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Decreased serum levels of free fatty acids are associated with breast cancer

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ABSTRACT

Background: Changes in the levels of lipids are associated with breast cancer (BC).
Methods: Disease-specific serum free fatty acids (FFAs) were quantified using chip-based direct-infusion nanoelectrospray ionization-Fourier transform ion cyclotron resonance mass spectrometry (CBDInanoESI-FTICR MS) in the negative ion mode. Multiple point internal standard calibration curves between the concentration ratios of fatty acids (i.e., $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, $C_{18:1}$, $C_{20:4}$, and $C_{22:6}$) to internal standards ($C_{17:1}$ for $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, and $C_{18:1}$, $C_{21:0}$ for $C_{20:4}$ and $C_{22:6}$) and their corresponding intensity ratios were established with a correlation coefficient of greater than 0.986.
Results: Data from 342 serum samples including 202 healthy controls and 140 BC patients indicate that serum concentrations of FFAs in patients with BC were significantly decreased compared with those in healthy controls. A panel of $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, $C_{20:4}$, and $C_{22:6}$ showed an excellent diagnostic ability to differentiate the patients with early stage BC from healthy controls, with the area under the receiver operating characteristics (ROC) curve of 0.953, a sensitivity of 83.3%, and a specificity of 87.1%.
Conclusion: Our findings suggest that these FFAs may be a valuable biomarker panel for the early-stage detection of BC.

1. Introduction

Breast cancer (BC) is the most common malignancy in females in the world [1]. Mammography is a widely used screening tool for this disease based on the ionizing radiation, along with a 10–15% false negative rate [2]. Other gold standards, such as histopathology and blood tests, are rarely used as risk assessment tools due to their high cost and poor sensitivity [3,4]. Cancer antigen 15-3 (CA15-3), HER2, and carcinoembryonic antigen as tumor markers have been used in the patients with BC for predicting BC recurrence and for evaluating BC treatment outcomes, but they are non-specific biomarkers for BC [5,6]. Ideal techniques for the early detection of BC should be low cost and minimally invasive, with a high diagnostic accuracy. Microarray-based gene expression profiling including microRNAs and DNA methylation was performed for the early detection of BC with high sensitivity and high throughput [7,8]. But its application in clinical routine test involves the complicated extraction of microRNA and DNA. Circulating tumor cells in the peripheral blood are used to diagnose BC, but rare-events detected in the peripheral blood restricted its implementation [9,10]. Downstream of genomics, transcriptomics, and proteomics metabolomics not only provides a novel insight into the changes in the metabolic status of living system but also improves our understanding of the pathogenesis.

Free fatty acids (FFAs) play a key role in many metabolic pathways. FFAs act as substrates in energy metabolism and mediators in signal transduction. Changes in blood FFA concentrations are indicators of a healthy status. Several potential biomarkers for diabetes mellitus [11–13], Alzheimer's disease [14], pancreatic cancer [15], and autism [16,17] were found relative to changes in the concentrations of FFAs in the peripheral blood. Recently, serum FFA profiles in BC patients were examined by gas chromatography/mass spectrometry (GC/MS), which demonstrated that changes in FFA concentrations were associated with BC [18]. However, fatty acids must be derivatized prior to GC/MS analysis due to their nonvolatile feature. It should be noted that time-consuming and complicated procedures for sample preparation are disadvantages of GC/MS and liquid chromatography/MS analysis.

In this study, we employed chip-based direct infusion nanoelectrospray ionization-Fourier transform ion cyclotron resonance mass

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spectrometry (CBDInanoESI-FTICR MS) in the negative ion mode to simultaneously quantify six serum FFAs from the patients with BC and age-matched healthy controls. Mann–Whitney U test was performed to compare healthy controls with the patients. Excellent diagnostic ability with the area under the receiver operating characteristics (ROC) curve (AUC) of 0.953, a sensitivity of 83.3%, and a specificity of 87.1% between healthy controls and patients with early-stage BC may provide a permission of early detection of BC.

2. Materials and methods

2.1. Chemicals and reagents

Palmitoleic acid ($C_{16:1}$), heptadecenoic acid ($C_{17:1}$), linolenic acid ($C_{18:3}$), linoleic acid ($C_{18:2}$), oleic acid ($C_{18:1}$), arachidonic acid ($C_{20:4}$), heneicosanoic acid ($C_{21:0}$), docosahexaenoic acid ($C_{22:6}$), and ammonium acetate (all purity > 99% except for $C_{22:6}$ with purity > 98%) were from Sigma-Aldrich Chemicals. Palmitic acid ($C_{16:0}$, purity > 99%) was from J&K (J&K Scientific Ltd.). HPLC-grade methanol, ethanol, and acetonitrile were from Fisher Scientific. The ultrapure water was supplied by a Milli-Q system (Millipore).

2.2. Participants

Characteristics of all participants are summarized in Table 1. 140 patients with BC from China–Japan Union Hospital (Changchun, China) were enrolled in this study, 36 of which were classified as early stage (stage I or II) and 24 as advanced stage (stage III or IV) based on the 7th edition of the Union for International Cancer Control (UICC) tumor–node–metastasis (TNM) classification. Evaluations of hematochemical parameters were also performed in this hospital. 202 healthy controls were also from China–Japan Union Hospital, with no obviously clinical abnormalities. All specimens were remaining sera after clinical laboratory examination. Serum samples from the patients were collected at diagnosis and all participants have given informed consents. This study was approved by the Ethics Review Board at the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences.

2.3. Study design

Serum samples from the patients were randomly classified into two groups: the training set (n = 68) and the validation set (n = 72). Healthy controls were randomly matched by age to the patients in the training set and the validation set, respectively.

2.4. Preparation of stock standard solutions

The internal standard (IS) stock solution including 83.3 μmol/l of $C_{17:1}$ and 33.3 μmol/l of $C_{21:0}$ was prepared in methanol, and was further diluted to proper concentrations until use. The stock standard solution of FAs that included $C_{16:1}$ (342.0 μmol/l), $C_{18:3}$ (81.7 μmol/l), $C_{18:2}$ (569.0 μmol/l), $C_{18:1}$ (836.0 μmol/l), $C_{20:4}$ (183.0 μmol/l), and $C_{22:6}$ (56.2 μmol/l) in methanol was further diluted by 500-fold with methanol/acetonitrile/5 mmol/l ammonium acetate in water (42/28/30, v/v/v) as STD1 (684.0 nmol/l $C_{16:1}$, 163.5 nmol/l $C_{18:3}$, 1138.0 nmol/l $C_{18:2}$, 1672.0 nmol/l $C_{18:1}$, 366.0 nmol/l $C_{20:4}$, and 112.3 nmol/l $C_{22:6}$). Then STD1 was further serially diluted by 2-, 10-, 20-, 50-, and 80-fold with methanol/acetonitrile/5 mmol/l ammonium acetate in water (42/28/30, v/v/v), which were named as STD2 (342.0 nmol/l $C_{16:1}$, 81.8 nmol/l $C_{18:3}$, 569.0 nmol/l $C_{18:2}$, 836.0 nmol/l $C_{18:1}$, 183.0 nmol/l $C_{20:4}$, and 56.2 nmol/l $C_{22:6}$), STD3 (68.4 nmol/l $C_{16:1}$, 16.4 nmol/l $C_{18:3}$, 113.8 nmol/l $C_{18:2}$, 167.2 nmol/l $C_{18:1}$, 36.3 nmol/l $C_{20:4}$, and 11.2 nmol/l $C_{22:6}$), STD4 (34.2 nmol/l $C_{16:1}$, 8.2 nmol/l $C_{18:3}$, 56.9 nmol/l $C_{18:2}$, 83.6 nmol/l $C_{18:1}$, 18.3 nmol/l $C_{20:4}$, and 5.6 nmol/l $C_{22:6}$), STD5 (13.7 nmol/l $C_{16:1}$, 3.3 nmol/l $C_{18:3}$, 22.8 nmol/l $C_{18:2}$, 33.4 nmol/l $C_{18:1}$, 7.3 nmol/l $C_{20:4}$, and 2.3 nmol/l $C_{22:6}$), and STD6 (8.5 nmol/l $C_{16:1}$, 2.0 nmol/l $C_{18:3}$, 14.2 nmol/l $C_{18:2}$, 20.9 nmol/l $C_{18:1}$, 4.6 nmol/l $C_{20:4}$, and 1.4 nmol/l $C_{22:6}$), respectively. The above-mentioned standard solutions were spiked with 1 μl of the IS solution with the final concentrations (83.3 nmol/l $C_{17:1}$ and 33.3 nmol/l $C_{21:0}$) to establish calibration equations, respectively. STD3 was also used as a quality control (QC) sample to examine the experimental stability. All standard solutions were freshly prepared and stored at 4 °C.

Table 1
Characteristics of the participants for the training and validation set.

	Training set		Validation set	
	Controls	BC	Controls	BC
	(n = 70)	(n = 68)	(n = 132)	(n = 72)
Age (years)				
Mean ± SD	53.3 ± 10.9	52.5 ± 10.8	52.9 ± 10.3	50.9 ± 9.2
Range	28–76	29–76	29–73	29–78
Stage (male/female)				
I & II (Early stage)				36
III & IV (advanced stage)				24

BC: breast cancer.

2.5. Sample preparations

FFAs were prepared as our own previous study. Briefly, 50 μl of serum was added into 950 μl of methanol/acetonitrile (3/2, v/v) to precipitate serum proteins. The mixture was vortexed for 30 s, and then stored at −20 °C overnight. After centrifuging at 19,000 ×*g* for 30 min at 4 °C, the supernatant was transferred into a new 1.5 ml tube. An aliquot of 20 μl supernatant was spiked with 1 μl of IS stock solution, and then 500 μl of hexane and 500 μl of water were added followed by vortex-mixing for 30 s. The resulting mixture was then centrifuged at 1500 ×*g* for 10 min. Organic phase was transferred into another glass vial and then air-dried. One milliliter of methanol/acetonitrile/5 mmol/l ammonium acetate in water (42/28/30, v/v/v) was added to redissolve the residues for mass spectrometric analysis.

2.6. Mass spectrometry

All mass spectra were acquired by a 9.4 T Apex-ultra™ hybrid Qh-Fourier transform ion cyclotron resonance mass spectrometer (FTICR MS, Bruker Daltonics) equipped with a NanoMate system including nanoelectrospray ionization (nanoESI) source and a cooling unit (Advion BioSciences). The cooling unit was set at 4 °C to reduce solvent evaporation of the samples. The NanoMate system consists of a 96-well plate, conductive pipette tips, and ESI-Chip with a 20 × 20 array of nozzle. The sample volume was 0.1 μl. A low delivery gas pressure of 0.7 psi and nanoESI voltage of −1.8 kV was applied to the nozzle to generate nanoelectrospray at a flow rate of about 100 nl/min.

A mass spectrum was accumulated by 10 full scans in the negative ion mode with time-domain sizes of 1 Mb (resolution: 200,000 at *m/z* 400). A mixture of $C_{15:0}$ (molecular weight, 242.22458 Da), $C_{17:0}$ (270.25588 Da), and $C_{21:0}$ (326.31848 Da) was employed to calibrate the mass spectrometer over the mass range of 150–400 Da.

2.7. Data processing

Mass spectra were obtained from ApexControl 3.0.0 (Bruker) in expert mode. DataAnalysis 4.0 (Bruker) was used for subsequent data processing. After isotopic deconvolution, peaks were transferred to Microsoft Excel for the following analyses. Serum FFAs were identified based on their observed accurate molecular masses and reliable isotope distributions. Absolute mass error was less than 0.00023 Da, and relative intensity error of observed to theoretical values was <2%. If FFA