor stage. Furthermore, as choline biochemistry is intimately involved with cellular proliferation, deregulated transporters or enzymes involved in choline handling may act as potential therapeutic targets. To our knowledge, this is the first study to show altered choline handling in endometrial cancer, and moreover this study uses the novel technique of HR-MAS MRS to illustrate specific alterations in choline metabolites in primary tissue specimens of endometrial carcinoma.

A number of studies have investigated the utility of HR-MAS MRS in cancer where the metabonomic phenotype was correlated with recognized prognostic features (13, 33, 35, 36). Overall these studies illustrate increased total Cho resonance signal at approximately 3.2 ppm in cancer compared with normal tissue consistent with our findings. PCho, GPC, and Cho all contribute to the total choline resonance and we report relative levels of PCho, GPC, and Cho consistent with previously reported values, with PCho being the main contributor to the total choline resonance. We reported an approximately 70% increase in PCho concentration in cancer compared with normal tissue. Consistent with previous publications in ovarian and breast cancer, we report a significant increase in the PCho/Cho ratio (11, 37) and nonsignificant increase in the PCho:GPC ratio in endometrial cancer. We hypothesize that the lack of a distinct PChoGPC switch, which is associated with oncogenic transformation in breast cancer (11), may not occur in endometrial cancer and that overexpression of GPCPD1 identified in endometrial cancer converts GPC via the deacylation pathway into free choline, which is again converted to PCho by CHKA (38, 39).

Considering the changes observed by MAS MRS, we investigated the gene expression of enzymes responsible for phosphatidylcholine anabolism (Kennedy pathway), catabolism (deacylation pathway), and choline substrate transporters to ascertain whether the changes in the choline peak in endometrial cancer observed on MAS resulted from the deregulated intracellular metabolism of choline or transport of exogenous choline into the cells. Choline flux can only be measured accurately in *vitro*, and as we utilized clinical samples, qRT-PCR was used to assess the expression of transporters. The changes in relative gene expression of enzymes involved in choline handling have been studied in a number of tumor types including breast and ovarian cancer, and these studies are consistent in reporting an increase in the expression and activity of CHKA in cancer cells compared with normal epithelial cells (9, 38). Activation of CHKA results in an increase in the product, PCho, and product:substrate ratio, PCho:Cho. Furthermore, we report an increase in the expression of CHKA in endometrial cancer compared with normal endometrial tissue by IHC. Of interest, we noted increase in CHKA expression not only in endometrial cancer, but also in hyperplastic endometrial tissue. Our TMA included both simple and hyperplastic endometrium.
complex atypical hyperplasia, the latter being premalignant and this may account for the increase in CHKA in these specimens. Although our approach illustrates tissue heterogeneity, the use of microdissection techniques and larger samples size may reduce the variation observed in the presented results. In a number of studies, CHKA has been shown to mediate cellular proliferation, transformation, and carcinogenesis, and mediation of this target using siRNA and small-molecule inhibitors suggests inhibition of tumor growth (11, 16, 18, 40, 41).

As indicated, we further observed in clinical samples and endometrial cancer cell lines, a significant increase in the mRNA expression of enzymes of the phosphatidylcholine deacylation pathway; GPCPD1, LYPLA1, and LYPLA2. Alterations of the deacylation pathway have been previously investigated for its contribution to the relative PCho pool (38, 42). Iorio and colleagues reported a 2- to 4-fold increased levels of GPC in a number of ovarian cell lines (38), but found no change in the activity of LYPLA1 and 2. Gene expression analysis conducted in breast cancer cell lines illustrated a reduction in the expression of LYPLA1 in cancerous cell lines compared with normal controls (9). Discrepancies may arise from the use of whole tissue extracts of endometrial tissue rather than cell lines. Furthermore, as we used whole tissue, we were only able to consider gene expression rather than enzyme activity. It is unclear from the literature whether any posttranslational modifications occur for these enzymes and in future studies, it would be important to assess the functional alterations of these enzymes in endometrial cancer. Nonetheless, on MAS, the levels of GPC were not significantly altered, despite the

Figure 5. CHKA is overexpressed in endometrial cancer and causes increased uptake of radiolabeled choline. A, representative sections of TMA showing CHKA expression in normal, hyperplastic, or endometrial cancer samples. Bar, 100 μm. B, cells were pulsed with [3H]choline for 1 hour and retained activity measured. Uptake of [3H]choline in human endometrial cancer cell lines HEC-1B and Ishikawa was compared with the human endometrial stroma cell line St-T1b (error, SEM; **, P < 0.01; *** P < 0.001). C, the aqueous metabolites were extracted from the three endometrial cancer cell lines, analyzed by MRS, and the choline metabolites measured. Data expressed as PCho/Cho ratio ± SEM and significant differences to the St-T1b cell line indicated (**, P < 0.05; ***, P < 0.01). D, relative mRNA expression of indicated enzymes involved in choline handling. Expression is shown relative to normal cell line (St-T1b) and statistical significance was determined by comparison of the cancer cell lines to St-T1b (error, SD; *, P < 0.05; **, P < 0.01).
increased expression of enzymes in the catabolic arm of choline handling the complementary activities of choline phosphorylation, and deacylation could contribute to the unique NMR profile observed in endometrial cancer.

The increase in the expression of CHKA in human endometrial cancer compared with normal endometrial tissue provides further support for the use of choline radiotracers in the staging of endometrial cancer, particularly in patients at high risk of lymph node involvement where the use of choline PET imaging may alleviate the need for staging lymphadenectomy and its accompanied adverse effects. A number of studies have investigated the utility of $2^{[18F]}$fluor-2-deoxy-D-glucose (FDG) PET in endometrial cancer (3, 43). These studies are limited by poor sensitivity because of the rapid excretion and accumulation of FDG within the bladder, the largest study reporting 74% sensitivity in detecting lymph node involvement (3). The use of MRS in assessing endometrial cancer is limited by poor spectral signal to noise ratio (7, 44). We have recently shown the feasibility of imaging using $[^{11C}]$choline PET/CT in detecting lymph node involvement in high-risk prostate cancer. Furthermore, we suggest a correlation between the expression of CHKA within prostate cancer specimens with $[^{11C}]$choline uptake (6). To this end, we showed differential uptake of $[^{1H}]$choline by endometrial cancer cells compared with normal stromal by 7- to 12-fold, highlighting the potential utility of choline radiotracers in imaging endometrial cancer. The utility of $[^{11C}]$choline PET/CT was demonstrated in a small study of 22 patients, where the accuracy of detecting lymph node involvement was 86%, increasing to 96% with both $[^{11C}]$choline PET/CT and MRI. However, the use of $[^{11C}]$choline is limited to institutions with onsite cyclotron given the short half-life of $^{11C}$ (20.38 minutes). The use of $^{18F}$-labeled choline tracers is felt to be limited by bladder uptake; however, this could be alleviated through the use of delayed phase imaging, with the novel, more metabolically stable $[^{18F}]$Fluoromethyl-$[^{1,2-2H}_4]$-choline or through the application of filtering techniques (45). Choline imaging by PET or MRS has become an established tool of preclinical and clinical research and small-molecule inhibitors of CHKA are currently in development.

Using an unbiased HR-MAS MRS approach, we identified additional metabolic patterns that illustrate a severely altered phenotype in endometrial cancer. The majority of these metabolites can be classed into proliferation-associated markers (e.g., various amino acids and the nucleobase uracil, which are indicative of high transcriptional and connected markers (e.g., various amino acids and the nucleobase uracil, which are indicative of high transcriptional and deacylation). Maintenance of intracellular redox homeostasis (glutathione), osmoregulation (inositols, taurine), and glucose and lipid metabolism (lactate, acetate, free Cho, PCho, GPC, fatty acids, and unsaturated lipids; ref. 46). Glucose and lipid metabolisms are tightly connected in cancer. Because of the Warburg effect, less glucose-derived metabolites are fed into the TCA cycle. Consequently, the required intermediates for generation of lipids need to be restored, and acetate, lactate, ketone bodies, and glutamine serve as substitutes (47). Interestingly, these metabolites were not increased in our dataset, but the end products, unsaturated lipids and fatty acids (summarized as lipids in Table 1), were among the most severely upregulated metabolites.

A limitation of this study is that the menopausal status of the patients studied was not known; however, based on the median age of the patients in the control cohort and those in the cancer cohort, it can be inferred that patients in the control cohort were likely to be partially premenopausal, whereas those in the cancer cohort were likely to be predominately postmenopausal. Changes in MRS spectra caused by cyclical changes of the endometrial tissue or with menopause remain an important question for further investigation.

In conclusion, using a multimodality approach, we have shown that the expression and activity of CHKA are increased in human endometrial cancer specimens compared with normal endometrium. These findings suggest that deregulated choline metabolism may represent a source of molecular targets with important diagnostic and therapeutic implications for the management of patients with endometrial cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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