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Measurement of salivary metabolite biomarkers for early monitoring of oral cancer with ultra performance liquid chromatography–mass spectrometry

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ABSTRACT

This study aimed to set-up an ultra performance liquid chromatography–electrospray ionization–mass spectrometry (UPLC–ESI–MS) method for the determination of salivary L-phenylalanine and L-leucine for early diagnosis of oral squamous cell carcinoma (OSCC). In addition, the diagnostic accuracy for both biomarkers was established by using receiver operating characteristic (ROC) analysis. Mean recoveries of L-phenylalanine and L-leucine ranged from 88.9 to 108.6% were obtained. Intra- and inter-day precision for both amino acids was less than 7%, with acceptable accuracy. Linear regression coefficients of both biomarkers were greater than 0.99. The diagnostic accuracy for both biomarkers was established by analyzing 60 samples from apparently healthy individuals and 30 samples from OSCC patients. Both potential biomarkers demonstrated significant differences in concentrations in distinguishing OSCC from control ($P < 0.05$). As a single biomarker, L-leucine might have better predictive power in OSCC with T1–2 (early stage of OSCC including stage I and II), and L-phenylalanine might be used for screening and diagnosis of OSCC with T3–4 (advanced stage of OSCC including stage III and IV). The combination of L-phenylalanine and L-leucine will improve the sensitivity (92.3%) and specificity (91.7%) for early diagnosis of OSCC. The possibility of salivary metabolite biomarkers for OSCC diagnosis is successfully demonstrated in this study. This developed method shows advantages with non-invasive, simple, reliable, and also provides lower detection limits and excellent precision and accuracy. These non-invasive salivary biomarkers may lead to a simple clinical tool for the early diagnosis of OSCC.

1. Introduction

In recent years, there is a growing interest among researchers to use salivary biomarkers in investigation of disease diagnosis, such as lung cancer [1], breast cancer [2], pancreatic cancer [3], oral cancer [4–6], sjögren's syndrome [7], etc. Oral cancer, one of the six most common human cancers, refers to all malignancies arising from the lips, the oral cavity, and pharynx [8,9]. The World Health Organization has reported oral cancer as having one of the highest mortality ratios amongst other malignancies with a death rate of 45% at five years from diagnosis [10]. Approximately 300,000 individuals worldwide are diagnosed with oral cancer annually. More than 90% of oral cancer is squamous cell carcinoma (OSCC). At present, once OSCC detected, it will be at advanced stage, which would generally result in a poor prognosis and a low survival rate. Therefore, early detection of OSCC as well as the screening of high risk populations with precancerous lesions remains to be an urgent need.

Currently, the most definitive method for oral cancer diagnosis and screening is a scalpel biopsy. It is time-consuming and needs extensive experience. In addition, it is also impractical to use imaging techniques for oral cancer screening, since they are expensive and insensitivity for small lesions [11]. Therefore, a number of molecular-based diagnostic markers have been used to detect the presence of OSCC with varying degrees of sensitivity and specificity. Compared with blood samples, using saliva for clinical diagnostics have attracted more and more research scientists and clinical doctors.

Human saliva, a multi-constituent oral fluid, is secreted primarily by three major glands namely parotid gland, submandibular gland and sublingual gland [12,13]. Generally, salivary glands produce about 1–1.5 L of saliva daily. It contains approximately 99% water with minerals, nucleic acids, electrolytes, mucus and proteins [14]. It is one of the most complex, versatile, and important body fluids, supplying a large range of physiological needs. Therefore, saliva is also called the “mirror of the body” or

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"a window on health status". Non-invasive collection is one of the great advantages of saliva as a diagnostic medium, especially when repeated samples must be taken for particular examinations. Additionally, it has the advantages of easy to store and inexpensive compared to blood sample collection [15].

In order to investigate biomarkers in saliva, recently developed technologies such as proteomics [16], transcriptomics [17] and metabonomics [18] have been explored. Metabolomics is the systematic study of small-molecular-weight substances in cells, tissues or whole organisms as influenced by multiple factors [19,20]. The major analytical techniques that are employed for metabonomics investigations are based on nuclear magnetic resonance spectroscopy [21,22] and mass spectrometry (MS) [23,24]. Sugimoto et al. use CE–TOF–MS to analyze saliva samples collected from both healthy and oral cancer persons, and more salivary metabolite marker candidates ($P < 0.05$ Steel–Dwass test) of oral cancer have been found [25]. HPLC–MS analysis was also performed to discriminate individuals with OSCC and oral leukoplakia (OLK) from healthy control and a total of 14 OSCC-related and 11 OLK-related metabolites were discovered [26]. In addition, a panel of three salivary metabolites including lactic acid, phenylalanine and valine were selected in combination yielded sensitivity of 94.6% and specificity of 84.4% in distinguishing OSCC from OLK by Wei et al. [27]. ^{1}H NMR-based metabonomic technique was used by Zhou et al. to differentiate the OSCC patients from the OLK patients and the controls by using PLS-DA analysis [28]. According to the research of Sugimoto, *P*-value of L-phenylalanine and L-leucine are < 0.005. L-phenylalanine is an essential amino acid and is a precursor of the neurotransmitters called catecholamines, which are adrenalin-like substances. L-leucine is one of branched chain amino acids, which is critical to human life and is particularly involved in stress, energy and muscle metabolism.

In this study, we aimed to develop and validate an assay for the simultaneous analysis of salivary L-phenylalanine and L-leucine using UPLC–ESI–MS with an intention to build criteria for diagnostics of OSCC. L-phenylalanine and L-leucine in combination yielded satisfactory accuracy for early OSCC prediction. Therefore, accurate detection of both of salivary biomarkers has important clinical value in early diagnosis of OSCC.

2. Materials and methods

2.1. Materials

Acetonitrile (HPLC grade) was purchased from Burdick & Jackson (USA). Distilled water was purified "in-house" using an ULUPURE system (Chengdu Ultrapure Technology Co., Ltd, Chengdu, China). Trifluoroacetic acid (HPLC grade) and ammonium formate were purchased from Adamas (Switzerland). Phenylisothiocyanate (PITC) was purchased from Alfa Aesar (Tianjin, China). Formic acid, triethylamine and *n*-hexane (Ke Long Chemical Reagent Factory, Chengdu, China) were used in this work. The stock standard solutions of L-phenylalanine and L-leucine (Sangon Biotech Co., Ltd, Shanghai, China) at a concentration of 1 mg/mL were prepared by dissolving L-phenylalanine and L-leucine in water with 0.1% formic acid, respectively. These solutions were stored at 4 °C and diluted to the required concentrations with water containing 0.1% formic acid prior to use.

2.2. Saliva specimens

Saliva samples were collected from a group of 30 OSCC patients (25 men and 5 women, 7 of stage I, 6 of stage II, 2 of stage III and 15 of stage IV), whose mean age was 62 years. The OSCC patients were all recruited from the West China Hospital of Stomatology, West China School of Stomatology, Sichuan University. There was no history of receiving medication and none had been treated with chemotherapy and radiotherapy. Diagnosis for all OSCC cases was based on clinical and histopathologic criteria. In addition, we obtained control samples from a group of 60 healthy individuals, cancer-free individuals with 35 males and 25 females. The Ethical Committee of the West China Hospital of Stomatology, Sichuan University, approved the protocol and all of the subjects signed an Ethical Committee consent form agreeing to serve as saliva donors for the experiments.

2.3. Saliva collection and preparation

A well-defined and standardized protocol was used for collection, storage, and processing of all the saliva samples. All the donors were asked to be on an empty stomach, without eating, drinking, smoking, or using oral hygiene products for at least 1 h prior to sample collection, and then rinse their mouth thoroughly with water. All samples were kept on ice during the process. Roughly 3 mL of clear unstimulated whole saliva was obtained. The samples, once collected, were centrifuged at 2500 rpm for 15 min at 4 °C. Thereafter, the supernatant was removed and a further centrifugation at 12,000 rpm for 20 min at 4 °C was performed to remove insoluble materials, cell debris, and food remnants. Finally, the samples were divided into 400 μL and frozen at −40 °C until the laboratory analyses.

The samples were thawed at room temperature first. To precipitate proteins, acetonitrile (800 μL) was added to the 400 μL saliva sample and the mixture was shaken for 60 s vigorously, and then was allowed to stand for 10 min. The obtained samples were centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant (1 mL) were used for amino acid derivation.

2.4. Amino acid derivation

Amount of 500 μL triethylamine (1 mol/L) and 500 μL PITC (0.1 mol/L) were added into the supernatant, and reacted for 1 h at room temperature after shaking 60 s vigorously. Then, 1.5 mL *n*-hexane was used as extractant for three times to remove unreacted PITC. After extraction, 250 μL 1.0 mol/L trifluoroacetic acid was added in the solution, and refluxed at 70 °C for 30 min. The final product (PTH-phe and PTH-leu) was centrifuged at 13,000 rpm for 20 min at 4 °C. The supernatant was filtered through a syringe filter (0.22 μm) for UPLC–TOF/MS analysis.

2.5. UPLC–ESI–MS

The UPLC–ESI–MS system consisted of a Waters ACQUITY™ Ultra Performance Liquid Chromatography system and a Micromass LCT Premier™ orthogonal accelerated time of flight mass spectrometer (Waters, Milford, USA). An ACQUITY UPLC™ BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm, Waters, Milford, USA) was used as the analytical column. The column was maintained at 45 °C. The flow rate of the mobile phase was 0.2 mL/min. Gradient elution was performed with the following solvent system: (A) 0.1% formic acid—water with 1 mM ammonium formate, (B) Acetonitrile; 5% A for 1.0 min, 5%∼10% A in 2.0 min, 10%∼25% A in 3.0 min, 25%∼50% A in 6.0 min, 50%∼90% A in 8.0 min, 90%∼5% A in 10.0 min and then holding at 5% A for 2 min. TOF/MS was operated with an ESI source with positive ion and W-geometry mode. The ionization parameters are listed as follow: capillary voltage, 3.0 kV; cone voltage, 100 V. "Aperture 1" voltage was set to 0 V. The flow rates of cone gas and desolvation gas were 40 and 700 L/h, respectively. The source temperature and desolvation temperature were 110 and 350 °C, respectively.