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Amino acid profile of saliva from patients with oral squamous cell carcinoma using high performance liquid chromatography

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Abstract: Oral cancer is the leading cause of death worldwide and it is the eighth most common cause of cancer death. Cancer cells utilize more glucose and amino acids than their benign counterparts. Diagnosis of disease via the analysis of saliva is potentially valuable, as the collection of fluid is associated with fewer compliance problems than the collection of blood. Hence, the present study was undertaken to evaluate the comprehensive amino acid profiling of saliva by high performance liquid chromatography (HPLC). The study group comprised 16 subjects, of whom eight were classified as having well-differentiated oral squamous (OSCC) cell carcinoma (Group I) and eight were classified as having moderately differentiated oral squamous cell carcinoma (Group II). Eight healthy individuals comprised the control group (Group III). The results showed increased salivary levels of all the amino acids in both groups of OSCC patients (Groups I and II) when compared with healthy controls (Group III). Hence, our study showed higher levels of all amino acids in the saliva of OSCC patients than in the saliva of healthy controls. The increased levels may serve as a "diagnostic and prognostic marker" for oral squamous cell carcinoma

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Introduction

Cancers of the oral cavity represent approximately 9.8% of all malignant neoplasms in India, and oral squamous cell carcinoma (OSCC) accounts for nearly 50% of all newly diagnosed cancers in India (1). Tumor markers that can be identified in saliva may therefore be useful for screening malignant diseases (2) Salivary proteins play an important role in the properties and functions of saliva (3) Detection of amino acid levels in serum and saliva is largely helpful in the early diagnosis and prognosis of various malignancies.

Salivary levels of various biochemical parameters have been measured in infectious diseases, hereditary diseases, autoimmune diseases, endocrine disorders, cancers and psychiatric disorders. Salivary parameters such as cancer antigen (CA)-125, cancer antigen (CA)-15-3, kallikrein, epidermal growth factor and p53 have been estimated as tumor markers in malignancies of the breast, ovary, lung and colon. However, the use of saliva in the diagnosis of oral cancer is still in its infancy, although the molecules mentioned above have been identified as potential tumor markers in OSCC (4).

Saliva contains 20 free amino acids (5). Some studies have demonstrated the derangement of tryptophan

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metabolism, which is reflected by an increase in tryptophan and its metabolites in the saliva of patients with oral squamous cell carcinoma (6). Thus, elevation of salivary amino acids may serve as a disease marker, as salivary levels are the preferred method for assessing the longterm adequacy and dynamics of amino acid utilization (7).

Saliva contains a large number of protein compounds, the structure and function of which have been studied by traditional biochemical techniques, including liquid chromatography, gel electrophoresis, capillary electrophoresis, nuclear magnetic resonance, mass spectrometry, immunoassays (e.g., RIA, IRMA, EIA, ELISA) and lectin probe analysis (8).

Chromatographic methods are classified according to their mobile and stationary phases. All forms of chromatography rely on differential solubility or absorption of compounds to separate molecules between a stationary phase and a mobile phase (9). High performance liquid chromatography (HPLC) was developed by Casaba Horváath at Yale University in 1966. It was popularized as a sophisticated improvement over open columns, and provided more precise and rapid separation. Detection of proteins, amino acids and nucleic acids is performed via UV absorbance (10). HPLC has advantages over other biochemical techniques, as it allows for fast and efficient separation and characterization of analytes within a given sample. It also helps in urgent diagnosis by providing modern, rapid and automated analysis of amino acid profiles for acute forms of disease with minimal effort and at reasonable cost (11).

Saliva is currently emerging as a diagnostic tool and has been used in the diagnosis of various diseases, including oral squamous cell carcinoma. Numerous studies have been performed in serum to confirm the changes in amino acid profiles in oral squamous cell carcinoma patients. In contrast, very few studies have used saliva as a diagnostic tool, and not many amino acids have been evaluated. Hence, in order to both highlight saliva as a potential diagnostic tool and analyze amino acid derangement in oral squamous cell carcinoma patients, the present study was undertaken to evaluate the comprehensive amino acid profile in saliva from oral squamous cell carcinoma patients using HPLC.

Materials and Methods

The study group comprised 16 subjects, of whom eight were classified as having well differentiated oral squamous cell carcinoma (Group I) and eight were classified as having moderately differentiated oral squamous cell carcinoma (Group II). Patients were free of systemic and local oral conditions. The control group comprised eight healthy individuals (Group III). The protocol was approved by the Ethics Committee and Institutional Review Board at Saveetha University, Chennai, India. The patients in the study group were diagnosed histopathologically as having squamous cell carcinoma during their visit to the department of Oral and Maxillofacial Pathology, Saveetha Dental College and Hospitals, Chennai. The control group comprised eight apparently healthy, age- and sex-matched subjects without any recent illness. The patients in the study group were ruled out for prior treatment of oral squamous cell carcinoma. All patients were informed about the aim of the study. Informed consent was obtained from both study and control groups.

Saliva sampling

Samples of unstimulated mixed saliva was collected from patients and controls each morning between 7 and 8 am, 10 min after mouth washing with MilliQ water using the spitting method. After the saliva (2 mL) was collected in a plastic container it was immediately mixed with 0.3 mL of acid citrate dextrose (ACD). The samples were then centrifuged at 2,500 rpm for 10 min and the supernatant was separated at -80°C. For each 1 mL of supernatant, 0.1 mL of trichloroacetic acid was added (TCA) in order to precipitate proteins, after which the samples were once again centrifuged for 10 min at 2,500 rpm. The separated supernatant samples were stored at 4°C, placed in an ice carrier box and transferred to the Department of Biochemistry, Sankara Nethralaya, Chennai, for HPLC analysis of amino acids.

Procedure

The Agilent 1100 HP from Agilent Technologies (Hewlett Packard, Strasse-8, Waldbronn, Germany) HPLC system was used in the present study. Standard (10 μ L) was mixed in 60 μ L of borate buffer and 10 μ L of reagent in a dilution vial and final mixing was carried out by a high shear, fast intensive mixer (Cyclomix, Hosokawa Micron Ltd., Runcorn, Cheshire, UK). From this mixture, 50 µL was injected into the HPLC system using Hamilton syringes. Each standard was individually run on the gradient noted in the LC (Liquid Chromatography) parameters program (reaction temperature: 40°C, pressure: 100 bars, column size: 3.0×150 mm, column bed volume: 0.64 mL, flow rate: 0.5 mL per minute, detection wave length: 338 nm and injection volume: 50 µL). Chromatograms were obtained. Two consecutive runs with same retention times were taken, and the means were used for plotting graphs in the calibration table. This

Table 1 Mean values for various amino acids and comparisons between the groups

Amino acids	Group I (WDSCC)	Group II (MDSCC) µmol/mL	Group III (Control) μmol/mL	Kruskal Wallis Test (P < 0.001)	Post-hoc Tukey HSD test $(P < 0.05)$		
	µmol/mL				А	В	С
Aspartic acid (AS)	0.241	0.601	0.035	0.000*	0.000*	0.004*	0.000*
Glutamic acid (GL)	0.168	0.447	0.222	0.001*	0.003*	0.750	0.016*
Serine (S)	0.187	0.515	0.050	0.000*	0.000*	0.014*	0.000*
Histidine (H)	0.300	0.527	0.225	0.006*	0.093	0.750	0.021*
Glycine (G)	0.288	0.552	0.065	0.000*	0.000*	0.001*	0.000*
Threonine (T)	0.435	0.726	0.157	0.003*	0.060	0.075	0.000*
Alanine (A)	0.178	0.567	0.096	0.000*	0.000*	0.081	0.000*
Arginine (AR)	0.220	0.592	0.047	0.000*	0.000*	0.001*	0.000*
Tyrosine (TY)	0.343	0.602	0.112	0.002*	0.028*	0.053	0.000*
Valine (V)	0.165	0.320	0.038	0.000*	0.015*	0.052	0.000*
Methionine (M)	0.162	1.253	0.012	0.000*	0.000*	0.005*	0.000*
Phenylalnine (P)	0.105	0.368	0.011	0.000*	0.000*	0.001*	0.000*
Isoleucine (I)	0.236	0.443	0.033	0.000*	0.001*	0.001*	0.000*
Leucine (L)	0.241	0.501	0.015	0.000*	0.000*	0.001*	0.000*
Lysine (LY)	1.368	0.472	0.837	0.013*	0.041*	0.29	0.546

WDSCC: Well differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous cell carcinoma

* stastistically significant (P < 0.05), A: difference between Group I and II, B: difference between Group I and III, C: difference between Group II and III

procedure was termed as calibration, and the obtained curve was referred to as the calibration curve. From the chromatogram, the area of the peaks were recorded, calibrated along with the standards and used for calculations. Results were expressed in terms of μ mol/mL.

Statistical analysis

The significance of the results obtained from the control and study groups were statistically analyzed using Kruscal-Wallis test and post-hoc test (Tukey HSD). Kruscal-Wallis test was performed for the simultaneous comparison of means from all the groups and post-hoc test was performed for comparisons between the means from two groups. *P* values of < 0.05 were considered to be statistically significant.

Results

The results of the present study showed higher salivary levels of all amino acids in both groups of OSCC patients than in healthy controls, except for glutamic acid (0.168 μ mol/mL), which was lower in well differentiated OSCC patients (Group I) than in controls (Group III) (0.222 μ mol/mL) (Table 1).

Statistical analysis of simultaneous comparison of three groups with a "*P*" value < 0.05 was statistically significant. (Table 1) The values for all the amino acids were found to be statistically significant (P < 0.05) when compared by post-hoc test (Tukey HSD) between Group

I and Group II, except for histidine (P = 0.093) and threonine (P = 0.060) (Table 1).

Discussion

Both excessive nitrogen loss and increased protein breakdown can be observed in oral cancer. Cancers cells utilize more glucose and amino acids than their benign counterparts. Peripheral proteins are broken down and amino acids are mobilized and transferred both to the visceral organs and to the tumor. These amino acids may be used for cell proliferation, energy expenditure (gluconeogenesis) and tumor growth (10). Studies have shown that amino acids and their derivatives serve as useful markers that reflect protein metabolism. In some situations, elevation of salivary amino acid levels can serve as a disease marker (7).

Of the 20 known essential and non-essential amino acids, 15 free amino acids (aspartic acid, glutamic acid, serine, histidine, glycine, threonine, alanine, arginine, thyrosine, valine, methionine, phenylalanine, isoleucine, leucine and lysine) were detected in the saliva of both groups. The remaining four sulphur-containing amino acids were not analyzed, as these amino acids have different detection peak values and are therefore difficult to differentiate for analysis. Collagen-related amino acids (proline, hydroxyproline and hydroxylysine) were not analyzed due to a lack of a suitable fluorescence detection facility. HPLC was selected over other biochemical methods for profiling of amino acids, as it is a sophisticated method and the high resolution ensures more rapid and precise separation. Its high sensitivity permits quantitative estimation in quantities smaller than pmol and it provides a modern, rapid and automated analysis with minimal effort and at reasonable cost.

In our study, the assay levels of amino acids histidine, threonine, valine, isoleucine, methionine, phenylalanine, leucine, lysine, tyrosine, arginine, alanine, glycine, serine and aspartic acid were significantly higher in both well-differentiated OSCC cases (Group I) and moderately differentiated OSCC cases (group II) than in healthy controls (Group III). Whereas the assay levels of glutamic acid were lower in well-differentiated OSCC (Group I) than in healthy controls (Group III).

The results of our study are consistent with previous studies by Sugimoto et al. (12), who reported markedly higher levels of salivary concentration of leucine, isoleucine, tryptophan, valine, threonine, histidine, alanine and phenylalanine in OSCC patients than in healthy individuals.

Laviano et al. (13) and Tankiewiez et al. (6) studied salivary free tryptophan levels in OSCC patients. They suggested that increased concentrations in oral cancer patients may be caused by amino acids produced by tumor cells, which may be involved in the regulation of carcinogenesis.

Mukherji et al. (14) detected certain amino acids in OSCC patients and found that their levels were higher in oral cancer patients than in controls, and they also noted elevated levels of various metabolites, including amino acids, in other tumors, such as colorectal and gynecologic carcinomas.

Analysis of squamous cell carcinoma cell line cultures suggests rapid membrane biosynthesis due to increased cell proliferation. In vitro studies using (2D) correlated spectroscopy revealed that a variety of metabolites, such as alanine, glutathione, histamine, isoleucine and leucine, were present at higher concentrations in OSCC patients than in controls (12).

The increased concentration of various amino acids seen in the tumor cells may be due to regulated protein synthesis in tumors, owing to their need for more protein synthesis caused by rapid cell proliferation. Increased activity of various amino acid transport systems may enhance protein synthesis in tumors. Furthermore, accessory pathways of protein synthesis may become activated in order to meet the demands of rapid cell proliferation in tumors (14).

The heterogeneous systems that transport amino acids

from blood to saliva via the salivary gland, or either the dependence or independence of small ions such as potassium and sodium, may alter the concentration of these ions because of water movement through paracellular routes or channels. Metabolism in the salivary gland itself might also have a major role in the differences in profiles between saliva and blood. However, further validation of these findings by comparing saliva profiles with blood and tissue profiles is needed to understand the reasons for different saliva amino acid profiles (12).

Amino acid transporters play an important role in supplying organic nutrients to cells. The L-Type amino acid transporter 1 (LAT1) is strongly expressed in oral cancer cells to support their continuous growth and proliferation. In addition, several reports have demonstrated that amino acid uptake increases during the proliferation process of cancer cells to support DNA and protein biosynthesis (12).

The expression levels of amino acid transporters ACST2 and LAT1 are elevated in primary human cancers, as cancer cells optimize their metabolic pathways by activating the extra- to intra-cellular exchange of amino acids, enhancing the secretion of amino acids into saliva. Peptides and amino acids are derived from various sources, including fragmented proteins, and the saliva metabolome profiles comprising these compounds may reflect the integrated results (12). Oral cancers are in direct contact with the saliva, and due to local tissue destruction, glycoproteins, which are an integral part of tumor cells, are released into saliva. These are hydrolysed by peptidases and proteases of the saliva, leading to an amino acid pool in saliva (4,15).

In our study, the assay levels of glutamic acid $(0.168 \ \mu mol/mL)$ were lower in well-differentiated OSCC (Group I) than in healthy controls (Group III). The decreased concentration of glutamic acid in oral cancer patients may be due to its utilization by tumor cells to meet the increased energy demand.

Detailed study of amino acid profiles may also help in understanding the mechanisms of metabolic derangement in oral cancer patients and improving their cancer cachexia. However, further studies are necessary to elucidate the potential of these profiles in monitoring therapy as well as the early detection of both malignancy and recurrence. This non-invasive study may thus pave the way for a new era in the field of oral cancer by simplifying early diagnosis and improving prognosis.

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