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Investigation of salivary free amino acid profile for early diagnosis of breast cancer with ultra performance liquid chromatography-mass spectrometry

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ABSTRACT

Background: Breast cancer (BC) is the second leading cause of cancer death in women worldwide. This study aimed at investigating salivary free amino acid (SFAA) profile to facilitate the early diagnosis of BC.
Methods: Unstimulated whole saliva from BC patients (n = 27) and randomly from healthy females (n = 28) were assayed with ultra-performance liquid chromatography–mass spectrometry (UPLC–MS). Receiver operating characteristic curve (ROC) was used to evaluate the diagnostic performance of each of the amino acid (AA) biomarkers, or SFAA profile index for BC screening.
Results: Concentrations of 15 SFAAs demonstrated significant differences ($P < 0.05$) between BC patients at stages I–II and healthy controls (HC). The area under the curve (AUC) values in HC vs BCs I–II based on single AA were from 0.695 to 0.866. The AUC for SFAA profile index combined Pro, Thr, His was 0.916 (sensitivity 88.2%, specificity 85.7%) in distinguishing HC from BCs I–II.
Conclusions: The diagnostic potentials of 15 SFAAs as early diagnostic biomarkers for BC were verified and the diagnostic accuracy was improved in the use of SFAA profile index. The detection of SFAA profile is expected to be applied for the preclinical screening of early stage of BC in the future.

1. Introduction

Breast cancer (BC), one of the most common malignant tumor, is the second leading cause of cancer death in women worldwide [1]; at least 1.38 million [2] cases of BC are expected to be newly diagnosed, and more than 410,000 women [3] die from it every year. Studies have demonstrated that early detection of locally recurrent BCs can significantly improve the survival rate [4,5]. Therefore, the early detection of BC, as well as screening of high risk populations with precancerous lesions, will be an effective strategy for reducing the BC incidence. At present, screening mammography is considered the gold standard for detection of BC; however, the sensitivity of this test is between 54% and 77% depending on the type of mammography [6]. Furthermore, exposed to radiation and being uncomfortable when having a mammogram, most women do not get annual mammograms. Therefore, it is necessary to find a straightforward screening test for BC that would ideally be noninvasive with high sensitivity and specificity. A number of molecular-based diagnostic markers have been used to diagnose the BC with varying degrees of sensitivity and specificity. Therefore, the increasing worldwide incidence of BC is in urgent need of finding new biomarkers to diagnose the BC effectively.

Metabolomics, a rapidly expanding area of scientific research, has been widely used in diagnosing disease, finding biomarkers, and studying disease mechanisms. Metabolomics is the systematic study of low molecular weight metabolites in cells, tissues or whole organisms as influenced by multiple factors [7]. The major analytical techniques for metabolomics studies are NMR spectroscopy [8,9], GC-MS [10], and LC-MS [11], etc. RPLC-MS/MS analysis was employed to discriminate individuals with BC from HC and a total of 12 BC related metabolites were discovered [11]. In addition, ^{1}H NMR-based metabolomics technique was also used by Slupsky et al. to differentiate BC patients from the ovarian cancer patients and the controls [12]. In recent years, there is growing interest among researchers to use salivary metabolite biomarkers in investigation of disease diagnosis, such as oral cancer [13], pancreatic cancer [14], lung cancer [15], and Sjögren's syndrome [16]. Our previous research has verified the potential of salivary metabolite biomarkers

Abbreviations: BC, breast cancer; HC, healthy control; AA, amino acid; SFAA, salivary free amino acid.

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applying for the early diagnosis of OSCC and four salivary biomarkers in combination yielded satisfactory accuracy (AUC = 0.997) for diagnosis [17]. Saliva, as an analytical matrix, has its own superiority in comparison to blood and urine. First, saliva collection is non-invasive which means it is convenient, safe and painless for patients. It reduces the risk of percutaneous injury in blood collection and avoids the embarrassing conditions in urine collection. Second, it does not need the medical personnel with professional training, which makes it cost-effective and appropriate for large-scale collection [18].

AAs, as the raw materials for protein synthesis and the product of protein metabolism, are ingested or synthesized endogenously. They play essentially physiological roles as both basic metabolites and metabolic regulators among the most important compounds for focused metabolomics. Patients with cancer are always in a hypermetabolic and hypercatabolic state, causing both the synthesis and decomposition of protein to be enhanced, which results in the change of AA concentration. The body relies on an intricate and sophisticated system to keep the concentrations of AAs in a dynamic balance between the processes of utilization or catabolism and ingestion or biosynthesis. Disease will cause the dynamic balance broken with the abnormal metabolic process of proteins and AAs. Studies have shown that the AAs used as potential biomarkers are different from diverse cancers, and the changes of AA concentrations in body fluids are significant for the cancer diagnosis [13,19–21]. Sugimoto et al. used CE–TOF–MS to analyze saliva samples collected from both HC and BC patients, and identified 28 salivary metabolites for BC ($P < 0.05$ Steel–Dwass test), Where 14 AAs had significant values ($P < 0.05$) [20].

Since AAs are strong polar metabolites, they are difficult to be well separated in a widely used reversed-phase (RP) chromatographic method for the week retention on the column. In general, the separation and determination of AAs by LC methods require derivatization of the functional groups with a complicated derivatization procedure [22,23]. To obtain a satisfactory chromatographic profile with good resolution, peak shape and reproducibility in high efficiency, an UPLC-MS method in the hydrophilic interaction chromatography (HILIC) mode was used for the analysis of this study. Ultra performance liquid chromatography (UPLC) could be considered as a new direction of liquid chromatography (LC). UPLC can obtain dramatic increases in resolution, sensitivity, and speed of analysis by using sub-2 μm particles and mobile phases at high linear velocities, and instrumentation that operates at higher pressures than those used in HPLC [24]. Hydrophilic interaction chromatography (HILIC) was a LC separation method, which provides different selectivities and better retention of polar analytes compared with reversion phase chromatography (RP-LC) approaches [25,26]. Its possible separation mechanism is based on the interaction of hydroxyl groups of the analytes with the hydrophilic polar stationary phase [25]. Besides, HILIC may also enhance the MS sensitivity due to the increased ionization efficiency with ESI process resulting from the low aqueous/high organic mobile phases used for chromatography [26].

2. Materials and methods

2.1. Reagents and materials

Acetonitrile (HPLC grade) was from Fisher. Distilled water was purified "in-house" using an ULUPURE system (Chengdu Ultrapure Technology Co. Ltd.). Ammonium formate was purchased from Adamas and formic acid (HPLC grade) was purchased from KeLong Chemical Reagent Factory. A mixed stock standard solutions of arginine (Arg), ornithine (Orn), citrulline (Cit), alanine (Ala), methionine (Met), glutamine (Gln), aspartic acid (Asp), phenylalanine (Phe), tryptonphan (Trp), proline (Pro), threonine (Thr), serine (Ser), histidine (His) leucine (Leu), valine (Val), glutamic acid (Glu) and lysine (Lys) (Sangon Biotech Co., Ltd.) at a concentration of 0.2 mg/ml were prepared by dissolving the AAs in water. The solutions were stored at 4 °C and diluted further to the desired concentration using a mixture of acetonitrile/water (75:25 v/v) prior to use.

2.2. Study participants

Twenty seven females with BC (5 at stage I, 12 at stage II, and 10 at stage III) were recruited from the Galactophore Department of China West Hospital of Sichuan University during 2013 to 2014. BC stage was established according to the Tumor Node Metastasis (TNM) staging system, promulgated by the American Joint Committee on Cancer (AJCC). The detailed clinical characteristics of BC patient samples used in this study are provided in Table 1. All patients were >18 years and the diagnosis was based on clinical and histopathological criteria. They had no histories of receiving medication or surgical operation and none of them had been treated with chemotherapy or radiotherapy before sample collection. Twenty eight female volunteers with no known medical history of malignancy or clinically significant breast disease were recruited as HC. All HC were >18 years. The study was approved by the Ethics Committee of Sichuan University and conducted in accordance with the Declaration of Helsinki. All of the subjects signed an informed consent form agreeing to serve as saliva donors for the experiments.

2.3. Saliva collection and preparation

Saliva samples were collected between 8:30 and 10:30 a.m. in a private room. All of the donors were refrained from smoking, eating, drinking or oral hygiene procedures for at least 1 h prior to saliva collection. Roughly 2 ml of clear unstimulated whole saliva was obtained. The samples, once collected, were centrifuged at 13,500 rpm for 20 min at 4 °C to remove any insoluble materials, cell debris, and food remnants. Finally, the resulting supernatants were immediately frozen at −40 °C until the laboratory analysis.

Before analysis, the saliva samples were thawed at room temperature. In order to precipitate the proteins, 800 μl of acetonitrile was added to 400 μl of aliquots of saliva in a 1.5 ml Eppendorf tube, followed by vigorous shaking for 1 min. The mixture was allowed to stand for 15 min and then centrifuged at 13,500 rpm for 20 min at 4 °C. The supernatant was filtered through syringe filters (0.22 μm, Jinteng) for the following analysis.

2.4. SFAAs analysis by HILIC–UPLC–MS

Analysis was conducted using an Aquity UPLC coupled to a Micromass LCT Premier™ orthogonal acceleration time-of-flight mass spectrometer (Waters). A BEH Amide column (100 mm × 2.1 mm i.d. with 1.7 μm particle size) was used. The temperature of column oven was maintained at 45 °C. The autosampler had a 10 μl loop and was operated in the full loop injection mode. The flow rate of the mobile phase was 0.2 ml/min. Separations were performed using binary gradient mobile phases, consisting of water with 10 mM of ammonium formate (A1) and acetonitrile/water (95:5) with 2 mM of ammonium formate (B1) at

Table 1
Clinical characteristics of patient samples in this study.

Characteristics	BC patients
Number of subjects (M/F)	27/0
Race	Chinese
Clinical stage	
Early stage (I–II)	17 (I: 5, II: 12)
Advanced stage (III–IV)	10 (III: 10, IV: 0)
TNM status	
Tumor states (T)	T1: 13, T2: 12, T3: 1, T4: 1
Regional lymph node status (N)	N0: 14, N1: 3, N2: 8;N3:2
Distant metastasis status (M)	M0: 27, M1: 0
Age	
(Mean ± SD)	51.5 ± 11.4
(Min-Max)	30-73