

## Salivary Metabolomics and Oral Carcinogenesis

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Received: April 25, 2016

Revised: June 15, 2016

Accepted: June 18, 2016

Published online: August 18, 2016

### ABSTRACT

Cancers of the oral cavity has received reasonable importance especially in developing countries due to its significant morbidity and mortality rates. Oral squamous cell carcinoma is the common type of cancer occurring either de novo or from pre-existing potentially malignant disorders like leukoplakia. Ever since the disease has been identified, efforts are made to understand its pathogenesis in order to identify methods for early diagnosis, evolve appropriate treatment strategies and improve prognosis. Though tobacco, alcohol and microbes are considered important risk factors for OSCC; these are usually accompanied by various genetic and epigenetic factors. Modern analytical technologies provide comprehensive methods for identification of these molecular changes which can serve as an important biomarker. Metabolomics forms a part of 'omic' group that also includes genomics, proteomics and transcriptomics which is associated with the identification and quantitation of small molecules involved in metabolic reactions. The identification of a wide range of metabolites in bodily fluids is considered as an important diagnostic tool for assessment of disease biomarkers. The role of saliva as an important diagnostic tool for biomarker analysis has been accepted as a safe alternative to blood owing to its easy and non-invasive nature of collection and its close proximity to the oral lesions. This review

provides an overview of the use of salivary metabolomics in OSCC and presents the current literature findings and future implications.

**Key words:** Salivary metabolomics; Oral cancer; Oral leukoplakia; Molecular diagnostics

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Sridharan G, Patankar SR. Salivary Metabolomics and Oral Carcinogenesis. *Journal of Tumor* 2016; 4(4): 450-455 Available from: URL: <http://www.ghrnet.org/index.php/jt/article/view/1696>

### INTRODUCTION

Metabolism is a set of processes catalyzing the production of energy and cellular building blocks by the cell utilizing the nutrients obtained from the environment. These building blocks and the biochemical intermediates generated during their production and utilization are collectively referred to as metabolites<sup>[1]</sup>. Metabolomics is the comprehensive analysis of all metabolites in a biological system<sup>[2]</sup>. Currently with the advent of sophisticated technologies, metabolomics has become a powerful tool to gain insight into various cellular functions. It enables the assessment of broad range of endogenous and exogenous metabolites which have an important role in biological systems thus serving as an attractive candidate to understand disease phenomenon. The human metabolome is composed of diverse group of low molecular weight structures like lipids, aminoacids, peptides, nucleic acid and organic acids, vitamins, thiols and carbohydrates<sup>[3]</sup>. Salivary metabolomics is an emerging field for identification of disease related biomarkers which may be helpful in early diagnosis as well as monitoring of oral cancer. The presence of wide range of biological components in human saliva, its close proximity to the lesion, the non-invasive nature of collection and decreased probability of spread of infection makes it an ideal bodily fluid for biomarker identification. Commonly employed analytical techniques for metabolomics include nuclear magnetic resonance (NMR) spectroscopy, Liquid chromatography/ mass spectrometry techniques, gas chromatography/ mass spectrometry among others.

Oral squamous cell carcinoma (OSCC) is a common malignancy of epithelial origin with high prevalence rate in developing countries of the world. The use of tobacco in smokeless and smoking form is one of the important determinants of OSCC with the common intra-oral site being the alveolo-gingivo-buccal complex<sup>[4]</sup>. In general, OSCC can arise *de novo* or more commonly from pre-existing oral lesions collectively referred to as oral potentially malignant disorders. Leukoplakia is a common oral lesion with a potential for malignant transformation to OSCC<sup>[5]</sup>. Despite diagnostics and prognostic advancements, the exact mechanism occurring at the cellular level in cancer development and malignant transformation in leukoplakia is still unclear. It is imperative to understand these molecular changes as not all tobacco users show the tendency to develop oral cancer and some patients with leukoplakic lesions of the oral cavity do not show malignant transformation for prolonged period of time.

It is a well-known fact that the development of oral malignancy is associated with a high rate of morbidity and mortality. The possible reason for this could be attributed to the delay in diagnosis of OSCC and oral leukoplakia; lack of awareness concerning the precursor lesions and absence of adequate screening and diagnostic modalities to predict the nature of these lesions. Currently much of the diagnosis is based on the histopathological examination of the tissue specimen and while it continues to be the gold standard of diagnosis, a reliable non-invasive diagnostic aid is the need of the hour. A range of diagnostic models have been used in the diagnosis of OSCC and oral leukoplakia which includes but not limited to assessment of tumor biomarkers in tissue and bodily fluids like serum, plasma and saliva. The assessment of salivary biomarkers is a recent development with increased interest for its use as a diagnostic and prognostic tool<sup>[6]</sup>. With the availability of the newer technologies, it has become easier to identify the biomarkers present in small quantities thus signifying the use of saliva as an important assessment media. Also, saliva is a complex fluid with numerous constituents such as protein, lipids, carbohydrates that contribute to the oral microflora. The close proximity of salivary fluids to the oral lesions and the relative non-invasive nature of collection make it an ideal tool for biomarker discovery.

This review attempts to enlighten the role of salivary metabolomics in identification of various biomarkers that are suitable for diagnosis and prognosis of oral potentially malignant disorders and oral carcinoma.

## ORAL LEUKOPLAKIA AND SQUAMOUS CELL CARCINOMA

Leukoplakia is one of the major forms of potentially malignant disorder that can be clinically characterized primarily as a non-scrapable white lesion of oral mucosa. The incidence in India is in the range of 1.3-2.1 per 1000 individuals depending on the prevalence of tobacco users. Literature studies have reported that the incidence of oral leukoplakia increased to 17 per thousand in tobacco users<sup>[7]</sup>. It is also reported that 80% of oral cancers in India were preceded by various potentially malignant disorders and oral leukoplakia was the commonest of precursor lesions among them<sup>[8]</sup>. Tobacco chewing and smoking has been identified as an important risk factor for oral cavity pre-cancer and cancer in India<sup>[9]</sup>. Molecular epidemiological studies have now provided evidence that an individuals' susceptibility to oral leukoplakia and cancer is modulated by both genetic and environmental factors<sup>[10]</sup>.

Oral squamous cell carcinoma (OSCC) is one of the most common epithelial malignancies with significant morbidity and

mortality and possesses a multifactorial etiology. Factors such as habits, genetic, environmental and their interactions and microorganisms have been implicated in its etiopathogenesis. Molecular and genetic techniques have enabled us to unravel some of the critical events associated with the development of oral cancer and pre-cancer<sup>[11]</sup>. Despite the diagnostic and therapeutic advances over the decades, the disease still remains a challenge for medical professionals with the five-year survival rate being 30%-50%<sup>[12]</sup>. The mortality rate associated with oral cancer is high because it is routinely discovered late, commonly after metastasis to lymph nodes or neck has already occurred<sup>[13]</sup>. Recent observations indicate that the clinical and histological appearance of oral mucosa may not truly depict the damage occurring at the genetic level. This phenotypic and genotypic disparity may account in part for the failure to establish effective screening and surveillance protocols based on traditional clinical and microscopic examination<sup>[12]</sup>. Carcinogenesis is multistep process involving initiation, promotion and progression and evidence indicates that these are driven by accumulation of specific gene alterations<sup>[14]</sup>. Initiation is an important step which results when a normal cell sustains a DNA mutation and a round of DNA synthesis results in a fixation of the mutation thereby producing an initiated cell. This is followed by an equally important step in promotion and the hallmark of this stage is the modulation of gene expression resulting in increased cell number either through cell division and/or decreased apoptosis<sup>[15]</sup>. An understanding of the molecular mechanisms involved in OSCC is helpful in providing a more complete picture of the ways in which tumor arise and advance and a rationale for novel strategies of cancer detection.

Tumor cells exhibit or produce biochemical substances referred to as tumor markers. These can be normal endogenous products that are produced at a greater rate in cancer cells or the products of newly switched on genes that remain quiescent in the normal cells<sup>[16]</sup>. Tumor marker may be present as intracellular substances in tissues or as released substances in circulating body fluids such as serum, urine, CSF, and saliva.

The call for the identification of disease specific biomarker has resulted in exponential rise in research related to detection of genomic, proteomic and transcriptomic markers for tumor diagnostics and prognostics. Studies have mainly focused on altered DNA methylation; changes in the RNA and protein levels; analysis of transcriptome and proteome levels. An important hallmark of cancer is the reprogramming of energy metabolism, which although was identified long back assumed significance recently in the light of technologies available for its identification<sup>[11]</sup>. The metabolic profiles can be altered by various pathological processes and global changes in these profiles may indicate the presence of a disease. Thus, it can be assumed that in addition to the genetic and protein modifications, changes do occur in the endogenous metabolites in patients suffering from oral leukoplakia and OSCC and these can serve as a complementary approach in the early detection of oral cancer.

## METABOLOMICS

Metabolomics or metabonomics can be defined as a systematic study of small molecular weight substances in cells, tissues or whole organisms as influenced by multiple factors<sup>[17]</sup>. It is one of the core disciplines of systems biology focusing on the study of low molecular weight organic and inorganic chemicals in biological system<sup>[18]</sup>. It enables the assessment of the levels of a broad range

of endogenous and exogenous metabolites which includes but not limited to a diverse group of low molecular weight structures such as lipids, amino acids, peptides, nucleic acids, organic acids, vitamins, thiols and carbohydrates. The assessment of the various metabolites is currently considered as a dependable tool for diagnosing disease, identifying new therapeutic targets and enabling appropriate treatments<sup>[3]</sup>.

Metabolomics offer a number of advantages. There are two important characteristics which enable this technique to be a highly sensitive and a rapid measure of the system phenotype. One being that the metabolome is the final downstream product of transcription and translation thereby being closest to the phenotype and secondly the dynamics of primary metabolism operates in timescales of seconds<sup>[19]</sup>. Metabolic analysis is typically characterized as two complementary methods namely the targeted and untargeted methods. Targeted approach focuses on identifying and quantifying selected metabolites such as substrate of an enzyme, direct products of a protein, a particular class of a compound or members of a particular pathway. While targeted approach is usually hypothesis driven, untargeted metabolites can generate new hypothesis for further tests by measuring ideally all the metabolites of a biological system<sup>[2]</sup>. The common analytical tools applied to metabolomics are nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC/MS) and liquid chromatography-mass spectrometry (LC/MS). The technology consists of two sequential steps: (a) an experimental technique based on MS or NMR spectroscopy designed to profile low molecular weight compounds and (b) multivariate data analysis<sup>[20]</sup>. The experimental strategy of metabolic profiling studies the metabolome in a holistic approach and from a point of limited biological knowledge. Powerful analytical techniques in combination with appropriate sample preparation is required to detect the numerous metabolites in a single metabolite and use of LC/MS has an advantage of simultaneous detection of many metabolites.

LC/MS is a coupling of liquid chromatography and mass spectrometry. Coupling of mass spectrometry to chromatographic techniques is desirable due to the sensitive and highly specific nature of MS compared to other chromatographic detectors<sup>[21]</sup>. Liquid chromatography in LC/MS is a modified and different from the conventional form of HPLC. The function of liquid chromatography is to separate the components of the mixture which will further be identified and quantified. Mass spectrometers operate by converting the analyte molecules to a charged ionized state, with subsequent analysis of the ions and any fragment ions that are produced during the ionization process on the basis of their mass to charge ratio<sup>[21]</sup>. MS typically is composed of three major parts namely ion source, mass analyzer and detector. While the ion source converts sample molecules into ions, the mass analyzer resolves these ions before they are measured by the detector. Due to diverse chemical properties of metabolites, it is often required to analyze the biological sample in both positive and negative modes under scan range of  $m/z$  50 to 1000 to maximize metabolome coverage<sup>[2]</sup>. This is then followed by pre-processing of the raw LC-MS data into a peak list for easy interpretation and comparison. After pre-processing, the LC-MS raw data are summarized by a peak list to which statistical analysis can be applied to detect those peaks whose intensity levels are significantly altered between distinct biological groups.

The potential of bodily fluids in the diagnosis of various human pathologies is highly appreciated and is reflected with the numerous publications related to this field. Metabolomics has also attempted to tap the utility of bodily fluids in the diagnosis of various diseases including cancer. A bodily fluid of importance with respect to oral

cavity lesions is saliva which has a pivotal role in identification of various metabolites associated with important oral lesions.

Saliva is composed of 99.5% water and 0.5% solid material which is inclusive of organic and inorganic constituents. The components present in the saliva could either be the inherent component of the saliva itself or the metabolites transferred from the plasma. The passage of plasma components into saliva involves several processes like, ultrafiltration through gap junctions between cells of secretory units, transudation of plasma compounds into oral cavity, from crevicular fluid or directly from oral mucosa and selective transport through cellular membranes by passive diffusion of lipophilic molecules or by active transport through protein channels<sup>[22]</sup>.

The source of information in saliva is largely derived from the variety of DNA's, RNA's and proteins present in the saliva. Salivary DNA represents the genetic information of the hosting human body, the oral microbiota and the infecting DNA-viruses. Salivary RNA provides information on the transcription rates of the host genes and those of oral microbiota. Salivary proteins represent genetic information and help to understand the translational regulation of the host body and the oral microbiota<sup>[23]</sup>. In addition, saliva is also useful in detection of range of potential markers that includes non-organic compounds, proteins, cell cycle markers (p16, p53 etc), growth factors (Epidermal growth factor, transforming growth factor etc), cell surface markers, DNA, mRNA, microRNA, oxidant and antioxidants among others<sup>[24]</sup>.

Saliva is a readily accessible and informative fluid making it ideal for early detection of various diseases. The identified principle salivary metabolites can be used as promising biomarkers for accurately predicting the probability of disease and to discriminate between healthy controls from disease groups.

## SALIVARY METABOLOMICS IN ORAL LEUKOPLAKIA AND ORAL SQUAMOUS CELL CARCINOMA

The metabolome is the complement of small molecule metabolites and it changes continuously and evaluating a single profile reflects the gene and protein expression. Metabolomic investigations can generate quantitative data for metabolites which helps in elucidating the metabolic dynamics related to disease states and drug exposure<sup>[25]</sup>. Ever since its discovery, metabolomics has been widely applied to various clinical conditions for identifying biomarkers. Some of the high profile metabolomics studies were conducted in the field of cancer research and the initial studies were performed using plasma, tissue and urine samples of patient suffering from prostate cancer<sup>[26]</sup>. The identification of biomarkers for the diagnosis of oral cancer is a relatively new field and is being currently investigated with the help of salivary metabolomics.

In a study performed to evaluate the salivary metabolomics as an approach to the diagnosis of oral squamous cell carcinoma, oral lichen planus and oral leukoplakia a total of 14 OSCC related, 13 OLP related and 11 oral leukoplakia related biomarkers were discovered<sup>[27]</sup>.

A study by Wang *et al* (2014) utilized reversed phased liquid chromatography and hydrophilic interaction chromatography based salivary metabolomics to investigate saliva samples from OSCC and healthy controls. Multivariate data analysis was performed to highlight discriminated variables and after refining the model identified 14 potential biomarkers for the early diagnosis of OSCC. Of these, eight biomarkers up-regulated in OSCC patients compared

with controls and six down regulated in OSCC group. The eight up-regulated biomarkers included lactic acid, hydroxyphenyllactic acid, N-nonanoylglycine, 5-hydroxymethyluracil, succinic acid, ornithine, hexanoylcarnitine and propionylcholine. The six down-regulated biomarkers were carnitine, 4-hydroxy-L-glutamic acid, acetylphenylalanine, sphinganine, phytosphingosine and S-carboxymethyl-L-cysteine<sup>[17]</sup>.

Wei *et al* (2011) performed a study to evaluate the salivary metabolome in oral leukoplakia and oral squamous cell carcinoma using HPLC-MS technique and a total of 14 OSCC related and 11 oral leukoplakia related biomarkers were discovered. Among these the most discriminant metabolites were  $\gamma$ -aminobutyric acid, phenylalanine, valine, n-eicosanoic acid and lactic acid<sup>[28]</sup>.

Capillary electrophoresis mass spectrometry based saliva metabolomics was performed by Sugimoto *et al* (2010) to identify the metabolomic profiles of oral, breast and pancreatic cancers. The study revealed that the marker pool used to discriminate between healthy individuals and oral cancer patients revealed 28 metabolites namely pyrrolinehydrocarboxylic acid, leucine plus isoleucine, choline, tryptophan, valine, threonine histidine, pipercolic acid, glutamic acid, carnitine, alanine, piperidine, taurine, glutamine, betalanine and cadaverine and two other metabolites<sup>[13]</sup>. The findings of the various studies in the literature are summarized in table 1. Table 2 provides the physiologic role and the probable association with oral leukoplakia and oral squamous cell carcinoma of few potential metabolites as described in the literature.

## CONCLUSION

Oral squamous cell carcinoma is a complex disease which may arise from potentially malignant disorders and their early diagnosis is generally associated with improved prognosis and survival rate. Currently, a lack of reliable diagnostic biomarkers results in delayed diagnosis and treatment thus leading to increased mortality and morbidity. Identification and characterization of oral cancer specific biomarkers is necessary which is possible in current scenario due to the presence of advanced technologies. Salivary diagnostics is emerging as an attractive tool and since OSCC is characterized by a series of biochemical and molecular alterations, a panel of several metabolite markers can be identified with the help of salivary metabolomics. Though still at a nascent stage, appropriate application of metabolomics may help in identification of potential biomarkers which along with the conventional diagnostic protocol can be used for early detection of potentially malignant disorders and oral cancer.

## ACKNOWLEDGEMENT

This manuscript is prepared and published as a part of the PhD program of Dr. Gokul Sridharan at Saveetha University, Chennai. The author thanks the faculty and management of Saveetha University, Chennai; the dean and management of YMT Dental College and Hospital, Navi Mumbai for required permission and facilities to carry out the necessary work.

**Table 1** Summary of the literature data on salivary metabolomics in oral potentially malignant disorders and oral carcinoma.

Sl. No	Author/year	Method of metabolite identification	Metabolites identified	Nomenclature of the metabolites
1	Yan <i>et al</i> 2008	HPLC-MS	14 OSCC related, 13 oral lichen planus and 11 oral leukoplakia related metabolites	Structure identification not done
2	Sugimoto <i>et al</i> 2010	Capillary electrophoresis- time of flight- mass spectrometry	28 metabolites identified to discriminate between oral cancer and healthy individuals	<ul style="list-style-type: none"> <li>• Pyrroline hydroxycarboxylic acid</li> <li>• Leucine plus isoleucine</li> <li>• Choline</li> <li>• Tryptophan</li> <li>• Valine</li> <li>• Threonine</li> <li>• Histidine</li> <li>• Pipercolic acid</li> <li>• Glutamic acid</li> <li>• Carnitine</li> <li>• Alanine</li> <li>• Piperidine</li> <li>• Taurine</li> </ul>
3	Wei <i>et al</i> 2011	Ultraperformance liquid chromatography coupled with quadropole/time of flight mass spectrometry	Identified 5 significant discriminant metabolites	<ul style="list-style-type: none"> <li>• <math>\gamma</math>-aminobutyric acid</li> <li>• phenylalanine</li> <li>• valine</li> <li>• <math>\eta</math>-eicosanoic acid</li> <li>• lactic acid</li> </ul>
4	Wang <i>et al</i> 2014	Reversed phase liquid chromatography and hydrophilic interaction chromatography combining with time of flight mass spectrometer	Eight potential metabolites were upregulated in OSCC patients and six were downregulated	Up-regulated metabolites <ul style="list-style-type: none"> <li>• lactic acid</li> <li>• hydroxyphenyllactic acid</li> <li>• N-nonanoylglycine</li> <li>• 5-hydroxymethyluracil</li> <li>• Succinic acid</li> <li>• Ornithine</li> <li>• Hexanoylcarnitine</li> <li>• Propionylcholine</li> </ul> Down-regulated metabolites <ul style="list-style-type: none"> <li>• Carnitine</li> <li>• 4-hydroxy-L- glutamic acid</li> <li>• Acetylphenylalanine</li> <li>• Sphinganine</li> <li>• Phytosphingosine</li> <li>• S-carboxymethyl-L-cysteine</li> </ul>

**Table 2** Physiologic role of potential salivary metabolites and their probable association with oral carcinoma.

Sl. No	Name of the metabolite	Physiologic role	Role in oral carcinogenesis
1	Lactic acid	End product of glycolysis	<ul style="list-style-type: none"> <li>• Cancer cells utilize aerobic glycolysis thus producing excess lactic acid<sup>[29]</sup></li> <li>• Impaired tricarboxylic acid production due to insufficient pyruvate supply which is a series of chemical reactions of importance in living cells<sup>[30]</sup>.</li> </ul>
2	Polyamines (cadaverine and putrescine)	Correlated with cell growth <sup>[31]</sup> .	<ul style="list-style-type: none"> <li>• Markedly higher in oral cancer patients</li> </ul>
3	γ-aminobutyric acid (GABA)	Utilized in transformation of glutamate to 2-oxo-glutarate	<ul style="list-style-type: none"> <li>• Glutaminolysis occurs to provide energy to tricarboxylic acid cycle thus leading to increased utilization of GABA</li> </ul>
4	η-eicosanoic acid		<ul style="list-style-type: none"> <li>• Increased risk of oral cancer may be correlated with ingestion of saturated fatty acids<sup>[32]</sup>.</li> </ul>
5	Ornithine	Amino acid produced in urea cycle by splitting urea from arginine <sup>[33]</sup> .	<ul style="list-style-type: none"> <li>• Increased expression of ornithine in OSCC than in healthy controls</li> </ul>
6	5-hydroxymethyluracil	Formed when cells are under oxidative stress	<ul style="list-style-type: none"> <li>• Elevated levels in OSCC due to change in redox state resulting in oxidative stress<sup>[34]</sup>.</li> </ul>
7	Sphinganine and phytosphinganine	Involved in ceramide synthesis. Ceramide is an important cellular signal for inducing apoptosis <sup>[35]</sup> .	<ul style="list-style-type: none"> <li>• Downregulated in OSCC.</li> </ul>
8	Carnitine	Essential facto in beta-oxidation of fatty acids and is synthesized from lysine and methionine <sup>[36]</sup> .	<ul style="list-style-type: none"> <li>• Lower in OSCC probably because fatty acid metabolism is downregulated</li> </ul>
9	Glutamic acid		<ul style="list-style-type: none"> <li>• Downregulated in OSCC due to its utilization by tumor cells to meet increased energy demand<sup>[37]</sup>.</li> </ul>

## COMPETING INTERESTS

The authors declare that they have no competing interests.

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