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Investigation and identification of potential biomarkers in human saliva for the early diagnosis of oral squamous cell carcinoma

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ABSTRACT

Background: Oral cancer is 1 of the 6 most common human cancers, with an annual incidence of >300,000 cases worldwide. This study aimed to investigate potential biomarkers in human saliva to facilitate the early diagnosis of oral squamous cell carcinoma (OSCC).
Methods: Unstimulated whole saliva obtained from OSCC patients (n = 30) and apparently healthy individuals (n = 30) were assayed with ultra-performance liquid chromatography–mass spectrometry (UPLC–MS) in hydrophilic interaction chromatography mode. The data were analyzed using a nonparametric Mann–Whitney *U* test, logistic regression, and the receiver operating characteristic (ROC) to evaluate the predictive power of each of 4 biomarkers, or combinations of biomarkers, for OSCC screening.
Results: Four potential salivary biomarkers demonstrated significant differences ($P < 0.05$) in concentrations between patients at stages I–II and the healthy individuals. The area under the curve (AUC) values in control vs OSCC I–II mode based on choline, betaine, pipecolinic acid, and L-carnitine were 0.926, 0.759, 0.994, and 0.708, respectively. Four salivary biomarkers in combination yielded satisfactory accuracy (0.997), sensitivity (100%), and specificity (96.7%) in distinguishing OSCC I–II from control.
Conclusions: Salivary metabolite biomarkers for the early diagnosis of OSCC were verified in this study. The proposed approach is expected to be applied as a potential technique of preclinical screening of OSCC.

1. Introduction

Human saliva is a multi-component oral fluid, which has high potential for the early diagnosis of diseases. In recent years, there have been many studies of disease diagnosis using salivary biomarkers in lung cancer [1], breast cancer [2], pancreatic cancer [3], oral cancer [4], Sjögren's syndrome [5], etc. Over 90% of oral cancers are oral squamous cell carcinoma (OSCC), which is one of the six most common human cancers, with an annual incidence of over 300,000 cases worldwide [6,7]. OSCC occurs in the lips, oral cavity, and pharynx, and has a relatively high rate of related morbidity. The World Health Organization has reported that OSCC has one of the highest mortality rates among other malignancies, with a 5-year mortality rate of approximately 50% [8]. Therefore, the early detection or prevention of this disease and the screening of high risk populations with precancerous lesions will be the most effective strategy.

The increasing worldwide incidence of OSCC urgently demands the discovery of new biomarkers. Saliva is a noninvasive and stress-free alternative to plasma and serum, and is widely accepted as a potential medium for clinical diagnostics. It also has the advantages of being simple to collect, easy to store, and less expensive compared with blood sample collection [9–11]. Saliva is secreted primarily by three major glands, i.e., the parotid gland, submandibular gland, and sublingual gland [12,13]. In general, the flow rate of unstimulated saliva is 0.3 ml/min. Saliva contains approximately 99% water as well as minerals, nucleic acids, electrolytes, mucus, and proteins [14]. It is one of the most complex, versatile, and important body fluids, which reflects a large range of physiological needs and information. Therefore, saliva is also known as the "mirror of the body".

At present, the standard method for OSCC diagnosis and screening is time-consuming and requires extensive experience. Therefore, modern high-throughput metabolomics approaches have been used extensively to observe the altered expressions of various metabolites in a range of cancers, including OSCC, with varying degrees of sensitivity and specificity. Metabolomics is a new platform for studying systems biology, which facilitates high throughput screening processes in the pharmaceutical industry and in clinical diagnosis [15,16]. The major analytical techniques used for metabolomics investigations are based on ^{1}H nuclear magnetic resonance (NMR) spectroscopy [17–19], LC–MS [20–22], and GC–MS [23]. Principal component analysis and orthogonal partial least squares discriminant analysis are used most frequently to screen for biomarkers of disease [24]. Sugimoto et al. used capillary electrophoresis mass spectrometry to discriminate individuals with oral cancer from healthy control and 28 salivary metabolite biomarker

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candidates ($P < 0.05$, Steel–Dwass test) were identified, where choline, betaine, pipecolinic acid, and L-carnitine had significant values of $P < 0.01$ [25]. Choline, a strong organic base, is an essential nutrient, which is supplied mainly by the diet. Abnormal choline metabolism is emerging as a metabolic hallmark associated with oncogenesis and tumor progression. This abnormal metabolism may be a result of enhanced membrane synthesis and degradation, which indicates the excessive proliferation of cancer cells [26]. Free choline is irreversibly oxidized to betaine. Thus, a high concentration of betaine may be observed in cancer patients. L-carnitine promotes the transport of fatty acids into mitochondria and plays an important role in energy metabolism. Pipecolinic acid is an intermediate product during the catabolism of lysine.

At present, the majority of LC metabolic profiling applications have been based largely on reverse-phase (RP) chromatographic methods. However RP–LC has limitations during the analysis of highly polar analytes because they are retained poorly on RP columns. Hydrophilic interaction chromatography (HILIC) allows different selectivities and the better retention of polar analytes compared with RP–LC approaches. The term HILIC was first introduced by Alpert [27]. HILIC is based on the partitioning of an analyte between a water-enriched layer of stagnant eluent on a hydrophilic stationary phase and a relatively hydrophobic bulk eluent, with a possible contribution from some type of dipole–dipole interaction [28].

In this study, we aimed to investigate and identify potential biomarkers in human saliva to facilitate the early diagnosis of OSCC. We systematically investigated a panel of 4 salivary metabolites, i.e., choline, betaine, pipecolinic acid, and L-carnitine. According to a nonparametric Mann–Whitney *U* test, the 4 potential biomarkers selected in this study demonstrated significant differences ($P < 0.05$) in concentrations between OSCC patients at stages I–II and the healthy individuals. Therefore, the accurate detection of these salivary biomarkers has important clinical value in the early diagnosis and identification of OSCC. The proposed approach is expected to be applied as a potential technique of preclinical screening of OSCC.

2. Materials and methods

2.1. Materials

Acetonitrile and methanol (HPLC grade) were from Fisher. Distilled water was purified "in-house" using an ULUPURE system (Chengdu Ultrapure Technology Co. Ltd.). Ammonium acetate (KeLong Chemical Reagent Factory) was used in this study. Betaine and choline were purchased from Damas-beta. Pipecolinic acid and L-carnitine were obtained from J&K Chemical Ltd. Stock standard solutions of the analytes were prepared at a concentration of 1 mg/ml by dissolving betaine, pipecolinic acid, L-carnitine, and choline in water. These solutions were diluted further to the desired concentration using a mixture of acetonitrile/methanol (75:25 v/v) prior to use.

2.2. Study participants

Saliva samples were collected from OSCC patients (n = 30) during 2012 and 2013 (twenty-five males and five females, clinical stage: 4 at stage I, nine at stage II, three at stage III, and 4 teens at stage IV), whose mean age was 55 y (range: 29–72 y). OSCC stage was established according to the Tumor Nodes Metastasis (TNM) staging system, promulgated by the American Joint Committee on Cancer (AJCC). The detailed clinical characteristics of OSCC patient samples used in this study are provided in Table 1. The disease status and staging of the OSCC patients were obtained from their clinical records. The OSCC patients were all recruited from the West China Hospital of Stomatology, West China School of Stomatology, Sichuan University. They had no histories of receiving medication and surgical operation and none had been treated with chemotherapy or radiotherapy before sample collection. For all the OSCC cases, the diagnosis was based on clinical and histopathological criteria. We obtained control samples from a group of healthy, age–gender matched cancer-free individuals (n = 30), i.e., 25 males and 5 females, whose mean age was 47 y (range: 25–69). The Ethical Committee of the West China Hospital of Stomatology, Sichuan University, approved the protocol. All of the subjects signed an Ethical Committee consent form and agreed to serve as saliva donors in the experiments.

Table 1
Clinical characteristics of patient samples used in this study.

Characteristics	OSCC patients
Number of subjects	30
Race	Chinese
Clinical stage	
Early stage (I–II)	13 (I: 4, II: 9)
Advanced stage (III–IV)	17 (III: 3, IV: 14)
TNM status	
Tumor states (T)	T1: 7, T2: 10, T3: 1, T4: 12
Regional lymph node status (N)	N0: 18, N1: 9, N2: 3
Distant metastasis status (M)	M0: 30, M1: 0

2.3. Saliva collection and preparation

Saliva samples were collected between 9:00 and 11:00 a.m. in a private room. All of the donors were asked to attend with an empty stomach and were asked to refrain from smoking, eating, drinking, or oral hygiene procedures for at least 1 h prior to sample collection. They rinsed their mouths thoroughly with water and approximately 2 ml of clear unstimulated whole saliva was obtained. The collected samples were centrifuged at 12,000 rpm for 20 min at 4 °C to remove any insoluble materials, cell debris, and food remnants. Finally, the samples were divided into 400 μl aliquots and frozen at −40 °C until the laboratory analyses.

Before the analysis, the saliva samples were thawed at room temperature. In order to precipitate the proteins, a mixture of acetonitrile/methanol (75:25 v/v, 800 μl) was added to 400 μl aliquots of saliva in a 1.5 ml Eppendorf tube, followed by vigorous shaking for 60 s. The mixture was allowed to stand for 10 min, before the samples were centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was used in the following analyses.

2.4. HILIC–UPLC–MS analysis

We performed the analysis using an Acquity™ UPLC (ultra-performance liquid chromatography) system, which was equipped with a 100 mm × 2.1 mm i.d. ACQUITY 1.7 μm/BEH Amide column and coupled to a Micromass LCT Premier™ orthogonal acceleration time-of-flight mass spectrometer (Waters). The column oven temperature was set 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. Isocratic elution was performed using the following solvent system: (A) 95:5 acetonitrile–10 mmol/l aqueous ammonium acetate, (B) 50:50 acetonitrile–10 mmol/l aqueous ammonium acetate; 65% A and 35% B for 7 min. TOF/MS was operated in the positive ion and W-geometry mode with 12,000 mass-resolving power.

In this study, independent reference Lock-mass ions were obtained via the LockSpray™ interface to ensure the mass accuracy during data acquisition. Leucine-enkephalin (Sigma-Aldrich, L9133, lot 095K5109; $[LE + H]^+$, m/z, 556.2771) was used as the reference compound. The LE solution was infused through the reference probe at a flow rate of 0.04 ml/min by a second LC pump (Waters). The mass accuracy was <10 ppm and exact mass data were acquired in the 50–700 m/z range with a 0.10 s scan time.