

Serum Advanced Oxidation Protein Products in Oral Squamous Cell Carcinoma: Possible Markers of Diagnostic Significance

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Abstract

Background: The aim of this study was to measure the concentrations (levels) of serum total proteins and advanced oxidation protein products as markers of oxidant mediated protein damage in the sera of patients with oral cancers.

Methods: The study consisted of the sera analyses of serum total protein and advanced oxidation protein products' levels in 30 age and sex matched controls, 60 patients with reported pre-cancerous lesions and/or conditions and 60 patients with histologically proven oral squamous cell carcinoma. One way analyses of variance were used to test the difference between groups. To determine which of the two groups' means were significantly different, the post-hoc test of Bonferroni was used. The results were averaged as mean \pm standard deviation. In the above test, P values less than 0.05 were taken to be statistically significant. The normality of data was checked before the statistical analysis was performed.

Results: The study revealed statistically significant variations in serum levels of advanced oxidation protein products ($P < 0.001$). Serum levels of total protein showed extensive variations; therefore the results were largely inconclusive and statistically insignificant.

Conclusion: The results emphasize the need for more studies with larger sample sizes to be conducted before a conclusive role can be determined for sera levels of total protein and advanced oxidation protein products as markers both for diagnostic significance and the transition from the various oral pre-cancerous lesions and conditions into frank oral cancers.

Keywords: Oral squamous cell carcinoma, Reactive oxygen species, Free radicals, Transformation, Pre-cancerous, Serum albumin

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Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common

malignant neoplasms worldwide. In India, OSCC is the most common cancer in males and the third most

common in females. Approximately 60,000 new cases of oral cancer are reported to occur every year in India, with tobacco consumption being the single most important risk factor for the development of oral cancers. Bursts of reactive oxygen species (ROS) have long been implicated as the prime form of damage to genetic material leading to non-lethal mutations that eventually form frank malignant lesions in tobacco users.¹

Oral squamous cell carcinoma has a much higher prevalence in the elderly age groups compared with the younger population. This higher prevalence among the elderly might result from an age related increase in the magnitude of free radical attacks, including ROS and reactive nitrogen species (RNS) that cause various DNA mutations and aberrations. It might also result from an age related reduction in the body's antioxidant defenses in addition to ROS and RNS or, both.^{2,3}

The development of cancer is multi-factorial depending on the extent of damage to DNA which, in turn, is proportional to the magnitude of reactive oxygen and nitrogen stresses. When this equilibrium is disturbed DNA damage occurs and cancer evolves.^{1,4-6}

In plasma, free thiol groups are quantitatively the most important scavengers of the various free radicals and are known to be located largely on various serum proteins, such as albumin. Advanced oxidation protein products (AOPP), formed as a result of irreparable oxidative damage to the proteins, are defined as novel, reliable markers of irreversible oxidative damage.

Despite tremendous advances in the diagnosis and the management of oral cancers, this group of cancers is considered to have the highest mortality and morbidity rates. Diagnostic adjuncts in use to aid early diagnosis of oral cancers either suffer from a lack of sensitivity in the initial stages of the malignant transformation or, are not so cost effective. In addition, biopsy, which is considered to be the gold standard in the diagnosis of oral cancers, suffers from lack of reliability depending on the choice of an appropriate specimen site from the sampled tumor area as well as the

pathologist's criteria for evaluation. The introduction of the concept of the field of cancerization has further questioned the significance of biopsy results in the approval or rejection of reports that confirm either dysplastic or, frank cancerous tissue changes. More so, clinical staging seems to correlate much better with the prognosis than histopathologic grading of the tumor.

The role of biochemical markers, on the other hand, provides convincing evidence of the changes occurring in the body which eventually develop into frank malignant degenerations. The alteration of serum chemistry and the outpouring of the various growth factors and cytokines as tumor markers in the initial stages of the ongoing process of malignant transformation is an added benefit in early diagnosis. At this time, tissue and cell level changes are not obvious to be taken as evidence. The role of serum AOPP as markers of oxidant mediated protein damage, an important part of the process of malignant transformation, if proven statistically significant, could be helpful as an important diagnostic adjunct in the early diagnosis of oral cancers. These markers can assist in possible early identification and even more significantly, in determining the pre-disposition of the various oral pre-cancerous lesions and conditions as they transform into frank oral cancers. Such biochemical markers can be considered to have an edge when compared with the other subjective, invasive and not so cost-effective procedures of risk assessment, diagnosis and prognostication of oral cancers.

The present study assessed the levels of serum AOPP in normal, healthy individuals and those afflicted with the various oral pre-cancerous lesions and conditions and individuals diagnosed with frank oral cancers.

Materials and Methods

Data source

The study was conducted in the Department of Oral Medicine and Radiology, Government Dental College and Research Institute, Bangalore for a period of three months, from January 2010 to

Table 1. Serum total protein results.

Group	Mean	Std dev	SE of mean	Min	Max	F	P-value
Control [n=30]	8.11	1.54	0.28	5.50	10.70		
Pre-cancerous/ pre-malignant [n=60]	8.65	3.00	0.39	1.80	19.30	2.850	0.061
Oral squamous cell carcinoma (OSCC) [n=60]	7.49	2.72	0.35	0.70	18.20		

No significant difference was observed between the three groups with respect to the mean serum total protein ($P>0.05$).

March 2010. The study group consisted of 60 new cases of clinically diagnosed and histologically proven poorly differentiated OSCC; 60 patients with pre-cancerous lesions and conditions that included 20 patients with speckled leukoplakia, 20 patients with erosive lichen planus and 20 patients with oral sub-mucous fibrosis; in addition to 30 age and sex matched healthy controls.

Method of data collection

None of the patients were on any treatment modality or, had any systemic condition, especially hepatic or renal disorders, which could have affected sera levels of proteins, prior to inclusion in the study. Controls and patients who were chronic alcoholics were also excluded from the study in order to rule out the probability of derangement of liver functions that could have contributed towards the variations in sera protein levels.

Methodology

Patients were informed in detail about the planned study and written informed consents were obtained. Patients were subjected to a detailed history and a thorough clinical examination using a specially prepared proforma. Participants underwent a thorough oral examination and routine hematological examination with assessment of serum total protein and AOPP levels to be used as baseline values prior to their inclusion in the study.

Hematoxylin and eosin stained histopathological slides were reviewed to include the cases of clinically diagnosed carcinoma buccal mucosa histologically proven as poorly differentiated

OSCC according to biopsy results from the Department of Oral Pathology, Government Dental College and Research Institute, Bangalore. Histopathological grading was re-confirmed by the Department of General Pathology, Bangalore Medical College and Research Institute and Associated Hospitals, Bangalore.

Since oral cancers are in an anatomic site that is easy to examine, the prognosis for treated lesions is supposed to be high. However, this is not usually the case as unfortunately many health care providers are not observant in their clinical examination of early, incipient lesions. That, coupled with the fact that oral cancers are usually asymptomatic, often delays the diagnosis and adversely affects the prognosis.

Poorly differentiated or, anaplastic OSCC with much cellular or, nuclear polymorphism and either little or, no keratin production may be so immature that it becomes difficult to identify the tissue of origin. Such a tumor often enlarges rapidly and metastasises early in its course.

The intention behind selective inclusion of histologically proven cases of poorly differentiated OSCC was to select cases where the sera levels of serum total protein and AOPP would provide an easy method to estimate response to treatment as well as an adjunct in predicting the disease course, the chances for metastasis and survival rate of the patients. Few studies have related these levels to aid with diagnosis and prediction of pathogenesis, progression, process of transformation from the pre-cancerous stage to frank carcinomatous changes, response to treatment, periodic assessment with treatment progress as well as the chances of metastasis and survival rates in relation to general body cancers.

Table 2. Serum advanced oxidation protein product (AOPP) results.

Group	Mean	Std dev	SE of mean	Min	Max	F	P-value	Sig diff between groups
Control [n=30]	0.08	0.03	0.01	0.04	0.15			1 vs. 2
Pre-cancerous/ pre-malignant [n=60]	0.37	0.09	0.01	0.08	0.53			1 vs. 3
Oral squamous cell carcinoma (OSCC)[n=60]	0.42	0.21	0.03	0.14	0.92	60.181	<0.001*	

*Denotes significant difference; The difference in mean serum AOPP levels between the three groups was statistically significant ($P<0.001$). Further, significant difference was observed between the controls and pre-cancerous/pre-malignant group ($P<0.001$) as well as the control and OSCC group ($P<0.001$).

Assessment of serum total protein and advanced oxidation protein products (AOPP)

Bio-chemical analysis of serum total protein and AOPP was done in the Department of Clinical Biochemistry, Bangalore Medical College and Research Institute and Associated Hospitals, Bangalore. Assessment of serum total protein was performed according to the Biuret method while AOPP was performed by spectrophotometry.

Collection of blood and serum separation

Following an overnight fasting period, 5 ml of venous blood was taken from selected patients from the ante-cubital vein using a sterile disposable syringe, between 8 and 10 am. Samples were obtained while patients were sitting. The samples were allowed to clot and serum was immediately separated by ultracentrifugation, taking full precautions to prevent hemolysis. The supernatant was discarded and the remainder of the sample was stored at -20°C .

Assay of serum total protein

Assessment of sera levels of total protein was performed by the Biuret method. Sera levels of total protein were expressed as g/dL. Biuret test is a chemical test used to detect the presence of peptide bonds. In the presence of peptides, a copper (II) ion forms a violet-colored complex in an alkaline solution. Several variants on the test have been developed.

Assay of advanced oxidation protein products

(AOPP)

Advanced oxidation protein products were measured by spectrophotometry. The assay was calibrated using chloramine-T and absorbance read at 340 nm on a microplate reader. Advanced oxidation protein products' concentrations were expressed as $\mu\text{mol/L}$ of chloramine-T equivalents. Current AOPP methods suffer from poor reproducibility and accuracy due to precipitation of lipids in plasma samples. Solubilization of plasma lipids was therefore carried-out before spectrophotometric analysis of AOPP levels in order to prevent both loss of lipoproteins due to precipitation and overestimation as a result of light scattering.

Statistical analysis

The results were averaged as mean \pm standard deviation for continuous data. One way analyses of variance (ANOVA) were used to test the difference between groups. P values less than 0.05 were considered statistically significant. The normality of data was checked using Kolmogorov-Smirnov and Shapiro-Wilk tests for significance before the statistical analysis was performed.

Results

The study revealed statistically significant variations in sera levels of AOPP ($P<0.001$). Sera levels of total protein showed extensive variations in the study, thus the results were largely inconclusive and statistically insignificant.

The mean values for serum total protein were similar between controls ($8.11\pm 1.54\text{g/dL}$), those with pre-cancerous lesions and conditions

(8.65 ± 3 g/dL) and OSCC (7.49 ± 2.72 g/dL). Although there were great variations in the minimum (1.8g/dL) and maximum (19.3g/dL) values for individuals with pre-cancerous lesions and conditions compared to the minimum (0.7g/dL) and maximum (18.2g/dL) values for those with frank oral cancers, the results were statistically insignificant ($P=0.061$; Table 1).

Advanced oxidation protein products also revealed marked variations between controls (0.08 ± 0.03 μmol/L), those with oral pre-cancerous lesions and conditions (0.37 ± 0.09 μmol/L), and frank oral cancers (0.42 ± 0.21 μmol/L) with a $P < 0.001$. Serum levels of AOPP ranged from a minimum of 0.08μmol/L to a maximum of 0.53μmol/L in patients with oral pre-cancerous lesions and conditions. In patients afflicted with oral cancers, the range was 0.14μmol/L (minimum) to a maximum of 0.92μmol/L (Table 2).

Discussion

Oxidative stress is a general term used to describe the steady state level of oxidative damage in a cell, tissue or, organ caused by ROS. This damage can affect a specific molecule or, an organism as a whole.⁷ Reactive oxygen species such as free radicals and peroxides represent a class of molecules that are derived from the metabolism of oxygen and exist inherently in all aerobic organisms. Most ROS are generated from endogenous sources as byproducts of normal and essential metabolic reactions, such as energy generation from mitochondria or, detoxification reactions that involve the hepatic microsomal enzyme system. Exogenous sources include exposure to cigarette smoke, environmental pollutants such as emission from automobiles and industries, excessive consumption of alcohol, asbestos, exposure to ionizing radiation, and the plethora of bacterial, fungal and viral infections.⁴

The role of oxygen free radicals in the initiation, promotion and progression of carcinogenesis and the protective role of anti-oxidant defenses has been the subject of much speculation with conflicting reports in the literature.⁸ In recent years, increasing experimental

and clinical data have provided compelling evidence for the involvement of oxidative stress in a large number of pathological states including cancers.⁹

The determinants of oxidative stress are regulated by an individual's unique hereditary factors as well as environment and characteristic lifestyle. Unfortunately, under the present day lifestyle, many people run an abnormally high level of oxidative stress that could increase their probability of early incidence of decline in optimum body functions and lead to a number of pathologies including cancers.¹⁰⁻¹⁹

Most free radicals are highly reactive and short lived.^{1, 3, 20} These free radicals have been proposed to be involved in both the initiation and promotion of multistage carcinogenesis by leading to DNA damage, activating pro-carcinogens and altering the cellular anti-oxidant defense mechanisms that otherwise counteract these lethal effects in normal living cells.^{3, 21}

Oxidative stress, however, is not always detrimental. Selective oxidative stress is sometimes desirable and can even possess therapeutic significance. Certain drugs including anti-malaria medications (chloroquine, quinine, mefloquine, primaquine and artemisinin), antibiotics (ciprofloxacin), anti-cancer agents (bleomycin and calcitriol), and iron chelators are included in the category of drugs that utilize oxidative stress for deriving therapeutic advantage.²²

Plasma is known to contain a wide range of important anti-oxidants including albumin, ascorbic acid and uric acid. In contrast, there are certain enzymes, such as super-oxide dismutase, glutathione and catalase, all of which are known to be important intra-cellular anti-oxidants. While ascorbate is an important extra-cellular anti-oxidant, albumin via its thiol groups provides approximately ten-fold greater anti-oxidant protection against various ROS and RNS held responsible for the genetic damage that eventually leads to the development of cancers.^{1, 3, 10, 20, 23-26}

The analysis of changes in serum total protein is a means of studying abnormalities in protein

metabolism in malignancies. Until recently, radical-induced damage to proteins has been considered primarily a chain-terminating process. It was thought that the products of the damage produced on the protein such as protein scission, cross-linking, and chemical modification of side chains, were relatively inert. These damaged materials are thought to be subsequently degraded by intra- and extra-cellular enzymes. It has, however, recently been demonstrated that two types of material, protein-bound reducing moieties (3, 4-dihydroxy phenylalanine) and protein peroxides, are capable of initiating further chemical reactions. Thus, the protein bound reducing moieties have been shown to be able to reduce transition metals resulting in redox cycling of these species which are normally present solely in their oxidized states. Protein peroxides, on the other hand, consume important cellular anti-oxidants and reductants such as ascorbates and glutathione via redox reactions.^{1, 27-32} For similar reasons, serum total protein in the current study came out to be statistically insignificant, which implied a role for several factors in protein metabolism in cancer patients.

There are several methods described in the literature for the estimation of proteins in sera. The Biuret method detects the presence of peptide bonds. This assay is based on copper ions binding to peptide bonds of protein under alkaline conditions to give a violet or purple color. The intensity of the charge transfer absorption band that results from the Cu-protein complex is linearly proportional to the mass of protein present in the solution. The Biuret reaction can be used to assay the concentration of proteins because peptide bonds occur with the same frequency per amino acid in the peptide. The chromophore or, light-absorbing center seems to be a complex between the peptide backbone and cupric ions. The intensity of the color, and hence the absorption at 540 nm, is directly proportional to the protein concentration according to the Beer-Lambert law. Several variants on the test have been developed recently to increase its sensitivity and its application for the estimation of proteins.

The Biuret method was used for the estimation of sera levels of total protein and albumin in the study since it has been considered to be one of the simplest and quickest methods for protein estimation. Also, this method is considered to be sensitive to the amino acid composition of the proteins and can be used to estimate proteins in crude extracts over a large range of concentrations.

As previously stated, cells can generally remove oxidized proteins by proteolysis. However certain oxidized proteins are poorly handled by cells, which may contribute to the observed accumulation and damaging actions of oxidized proteins during aging and various other pathologies, even cancers.^{10-19, 23, 33-35}

Advanced oxidant protein products, first described by Witko-Sarsat et al. (1996), have been hypothesized to activate the endothelial cells and to a lesser extent, fibroblasts to generate ROS.^{6, 36, 37} Advanced oxidation protein products generated by different oxidation patterns lead to the production of either NO or, H₂O₂ which suggests their role in the generation of different types of ROS that set a cascade of reactions with a potential to damage cellular micro-molecules, eventually turning into frank oral cancers.^{32, 38}

The level of AOPP in our study ranged from a minimum of 0.08 μmol/L to 0.53 μmol/L in patients diagnosed with potentially malignant pre-malignant/pre-cancerous lesions and as high as 0.92 μmol/L in patients diagnosed with OSCC, yet negligible in controls with a range from 0.04 μmol/L to 0.15 μmol/L.

Conclusion

Reactive oxygen and nitrogen stresses have long been implicated in the genesis of oral cancers. There is sufficient literature that shows convincing evidence in the use of anti-oxidants as chemopreventive agents to stop the conversion of the various oral pre-cancerous lesions and conditions into frank oral cancers. The results of this research emphasize the need for more studies to be conducted for the assessment of sera levels of total protein and AOPP to justify these parameters as relevant diagnostic adjuncts, to assess their

roles in pathogenesis and their impact on the prognosis of OSCC. This would provide a scientific ground for the use of the various chemopreventive strategies in controlling damage at genetic and molecular levels to prevent the ongoing transition of the various oral precancerous lesions and conditions into frank oral cancers.

Contributions from the author

Literature search, manuscript preparation, manuscript editing and manuscript review.

Ethical Declaration

The study has been approved by the Ethical Committee appointed by the Government Dental College and Research Institute, Bangalore and Bangalore Medical College and Research Institute and Associated Hospitals, Bangalore. The study was performed in accordance with the ethical standards laid down in the 1975 Declaration of Helsinki and its later amendments in 2000, after written informed consents were obtained from the patients for their inclusion in the study. Details that might have disclosed patients' identities have been omitted.

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