Comparison of NMR Spectroscopy and Proton MR Spectroscopy for Quantification of Choline Metabolites in Breast Cancer

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The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

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June 2012

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June 2012

ACKNOWLEDGEMENTS

First of all, I express my gratitude to my thesis supervisor, professor Eun-Kyung Kim, for her supervision and encouragement to successfully complete this thesis.

I give my appreciation to professors Min Jung Kim, Dong-hyun Kim, Seung Il Kim, and Ja Seung Koo who gave me experienced advices and warm supports. I also thank to professors Jin Young Kwak and Hee Jung Moon for great support and encouragement in performing this study.

I would like to express my gratitude to my parents and family members for their continuous caring and love throughout my life.

Written by Ji Soo Choi

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ABSTRACT

Comparison of NMR Spectroscopy and Proton MR Spectroscopy for Quantification of Choline Metabolites in Breast Cancer

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(Directed by Professor Eun-Kyung Kim)

Purpose: To examine correlation between nuclear magnetic resonance (NMR) spectroscopy using biopsy specimen and magnetic resonance (MR) spectroscopy for quantification of choline metabolites in breast cancer and to assess clinical role of these spectroscopy for breast cancer research.

Materials and Methods: After institutional review board approval and informed consent were obtained for this study. Breast MR spectroscopy (3.0T) was performed in patients with biopsy-proven malignant lesions measuring 1cm or larger at imaging. The total choline (tCho) and signal to noise ratio (SNR) of tCho peak were quantified by single-voxel ^{1}H MR spectroscopic data. Concentrations and metabolic ratios of multiple choline metabolites were estimated by NMR spectroscopy (11.7T) using biopsy specimens. NMR spectroscopic values were then compared with MR

spectroscopic values. Additionally, NMR and MR spectroscopic values were compared according to clinicopathologic variables.

Results: A total 34 patients (age range, 34-68 years) with 36 malignant lesions (mean size, 29.7mm) were imaged. NMR spectroscopy quantified and discriminated choline metabolites in all samples of 36 lesions, however, MR spectroscopy quantified that in 32 lesions. The free choline (Cho)/Creatine (Cr) on NMR spectroscopy was significantly correlated with normalized tCho (r=0.354, p=0.04) and SNR of tCho peak (r=0.403, p=0.03) on MR spectroscopy. In addition, the glycerophosphocholine (GPC)/Cr (r=0.412, p=0.02) and tCho/Cr (r=0.353, p=0.04) significantly correlated with SNR of tCho peak, We found several metabolite markers (Cho, Cr, taurine, s-inositol, m-inositol, tCho, phosphocholine (PC), glycine, Cho/Cr, tCho/Cr, PC/Cr) on NMR spectroscopy to correlate with histopathologic prognostic factors [ER (estrogen receptor), PR (progesterone receptor), HER2 (a receptor for human epidermal growth factor), triple negativity, Ki-67, and poor prognosis group (detection of axillary lymph node metastasis, tumor size with diameter >2cm, or negative for ER or PR)].

Conclusion: MR spectroscopic values relatively correlate with several NMR spectroscopic values in breast cancer, indicating MR spectroscopy may be used to aid in vivo assessment of choline metabolites of breast cancer. NMR spectroscopic values were correlated with the histologic prognostic markers (ER, PR, HER, triple negativity, Ki-67, prognosis group), indicating NMR spectroscopy may be helpful for detection of reliable markers of characterization of breast cancer.

Key words: spectroscopy, MRI, NMR, choline, breast

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I. INTRODUCTION

Breast cancer is one of common cancer and the important cause of cancer related deaths among women globally.[1, 2] Early diagnosis of breast cancer is crucial for successful treatment and screening program using mammography, ultrasonography, and magnetic resonance imaging (MRI) has been effected in industrialized countries.[3-6] In addition to early detection of breast cancer, clinical and histologic assessment of breast cancer is also important to treatment management. The presence of hormone receptors is favorable, as appropriate hormone therapy can suppress the growth of such tumors. Patients with large tumors and axillary lymph node metastasis are considered to have high risk of

tumor recurrence. These patients need more extensive treatment. Identification of reliable markers to improve diagnostic accuracy and to predict prognosis, would be an important accomplishment.

High-resolution nuclear magnetic resonance (NMR) spectroscopy has been applied in studies of various human tissues and diseases.[7-10] NMR spectra of tissue sample consist of multiple peaks that provide information on their metabolic composition. Metabolic composition in the tumor assessed with NMR spectroscopy suggested as promising tools in diagnosis and characterization of cancer.[11-13] Recent studies using NMR spectroscopy have also reported the choline-containing compounds of breast cancer tissue, including glycerophosphocholine (GPC), phosphocholine (PC), free choline (Cho), and taurine (Tau) also show different concentration and distribution according to clinical parameters.[14, 15]

In vivo proton magnetic resonance (MR) spectroscopy is a noninvasive method that can provide metabolic information of tumor, although NMR spectroscopy needs invasive procedure to acquire tissue sample for analysis. MR spectroscopy of breast can improve specificity of contrast-enhanced breast MRI by detection of elevated levels of choline metabolites, which has been associated with breast malignancy.[16-21] Also, MR spectroscopy may have potential to monitoring treatment response in patients receiving neoadjuvant chemotherapy due to locally advanced breast cancer. Several groups observed that presence of total choline (tCho) peak before treatment and its reduction/absence after treatment.[22, 23] However, most studies about MR spectroscopy have used 1.5-T MR scanner. At high field strength (≥3.0 T), MR

spectroscopy may provide more qualitative assessment of choline metabolites in breast cancers.

We hypothesized that 3.0-T MR spectroscopy may correlate with high-resolution NMR spectroscopy and comparison of the two modalities may help to identify reliable marker for characterization of breast cancer. Therefore, the purpose of this study was to examine correlation between NMR spectroscopy using biopsy specimen and 3.0-T MR spectroscopy for quantification of choline metabolites in breast cancer and to assess clinical role of these spectroscopy for breast cancer research.

II. MATERIALS AND METHODS

1. Patients

This study was approved by the institutional review board, and informed written consent was obtained from each patient prior to the study. Between August 2010 and February 2011, 38 female patients (mean age 52.2 years; age range 34-68 years) with 40 breast lesions assessed as Breast Imaging Reporting and Data System (BI-RADS) 4c or 5 and larger than 1cm in diameter at mammographic or US imaging were included. All patients underwent ultrasound (US)-guided core-needle biopsy and subsequent breast MRI and single-voxel ^{1}H MR spectroscopy. After examination of MR spectroscopy, NMR spectroscopy was performed using core biopsy specimen of each patient. Of these, 4 patients were excluded because of technical failure (insufficient

water and/or fat suppression). Remaining 34 patients with 36 breast lesions fulfilled following inclusion criteria: 20 years of age or older; breast lesion with diameter more than 1 cm on MRI; breast lesion pathologically diagnosed as malignancy by core biopsy; no pregnancy; no history of breast cancer or previous breast surgery including breast implants; no contraindications for MRI and/or contrast agents.

2. MRI and ^{1}H MR spectroscopy

MRI was performed using a 3.0-T system (TIM Trio; Siemens Healthcare, Erlangen, Germany) with a dedicated, bilateral breast coil (Siemens Healthcare). All patients were examined in a prone position. A single-voxel ^{1}H MR spectroscopy was added to routine protocol of breast MRI. The MRI protocol included 3D coronal T1-weighted turbo fast low-angle shot sequence (TR/TE, 326/2.6 ; field of view 360 × 360 mm; section thickness 3 mm), transverse T2-weighted turbo spin-echo imaging (TR/TE, 4360/82 ; field of view 360 × 360 mm; section thickness 3 mm), and dynamic contrast-enhanced MRI using volumetric interpolated breath-hold examination (VIBE) sequence (TR/TE, 280/2.6 ; field of view 360 × 360 mm; section thickness 3 mm). The T1-weighted fast low-angle shot image was reconstructed to sections in all three dimensions for accurate positioning of the MR spectroscopic voxel.

Single-voxel ^{1}H MR spectroscopy was performed before contrast agent injection to avoid any influence of the contrast agent on the detected tCho signal.[24] A point-resolved spectroscopic sequence was used for MR spectroscopy (TR/TE 1500/100; voxel size, 15 × 15 × 15 mm; acquisitions 128;

spectral width 1400 Hz; data points 1024; time of acquisition, 4 minutes). Voxel placement was performed by a technician, who was supervised by one radiologist with 9 years of experience (M.J.K). In large tumor with heterogeneous nature, a voxel was placed in the homogeneous area of the lesion while avoiding areas of necrosis, hemorrhage, or fibrosis. Automatic shimming was performed first, followed by manual shimming on the water resonance for optimization of the homogeneity within each volume of interest. Water peak line-widths of 15-25 Hz (full width at half-maximum) were typically achieved. The spectra were acquired with water suppression by applying three chemical-shift selective excitation pulses[24] and fat attenuation by frequency-selective suppression pulses. The MR spectroscopy scan time was approximately 7 minutes. The total scan time for MR spectroscopy including the additional time for voxel placement and shimming was less than 10 minutes.

The jMRUI software package[25] was used for time-domain analysis. In order to measure the tCho (the sum of PC, GPC, and Cho) peak from the water and fat suppressed spectrum, we performed a preprocessing that consisted of zero-filling of 2048 points, Gaussian apodization of 5 Hz, Fourier transformation, and phase correction of the transformed spectrum. Removal of residual water (e.g., 4.0 – 6.0 ppm) and fat (e.g., 0.0 – 2.7 ppm) components of the signal was performed in a preprocessing step using the Hankel-Lanczos singular value decomposition (HLSVD).[26] AMARES (Advanced Method for Accurate, Robust and Efficient Spectral fitting)[27], a widely used quantitation tool for MRS data, was employed to fit the spectra. In this study, a Gaussian lineshape model was chosen for quantifying the tCho peak. Soft constraints were imposed for a faster

and more accurate quantitation during spectral fitting. Linewidths for the tCho peak were allowed to vary between 1 and 10 Hz. The frequency constraint range was restricted to ± 0.2 ppm (e.g., 3.12 – 3.32 ppm). After the zero and first order phases were switched off, the frequency-selective option[28] was applied, weighting the first 20 points of the time domain signal by the first quarter of a squared sine function. tCho signal-to-noise ratio (SNR) was measured as the ratio of the peak integral of the signal in the tCho frequency range in one voxel localized within the lesion, relative to the background noise level lower than 0.0 ppm (where no signals are expected) in the same voxel.

Normalized tCho was obtained using the water in a cylindrical bottle phantom (height 4.0 cm and diameter 2.5 cm) as an external reference.[23] The phantom contained 1.25 g of $NiSO_4 6H_2O$ per 1,000 g of H_2O and was placed on the dorsal side of the breast coil. ^{1}H MR spectroscopy of the phantom was performed after examination of the breast lesion. The spectroscopic voxel size was 7 × 7 × 15 mm^3, and the spectra were acquired without water suppression. The tCho in the malignant breast lesion was calculated according to the following equation:

$$Scaling\ factor = \frac{10^6}{MW_{H_2O}} \times \frac{n_{H_2O}}{n_{tCho}} \times \frac{S_{tCho}}{S_{H_2O}} \times \left(\frac{f_{T_1 H_2O}}{f_{T_1 tCho}} \times \frac{f_{T_2 H_2O}}{f_{T_2 tCho}} \right) \times \frac{coilsens_{H_2O}}{coilsens_{tCho}}$$

where S_{tCho} is the signal amplitude of the tCho, and S_{H_2O} is the signal amplitude of the water in the external reference phantom. The terms n_{H_2O} and n_{tCho} represent the number of ^{1}H nuclei in each respective molecule. The scaling factor can be changed to molar concentration by correcting for the

number of ^{1}H nuclei per molecule and the molecular weight of water, MW_{H_2O}. The parameters f_{T_1} and f_{T_2} are the correction factors for T_1 and T_2 relaxation times: $f_{T_1} = 1 - \exp(-TR/T_1)$ and $f_{T_2} = \exp(-TE/T_2)$. T_1 relaxation times were 1513 ms for tCho and 746 ms for water; T_2 relaxation times were 269 ms for tCho and 97 ms for water.[29] The coil sensitivities $coilsens_{H_2O}$ and $coilsens_{tCho}$ are the signal intensity of the external reference phantom and the signal intensity within the breast imaging area.

3. ^{1}H NMR spectroscopy using biopsy specimen

The US-guided biopsies were performed with a 14-gauge dual-action semiautomatic core biopsy needle (Stericut with coaxial guide; TSK Laboratory, Tochigi, Japan) by one of 4 radiologists (with 6-13 years of experience). In large cancers with heterogeneous nature, homogeneous solid area was targeted for biopsies. Mean number of tissue samples of US-guided core biopsies was 6 (range 5-8) core samples. For NMR spectroscopy, one core tissue sample was put in cryogenic vials and immersed in liquid nitrogen immediately after biopsy. Samples were stored at -162 °C from 1 to 5 months before NMR spectroscopy.

NMR spectroscopy of the tissue samples were performed with an NMR spectrometer (Agilent, VNMRS 500) operating at a proton NMR frequency of 500.13 MHz (11.74T). Temperature was set to 19°C after calibration with methanol. Examination time of each sample was approximately 1 hour. Frozen samples were thawed in NMR laboratory, weighed, and placed into an HR-MAS (high-resolution magic angle spinning) nano-probe® (Agilent, Walnut

Creek, CA). The total volume of the sample cell is 40 ml, and an average of 7.5 mg core-biopsy samples were put in the cell with the remaining volume filled with D_2O containing 0.01% trimethylsilyl propionic acid (TSP). The probe was an inverse-detection type and equipped with single Z-gradient coil. The spectra were taken with CPMG pulse sequence to impose a T2 filter. The total T2 delay was set to 290 msec and the sample was spun at 2 KHz. The spectra were acquired with total complex points of 16 K, sweep width of 7961 Hz, and 1024 transients. The 90 degree pulse was calibrated with each sample on water resonance. Water signal was saturated using weak power continuous wave during the recycle delay. Each free induction decay signal is processed and analyzed using ACD software (Advanced Chemistry Development, Toronto, Ontario, Canada). Post-processing consisted of Fourier transformation, phasing and baseline correction. Chemical shift referencing was achieved relative to the creatine signal at 3.04 ppm. Metabolite peak amplitudes were measured by fitting a Voigt (e.g., Gauss + Lorentz) lineshape function. The integration values were normalized to the number of contributing protons per molecule and to tissue weight. Quantification was performed by comparing the integrated TSP signal with the signal of interest in the tumor spectrum. Absolute concentrations are given as means±SD in μmol/g wet weight.

4. Histopathologic analysis

All 36 lesions were pathologically diagnosed as malignancy by core biopsy prior to treatment. Final diagnosis was established with surgery in 29 patients with 31 malignant lesions (two patients with two malignant lesions). In five

patients with five malignant lesions, final diagnosis was confirmed by core biopsy without surgery. Final histopathologic results were used as reference standard. Informations about pathologic variables including histologic grade, estrogen receptor (ER), progesterone receptor (PR), HER2 (a receptor for human epidermal growth factor), and Ki-67 were based on final patients' pathologic reports.

All tissues were fixed in 10% buffered formalin and embedded in paraffin. All archival hematoxylin and eosin (H&E)-stained slides for each case were reviewed by experienced pathologists. Histologic grade of the tumor was determined according to the modified Bloom-Richardson classification.[30] Immunohistochemical (IHC) analyses for ER, PR, HER-2, and Ki-67 were performed on tissue blocks. Briefly, 5 μm-thick sections were obtained with a microtome, deparaffinized and rehydrated. After treatment with 3% hydrogen peroxide solution for 10 minutes to block endogenous peroxidases, the sections were pretreated in 10 mM citrate buffer (pH 6.0) for antigen retrieval in a microwave oven for 20 minutes. After incubation with primary antibodies against ER (clone SP1, 1:100; Thermo Scientific, Fremont, CA, USA), PR (clone PgR 636, 1:50; DAKO, Glostrup, Denmark), HER-2 (polyclonal, 1:1500; DAKO), and Ki-67 (1:400 dilution; Novocastra, Newcastle, U.K.), immunodetection was performed with biotinylated antimouse immunoglobulin, followed by peroxidase-labeled streptavidin using a labeled streptavidin biotin kit with 3,3'-diaminobenzidine chromogen as substrate. Slides were counterstained with Harris hematoxylin. ER and PR positivity was defined as more than 10 fmol/mg cytosol protein, or as 10% or more nuclear IHC staining.

IHC stain results of HER-2 were scored by counting the number of cells positively stained on the membrane and expressed as a percentage of total tumor cells. HER-2 staining was scored as follows: 0, membrane staining in <10 % of tumor cells; 1+, faint or incomplete membrane staining in >10% of cells; 2+, weak or moderate complete or incomplete staining in >10% of cells; 3+, strong complete membrane staining in >10% of cells. Tumors scored as 3+ were HER-2 positive cases whereas tumors with 0 to 1+ were regarded as negative cases. Borderline cases (2+) required further investigation using fluorescence in situ hybridization to assess whether they show gene amplification. Triple negativity was defined as lack of expression of ER, PR, and HER2. IHC staining of Ki-67 was scored by counting the number of cells positively stained nuclei and expressed as a percentage of total tumor cells. Staining results for Ki-67 were classified as follows: Group 1: ≤10%, Group 2: 10-29%, and Group 3: ≥30%.[31]

5. Data and Statistical analysis

From review of medical records, patients' clinicopathologic data were collected. Clinicopathologic variables included pathologic type of tumor, lymph node metastasis at the time of diagnosis, tumor size, histologic grade, status of ER, PR, HER2 and Ki-67 expression, and triple negativity (Table 1). According to IHC staining results, tumors were classified into four subtypes: luminal A (ER+ and/or PR+, HER2-, Ki-67 <14%), luminal B (ER+ and/or PR+, HER2-, Ki-67 ≥14% / ER+ and/or PR+, HER2+, any Ki-67), HER2-expressing (HER2+, ER-and PR-), and basal-like (ER- and PR-, HER2-). Tumor size was based on final pathologic results (n=22). However, tumor size measured with

MRI was used when the patients received neoadjuvant chemotherapy before surgery (n=9) or did not undergo surgery (n=5).

Table 1. Clinicopathologic data on 34 patients with 36 breast cancer in this study

Clinicopathologic variables	**Patients (%)**	**Lesions (%)**
Histologic grade		
Low (Grade 1-2)	13 (38.2)	15 (41.7)
High (Grade 3)	6 (17.7)	6 (16.6)
N/A	15 (44.1)	15 (41.7)
Tumor size		
≤2 cm	15 (44.1)	17 (47.2)
>2 cm	19 (55.9)	19 (52.8)
ER status		
Negative	6 (17.7)	6 (16.6)
Positive	26 (76.4)	28 (77.8)
N/A	2 (5.9)	2 (5.6)
PR status		
Negative	18 (52.9)	18 (50.0)
Positive	14 (41.2)	16 (44.4)
N/A	2 (5.9)	2 (5.6)
HER2 status		
Negative	27 (79.4)	29 (80.5)
Positive	5 (14.7)	5 (13.9)
N/A	2 (5.9)	2 (5.6)
Triple negativity		
Yes	4 (11.7)	4 (11.1)

No	28 (82.4)	30 (83.3)
N/A	2 (5.9)	2 (5.6)
Ki-67		
Low (Group 1-2)	24 (70.6)	26 (72.3)
High (Group 3)	6 (17.7)	6 (16.6)
N/A	4 (11.7)	4 (11.1)
Molecular subtype		
Luminal A	14 (41.2)	15 (41.7)
Luminal B	11 (32.3)	12 (33.3)
HER2 negative	8	9
HER2 positive	3	3
HER2-expressing	2 (5.9)	2 (5.6)
Basal-like	4 (11.8)	4 (11.1)
N/A	3 (8.8)	3 (8.3)
Lymph node metastasis		
Negative	22 (64.7)	24 (66.7)
Positive	12 (35.3)	12 (33.3)
Prognosis		
Good	7 (20.6)	9 (25.0)
Poor	25 (73.5)	25 (69.4)
N/A	2 (5.9)	2 (5.6)

N/A: not available

The correlations between the values of NMR spectroscopy (Metabolite Concentration: tCho, Cho, PC, GPC, Tau, glycine (Gly), myo-inositol (m-Ins), scyllo-inositol (s-Ins), creatine (Cr) / Metabolic Ratio: Cho/Cr, PC/Cr, GPC/Cr, GPC/PC, GPC/Cho, PC/Cho) and those of MR spectroscopy (tCho concentration, signal to noise ratio (SNR) of tCho peak) were determined with Pearson's correlation coefficients.

Additionally, MR and NMR spectroscopic values were compared according to clinicopathologic variables using Student's *t*-test. Tumors were grouped by size based on diameter ≤ 2cm or > 2cm. In addition, tumors were grouped by prognosis. Good prognosis group was defined by no spread to axillary lymph nodes, tumor size with diameter ≤ 2cm, and positive for ER and PR. Poor prognosis group was defined by detection of axillary lymph node metastasis, tumor size with diameter >2cm, or negative for ER or PR. Statistical difference of MR and NMR spectroscopic values between molecular subtypes could not be examined due to a small number of HER2-expressing subtype (n=2)

Statistical analysis was done with SAS for Windows, version 9.0 (SAS Institute, Cary, NC, USA). Statistical significance was defined as a *P*- value < 0.05.

III. RESULTS

Mean tumor size was 29.7 mm (range, 10-80 mm). Most common tumor type was invasive ductal carcinoma (n=30), and other cancers were 3 DCIS, 1

mucinous carcinoma, 1 tubular carcinoma, and 1 invasive micropapillary carcinoma.

NMR spectroscopy quantified and discriminated multiple Cho metabolites in all 36 lesions (Table 2). Mean and median values of tCho concentration were 1.95 μmol/g (range, 0.06-6.55) and 1.46 μmol/g (interquartile range 0.61-3.0). Mean and median values of PC concentration were higher than those of Cho or GPC. MR spectroscopy detected and quantified Cho in 32 lesions (Fig. 1) of 30 patients. In the remaining four invasive ductal carcinomas, there was no resonance peak at 3.12-3.32 ppm. These four cancers showing no Cho peak were characterized by HER2 negativity (n=4), ER positivity (n=4), PR positivity (n=3), and low level of Ki-67 (n=3). Mean and median values of tCho quantified by MR spectroscopy were 1.27 (range, 0.20-4.05) and 0.9 (interquartile range 0.48-1.87), respectively. Mean and median values of tCho peak SNR on MR spectroscopy were 5.2 (range 0.8-22.5) and 3.35 (interquartile range 2.05-7.65), respectively.

Table 2. NMR spectroscopic values for 36 breast cancer specimens

Metabolite concentration (mmol/kg)		**Metabolic ratio**	
Metabolite	**Median (Mean±SD)**	**Ratio**	**Median (Mean±SD)**
Cho	0.29 (0.36±0.28)	Cho/Cr	1.53 (1.76±0.99)
PC	0.88 (1.34±1.26)	PC/Cr	1.57 (1.77±1.20)
GPC	0.19 (0.25±0.24)	GPC/Cr	0.83 (1.06±0.75)
tCho	1.46 (1.95±1.63)	tCho/Cr	3.86 (4.60±2.17)
Cr	0.61 (0.66±0.47)	GPC/PC	0.60 (0.73±0.61)
Gly	0.62 (0.96±1.17)	GPC/Cho	0.55 (0.72±0.63)
Tau	1.56 (1.83±1.41)	PC/Cho	1.20 (1.28±1.08)
s-Ins	0.44 (0.56±0.58)		
m-Ins	0.53 (0.86±0.88)		

Data represent median (mean ± standard deviation)

Cho: choline, PC: phosphocholine, GPC: glycerophosphocholine, tCho: total choline, Cr: creatine

Tau (taurine), Gly (glycine), m-Ins (myo-inositol), s-Ins (scyllo-inositol)

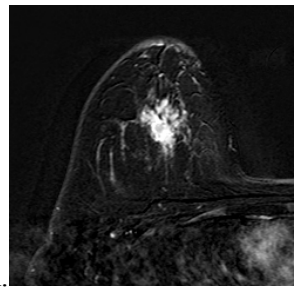
Figure 1. 38-year-old woman with invasive ductal carcinoma (tumor size 37mm, triple negative, strongly positive Ki-67)

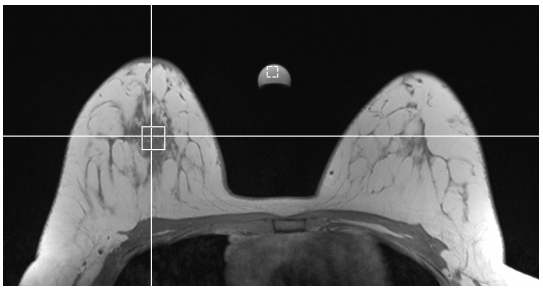
A. Contrast-enhanced MR image shows enhancing mass of the right breast.

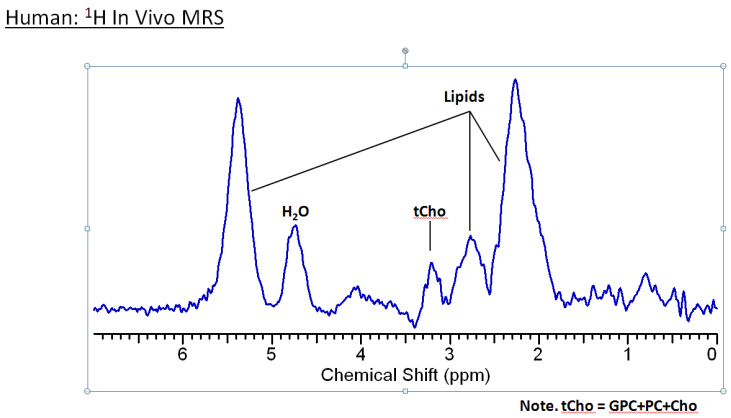
B. Precontrast T1-weighted image was used for voxel placement within the tumor and external reference (cylindrical bottle phantom)

C. Single-voxel MR spectrum shows Cho resonance peak at 3.2 ppm. tCho concentration measured with MR spectroscopy was 1.01 mmol/kg.

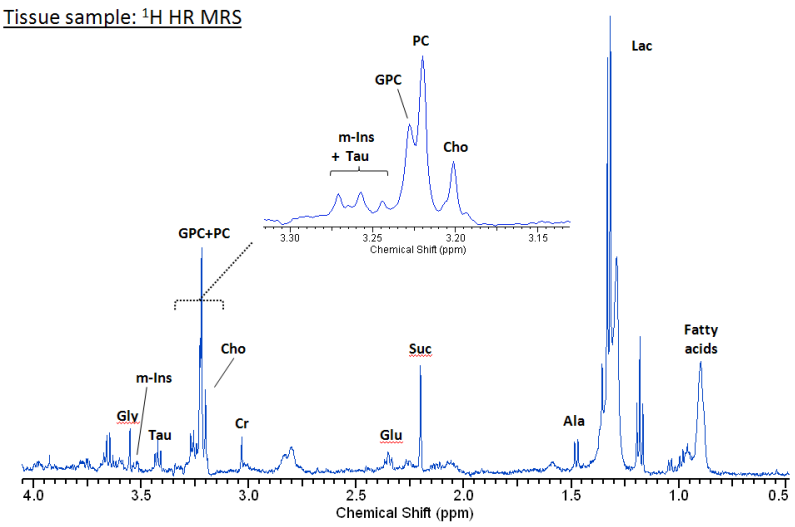
D. NMR spectrum using the biopsy specimen show the peak of each choline metabolite showed more clarity, compared with MR spectrum. tCho concentration measured with NMR spectroscopy was 6.5 mmol/kg.

A. 

B. 

C. 

D.



Note. Lac, lactate; Ala, alanine; Glu, glutamate; Cr, creatine; Cho, free choline; GPC+PC, glycerophosphocholine + phosphocholine; tCho, total choline-containing compounds (e.g., tCho = GPC+PC+Cho);Tau, taurine; m-Ins, myo-inositol; Gly, glycine.

Several metabolic ratio obtained with NMR spectroscopy were correlated with MR spectroscopic values. The Cho/Cr on NMR spectroscopy was significantly correlated with the normalized tCho (r=0.354, p=0.04) and SNR of tCho peak (r=0.403, p=0.03) on MR spectroscopy. In addition, the GPC/Cr (r=0.412, p=0.02) and tCho/Cr (r=0.353, p=0.04) significantly correlated with SNR of tCho peak. Other NMR spectroscopic values did not correlate with MR spectroscopic values. Also, there was no significant correlation between the values of tCho measured with MR spectroscopy and NMR spectroscopy.

As shown in Table 3, we found several metabolite markers on NMR spectroscopy correlate with histopathologic prognostic markers. ER negative cancers showed higher Cho concentration than ER positive cancers (p=0.03). PR negative cancers showed higher concentrations of Cho, Cr, and Tau than

those of PR positive cancers (p=0.01, p=0.02, and p=0.02, respectively). Between HER2 positive and negative groups, concentrations of Tau, s-Ins, and m-Ins of HER2 positive cancers were significantly higher than those of the HER2 negative cancers (p=0.01, p=0.03, and p=0.01, respectively). Triple negative cancers showed higher Cho concentration and higher value of Cho/Cr, tCho/Cr than those of non-triple negative cancers. Cancers strongly positive for Ki-67 showed higher concentrations of tCho and PC (p=0.01), and higher value of PC/Cr than those of cancers weekly positive for Ki-67 (p=0.01). Poor prognosis group showed higher Gly and s-Ins concentrations than those of good prognosis group (p=0.02 and p=0.01, respectively). Histologic grade, tumor size, and lymph node metastasis did not correlate with NMR spectroscopic values. MR spectroscopic values were not significantly different according to histopathologic parameters.

Table 3. Correlation with histopathologic parameters with value of NMR spectroscopy

Metabolite or Metabolic ratio	ER			PR			HER		
	Negative (n=6)	Positive (n=28)		Negative (n=18)	Positive (n=16)		Negative (n=29)	Positive (n=5)	
	Mean (SD)	Mean (SD)	*P*	Mean (SD)	Mean (SD)	*P*	Mean (SD)	Mean (SD)	*P*
Cho	**0.60 (0.17)**	**0.34 (0.28)**	**0.03**	**0.50 (0.28)**	**0.25 (0.23)**	**0.01**	0.37 (0.30)	0.47 (0.20)	0.50
PC	2.18 (1.78)	1.16 (1.05)	0.07	1.61 (1.31)	1.02 (1.03)	0.17	1.33 (1.38)	1.96 (0.63)	0.32
GPC	0.27 (0.20)	0.25 (0.24)	0.80	0.30 (0.25)	0.19 (0.18)	0.15	0.23 (0.24)	0.39 (0.23)	0.19
tCho	3.06 (2.05)	1.75 (1.43)	0.07	2.42 (1.65)	1.47 (1.42)	0.08	1.94 (1.75)	2.83 (1.03)	0.28
Cr	0.82 (0.37)	0.64 (0.47)	0.39	**0.84 (0.47)**	**0.48 (0.36)**	**0.02**	0.60 (0.41)	1.04 (0.62)	0.06
Tau	2.60 (1.10)	1.71 (1.45)	0.17	**2.42 (1.48)**	**1.25 (1.06)**	**0.02**	**1.54 (0.95)**	**3.32 (2.11)**	**0.01**
s-Ins	0.55 (0.28)	0.63 (0.67)	0.78	0.59 (0.27)	0.64 (0.85)	0.85	**0.43 (0.30)**	**0.82 (0.27)**	**0.03**
m-Ins	1.22 (0.39)	0.83 (0.97)	0.35	1.17 (0.97)	0.58 (0.72)	0.06	**0.71 (0.65)**	**1.75 (1.48)**	**0.01**
Gly	1.18 (0.79)	0.97 (1.29)	0.72	1.14 (1.22)	0.86 (1.23)	0.52	0.78 (1.00)	1.99 (1.96)	0.24
Cho/Cr	2.53 (1.30)	1.68 (0.88)	0.06	2.01 (1.15)	1.62 (0.77)	0.27	1.99 (1.12)	1.46 (0.30)	0.31
PC/Cr	2.50 (2.12)	1.63 (0.96)	0.11	1.78 (1.46)	1.80 (0.94)	0.96	1.78 (1.35)	2.16 (0.95)	0.56
GPC/Cr	1.01 (0.70)	1.09 (0.79)	0.82	1.09 (0.88)	1.06 (0.64)	0.91	1.07 (0.81)	1.22 (0.76)	0.71
tCho/Cr	6.05 (3.12)	4.41 (1.91)	0.10	4.89 (2.60)	4.49 (1.70)	0.61	4.85 (2.43)	4.85 (1.85)	0.99

Metabolite or Metabolic ratio	Triple negativity			Ki-67			Prognosis		
	Negative (n=29)	Positive (n=4)		Low (n=26)	High (n=6)		Good (n=9)	Poor (n=25)	
	Mean (SD)	Mean (SD)	*P*	Mean (SD)	Mean (SD)	*P*	Mean (SD)	Mean (SD)	*P*
Cho	**0.35 (0.27)**	**0.65 (0.20)**	**0.04**	0.35 (0.29)	0.49 (0.21)	0.28	0.24 (0.24)	0.43 (0.28)	0.07
PC	1.21 (1.03)	2.30 (2.29)	0.10	**1.08 (1.07)**	**2.60 (1.43)**	**0.01**	1.27 (1.27)	1.45 (1.29)	0.72
GPC	0.25 (0.23)	0.26 (0.24)	0.93	0.23 (0.24)	0.37 (0.17)	0.20	0.24 (0.23)	0.27 (0.24)	0.76
tCho	1.81 (1.40)	3.21 (2.62)	0.10	**1.66 (1.47)**	**3.46 (1.71)**	**0.01**	1.75 (1.68)	2.16 (1.62)	0.53
Cr	0.86 (0.75)	0.59 (0.20)	0.74	0.62 (0.48)	0.94 (0.32)	0.14	0.51 (0.45)	0.77 (0.46)	0.15
Tau	1.87 (1.49)	2.07 (0.90)	0.79	1.79 (1.53)	2.34 (1.19)	0.43	1.27 (1.12)	2.17 (1.43)	0.12
s-Ins	0.66 (0.64)	0.39 (0.11)	0.41	0.63 (0.67)	0.58 (0.30)	0.86	**0.25 (0.18)**	**0.73 (0.64)**	**0.01**
m-Ins	0.87 (0.95)	1.12 (0.45)	0.62	0.86 (1.02)	1.05 (0.30)	0.66	0.54 (0.44)	1.03 (0.98)	0.06
Gly	1.04 (1.28)	0.83 (0.41)	0.75	1.03 (1.33)	0.99 (0.86)	0.93	**0.51 (0.30)**	**1.18 (1.34)**	**0.02**
Cho/Cr	**1.68 (0.85)**	**2.98 (1.41)**	**0.01**	1.77 (0.91)	1.60 (0.62)	0.65	1.71 (1.01)	1.83 (1.03)	0.76
PC/Cr	1.66 (0.91)	2.73 (2.68)	0.10	**1.51 (0.92)**	**2.97 (1.92)**	**0.01**	2.17 (0.95)	1.68 (1.30)	0.31
GPC/Cr	1.08 (0.77)	1.07 (0.88)	0.99	1.01 (0.78)	1.33 (0.87)	0.38	1.32 (0.64)	1.01 (0.79)	0.30
tCho/Cr	**4.42 (1.85)**	**6.80 (3.73)**	**0.04**	4.30 (1.85)	5.91 (3.22)	0.11	5.20 (1.52)	4.52 (2.38)	0.43

*Other NMR values (GPC/Cho, GPC/PC, PC/Cho) were not significantly difference according to histopathologic parameters.

Bold value indicates statistically significant ($P<0.05$)

IV. DISCUSSION

The main purpose of the most previous studies using breast ^{1}H MR spectroscopy was to evaluate diagnostic performance for differentiating between malignant and benign lesions.[16-22, 32-35] These studies have reported MR spectroscopy either alone or in conjunction with contrast-enhanced MRI improves diagnostic accuracy of breast MRI and it may be useful in reducing the number of lesions detected at MR imaging that require biopsy. One of them showed addition of MR spectroscopy to breast MRI would have increased positive predictive value of biopsy from 35% to 82%.[32] These studies with 1.5 T had a various range of detectability of tCho peak from 70 to 100% in breast malignancy, which means that 0 to 30 % of malignancy showed no tCho peak. In present study, single-voxel ^{1}H MR spectroscopy using 3.0-T MR scanner detected tCho peak in 90% of breast cancers (32/36). This rate of cancer showing tCho peak was also within the above-mentioned range (70-100%). Although another study by Gruber et al. showed 3.0-T MR spectroscopy provided sensitivity of 97% using tCho peak SNR value for differentiation of malignant and benign breast lesions,[35] about a third of breast cancers showed no tCho peak on 4.0-T MR spectroscopy according to the graph presented in the study by Bolan et al., who did not show exact number of that cancers.[21] Therefore, our results would be within the range of detectability of tCho peak in breast cancers with high-tesla MR spectroscopy (≥3.0 T). At high field strength (≥3.0 T), tCho signal would be more detected in benign lesion or normal tissue,[21, 36] which may increase false-positive cases. However, we could not

determine the effect of tCho signal in non-cancer tissue for diagnostic accuracy of high-tesla MR spectroscopy, because this study did not evaluate benign lesions. Therefore, further study may be needed about the appropriate levels of tCho peak for differentiation malignant from benign breast lesion in MR spectroscopy at high field strength.

In this study, all four cancers showing no tCho peak on MR spectroscopy were HER2 negative and ER positive, and the three cancers showed low level of Ki-67. This result is in agreement with the previous report by Tse et al. that false-negative cancers on MR spectroscopy showed no HER2 overexpression.[33] This finding means HER2 negative, low-grade, and low-proliferative breast cancer may correlate with false-negative results on MR spectroscopy, which is supported by the observation by Aboagye et al. who found forced HER2 overexpression in a tumor cell line resulted in a dramatic increase in levels of choline metabolites.[37] However, the proportion of HER2-negative cancers in this study was too high (80.5%, 29/36) to confirm this hypothesis.

To our knowledge, no study has directly compared MR spectroscopy and NMR spectroscopy for quantification of choline metabolites of breast cancer. We quantified choline metabolites using both MR spectroscopy and NMR spectroscopy and compared those in the current study. In the previous in vivo ^{1}H MR spectroscopy studies, the measured tCho concentration levels were within a range of 0.3 – 10.00 mmol/kg.[18, 21, 38, 39] The range of tCho concentration (range, 0.06-6.55 μmol/g) acquired with 11.7-T NMR spectroscopy using core-biopsy specimen was also consistent with values reported in previous study that used surgical specimen of breast cancer for

NMR spectroscopy.[14] Our result showed no statistically significant correlation between the values of tCho using MR and NMR spectroscopy. This finding may result from tumor heterogeneity and TSP (internal reference for NMR spectrum). We included breast cancers with variable size, and tumors with more than 3cm in diameter were 12 cases (33.3%). Larger breast cancers have heterogeneous histologic features, which may induce variation in tCho concentration of breast lesions. In the study by Bolan et al., single-voxel MR spectroscopic data acquired from different region of the same 3-cm breast cancer showed different tCho concnentrations.[21] Therefore, single-voxel location of MR spectroscopy and core-biopsy specimen used for NMR spectroscopy may not represent the metabolic composition of the large tumors. The variations of the absolute tCho concentration may be due to TSP. It was added as a standard for metabolic concentration estimates into NMR sample cell, which has potential to bind to plasma proteins.[40] If TSP binds to proteins in tissue samples, metabolites can be overestimated by the method used in present study. Actually, we found that tCho concentration using NMR spectroscopy tended to have higher value than normalized tCho value of MR spectroscopy in each patient.

Several metabolic ratios (Cho/Cr, GPC/Cr, tCho/Cr) acquired with NMR spectroscopy showed significant correlation with MR spectroscopic values, although there was no significant correlation between the values of tCho on MR and NMR spectroscopy. Besides absolute value of choline metabolite concentration, metabolic ratios using Cr and Cho metabolites have used to know difference of tissue composition of breast cancer in previous studies.[8, 14, 15] These groups showed that metabolic ratios of breast cancer tissue differed

according to histopathologic grade or tumor cell fraction. Especially for heterogeneous tumor, metabolic ratios may be more consistently available markers than absolute concentration of choline metabolites. Although we could not directly compare metabolic ratios of the two spectroscopic methods because we could not calculate concentrations of Cr and individual choline metabolite on MR spectroscopy, our result means partial correlation between NMR spectroscopic values and MR spectroscopic values. Consequently, this finding indicates MR spectroscopy may be useful for in vivo assessment of choline metabolites of breast cancer.

In breast cancer research, most of previous studies employing high-resolution NMR spectroscopy have used surgically obtained tissue specimen.[8, 14, 15] Therefore, their results could not affect diagnosis of breast tumors or surgical decision of breast cancers. We conducted NMR spectroscopy using 14-gauge core biopsy specimen. US-guided core needle biopsy is most-used procedure for diagnosis of suspicious breast lesion, therefore NMR spectroscopic values using US-guided core biopsy specimen can be clinically applicable for diagnosis or characterization of breast cancers to decide treatment options such as neoadjuvant chemotherapy.

High-resolution NMR spectroscopy quantified choline metabolites in all 36 cancer samples of our patient group. 11.7-T NMR spectra discriminated and quantified multiple choline metabolites including PC, GPC, and Cho, although 3.0-T MR spectra did not discriminate above-mentioned choline metabolites, which contribute to tCho peak signal. Concentrations of PC, GPC, and Cho were consistent with previously reported values, and PC was main contributor

of tCho peak signal.[14, 15] In addition, we found that some NMR spectroscopic values (Cho, PC, Tau, s-Ins, m-Ins, Gly, Cho/Cr, tCho/Cr, PC/Cr) significantly correlated with clinicopathologic parameters (ER, PR, HER2, and Ki-67 status, triple negativity) and poor prognosis group. In other words, our results suggest that the aggressiveness and proliferative activity of breast cancer were correlated with NMR spectroscopic values using core-biopsy specimen. Therefore, it is expected NMR spectroscopic values may be used as reliable markers for prediction of prognosis in breast cancer patients. For confirmation the relationship between NMR spectroscopic values and prognosis, future study about whether NMR spectroscopic values are independent factors for prognosis of breast cancer would be needed for large numbers of cancers.

Among the NMR spectroscopic values significantly correlated with clinicopathologic parameters in this study, PC and Cho were already well-known marker for breast cancer.[14, 15, 41-43] On the other hand, it has recently reported elevated levels of Tau and Gly were associated with breast cancer tissue.[14, 15, 44] Tau is an amino acid involved in many essential biological functions such as antioxidation, membrane stabilization, and cell shrinkage during apoptosis, while Gly is an amino acid known as a precursor to proteins.[45-48] Cao et al. reported treatment response of breast cancer patients receiving neoadjuvant chemotherapy was best predicted by Tau among multiple choline metabolites obtained with HR-MAS MR spectroscopy.[44] Sitter el al. reported Gly concentration was significantly higher in breast cancers larger than 2 cm compared to with smaller cancers.[14] Although the precise mechanisms of these findings are not known, our study also showed higher concentrations of

Tau and Gly were associated with PR negative / HER2 positive cancers and poor prognosis group, respectively. Accordingly, we suggest that Tau and Gly may be candidates for reliable marker for prediction of treatment response or poor prognosis in therapeutic monitoring of breast cancer patients. Further studies for assessment of most reliable marker among the multiple HR-MAS MR spectroscopic values will be needed for breast cancer research.

This study has some technical limitations. We used single-voxel MR spectroscopy used in most of published reports. Single-voxel imaging covers smaller region of the tumor, compared to multivoxel MR spectroscopic imaging. Therefore, the correct positioning of the voxel within a tumor requires experience and skill in single-voxel MR spectroscopy.[32] Multivoxel MR spectroscopic imaging has advantages of high spatial resolution and large spatial coverage over single-voxel MR spectroscopy. This approach can be advantageous for the diagnosis and characterization of heterogeneous or multicentric/multifocal tumors.[49] However, multivoxel spectroscopic imaging has the disadvantage that the voxel shape may be less well defined than single-voxel imaging.[50] This may result in the contamination of spectral data from individual voxels by large-amplitude signals from surrounding voxels containing fat. In recent studies, multivoxel MR spectroscopy using 3.0-T unit showed good performance for differentiation between malignant and benign breast lesions.[34, 35] If further research using multivoxel 3.0-T MR spectroscopy performed for comparison of MR spectroscopy and NMR spectroscopy, there is a possibility that these two modalities will show more significant correlation than our results. In this study, the normalized tCho signal was calculated using

external reference method.[23, 39, 51] External reference method requires correction of partial volume effect of adipose tissue in the voxel and separate calibration experiments.[39] On the other hand, internal reference method using intravoxel water as a reference automatically compensates for partial volume effect and does not need separate calibration.[21, 38] A potential limitation of this method was a possibility that water content of the voxel may change under varying pathologic conditions.[52] For this reason, we used external reference method in this study. However, these two reference methods have a possibility of errors being occurred between quantified values and the actual values in the patients, because the T1 and T2 values of Cho used were the values cited. Third, adjustments of the acquisition parameters are important to obtain optimal spectral data in both MR and NMR spectroscopy. Especially for MR spectroscopy, shimming and water suppression are particularly important.[50] We tried to adjust the acquisition parameter on each case when undertaking MR and NMR spectroscopy in this study. Finally, we could not examine the association between spectroscopic values obtained with MR or NMR spectroscopy and molecular subtypes of breast cancer. Molecular subtypes of breast cancer are considered to play important roles in predicting prognosis and determining treatment modalities.[53, 54] Therefore, further study would be needed for evaluation of the association between molecular subtypes and spectroscopic values.

V. CONCLUSION

In conclusion, MR spectroscopic values relatively correlate with several NMR spectroscopic values in breast cancer, indicating MR spectroscopy may be used to aid in vivo assessment of choline metabolites of breast cancer. Metabolites/Metabolic ratio obtained using NMR spectroscopy were correlated with the histologic prognostic markers (ER, PR, HER, triple negativity, Ki-67, prognosis group), indicating NMR spectroscopy may be helpful for detection of reliable markers of characterization of breast cancer.

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ABSTRACT (IN KOREAN)

유방암의 콜린 대사물질 정량화에 있어서 자기 공명 영상을 이용한 분광법과 핵자기 공명 분광법의 비교

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본 연구는 침 생검(core needle biopsy)으로 얻은 유방암 조직을 이용한 핵자기 공명 분광법(nuclear magnetic resonance spectroscopy)과 생체 내 자기 공명 영상을 이용한 분광법(in vivo proton magnetic resonance spectroscopy)으로 유방암 조직에서 발현되는 콜린(choline) 대사물질(metabolites)을 정량화한 후, 두 값을 비교하여 상호 연관성이 존재하는지 알아보고자 하였다. 동시에 유방암 연구에 있어 이 두 분광법의 임상적 역할에 대해 평가하고자 하였다. 이 연구는 전향적 연구로 임상시험위원회(institutional review board)의 승인을 받았다. 영상에서 1cm 이상의 크기를 보이며 침 생검을 통해 유방암으로 진단받은 환자들을 대상으로 단일 복셀(single-voxel) 자기 공명 영상(3.0-T)을 이용한 분광법을 시행하였다. 이를 통해 전체 콜린 수치(tCho)와 전체 콜린 높이(tCho peak)의

신호 대 잡음비(signal-to-noise ratio)를 구하였다. 위 환자들에게 얻은 침 생검 조직에 대해 핵자기 공명 분광법(11.7-T)을 시행하여 여러 콜린 대사 물질의 농도와 대사 비(metabolic ratio)를 구하였다. 각각의 분광법으로 구한 값 사이의 연관성을 평가하기 위해 통계 분석을 시행하였고, 두 값들이 임상병리학적 변수들(clinicopathologic variables)에 따라 차이가 있는지 알아보기 위해 추가 분석을 시행하였다. 총 34명의 환자(나이 범위, 34-68세)에서 발견된 36개 유방암(평균 크기, 29.7mm)을 분석하였다. 핵자기 공명 분광법은 모든 유방암에서 콜린 대사물질을 정량화하고 구분하였으나, 자기 공명 영상을 이용한 분광법은 32개의 유방암의 콜린 대사물질을 정량화하였다. 핵자기 공명 분광법으로 얻은 Cho(free choline)/Cr(creratine) 대사 비는 자기공명영상을 이용한 분광법으로 얻은 전체 콜린 수치(r=0.354, p=0.04)와 전체 콜린 높이의 신호 대 잡음비(r=0.403, p=0.03) 모두와 유의한 상관성을 보였다. GPC(glycerophosphocholine)/Cr (r=0.412, p=0.02)과 tCho/Cr(r=0.403, p=0.03) 대사 비는 전체 콜린 높이의 신호 대 잡음비(r=0.403, p=0.03)와 유의한 상관성을 보였다. 핵자기 공명 분광법으로 얻은 값 중 Cho, Cr, taurine, s-inositol, m-inositol, tCho, PC(phosphocholine), glycine, Cho/Cr, tCho/Cr, PC/Cr 값은 유방암의 임상병리학적 변수들[ER(estrogen receptor), PR(progesterone receptor), HER2(a receptor for human epidermal growth factor), triple negativity, Ki-67, poor prognosis group (detection of axillary lymph node metastasis, tumor size with diameter >2cm, or negative for ER or PR)]과 유의한 상관성을 보이는 것을 알 수 있었다. 본 연구 결과, 자기 공명 영상을 이용한 분광

법 값은 유방암 조직을 이용한 핵자기 공명 분광법 값 중 일부와 상관성을 보였으며, 이는 자기 공명 영상을 이용한 분광법이 유방암의 콜린 대사 물질을 생체 내에서 측정하는 데 있어 도움이 될 수 있음을 의미한다. 핵자기 공명 분광법으로 얻은 값은 조직학적 예후인자(ER, PR, HER, triple negativity, Ki-67, prognosis group)들과 유의한 상관성을 보였으며, 이는 핵자기 공명 분광법이 유방암을 특성화(characterization)할 수 있는 믿을만한 표지자를 찾는 데 도움을 줄 수 있음을 의미한다.

핵심되는 말: 분광법, 유방, MRI(자기 공명 영상), NMR(핵자기공명), 콜린(choline)