Protein Thiols and Butyrylcholinesterase in Saliva of Oral Cancer Patients

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Abstract Oral squamous cell carcinoma is one of the most common malignancies recognized. Biomarkers which can predict presence of cancer and its progression can help in better management of these disorders. Over production of lipid peroxidation byproducts and disturbances in antioxidant defense system have been implicated in the pathogenesis of several diseases including oral cancer. Studies have shown a correlation of butyrylcholinesterase (BChE), with tumourigenesis, cell proliferation and cell differentiation. Earlier we have observed a significant elevation in plasma BChE and protein thiols in oral cancer patients which correlated well with stages of cancer. As it was not clear whether the above markers will be altered in saliva of oral cancer patients this study was undertaken. Institutional Ethics Committee gave permission to carry out this study. Total of 55 subjects comprising healthy controls ($n = 30$) and biopsy proven oral cancer patients ($n = 25$) consented to participate in this study. Salivary samples from cases were taken before any definitive treatment. Protein thiols and BChE were estimated in salivary samples using validated assay methods. Oral cancer patients had a significant increase in pre-treatment salivary BChE levels ($p \leq 0.001$)

and a significant decrease ($p \leq 0.001$) in salivary thiols as compared to respective values in controls. Salivary protein thiols and BChE may have a role in pathophysiology of oral cancer. Saliva can be used as a potential non-invasive screening tool in oral cancer patients.

Keywords Oral squamous cell carcinoma · Saliva · Protein thiols · Butyrylcholinesterase

Introduction

Oral cancer is the eighth most common cancer worldwide [1] and its prevalence is high among men in India [2]. Identification of molecular markers which can predict disease progression (prognostic markers) is necessary for better management of these disorders [3]. A correlation of cholinesterase with tumourigenesis, cell proliferation and cell differentiation has been observed [4, 5]. Studies have shown that levels of Butyrylcholinesterase (BChE) can be used as a biochemical marker in the management of head, neck and cervical cancer [6]. Earlier we have observed a significant elevation in plasma BChE in oral cancer patients which correlated well with stages of cancer [7]. As it was not clear whether the above markers will be altered in saliva of oral cancer patients this study was undertaken.

Materials and Methods

Ethical clearance for this study was obtained from the Institutional Ethics Committee. Thirty healthy human volunteers (control) and biopsy proven oral squamous cell carcinoma patients ($n = 25$) who had not gone under treatment consented to participate in this study.

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Inclusion Criteria

For cases: Patients of either sex between 35 and 60 years with biopsy proven OSCC stage 2–4.

For controls: Healthy subjects between the ages of 35 and 60 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 Oral squamous cell carcinoma (OSCC); patients with serious medical and surgical illness; patients on long-term medication.

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

The subjects were instructed not to eat, drink or smoke for 1 h before sampling. Unstimulated saliva was collected in sterile containers after allowing saliva to pool in the floor of the mouth for 5 min by leaning forward and letting saliva drain into a sterile container. Since the salivary composition show diurnal variations, all the samples were collected between 9.00 to 12.00 am. Immediately after collection, aliquot of the sample was taken in 2 ml eppendorf tube and centrifuged for 10 min at 8000 rpm in cold centrifuge. The supernatant, free of debris was used for analysis of the parameters.

Thiol Assay

Salivary protein thiol activity as a measured based on the reactivity of protein thiol with the 5,5'-dithiobis, 2-nitrobenzoic 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 412 nm [8]. Values were expressed in $\mu\text{mol/l}$.

Butyrylcholinesterase Assay

Acylthiocholine was hydrolysed by BChE to corresponding fatty acid and thiocholine. The rate of formation of thiocholine was monitored by continuous reaction of thiol group with 5,5'-dithio-bis-(nitro-benzoic acid)-DTNB to form a yellow anion which was measured spectrophotometrically at 410 nm [9]. Values were expressed in IU/l.

Statistical Analysis

Statistical analysis was performed using SPSS version 16. The values were expressed in mean and standard deviation

Table 1 Salivary protein thiols and BChE in controls and oral cancer patients

Variable	Control ($n = 30$)	Case ($n = 25$)
Protein thiol ($\mu\text{mol/l}$) (median (IQR))	54.1 (40.3, 78.1)	11.3 (4.5, 20.1)*
Pseudocholinesterase (IU/l) (mean $\pm$ SD)	12.67 $\pm$ 5.76	28.55 $\pm$ 7.47**

* $P < 0.001$ (Mann–Whitney test); ** $P < 0.001$ (independent student t test)

Fig. 1 Box plot representing the summary of protein thiols between controls and cases

for BChE and protein thiol were expressed in median (IQR) and p value < 0.05 was considered to be statistically significant.

Results

In this study, subjects were aged between 35 and 60 years of either sex. Of them, 25 were biopsy proven oral cancer patients (stage 2, 3 and 4) and 30 were healthy controls. Thus, a total of 55 subjects participated in this study. The control group comprised of age and gender-matched healthy subjects. Among oral cancer patients, 22 were males and 3 were females. Among the oral cancer patients group, pre-treatment salivary BChE levels were significantly elevated ($p \leq 0.001$) and levels of protein thiols were significantly decreased ($p \leq 0.001$) compared to those of controls (Table 1; Figs. 1 and 2).

Discussion

As saliva has long been tested as a valuable tool for drug monitoring, screening diagnosis various systemic diseases

Fig. 2 Error bar representing the summary of pseudocholinesterase between controls and cases

including cancer, this study was done to understand whether salivary protein thiols and BChE will be altered in oral cancer patients [9, 10]. We have observed a significant alterations in salivary protein thiols and BChE levels indicating the importance of saliva as an efficient tool of screening such disorders.

As per World Health Organization report oral cancer is having one of the highest mortality ratios amongst other malignancies with a death rate at 5 years from diagnosis at 45 % [11]. This high morbidity rate is attributed to the late diagnosis of the disease [12]. Because of lack in national screening programs together with a lack of definitive and satisfactory biological markers [13] for early oral cancer detection has resulted in late stage diagnosis of oral cancer. In this regard, saliva as a diagnostic fluid meets the demands for inexpensive, noninvasive, and accessible diagnostic methodology.

Studies have shown that serum BChE levels which were low in all patients with epithelial malignancies like head and neck and uterine cervix cancers which started increasing after radiotherapy. It was observed that patients with no detectable/visible disease activity at 6 months follow-up had BChE values in the normal range [14]. Abnormal expression of both BChE and AChE, and in vivo amplification of their genes have been observed neoplasms such as meningioma, glioma and acoustic neurinomas, lung cancers, mega karyocytopoietic disorders, leukemias and ovarian tumours [15]. AChE and BChE also modulate cell adhesion in human neuroblastoma cells [16]. In a variety of human tumours, BChE genes are amplified, mutated and/or aberrantly expressed [17, 18]. 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 anchor

age independent cell growth which helps in cancer metastasis [15]. We also had in our earlier study found increasing levels of plasma BChE with advancing oral cancer [7]. This study also in accordance with our previous study which showed a significant increase in salivary BChE in cancer patients as that of controls.

Free thiols in biological systems have important roles. Oxidatively modified thiol groups of cysteine residues are known to modulate the activity of a growing number of proteins. Generation of reactive oxygen species (ROS) plays an important role in maintaining cancer phenotype due to their stimulating effects on cell growth and proliferation. Formation of tumors involves an accumulation of lesions in genes important for the regulation of cell proliferation, differentiation and death [19–21]. Levels of protein thiols and protein carbonyls in serum of brain tumor patients have been studied as an indicator of protein damage due to oxidative stress and significant alterations in their levels have been observed [22]. Many studies have observed altered protein thiols including alterations in the glutathione levels in various disease conditions with oxidative stress [23, 24]. In this study, the salivary protein thiols were significantly decreased in the oral cancer patients when compared to healthy controls. Similar reports were available in studies on salivary and serum antioxidants levels (peroxidase, superoxide dismutase, salivary total antioxidant status) in type 1 diabetes mellitus patients where altered antioxidant levels were observed indicating oxidative stress [25].

Conclusions

Saliva can be an inexpensive, noninvasive, and accessible diagnostic tool for screening of oral cancer patients. Salivary BChE and protein thiol levels are appropriate indicators of oxidative stress in oral cancer patient.

Limitations

The findings of our study cannot be generalized as the sample size is very small. Group characteristics included in the exclusion criteria are confounders for salivary BChE and protein thiol levels. So, if these subjects were included in the study, there could have been a possibility of a different result from the present.

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