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Original Research Article

Salivary Metabolomics As Markers Of Progression, Prognosis And Effectiveness Of Therapy In Oral Leukoplakia And Oral Cancer: A Review

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Abstract
The overall goal of the review is to discuss about establishing a panel of salivary metabolomics markers which may be useful as a non-invasive prognostic markers of oral precancerous and cancerous conditions. The non-invasive panel of salivary metabolomics may be unique in predicting the prognosis of oral pre-cancer and cancerous states, progression of the disease states as well as the effectiveness of treatment. A future research study may be planned to develop a panel of salivary metabolomics for predicting the prognosis of oral leukoplakia as well as oral cancer. It may also predict association of salivary metabolomics with the treatment effectiveness oral cancer.
A non-invasive and cost-effective salivary metabolomic parameters may be developed for predicting the prognosis of oral leukoplakia and oral cancer. Sensitivity, specificity and predictive values of salivary metabolomics panel may be compared as routine histopathology in predicting the transformation of precancerous to cancer may be established.

Key words: metabolomics, saliva, leukoplakia, oral cancer

BACKGROUND
Oral cancer has a tendency to be detected at late stage which is detrimental to the patients because of its high mortality and morbidity rates. Early detection of oral cancer is therefore important to reduce the burden of this devastating disease.

The earliest detectable morphologic changes of oral cancer are the appearance of the 'precancerous' lesions, of which the most common ones are leukoplakia and erythroplakia. Oral leukoplakia, a white lesion in the mucosa of the oral cavity, represents the most common precursor lesion of oral squamous cell carcinoma and its prevalence varies between 0.1% and 0.5%[1].

There is general consensus that the clinical stage at the time of diagnosis is the most important predictor of recurrence and mortality in oral cancer patients. The time to diagnosis is influenced by multiple clinical and sociodemographic variables, including patient reluctance to consult a health-care professional due to lack of access to health care, especially in patients with low socioeconomic status, as well as professional delay in diagnosing and treating the disease[2]. Studies have shown that dentists and other health-care providers are in desperate need of systemic educational updates in oral cancer prevention and early detection, as they are remiss in the provision of oral examinations and in the detection of early oral cancers. Clinicians can improve patients' survival rates if a cancerous lesion is detected at an early stage, or if a precursor lesion (dysplasia) is

discovered and treated prior to malignant progression [3]. There are other pathological conditions that are considered precancerous including oral lichen planus and oral submucous fibrosis.

A major challenge for early diagnosis of the at-risk tissue is our limited ability to differentiate oral precancerous lesions at high risk of progressing into invasive SCC from those at low risk[4]. Thus, the prevention of oral cancer and its associated morbidity and mortality, hinges upon the early detection of oral precancerous lesions, allowing for histological evaluation and subsequent treatment depending on the stage of diagnosis. Early detection and screening for oral cancer has the potential to decrease the morbidity and mortality of disease, but methods for screening have not been proven successful. Although a typical routine oral cancer examination requires a 90-s visual and tactile examination, too few practitioners and dentists in particular are conducting these exams [5].

The malignant potential of the above mentioned oral lesions cannot be accurately predicted solely on the basis of their clinical characteristics, histological evaluation is essential for all suspicious lesions. Unfortunately, histological findings only indicate that a given lesion may have malignant potential (dysplasia), and cannot be used for the prediction of malignant changes. Thus, the presence of dysplasia only indicates that an oral lesion may have an increased risk of malignant transformation. Molecular biomarkers capable of identifying the subset of lesions likely to progress to cancer are being widely investigated including genetic and epigenetic alterations observed in oral mucosal precancerous lesions [6].

NONINVASIVE TOOLS FOR EARLY DETECTION

Recent advancements in oral cancer research have led to the development of potentially useful diagnostic tools at the clinical and molecular level for the early detection of oral cancer. The gold standard for oral cancer diagnosis remains tissue biopsy with histological assessment, but this technique needs a trained health-care provider, and is considered invasive, painful, expensive and time consuming [5]. Recent clinical diagnostic tools for early detection of oral cancer include tolonium chloride or toluidine blue dye, Oral CDx brush biopsy kits, salivary diagnostics and lastly optical imaging systems [7,8].

All these methods have their own advantages and disadvantages but unfortunately these non-invasive tools have failed in their practical implication in the community setup, as patients are still being diagnosed in advanced stages of oral cancer [9,10].

SALIVA AS A DIAGNOSTIC TOOL

Saliva from patients has been used in a novel way to provide molecular biomarkers for oral cancer detection. Saliva is a mirror of the body, reflecting virtually the entire spectrum of normal and disease states and its use as a diagnostic fluid meets the demands for an inexpensive, non-invasive and accessible diagnostic tool. Discovery of analytes in saliva of normal and diseased subjects suggests a very promising function of saliva as a local and systematic diagnostic tool[11,12] The ability to analyze saliva to monitor health and disease is a highly desirable goal for oral health promotion and research. So far, saliva has been used to detect caries risk, periodontitis, oral cancer, breast cancer, salivary gland diseases and systemic disorders such as human immunodeficiency virus and hepatitis C virus [13]. However, due to lack of knowledge of disease markers and an overall low concentration of these markers in saliva when compared to serum, the diagnostic value of saliva has not been fully realized. However, nowadays, highly sensitive and high-throughput assays such as DNA microarray, mass spectrometry and nanoscale sensors can measure protein and RNA markers at low concentrations in saliva, thus expanding the utility of saliva as a diagnostic tool [14, 15].

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Salivary metabolomics is an emerging approach for the diagnosis or screening of oral cancers, including OSCC, leukoplakia, and lichen planus [16]. Saliva is an ideal biofluid with vast information reflecting the systemic health status that could be used to detect various diseases [16, 17]. Applying salivary metabolites is plausible since these molecules may be transferred into saliva by various cells, including OSCC, present in the oral cavity and salivary glands; moreover, saliva allows non-invasive analysis [17].

Few studies have revealed the rational mechanism underlying the relation between salivary biomarkers and the host tumor metabolism. For instance, Sridharan *et al*. profiled the metabolite concentrations in serum and saliva samples from patients with oral cancer [18,19]; these samples were collected from different cohorts and individual variation was considered, but no overlap was observed among the potential biomarkers in these samples. Ishikawa *et al.* also analyzed the metabolomic profiles in saliva samples collected from patients with oral cancer, as well as in their oral tissues and adjacent paired control tissues, and confirmed that 17 metabolites were consistently elevated in the saliva and tumor tissues [20]. These metabolites included amino acids, such as valine, tryptophan, and threonine, and other several metabolites. Spermidine in the polyamine pathway and various metabolites, including choline, methionine, and *S*-adenosyl methionine, that are present in a pathway upstream of the polyamine synthesis pathway were also elevated [20]. Hsu *et al.* conducted both metabolomic and transcriptomic analyses of tumor tissues and adjacent paired tissues from patients with OSCC and found that polyamine pathways including putrescine, spermidine, spermine, and their acetylated forms were elevated in tissue samples [21]. Elevation of intermediate metabolites in the polyamine pathway was consistently observed in the oral cancer tissues in both data [20, 21].

The link between salivary biomarkers and tumor tissues distant from the oral cavity has been both analyzed in *vitro* and in *vivo*. Lau *et al.* found that breast cancer-derived exosome-like molecules disturbed the transcriptional expression in salivary glands [22]. Further, discriminatory salivary biomarkers for pancreatic cancer were discovered using a mouse model implanted with the pancreatic cancer cell line Panc02, and these showed decreased discrimination ability when mice were implanted with cells in which exosome biogenesis was suppressed [23]. These results indicate that cancer-derived exosome-like vesicles interact with salivary glands, resulting in the secretion of salivary components. The relationships between cancer metabolism and metabolites in other biofluids have also been investigated. For instance, elevation of salivary polyamines in patients with breast and pancreatic cancers were reported [24-27]. Activation of the polyamine synthesis pathway in cancer cells is well known. The first rate-limiting enzyme of the polyamine synthesis pathway, ornithine decarboxylase (ODC), is negatively regulated via adenomatous polyposis coli (APC), a tumor suppressor gene [28]. The loss of a functional APC activates ODC through the upregulation of the oncogene MYC [29]. Moreover, the upregulation of spermine/spermidine acetyltransferase (SAT1) promotes the acetylation of the polyamines and these metabolites are secreted to surrounding environment [30]. A positive correlation between polyamine concentration levels in the urine and tumor volume in patients with pancreatic cancer has been reported [31]. Aberrance in arginine and its downstream pathways, such as the ornithine and polyamine pathways, has been frequently reported in various cancer cells. Hu *et al*. observed consistent changes in the metabolites in these pathways in blood and urine samples collected from patients with breast cancer [32]. Overall, these results indicate the need for collecting saliva, blood, and tissue samples, and investigating whether they demonstrate consistent metabolite changes.

Salivary metabolomics analysis has been used for cancer detection, as well as for predicting and monitoring the therapy response. Yatsuoka *et al.* found that salivary hypotaurine could predict medication-related osteonecrosis of the jaw in cancer patients treated with bone-modifying agents

[33]. Although the metabolomic profiles of other biofluids have been used for predicting the efficacy of adjuvant treatments such as chemotherapy [34], there are few reports of these applications using saliva.

To our knowledge, the identification of the prognostic biomarkers of leukoplakia and other precancerous conditions using salivary metabolomics has not been reported. Such a study may be planned with an aim to develop a panel of markers in saliva,

SALIVA COLLECTION AND SAMPLE PREPARATION

The protocol for saliva collection as described previously [16]. Briefly, before saliva collection, a skilled dentist and dental hygienist has to check the oral hygiene of all participants. Remarkable dental plaque and calculus deposits to be removed using a toothbrush without dentifrice and ultrasonic scaling at ≥ 3 h before saliva collection. Subjects have to refrain from eating and drinking for ≥ 1.5 h before saliva collection. The participants have to rinse their mouths with water before sample collection and split their saliva into 50 cc Falcon tubes in a paper cup filled with crushed ice. Subsequently, approximately 3 mL of unstimulated whole saliva has to be collected for approximately 5 min. Finally, the samples needs to be aliquoted into smaller volumes and stored at -80°C.

PRECAUTIONS TAKEN

However, salivary metabolic profiles are sensitive to various factors irrelevant to the disease, and the biomarkers should be robust to withstand these. Pre-conditioning of sample collection (such as fasting duration), sampling methods (stimulated or unstimulated), sampling timing, and storage conditions also affect the salivary metabolite profiles. Therefore, saliva donors will be instructed to strictly follow the SOPs at the marker discovery stage to minimize unexpected bias.

METABOLOMIC ANALYSIS OF SALIVA

Salivary metabolites like amino acids, such as valine, tryptophan, and threonine, and other several metabolites. Spermidine in the polyamine pathway and various metabolites, including choline, methionine, and *S*-adenosyl methionine, that are present in a pathway upstream of the polyamine synthesis pathway are the ideal candidates to be analyzed by LCMS.

Various studies have compared specific disease samples and healthy controls; therefore, evaluation of the specificity of discovered markers is still lacking. Using a combination of multiple metabolite concentration patterns can solve these problems. For example, multivariate statistical methods and machine learning classification methods could help enhance data specificity when data for various diseases are available. In particular, the health condition in the oral cavity, such as caries and PD, has been shown to change salivary metabolomic profiles. Subjects with leukoplakia would have significantly skewed the metabolomic profiles. Ideally, cancer-specific biomarkers are robust against the routine detection methods.

OUTCOME MEASURES

Salivary metabolites like amino acids, such as valine, tryptophan, and threonine, and other several metabolites. Spermidine in the polyamine pathway and various metabolites, including choline, methionine, and *S*-adenosyl methionine, that are present in a pathway upstream of the polyamine synthesis pathway.

SIGNIFICANCE OF THE STUDY AND EXPECTED OUTCOME

Salivary cancer screening is promising and provides various advantages including safe and non-invasive sample collection. A simplified method of sample storage and transfer can allow self-

sampling without limitation of place, which facilitates frequent testing and enhances the chance of detecting oral cancer early. This may be beneficial for patients with oral cancer, as early detection allows for a wide range of treatment choices and improved prognosis.

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