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Evaluation of Serum of Breast Cancer Patients using High Resolution Magic Angle Proton Magnetic Resonance Spectroscopy (HR-MAS): A Search for Possible Biomarker?

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Abstract

Background: Early diagnosis is an important factor for successful outcome in breast cancer. Current existing prognostic and predictive tool like ER, PR and HER2 status have main utility to guide whether a patient should or should not receive adjuvant endocrine or targeted therapy and situation has become more complex after the discovery of genomic tests like oncotype Dx etc. So the need to enhance the understanding of the disease process and treatment response, a hunt for suitable tumor marker is still on.

Sample collection: 5-7 ml fasting peripheral venous blood was withdrawn from newly diagnosed breast cancer patients. The subjects were properly matched in terms of age, sex, dietary habits and other parameters. The blood was centrifuged and resultant supernatant serum was put into the 3 ml ependorf tube and the specimen was immediately snap frozen in liquid nitrogen and was transferred to the HRMAS lab where it was stored at -80ºC.

HRMAS experiment: The collected samples (Malignant 32, Benign 32 and Healthy Control 28) were thawed and subjected to 800 MHz HRMAS spectrometer. The serum samples were recorded in native form using TSP as an internal standard and a coaxial insert. The spectra were acquired using 1D single pulse and Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with water suppress [PRESET- 90 degree- (δ-180°-δ)n-Aq] with a echo time of 40 ms. The HRMAS findings were correlated with the standard histopathological report.

Results: The 3D scattered PCA score plot of serum (explaining 83% of the total variance showed distinct group separation among the healthy, fibroadenoma and malignant tumor samples. The PLS-DA model generated considering all the three groups showed a predictive ability of 78% which proves that the model was robust enough for group differentiation. The X-loading plot (PC-1 which explained 69% of total variance) of the cases, exhibited positive loadings of lactate, succinate and alanine in serum. When compared with healthy controls, while glucose was found to be down-regulated in almost all the fibroadenoma and malignant cases. Thus, glucose along with lactate appeared to be the major confounders between the two groups *viz*. healthy and tumor groups.

Conclusion: All malignant tumors showed up regulation of lactic acid, acetate and choline containing compounds while down regulation of glucose and lipids. The study did provided evidence for the clinical use of these identified metabolites. However, future studies involving large sample size using sequential samples should be carried out.

Keywords: Breast cancer; Serum; HRMAS

Introduction

Cancer of the breast in women is a major health burden worldwide. In the developed countries of the West, breast cancer is the single largest cause of death in women between the ages of 35 and 54, with a mortality rate of 28.4 per 100000 females per year in UK [1] and 20.7 per 100,000 (age-standardized mortality rates) women in US [2]. It accounts for 25% of all malignant disease in woman and approximately one in twelve women in America and UK will suffer from some kind of breast diseases during their lifetime [3]. In India, cancer of the breast has been replacing cancer of cervix as the leading site of cancer in most urban population based cancer registries [4]. Since the early detection of breast cancer improves the prognosis in most cases, regular screening of an increasingly large proportion of the female population has been widely advocated [5,6].

Mammography and ultrasound imaging are established as the main non-invasive diagnostic procedure used in the identification of breast cancer in screening population and for further evaluation in already identified cases. Unfortunately, these techniques provide rather limited sensitivity and specificity. Over the past few year contrast material-enhanced breast magnetic resonance [7-10] (MR) imaging has evolved and has become an important tool in the evaluation of breast abnormalities, with a reported sensitivity as high as 94% -100% and specificity in the range of 37%-97% but unfortunately benign tumors, such as fibro adenomas also enhance to various levels from minimal to intense [11,12], therefore a reliable discrimination cannot be made on the basis of enhancement alone. Similarly 20% of Fine needle aspiration cytology (FNAC) may yield inadequate aspirate and may demonstrate a false negative rate of 6.4% [13] in palpable lumps.

At present no definite etiology and tumor markers are available for breast cancer and ER/PR receptor, Her 2 nu etc. are rather poor markers of prognosis as the prognostic significance of ER, PR and Her 2 nu status is limited and its optimal use is as a predictive marker for benefit of adjuvant anti estrogen and targeted therapy. Similarly, to determine

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response to treatment, the standard therapeutic practice to measure clinical, radiological and/or serum markers of disease 6–9 weeks after treatment initiation are missing till now and there are no biomarkers to predict the toxicity.

There may be significant reduction in mortality by early detection of the disease. Regular self-examinations and yearly mammograms are the most common tools used in the early diagnosis of breast cancer but neither the currently available tools detects the disease in pre-clinical stage nor provide any metabolic and biochemical information. The present study was planned to assess the metabolic profiling of the serum samples which would allow us to observe the biological role of different small molecular weight metabolites in differentiating benign and malignant groups and staging of breast cancer using proton HR-MAS.

Objective

1. To assess the N.M.R. spectra of serum of the patient of malignant, benign and healthy individual.
2. To correlate the N.M.R. Spectroscopy finding with standard histopathology report.

Methodology

This present cross-sectional comparative study was conducted in Department of General Surgery, King Georges Medical University Hospital, Lucknow, UP, India and in collaboration with Centre of biomedical research, Sanjay Gandhi Postgraduate Institute of Medical Sciences campus, Lucknow from September 2014 to August 2015. A total of 72 morning serum samples obtained after overnight fasting were collected. All the serum samples of the three groups were studied for metabolic differences by using 800 MHz HRMAS spectroscope.

Inclusion criteria

All fresh cases of malignant/benign breast lump.

Exclusion criteria

Patient with chemotherapy/radiotherapy and M.R.M./B.C.T.

Material

800 MHz N.M.R. Spectroscopy.

Nuclear magnetic resonance, or NMR as it is abbreviated by scientists, is a phenomenon which occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. Some nuclei experience this phenomenon, and others do not, dependent upon whether they possess a property called nuclear spin. Spectroscopy is the study of the interaction of electromagnetic radiation with matter. Nuclear magnetic resonance spectroscopy is the use of the NMR phenomenon to study physical, chemical, and biological properties of matter. As a consequence, NMR spectroscopy finds applications in several areas of science [12].

HRMAS does not generate an image of the tumor directly, but the spectroscopic data can be obtained from a well-localized area. Thus the biochemical information obtained from HRMAS can be interpreted in relation to a defined anatomical location, and images of metabolite distributions can be generated. In using HRMAS, the aim is to identify surrogate biochemical markers of cellular transformation, thus differentiating benign from malignant lesions and potential identifying different tumor types. Prognostic and diagnostic information is also sought from the spectrum of malignant tumors.

NMR experimental conditions and analysis

The serum from blood samples were collected and stored at -80°C until analyzed. After collection, the samples were thawed and subjected to NMR acquisition. The serum samples were recorded in native form using TSP as an internal standard and a coaxial insert. For stabilization of the binary pH, the urine samples were mixed with phosphate buffer. The NMR experiments were performed using a Bruker BiospinAvance III 800 MHz NMR (BrukerGmBH, Germany) spectrometer equipped with a 5mm Triple resonance inverse (TCI) $^{1}H/^{13}C/^{15}N$ cryoprobe with a Z-shielded gradient and standard vertical bore, operating at a proton frequency of 800.21MHz (18.8 T) [1]. H NMR spectra of serum samples were acquired with water pre- saturation at 300 K with 128 scans and 4 dummy scans and an acquisition time of 2.55 s. The spectra were acquired using 1D single pulse and Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with the following experimental parameters: spectral width of 12820.5 Hz, time domain data points of 64 K, effective 90 degree flip angle, 9.0 μs, relation delay 5.0 s, acquisition time of 2.55 s, 128 number of scans with 4 dummy scans with a total recording time of approximately 17 minutes. CPMG pulse sequence with water suppresses [PRESET- 90 degree- (δ-180°-δ) n-Aq] with an echo time of 40 ms was performed. All the spectra were processed by applying a line broadening of 0.3Hz to the FID prior to Fourier Transformation.

Statistical analysis

Data comprising H NMR spectra recorded for serum samples were phased, baseline corrected, aligned, and then subjected to multivariate analysis. The spectra were reduced to discrete chemical shift regions between 0.7-9.2 ppm for urine and 0.7-8.7 ppm for serum (excluding regions responsible for water (4.74-5.20 ppm) by digitization, and were binned into uniform buckets of 0.01 ppm. These buckets were scaled to the sum of their total intensities to integrate peak area. The buckets were normalized and the resulting data matrices were exported to Microsoft Excel 2007 (Microsoft Corporation USA) and then to 'The Unscrambler' software package (Version 10.01, Camo USA, Norway) for unsupervised multivariate PCA and supervised PLS-DA. The statistical model(s) of PCA and PLS-DA were generated using a full cross validation [1].

Results

In this study total 72 samples were analyzed and 24 samples from each group normal, benign and malignant were collected and 35 metabolites were analyzed. At the time of presentation 15 patients had locally advanced breast cancer, 3 had stage I, 5 stage II and one stage IV. The mean age in malignant cases was 46 years and 32 years in benign lesions ($p>0.08$).

All the patients having malignant diseases were confirmed histopathologically as infiltrating. Similarly in 24 cases of benign diseases 16 were being confirmed histopathologically as fibro adenoma, 4 as chronic mastitis, 4 as benign phylloides tumor.

Metabolomic profiles

Thirty five metabolites were identified in the NMR spectra of serum The H NMR spectra along with the assignments of metabolites of serum are shown in figure 1. The metabolites were characterized on the basis of their splitting pattern as reported in literature and also by comparison with their standard NMR spectra as reported in Biological Magnetic Resonance Bank (BMRB, www.bmrb.wisc.edu) and Human Metabolome Database (HMDB, www.hmdb.ca/the) (Figure 1).

Multivariate analysis

The 3D scattered PCA score plot of serum explaining 83% of the total variance showed distinct group separation among the healthy, fibro adenoma and malignant tumor samples. The loading plot (PC-1) demonstrated. The PLS-DA model generated considering all the three groups showed a predictive ability of 78% which proves that the model was robust enough for group differentiation. The X-loading plot (PC-1 which explained 67% of total variance) of the cases, exhibited positive

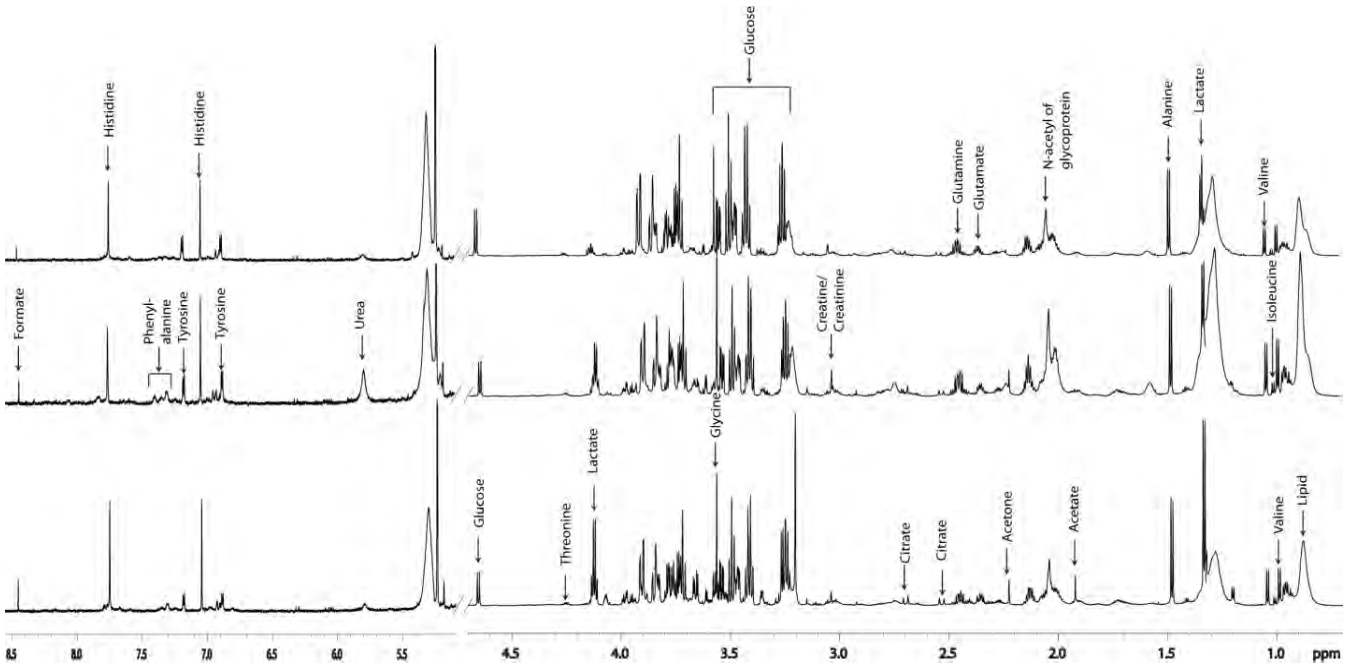

Figure 1: Assignments of various metabolites.

loadings of lactate, succinate and alanine in serum when compared with healthy controls, while glucose was found to be down-regulated in almost all the fibro adenoma and malignant cases. Thus, glucose along with lactate appeared to be the major confounders between the two groups viz. healthy and tumor groups. A PLS-DA model was then built using the three groups as Y-variables. Statistical models from supervised multivariate data analysis were validated by random permutation of the response variable resulting in R_2value of 0.82 and Q_2 values of 0.78 (Figure 2).

Analysis

All malignant cancer 100% show up regulation of lactate, acetate and choline (Figure 2b at 3.2 ppm) containing compound and down regulation of glucose (Figure 2b near 4.05 ppm) and lipid. While in case of fibro adenoma only 17 case (70%) show up regulation of lactic acid and acetate and choline containing compound and down regulation of glucose and lipid.in case of healthy individual. Only 2 case (8.33%) shows up regulation of lactic acid, acetate and choline containing compound and down regulation of glucose and lipid, rest all metabolites were not significantly changed (Table 1).

Discussion

Over last many years various imaging modalities like mammography, MRI were used for differentiation of cancer from benign lesions but with advancing time role of immunocytochemistry and biochemical markers started being studied. Recently molecular biological techniques like PCR (polymerase chain reaction) enable us to augment improved methods for tumor diagnosis and also to predict response to existing therapies. Breast cancers like other forms of malignancies are thought to progress by accumulation of a series of genetic and phenotypic changes in the pathways regulating cellular proliferation, differentiation, death (apoptosis or necrosis), DNA repair, tissue compartmentalization and responses to therapy.The spectra from serum of all the patients in this pilot study were compared between benign and normal serums. We have found markedly elevated levels of metabolites in cancer and benign breast samples. In cancerous cells anaerobic glycolysis was the major energy metabolism and hence high conc. of lactate which may be attributed to quantity of necrosis whereas in noninvolved tissue showed high levels of acetate depicting aerobic glycolysis. The normal serum spectrum represents a perfect balance of aerobic and anaerobic metabolism which is clearly evident as the concentration of ketone bodies (acetate, oxaloacetate, acetoacetate) which are in good concentration with respect to lactate and they are the major metabolites in NMR spectrum whereas in breast cancer no ketone bodies were observed and lactate was one of major metabolites in NMR spectrum. Spectra from the cancerous serum revealed high concentration of cytosolic amino acids consisting glycine, glutamine, alanine and glutamate, when compared to benign which constitutes the cytosolic amino acid pool required for cell proliferation. The significantly elevated levels of these amino acids indicate a high protein proliferation rate in pathogenic tissues. These findings of malignant serum have not been reported yet however, metabolic differences in various tissue samples indicating altered metabolism in cancerous cells has been described by previous workers [14,15] and these study of H NMR spectra of PCA extracted breast specimens provided a comprehensive window into the metabolic activities of the tissue.

All choline metabolites choline, phosphocholine and glycerophosphocholine, were detected. In few samples glycerophosphocholine was seen as a low field shoulder on the phosphocholine peak. Moreover, there was an increased trend of choline and other choline groups in the malignant tissue thus indicating high cellularity in such tissues. Two recent studies have also shown concurring evidence that magnetic resonance spectroscopy can be used to identify malignant lesions of the breast by detecting the presence of choline metabolites [16,17]. A large increase in the cellular concentration of phosphocholine is one of the earliest responses of tumor cells to growth factor proteins and breast cancer cells contain at least 10 times more phosphocholine than do normal mammary epithelial cells [18].

Contrary to the above findings a choline peak was has been detected in tissue by various workers in lactating volunteers [19]. In fact, the state of lactation is associated with increased choline metabolism because of the need to nourish the newborn with large amounts of choline (supplied in the milk predominantly as phosphatidylcholine, phosphocholine, glycerophosphocholine, and free choline). This increased activity of choline metabolism may be the biochemical basis for the composite choline detected in lactating breast tissue [19]. This finding point out the limitation of the use of the composite choline

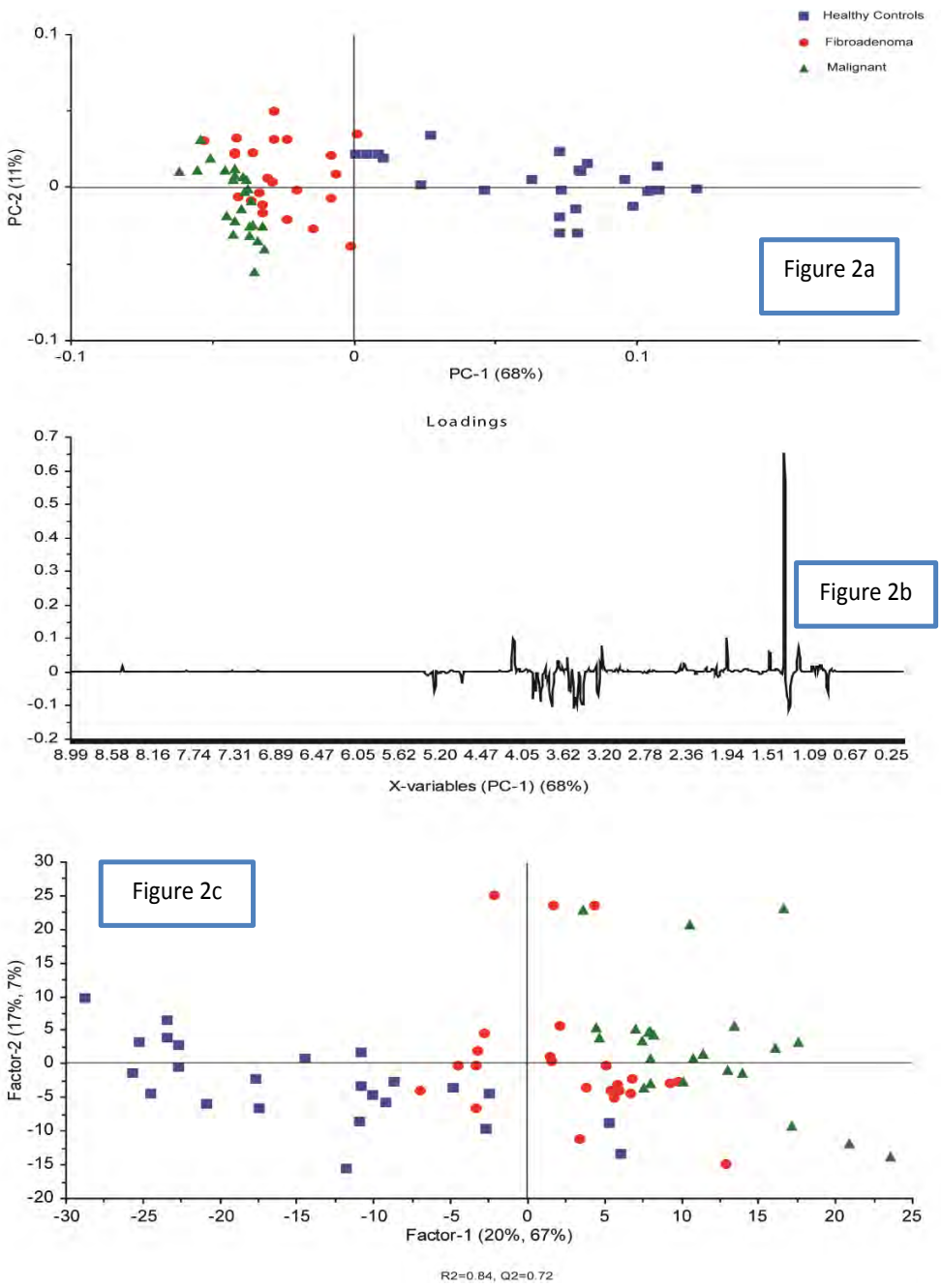

Figure 2: 3D scattered PCA score plot of serum.

Table I: Regulations with malignant, benign and healthy individual.

Regulations	Malignant (n=24)		Benign (n=24)		Healthy (n=24)	
	No.	%	No.	%	No.	%
Up regulation of lactate	24	100	17	70.83	2	8.33
Up regulation of acetate	24	100	17	70.83	2	8.33
Up regulation of choline containing compounds	24	100	17	70.83	2	8.33
Down regulation of glucose	24	100	17	70.83	2	8.33
Down regulation of lipids	24	100	17	70.83	2	8.33

signal as a marker for breast cancer and these results have been confirmed by other workers [20].

The *in vitro* studies to date is sufficient to indicate that the MRS studies of breast serum has not only emerged as novel minimal invasive tool to characterize tumor tissue, but is also enough competent to provide some insight about the tumors biochemical kinetics and possible tumor markers but unfortunately the available literature of *ex vivo* technique is short on the subject of etiology, prognosis and treatment monitoring. Although metabolites like choline, myo-inositol, glycine and glutamate can be used as a marker of malignancy. Unfortunately none of the metabolites of serum could be used to differentiate the malignant from the normal tissue and thus the evolving tool of spectroscopy may not

be of much value in field. The study being the pilot study and the small sample size prevent us in calculating the diagnostic statistics such as sensitivity, specificity etc.

The difference in the metabolic profile in the serum of malignant and benign disease has not been reported till now. The present study on serum demonstrates that the technique of HRMAS is capable of producing high resolution spectra in patients of benign and malignant breast disease. This novel technique may provide evidence leading to the establishment of biochemical criteria that allow a more accurate, rapid, objective means diagnosis and prognosis of human breast cancer. In addition to its utility in the diagnosis and prognosis the technique may also provide some evidences for biological marker, in the form of various lipoproteins (HDL,LDL,VLDL) which can be used as a new treatment monitoring and screening tool.

Conclusion

We conclude that by analyzing the global spectra of various metabolites of different types of serum, by the technique HRMAS we can correlate the rising or falling trends in the concentrations of different metabolites with the changes occurring in benign and malignant breast lesions. HRMAS still being an experimental diagnostic tool studies involving much higher resolution NMR tool and large sample size need to be undertaken to discover the unexplored potentials of HRMAS in medical sciences.

Conflict of Interest

Authors have no conflict of interests to declare.

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