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Implication of prophetic variables and their role in the development of oral squamous cell carcinoma (OSCC).

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Abstract

Background and objectives: Oral squamous cell carcinoma (OSCC) is regarded as a catastrophe of modern age which affecting millions of people around the globe without any age, gender and race discrimination. Due to low survival, late detection, poor prognosis and accelerated mortality rates, this carcinoma has attained profound attention worldwide. In this regard, efforts are being made to identify more specific biomarkers for OSCC in order to increase survival rates. As lipid peroxidation, oxidative, nitrosative and inflammatory stresses are key factors in oral cancer development, but there are still some unresolved issues and challenges for them to be valid, reliable and reproducible biomarkers of OSCC. Keeping in view the said facts, present study is designed to determine associations of various biochemical, inflammatory and oxidative components with OSCC oncology in humans.

Methodology: Lipoxidative profile (AGEs, AOPPs, MDA and NO), antioxidant profile (SOD, GSH, GPx, GR, Vitamin A, Vitamin E and CAT) and inflammatory profile (IL-1, TNF-α, MMP-2, MMP-9 and MMP-11) were biochemically assessed from the venous blood of fifty oral squamous cell carcinoma (OSCC) patients and twenty healthy controls. The results of all parameters were analyzed by independent sample t-test.

Results: We assessed various biochemical, inflammatory and antioxidative parameters in sera of 50 OSCC patients versus 20 healthy controls. Percent (%) fold increase of MDA, AGEs, AOPPs, IL-1, TNF-α. MMP-2, MMP-9 and MMP-11 were found to be 64.85, 65.52, 68.28, 37.72, 15.97, 9.62, 42.12, 15.42 and 30.35, respectively in carcinoma victims as compared to the controls. Whereas, statistical analyses revealed that GSH, SOD, CAT, Vitamin-A, Vitamin-E, and GRx were significantly ($p<0.05$) decreased i.e. 73.59, 72.34, 77.57, 26.49, 17.24 and 57.67%, respectively except GPX which was 75.57 times increased in OSCC patients relative to their control counter parts.

Conclusion: The results conclude that lipoxidative profile (AGEs, AOPPs, MDA and NO), antioxidant profile (SOD, GSH, GPx, GR, Vitamin A, Vitamin E and CAT) and inflammatory profile (IL-1, TNF-α, MMP-2, MMP-9 and MMP-11) are the major contributors and play a key role in the development of oral squamous cell carcinoma (OSCC). Theranostic management of antioxidative profile may contribute to alleviate the oxidative burden in OSCC patients which seems to be a major aggravating factor in disease progression.

Keywords: Matrix metalloproteinase, Advanced glycation end products, Advanced oxidation of protein products, Antioxidants.

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Introduction

Oral squamous cell carcinoma (OSCC) has become a worldwide dilemma, affecting men more than women as the former are more habitual users of notorious tobacco and tobacco products. Annually, Pakistan alone has about 10% cases of oral cancer. The major factors responsible for OSCC are the use of alcohol, tobacco and its related products [1,2]. Oral cancer (OC) is a gradual process comprising tumor invasion and metastasis, onset of inflammation and lastly, triggering angiogenesis. Carcinogenesis occurs due to the interaction between cancerous cells and those in vicinity. The interplay of inflammatory cells, reactive oxygen and nitrogen species (RONS) and matrix metalloproteinases (MMPs) sets off OSCC. Consequently oxidative stress and inflammation team up in OSCC where both have the uncanny potential to trigger one another and initiate OSCC.

Oxidative stress is the primary perpetrator in numerous diseases including cancer. The multifaceted signaling molecule nitric oxide (NO) has both tumor promoting and tumor suppressive properties. It also indirectly facilitates oxidative and nitrosative stress [3,4]. Advanced glycation end products (AGEs) and advanced oxidation protein products (AOPPs) levels provide a picture of the extent of oxidative stress in the body. Sugars undergo various chemical changes during aging, forming potential compounds that react with biomolecules resulting in modified sugars which are capable of triggering oxidative stress and inflammation [5,6]. These modified sugars can also arise from lipid peroxidation of bio-membranes which are rich in poly-unsaturated fatty acids (PUFA). Lipid peroxidation marker (MDA) specifically depicts the damage done to lipid membranes [7]. During stress and aging, reactive oxygen and nitrogen species are formed which react with bio-molecules, causing them to be modified. These modified compounds have the potential to cause oxidant-antioxidant imbalances and trigger inflammation. An excessive inflammation eventually leads to cancer onset [8].

Recently, over-expressed matrix metalloproteinases (MMPs) have been found to be the culprits behind various carcinomas including OSCC. The MMPs have multifunctional roles in releasing inflammatory molecules while inflammatory mediators also trigger the release of MMPs [9]. Although oxidative stress, nitrative stress, nitrosative stress and lipid peroxidation contribute to cancer initiation and progression in its own ways, the nature bestowed the cell with antioxidant enzymes makes it capable of hindering cancer onset. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxide (GPx) and glutathione reductase (GR) are part of enzymatic antioxidants whereas reduced glutathione (GSH), vitamins A, C and E are part of non-enzymatic antioxidants [10]. A large amount of literature suggests that low activities of antioxidant enzymes are the reason behind cancer onset.

Biomarkers have shown potential for diagnostic utility in numerous diseases. The oxidative stress (OS) and inflammatory biomarkers depicting early diagnosis are now in demand due to their increased specificity and sensitivity. Early detection via these tumor markers offers new hope to overcome OSCC by using better treatment modalities [11]. The present study was carried out to evaluate the lipoxidative stress biomarkers and inflammatory biomarkers in oral squamous cell carcinoma patients and compared them with healthy controls to assess their effective role in oral cancer disease. Moreover an additional purpose of this study was to establish the prognostic ability of these biomarkers in OSCC.

Materials and Methods

Study subjects and collection of blood samples

Study subjects comprised of oral squamous cell carcinoma patients (n=50) and normal healthy (n=20) persons. These healthy subjects without their tobacco history were recruited as controls. Participants were informed for blood sample collection on specific days in the morning at University Teaching Hospital, the University of Lahore, Pakistan. Then, a total of 5 mL venous blood sample was taken in gel containing vacutainer without anticoagulant from each subject. The blood of each person was allowed to clot at room temperature for about 30 minutes and then was centrifuged and stored at -70°C in eppendorf tubes for subsequent uses. This study was approved by ethics committee of the University of Lahore, Pakistan, and informed consent was also obtained from each participant. All chemical reagents of analytical grades were purchased from sigma/Invitrogen Chemical Co. (St. Louis, Mo, USA).

Inclusion and exclusion criteria

Subjects who were clinically diagnosed with oral cancer by biopsy and pathological exam (CA-125) were included in the study. Participants having systemic diseases, alcohol addiction or taking any type of medication were excluded from the study.

Estimation of biochemical variables

Catalase was measured by the method of Aebi [12]. Lipid peroxidation was evaluated by Ohkawa [13]. Superoxide dismutase (SOD) was determined the method of Kakkar [14]. Advanced oxidation protein products (AOPPs) were evaluated with method of Witko-Sarsat [15]. AGEs were estimated by Goldin method [16]. Estimation of GSH was as per defined methods of Moron and Tietze [17,18]. Glutathione reductase (GR) was evaluated by using method of [19] with the help of spectrophotometer. While Glutathione peroxidase (GPx) was determined by the help of spectrophotometer and buffer/ enzyme reagent [20]. NO was determined by using grease's reagent by the method of Bredt and Snyder [21]. Vitamin E was evaluated in samples by the Emmerie-Engel reaction as reported by [22]. Vitamin A was determined by the method of spectrophotometer as an ingredient of pharmacopeial preparation as adopted in 1956 IUPAC US Pharmacopeial Forum [23]. Interleukin, MMPs and TNF-α were estimated by the help of commercially available ELISA kits (BioVendor).

Statistical analysis

SPSS version 16.0 was used to apply Independent sample T-Test. Results of statistical analysis were expressed as mean ± S.D where $p<0.05$ was considered statistically significant, $p<0.01$ as highly significant and $p<0.001$ as very highly significant.

Results

The results of all variables are summarized in Table 1. Pearson S' Correlation coefficients of different important variables are presented in Table 2. In all, 70 individuals including 50 oral squamous cell carcinoma (OSCC) patients and 20 healthy controls were considered for stress, inflammatory and antioxidant biomarkers study. Demographic distribution of samples in diseased and controlled groups is explained in Table 1. In addition to this correlations among key biochemical parameters are also given in Table 2.

Table 1. *Demographic distribution of samples.*

Sex	Controls	Subjects
Male	15	30
Female	5	20
Smokers		
Yes	10	27
No	10	23
Betal Quid		
Yes	5	26
No	15	24
Alcohol Consumption		
Yes	0	0
No	20	50
Systematic Disease		
Yes	0	0
No	20	20
Medication		
Yes	0	0
No	20	50

Circulating stress biomarkers profile of oral cancer patients vs. control

An overall increasing trend was observed in MDA, NO, AGEs and AOPPS. MDA levels measured between OSCC patients and controls were 3.87 ± 0.017 nmol/ml *vs.* 1.36 ± 0.0013 respecitively. The NO stress biomarkers exoression was higher (55.66 ± 3.11 Um/L *vs.* 19.19 ± 1.76 Um/L) in patients and controls respectively. AGEs level recorded in patients and controls as 2.68 ± 0.09 U/mL and 0.85 ± 0.001 U/mL. Significant increase was seen in AOPPS level in patients (133.36 ± 12.17 ng/ml) as compared to controls (83.05 ± 7.13 ng/ml). On the other hand, a decreasing trend was observed in GSH levels between diseased patients and controls (2.58 ± 0.031 mg/dl *vs.* 9.77 ± 1.37 mg/dl). All stress biomarkers exhibited statistically highly significant values ($p<0.05$).

Inflammatory biomarkers profile of oral cancer patients vs. control

All inflammatory biomarkers studied showed raised levels in oral cancer patients. The mean value of IL-1 in control and diseased is 5.68 ± 0.913 pg/ml and 6.76 ± 1.09 pg/ml respectively. The mean value of TNF-α in healthy and diseased individual is 29.57 ± 1.43 pg/ml and 32.72 ± 3.17 pg/ml respectively. The mean value of MMP-2 in controls is 32.59 ± 3.77 pg/ml and that of diseased patients is 56.31 ± 4.41 pg/ml. In addition the mean value of MMP-9 in healthy individuals is 51.14 ± 7.27 pg/ml and that of oral cancer patients is 60.46 ± 8.79 pg/ml. whereas the mean MMP-11 levels in controls were 45.60 ± 2.47 pg/ml and that of oral cancer patients are 65.47 ± 8.71 pg/ml. All inflammatory biomarkers studied showed highly significant levels in oral cancer patients ($p<0.05$).

Antioxidant biomarkers profile of oral cancer patients vs. control

An overall decreasing trend was observed in all antioxidants studied in OSCC patients (SOD, CAT, Vitamin E, Vitamin A and GRx) except GPx which showed a higher level. In controls

the mean value of SOD was found to be 0.47 ± 0.0013 ng/ml and in diseased patients showed a mean value of 0.13 ± 0.0077 ng/ml. The mean value of CAT in controls is 4.28 ± 0.791 µmol/mol and that of OC patients is 0.96 ± 0.0021 µmol/mol. The mean value of antioxidant vitamin E in controls was 0.29 ± 0.001 µg/ml whereas in OC individuals the mean value was 0.24 ± 0.017 µg/ml. The vitamin A mean value in controls was 62.09 ± 7.78 µg/dl while in diseased individuals it was 45.64 ± 3.79 µg/dl. The mean value of GRx for controls was found to be 7.30 ± 1.53 µg/ml while oral cancer patients showed a mean of 3.09 ± 0.73 µg/ml. Whereas in contrast the mean value for GPx were found to be raised in patients as 6.51 ± 1.11 µg/ml, while in controls was 1.59 ± 0.0037 µg/ml.

Table 2. Pearson correlation coefficients of different variables having role in the development of oral squamous cell carcinoma (OSCC).

Variables	GSH	CAT	SOD	MDA	GPx	GR	Vit-E	Vit-A	NO	TNF-α	IL-1 α	AOPPs	AGEs	MMP-2	MMP-9	MMP-11
GSH	1	-0.235	0.196	-0.568	-0.195	-0.349	0.126	0.111	0.135	0.119	0.195	-0.369	0.159	0.169	-0.269	0.193
CAT		1	0.135	-0.462	0.158	0.235	0.235	0.235	0.156	0.252	0.236	0.235	0.265	0.235	0.235	0.235
SOD			1	-0.226	0.426	0.356	0.426	0.225	0.154	0.336	0.165	0.254	0.125	0.256	0.426	0.356
MDA				1	0.235	-0.426	-0.235	-0.326	0.426	0.326	0.226	0.426	0.235	0.135	0.265	0.261
GPx					1	0.235	0.235	0.325	0.235	0.235	-0.235	0.231	0.563	0.095	0.432	0.235
GR						1	0.254	0.26	0.251	0.425	0.265	0.325	0.265	-0.265	0.251	0.325
Vit E							1	0.426	0.235	0.245	0.032	0.235	0.426	0.231	0.235	0.325
Vit A								1	0.325	0.123	0.562	-0.165	0.225	0.135	0.235	0.326
NO									1	0.329	0.564	0.359	0.326	0.546	0.425	0.356
TNF-α										1	0.452	0.356	0.256	-0.235	0.256	0.302
IL-1											1	0.225	0.325	0.323	0.325	0.235
AOPPs												1	-0.325	0.235	0.235	0.235
AGEs													1	0.235	-0.239	0.426
MMP-2														1	0.442	-0.523
MMP-9															1	0.235
MMP-11																1

Discussion

Oral squamous cell carcinoma (OSCC) has become an enigma for underdeveloped countries due to poor survival rates and late diagnosis [24]. The tumor microenvironment comprises cancer cells and associated stromal components. During the course of inflammation, myriad interleukins, cytokines, chemokines, MMPs and ROS are secreted which activate multiple transcription factors, thereby initiating signaling cascades. The increment of certain growth factors leads to angiogenesis followed by epithelial-mesenchymal transition (EMT) and finally progression to OSCC. The novel OS marker AGEs and AOPPs have emerged as key players in triggering OC as they are involved in elevating tumor burden via release of more noxious oxidants. In the present study, oral cancer individuals showed significantly increased levels of AGEs and AOPPs as compared to the healthy controls, implying that oxidative stress has caused considerable damage to cellular carbohydrates, proteins and lipids by modifying them, forming AGEs that primarily trigger inflammation. Moreover AGEs further increase oxidative stress by releasing more reactive oxygen species (ROS). The AGEs activate the NOx which finally lead to the activation of myeloperoxidase (MPO) system. The MPO causes release of hypochlorous acid which in combination with albumin forms modified proteins. AOPPs further trigger NADPH oxidase contributes to oxidative stress as well as inflammation [25]. Interestingly, the oral cancer patients showed aggressive levels of AOPPs as compared to those of AGEs. This provides strong evidence that AOPPs has the major role in triggering inflammatory mediators in the OC patients.

Lipid peroxidation and nitrative stress are the major outcomes of oxidative stress. Significantly increased MDA level in serum of the oral cancer patients provide evidence that during oral cancer, apart from the free radicals produced by cancer causing agents, the OSCC tumor itself contributes towards a major portion of free radicals generation which eventually leads to increased lipid peroxidation levels. Hence oxidative stress leads to lipid peroxidation. The present study results are in accordance with those reported by [26,27]. Significantly increased levels of nitric oxide in the OC patients strongly suggest the unexplained massive production or defective regulation as contributors of cancer onset. Therefore, the more nitric oxide available, the more it would react with superoxide ion or molecular oxygen to form peroxynitrite resulting in

DNA damage. The release of NO from various inflammatory cells and greater presence of iNOS in the oral cancer individuals are also key factors in causing increased nitrosative stress [28,29]. Hence, in the long run, NO contributes towards nitrosative stress, oxidative stress and NO-mediated lipid peroxidation. Additionally, large amounts of NO have also been linked to excessive pain in OC patients as large quantities of this signaling molecule leads to activation of pain receptors. Moreover, our results further strengthen the fact that NO has a tumor promoting function in oral cancer.

To minimize the effects of oxidative stress, the body has a natural protective antioxidant mechanism comprising of enzymatic and non-enzymatic antioxidants. SOD, GPx, GR and Catalase are enzymatic while GSH, vitamin A and E are non-enzymatic antioxidants. SOD plays a crucial role in preventing diseased states by converting highly reactive superoxide free radicals to hydrogen peroxide which in turn processed into water and molecular oxygen [30]. Significantly reduced levels in oral cancer patients may be due to the production of extremely large amounts of endogenous superoxide ions in the body of patients which may lead to increased reaction of superoxide with NO to form peroxynitrite instead of converting superoxide to hydrogen peroxide. Also reduced levels of SOD may have led to more production of superoxide ion and hydrogen peroxide. An increase in lipid peroxidation may have also contributed in reduced SOD levels [31,32]. The CAT enzyme primarily catalyzes the potent ROS hydrogen peroxide efficiently into water and oxygen in biological systems. The CAT levels in our study were found to be significantly reduced in the patients as compared to the healthy individuals. This suggests that reduced levels of catalase would lead to excessive accumulation of hydrogen peroxide in the system, hence contributing towards oxidative stress. It has been revealed that the reasons behind the deficiency of catalase enzyme in OC subjects might be because of the production of large quantities of superoxide from molecular oxygen and presence of greater amounts of NO or NO products. In addition, circulating free radicals may cause exhaustively low levels or reduced activities of SOD and GPx which may altogether contribute to decreased CAT levels in the oral cancer patients [33].

The GSH levels may have been utilized to eliminate circulating stress markers or the tumor may take it up as nutrition [20,21]. Moreover, greatly reduced levels failed to regenerate the antioxidants alpha-tocopherol and ascorbic acid in the body leading to compromised state [34]. GPx is largely involved in catalyzing the decomposition of hydrogen peroxide [35,36]. The levels of GPx in the OSCC patients greatly increased in our study which suggests that in the presence of SOD and CAT deficiency in OC patients, GPx comes into action so as to combat oxidative stress and scavenge hydrogen peroxide to less toxic molecules [37]. In addition, it also points towards an important complication that enhanced activity of GPx converts the available GSH to oxidized form leaving behind insufficient active GSH levels(GSH *vs.* GPx=-0.195). GR is involved in transforming oxidized form of glutathione (GSSG) to its reduced form (GSH). The present study results were contradicted with those of [38] suggesting that in the absence of sufficient SOD and CAT levels, the available reduced GSH was excessively utilized by GPx to vanquish hydrogen peroxide. This contributed to a major disturbance in the normal GSH to GSSG ratio, causing GSSG to out pass GSH levels hence putting burden on GR to compensate this anomalous shift. Although GR tries to restore the GSH:GSSG ratio by converting oxidized GSH back to reduced GSH but in the long run its own levels exhaust due to an excessive amount of reactive oxygen species (GSH *vs.* GR=-0.349).

The effective role of vitamins as antioxidants has been established in multiple diseases. Vitamin A plays a pivotal role in normal immune functions. Usually, retinoic acid, a form of vitamin A, is produced by macrophages and dendritic cells of mucosa and associated lymph nodes. During the course of inflammation, the conversion of CD4 T-lymphocytes into T-helper cells leads to production of various pro-inflammatory cytokines [39,40]. Decreased levels in patients suggest that they are more liable to encounter infections. Moreover reduced vitamin A levels indicate that more pro-inflammatory cytokines are being released leading to inflammation and hence triggering oxidative stress via NADPH oxidase but the present study shows no interaction between vitamin A and inflammatory mediators. The non-toxic, lipid soluble Vitamin E actively eliminates the damage induced by hydroxyl radicals. Primarily, this antioxidant finds its residence in cellular membranes and lipoproteins where it functions to hinder lipid peroxidation. Decreased vitamin E levels are associated with inflammation and accumulation of hydroxyl radicals resulting in more lipid peroxidation, thereby disturbing the cellular integrity and eventually leading to enhanced chances of oral cancer development. The same trend was also observed in some other studies [41,42].

1L-1alpha is a crucial pro-inflammatory cytokine detectable in both normal and deregulated inflammatory processes. Under normal conditions, IL-1 α plays a significant role in wound healing and tissue repair but in diseased states the same cytokine causes disaster in the body in the form of various chronic diseases [43]. Significantly increased levels in the oral cancer patients give an evidence of deregulated inflammation. The results provides a strong evidence that various inflammatory cells including macrophages, neutrophils and fibroblasts upon activation by tobacco, alcohol or stress release this cytokine in large quantities. Therefore, the role of IL-1α in OSCC is disease onset, tumor progression and metastasis. In our patients enhanced oxidative stress resulted in activation of this cytokine. Our results are analogous to some earlier published reports [44,45]. Tumor necrosis factor-alpha (TNF-α) is a versatile cytokine having pro-angiogenic and pro-inflammatory functions. Its role in carcinogenesis has been found where it acts in each stage of carcinogenesis. The TNF-α is a regulator of angiogenesis where it can promote and hinder tumor formation. It has tumor necrotic and tumor promoting activities in the body [33]. The results of the present study show increased levels in the oral cancer patients as compared to the controls.

MMPs are good biomarkers of oral squamous cell carcinoma [46,47] explain the role of various MMPs as ECM digesters has been known in different diseases thus assist in tumor invasion and release of inflammatory markers (MMP 2 *vs.* IL-1α=0.323 and MMP 11 *vs.* TNF-α=0.302). Our results show increased levels of MMP 2, MMP 9 and MMP 11 in the oral cancer patients; this coincides with the results of [48,49]. Increased levels of these MMPs suggest the unique role of carcinoma-associated fibroblasts (CAFs) in oral cancer by the release of MMP 2 *via* αVβ6 pathway to up regulate MMP 9 levels, thereby promoting ECM degradation and angiogenesis. Moreover, the release of MMP 11 aids in the conversion from precancerous to malignant form. The enhanced levels of MMP 9 in oral cancer patients point towards its role in aggressive cancers. Enhanced amounts of these metalloproteinases pinpoints the fact that greater levels are either due to the over expression of MMPs by cancerous cells or the increased inflammatory cytokine expression, the more ECM degradation will occur hence leading towards tumor invasion and angiogenesis.

Conclusion

In conclusion, present results indicate an imbalance in defensive mechanism in OSCC patients with retarded antioxidant potential and enhanced inflammatory events. Furthermore, evaluations of serum levels of various enzymes involve in antioxidative pathways were decreased in OSCC patients relative to their control counter parts. It is also emphasized that further investigation on MMPs, inflammatory and lipoxidative enzymes might be helpful to provide a new therapeutic clue for the ongoing research struggles in perspective of oral squamous cell carcinoma related biomarker discovery in future.

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