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Using ROC (Receiver Operating Characteristic) Curve to Determine Significance of Selected Biochemical Parameters in Patients with Cancer of Oral Cavity

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ABSTRACT

Objective: The objective of this study was to estimate the salivary level of protein thiol, Butyrylcholinesterase and phosphodiesterase and ceruloplasmin in newly diagnosed patients with cancer of oral cavity.

Methods: In total, 115 individual enrolled in this study. Salivary samples were collected from patients with cancer of oral cavity (n=65) before any definitive treatment and 50 healthy individual were included in this study. In total 115 salivary samples were collected and a selected salivary parameter assessed spectrophotometrically. It was also checked for pH and salivary flow rate was noted down.

Result: There was a significant increase ($p < 0.001$) in salivary Butyrylcholinesterase and phosphodiesterases levels in patients with oral cavity cancer (n=65) when compared to controls (n=50). Protein thiol is significantly decreased ($p < 0.001$) in cases when compared to controls. Ceruloplasmin did not show any significant change. We plotted ROC (Receiver operating characteristic) curve to analyze a possible strategy for demarcation of biochemical parameters in patients with cancer of oral cavity. We found specificity of above 80% and sensitivity of more than 90% for Butyrylcholinesterase and sensitivity was above 73% for phosphodiesterase.

Conclusion: Our study shows that protein thiols, phosphodiesterase and Butyrylcholinesterase might have a role in pathogenesis of oral cancer and saliva can be effectively used as a non-invasive tool for evaluation of such cases.

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Introduction

Cancer of oral cavity is one of the most common cancer among the male population of India and has tremendous effect on quality of life. According to the world health organization cancer involved in oral cavity is the sixth commonest in the developing countries¹⁻³. That involved 3% of all types of cancer. They are located in the oral cavity in 48% of cases, and 90% of cases are oral squamous cell carcinoma⁴. They are sometimes preceded by precancerous lesions, such as leukoplakia and erythroplakia. More than 300,000 new cases of oral squamous cell carcinoma are diagnosed annually⁵.

In India, the age standardised incidence rate of oral cancer is 12.6 per 100,000 population⁶. The term 'oral' cancer includes cancers of the lip, tongue, gingiva, oral mucosa, oropharynx and hypopharynx⁷. The most current information on the pathogenesis of head and neck squamous cell carcinoma (HNSCC) has been obtained from the studies of oral cancer, likely because oral cancer is the most frequently diagnosed HNSCC. Additionally, oral premalignant lesions are the most commonly diagnosed. Oral leukoplakias are visible precursor lesions that are macroscopically recognizable^{8,9}. However, there are several studies indicating that many precursor changes in the oral mucosa are not clinically visible.

In general, cancers including HNSCC arise from the accumulation of genetic and epigenetic changes and abnormalities in cancer-associated signalling pathways, causing the acquisition of cancer-related phenotypes that have previously been summarized by Hanahan and Weinberg¹⁰.

Oxidative stress could result in many types of cancer and it can contribute in many types of premature cell death leading to many types of major complication¹¹. The level of protein thiols in the body indicates

antioxidant status. The source of salivary proteins, other than those secreted, is albumin. Since oxidation of albumin related thiol groups has no adverse biological consequences, it has been proposed to act as a sacrificial antioxidant. Hence, salivary protein thiol levels are indicative of the oxidative stress status¹²⁻¹⁴. Ceruloplasmin is an important of extracellular antioxidant. Lack of circulating serum ceruloplasmin was observed in patients with neurodegeneration. Animal experiments showed that lack of ceruloplasmin was associated with redox injury in the brain¹⁵. The enzyme PDEs and Butryrylcholinestrase shown to involve in cellular differentiation, apoptosis, tumor invasion Angiogenesis^{16,17}. Since angiogenesis is a hallmark of many types of cancer as well as pre-cancerous condition, hence study of markers involved in newly formed blood vessel is of prime importance^{18,19}. The purpose of this study was to estimate the salivary level of protein thiol, Butryrylcholinestrase and phosphodiesterase and ceruloplasmin in newly diagnosed patients with cancer of oral cavity as well as control and to plot a receiver operating characteristic curve (ROC) for validity of the biochemical parameters in saliva of oral cancer patient.

Method

The study was carried out after obtaining approval from the Institutional Ethics Committee. The subjects were oral squamous cancer cases of either sex admitted under the Department of Radiotherapy and Oncology, Kasturba Hospital, Manipal. Age and sex matched healthy controls were local people from the same geographical area. Both the controls and cases were included in the study after obtaining informed consent.

Study design

Prospective, case control study.

Inclusion criteria

For cases

Patients of either sex between 26-75 years with biopsy proven OSCC stage 2-4 before definitive treatment.

For controls

Healthy subjects between the ages of 26 and 75 years, who underwent an oral examination to rule out all possible benign or malignant oral diseases.

Exclusion criteria

For cases

Oral cancers which are not squamous cell carcinomas; post-surgery, post chemotherapy or post radiotherapy patients of oral cancer; recurrent cases of squamous cell carcinoma; patients with malignancy other than OSCC; patients with serious medical and surgical illness; patients on long-term medication.

For controls

Subjects with coexisting diseases; subjects on long-term medications.

Butyrylcholinesterase (BChE) assay

Principle

Acetylthiocholine is hydrolysed by BChE to corresponding fatty acid and thiocholine. The rate of formation of thiocholine can be monitored by continuous reaction of thiol group with 5, 5'-dithio-bis-(nitro-benzoic acid) – DTNB to form a yellow anion that can be measured spectrophotometrically at 410nm²⁰.

Procedure

In a clean dry cuvette, 2.9ml of 5, 5'-dithio-bis 2-nitrobenzoate (DTNB) solution was taken and to this 0.1ml of butyrylthiocholine solution was added and then to this mixture, 20µl of serum sample was added. After mixing by inversion, absorbance was read at wavelength 410nm. Enzyme activity was calculated by absorption coefficient of the product of chemical reaction, 5-thio-2-nitro-benzoate (1.36 x mmol⁻¹ x min⁻¹ x cm⁻¹).

Principle of phosphodiesterases (PDE) Assay

Paranitrophenyl phosphate (4-nitrophenyl phosphate) is hydrolyzed by PDE to 4-nitrophenol (4-hydroxynitrobenzene) and inorganic phosphate. The yellow color formed due to liberation of 4-nitrophenolate at pH 9 was measured spectrophotometrically at 400 nm²¹.

Procedure

In a clean dry test tube, 1 ml of assay mixture containing 500 µl of Tris HCl, 100 µl of MgCl₂, 100 µl of paranitrophenyl phosphate, and 300 µl of distilled water was taken. The mixture was incubated at 37°C for 5 minutes. Then, to this mixture, 10 µl of saliva (10 µl of double distilled water in case of blank) was added. The mixture was again incubated at 37°C for 10 minutes. 2 ml of NaOH with EDTA was added to each test tube to stop the reaction. Activity was calculated using molar absorption coefficient of the product of chemical reaction, 4-nitrophenol²¹.

Thiol assay

Salivary protein thiol activity as a measured based on the reactivity of protein thiol with the 5, 5 dithiobis, 2-nitro-benzoic acid (DTNB/Ellman's reagent), as it reacts with protein thiol to give mixed disulfide and an aromatic thiol. (p-nitrothiophenol

anion) is responsible for change in color, which was measured spectrophotometrically at 412nm²². Values were expressed in $\mu\text{mol/l}$.

Ceruloplasmin

Ceruloplasmin is an oxidase that can catalyze oxidation of paraphenyldiamine to form a coloured complex which is read at 546 nm²³.

Result

In present study, patient age were between 30-58 years of either sex. Among the patients (n=65) were biopsy proven oral cancer (stage 2, 3 and 4) and 50 were healthy controls. Thus a total of 115 subjects participated in this study. The control group compromised age and gender matched healthy subjects. Among diagnosed oral cancer patients 63 (96%) were male and 2 (4%) were female. Since there was a large variation in individual value of protein thiol hence it is been expressed in median IQR. The level of salivary protein thiol in oral cancer patient were expressed in the form of median interquartile range 23.4 (10.3, 36.5) $\mu\text{mol/l}$. Salivary level of protein thiol was significantly lower than those in control group 61.2 (39.4, 82.3) $\mu\text{mol/l}$, in comparison between the test and control, protein thiol was found to be highly significant $P < 0.001$.

After analyzing of saliva, it is found that Butyrylcholinesterase and phosphodiesterases are significantly increased in patients 31.43 ± 6.31 IU/l and 74.34 ± 21.87 $\mu\text{mol/l}$ respectively. When these values are compared with controls 15.67 ± 2.1 IU/l and 17.27 ± 3.4 $\mu\text{mol/l}$ respectively and these values are found to be highly significant ($p < 0.001$). The concentration of protein thiol found to be decreased significantly ($p < 0.001$) in patient that indicates involvement of oxidative stress in oral cancer patients and the value is

shown in table 1. A cut off point, sensitivity and specificity of test for Butyrylcholinesterase as well Phosphodiesterases are represented in table 2. A receiver operating characteristic curve (ROC) for validity of the biochemical parameters in saliva of oral cancer patient plotted is shown in figure 1. The sensitivity and specificity for Butyrylcholinesterase was 96% and 83.3% and sensitivity and specificity for Phosphodiesterases was 73.1% and 69%.

Discussion

Several investigation have been carried out on tumour tissue and peripheral blood to identify the aetiology of cancers and to establish tumour markers as an adjunct for establishing the diagnosis and prognosis of disease. In this respect, many studies have shown serum alkaline phosphate, serum amylase, serum lactate dehydrogenase, CEA, serum calcium, serum magnesium, serum copper, serum zinc, and the copper/zinc ratio in various malignancies as possible diagnostic and prognostic biochemical markers²⁴⁻²⁷.

Human oral cancer is the sixth largest group of malignancies worldwide. Seventy percent of oral cancers appear from premalignant lesions. The process of formation of oral cancer results from multiple sites of premalignant change in the oral cavity (field cancerization).

This study shows that patients with cancer of oral cavity have high salivary concentration of Phosphodiesterases as well as Butyrylcholinesterase. The Phosphodiesterases are responsible for the hydrolysis of the second messengers, with a fundamental role in the transduction of the intracellular signals. Variations in Phosphodiesterases activity have been found in different pathologies, and they have also been correlated to different pathophysiological mechanisms, such as

cellular differentiation, apoptosis, and tumor invasivity.

Most OSCCs develop in fields of pre-cancerized epithelium in which there is clonal expansion of phenotypically normal but genetically altered keratinocytes. These genetically unstable precancerous keratinocytes manifest aneuploidy, gain or loss of chromosomal material, or alterations in the sequences of nucleotides. The genomic instability favour further acquisition of genetic alterations leading to growth superiority or inferiority of the affected cells. The genetically advantaged cells may ultimately acquire a cancerous phenotype. In a variety of human tumours, BChE genes are amplified, mutated and/or aberrantly expressed²⁸.

BChE affects cell proliferation by virtue of its anti-apoptotic effects which may support early stages of tumourigenesis. It also plays a role in the later stages of transformation by enhancing anchorage independent cell growth which helps in cancer metastasis²⁹. In this study we found increasing levels of salivary BChE as well as Phosphodiesterases in advancing OSCC.

ROS are tumorigenic by virtue of their ability to increase cell proliferation, survival, cellular migration and also by inducing DNA damage leading to genetic lesions that initiate tumorigenicity and sustain subsequent tumor progression. As shown by earlier studies, loss of antioxidant capacity of cell in early dysplasia can trigger initiation and progression of cancer³⁰.

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Table 1. Biochemical parameters in saliva of patient with oral cancer

Parameters	Controls (n= 65)	Oral cancer patients (n=50)
Protein thiol ($\mu\text{mol/l}$)	61.2 (39.4, 82.3)*	23.4 (10.3, 36.5)*
Butyrylcholinesterase (IU/l)	15.67 \pm 2.1**	31.43 \pm 6.31**
Phosphodiesterases ($\mu\text{mol/l}$)	17.27 \pm 3.4***	74.34 \pm 21.87***
Ceruloplasmin (mg/L)	5.1 \pm 1.2	6.5 \pm 2.5

P<0.001*, p<0.001**, p<0.001***

Table 2. Represent the cut off, sensitivity and specificity of test for Butyrylcholinesterase and Phosphodiesterases

Variable	Cut off	Sensitivity	Specificity	Area under the curve
Butyrylcholinesterase (IU/l)	17.76	96%	83.3%	0.949
Phosphodiesterases ($\mu\text{mol/l}$)	17.1	73.1%	69%	0.769

