Metabolomics of Breast Cancer Serum using 1H Nuclear Magnetic Resonance Spectroscopy

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SYNOPSIS: A novel combining approach of ^{1}H NMR spectroscopy of serum metabolite profile and linear multivariate discriminant function analysis (DFA) was carried out to identify the differential biomarkers of breast cancer (BC). The study was conducted on 60 healthy women and women with breast disorders-mainly malignant (n = 60) and fibroadenoma and cystosarcoma phylloides (n = 65). DFA reveals that ^{1}H NMR measured metabolites can differentiate (99.9%) not only between healthy controls and breast disordered samples but also malignant and non-malignant breast tumors (99%). This novel approach may serve as a rapid and promising surrogate diagnostic probe for screening breast disorders.

INTRODUCTION: Breast cancer (BC) is the most common and a significant cause of death in women (1-2). Breast cancer diagnosis is based upon fine needle aspirates, histological type and grade, lymph node status, expression of hormone receptors, MRI and X-ray mammogram (3-4). These approaches have various shortcomings such as invasive and painful procedure for samples collection, less sensitivity and specificity, outcome is much arguable issue, and very expensive. Scientific validation is required to evaluate the serum biochemistry in benign and malignant breast tissue, to reveal new biomarkers which remain unexplored to date. Therefore, the present study was designed to (a) quantitatively measure the absolute concentration of metabolites in serum and (b) determine the signature biomarkers of benign and malignant breast using linear discriminant function analysis (DFA).

MATERIALS AND METHODS: A 2 ml peripheral venous blood sample was obtained and serum was separated using standard protocol. A total of 185 serum from control (n=60), malignant (n=60), fibroadenoma (n=40) and cystosarcoma phylloides (n=25) subjects were collected and analyzed using ^{1}H NMR spectroscopy. Final diagnosis was achieved after consideration of mammography and biopsy investigations. The NMR experiments were performed on a Bruker Avance 400 MHz spectrometer using 5-mm broad band inverse probehead. 500µl of serum samples were taken in 5-mm NMR tubes. Before obtaining the NMR spectra, a sealed capillary containing pre-calibrated 0.75% trimethyl silyl propionic acid sodium salt (TSP) deuterated at CH_2 groups dissolved in deuterium oxide was inserted into the NMR tube. For all the specimens, 1D ^{1}H NMR experiments were performed using Carr-Purcell-Meiboom-Gill sequence with water suppression by pre-saturation at 25 ^{0}C. The parameters used were: spectral sweep width, 8000 Hz; data points, 32 K; pulse angle: 90^o; total relaxation delay of 5s; number of scans: 64; and line broadening: 0.3 Hz. The concentrations of metabolites were obtained using the integral area of the respective metabolite marker signal with reference to the integral area of TSP. Statistical significance for NMR derived metabolites was determined by univariate analysis (one-way ANOVA) followed by a *post hoc* Student-Newman-Keuls multiple comparisons test. The data were subjected for multivariate discriminant function analysis (DFA) with a stepwise-forward variable selection procedure in order to define important variables for differentiation of breast disorder patients from controls, followed by discrimination of benign and malignant patients based on the discriminant function coefficient values. On the basis of DFA we have constructed a classification model comprising four separate sets of phases; (1) CS vs. BBS + CBS, (2) CS vs. BBS, (3) CS vs. CBS and (4) BBS vs. CBS where CS is control serum, BBS is benign breast tumor serum, and CBS is cancer breast tumor serum.

RESULTS: Among several resonances; seventeen metabolites mainly (overlapping triplet of methyl group of HDL, LDL and VLDL), branched-chain amino acid (BCAA), alanine, acetate, glutamate, glutamine, creatine/creatinine, choline, glycerophosphocholine (GPC), glycine, lactate, threonine, glucose, tyrosine, histidine, phenylalanine and formate were quantified and subjected to statistical analysis. Out of 17 NMR measured metabolites, when only choline, alanine, threonine, GPC, lactate, glucose, and tyrosine were chosen based on their discriminant function coefficients, and DFA was performed for classification of CS and BBS+CBS (breast disorder) samples, overall 99.9% of breast disorder cases were classified with 99% sensitivity and 99% specificity (Wilks' Lambda, 0.068; p<0.0001) (Fig. 1). For classification of CS vs BBS samples, among 17 metabolites when only choline, glycine, BCAA, tyrosine, acetate, threonine, glucose, phenylalanine, and methyl group were chosen based on their discriminant function coefficients and the DFA was carried out, and result exhibited 99.9% cases could be classified, with 94% sensitivity and 96% specificity (Wilke's Lambda, 0.016; p<0.0001). Similarly, when CS cases were compared with the CBS; choline, GPC, acetate, methyl group, and alanine were chosen based on their discriminant function coefficients and DFA was performed, and the results exhibited that 99.9% of CBS cases were classified with 92% sensitivity and 93% specificity (Wilke's Lambda, 0.041; p<0.0001). Similarly, when BBS cases were compared with CBS, results reveal that overlapping methyl group, choline, acetate, glycine, histidine, tyrosine, phenylalanine were able to classify 99% of CBS cases with 96% sensitivity and 94% specificity (Wilke's Lambda, 0.110; p<0.0001).

DISCUSSION: This study exhibited the NMR observed metabolites as a 'method of choice' for a quick differential diagnosis of BC. DFA derived classification model defined that choline, alanine, threonine, GPC, lactate, glucose, and tyrosine were necessary to separate out the breast disorder (BBS+CBS) cases from the CS. Choline, glycine, BCAA, tyrosine, acetate, threonine, glucose, phenylalanine, and methyl group were able to segregate BBS from CS. Choline, GPC, acetate, methyl group, and alanine were vital metabolites to segregate CBS from CS and methyl group, choline, acetate, glycine, histidine, tyrosine, phenylalanine were the vital descriptors for the segregation of BBS from CBS. In order to check the accuracy of the prediction of this DFA model, the prediction possibility of the classifications was assessed with a 75/25 data split, by using 75% of the data in each category as training sets and the remaining 25% as test sets. The classifications of 99.1, 99.2, 90.9, and 96.5% were obtained for the test set groups containing CS vs. BBS+CBS, CS vs. BBS, CS vs. CBS, and BBS vs. CBS, respectively, followed by classifications of 99, 98.5, 89.8, and 91.2% for the training set groups, respectively. Results indicated that the prediction accuracy of BBS and CBS using NMR variables is very good and is simple, rapid, sensitive and comprehensive screening method for BC.

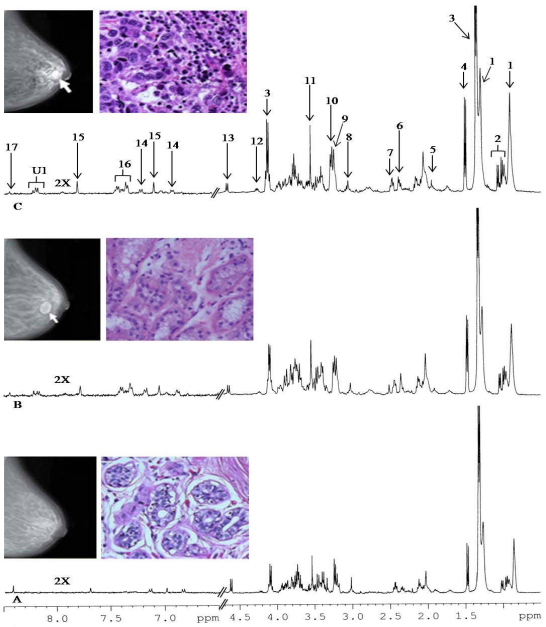

Fig.1: Typical ^{1}H NMR spectra of (A) CS, (B) BBS, and (C) CBS samples. 1, methyl group of HDL, LDL and VLDL; 2, BCAA; 3, lactate; 4, alanine; 5, acetate; 6, glutamate; 7, glutamine; 8, creatine/creatinine; 9, choline; 10, glycerophosphocholine; 11, glycine; 12, threonine; 13, β-glucose; 14, tyrosine; 15, histidine; 16, phenylalanine; 17, formate; 18, U1 (unassigned). The corresponding X-ray mammogram and histopathology evaluation are shown alongside of each NMR spectrum.

References: (1) CA Can J Clin. 2011; 61; 69-90. (2) Sal Pub Mex. 2011; 53; 372-384. (3) Ann Oncol. 2007; 18; viii3–viii7. (4) Acta histochemica. 2005; 107; 87-93.