Estimation of Serum Leptin, Adiponectin, and Malondialdehyde Levels in Tobacco-Induced Oral Squamous Cell Carcinoma: ELISA-Based Study

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Abstract

Background  Tobacco, a harmful, addictive chemical is responsible for occurrence of oral cancer by triggering inflammation and lipid peroxidation. The aim of the present study is to explore if there exists any difference in serum levels of inflammatory biomarkers such as leptin, adiponectin, and lipid peroxidation marker malondialdehyde (MDA) and also its correlation with oxidative stress in cases of tobacco-induced oral squamous cell carcinoma (OSCC) as compared with tobacco users having no lesion.

Materials and Methods  Enrolled participants in this study included a total of 84 subjects (all males and 28 in each group) inclusive of healthy individuals, tobacco users with no lesion, and oral cancer cases. Serum leptin, adiponectin, and MDA levels were measured via enzyme-linked immunosorbent assay method in all subjects.

Results  A significant reduction in serum leptin and adiponectin levels in patients with OSCC was observed when compared with tobacco users with no lesions and healthy persons (control). For serum MDA, findings were comparable in control and tobacco consumers with no lesions groups but significantly higher in OSCC cases. The correlation between serum adiponectin, leptin, and MDA levels with body mass index (BMI) was highly significant. In addition, comparison of BMI with serum markers and histopathological grades of OSCC showed significant difference.

Conclusion  These present study observations suggest that reduced adiponectin and leptin and elevated serum MDA could serve as valuable markers for both preventive and clinical intervention, and may deserve further investigation for the early diagnosis, treatment, and prognosis of OSCC.

Keywords
► serum
► leptin
► adiponectin
► malondialdehyde
► oral squamous cell carcinoma
► BMI

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Introduction

Cancer is a modern epidemic with ever-increasing prevalence and burden on health organizations all over the globe. The International Agency for Research on Cancer predicted that India’s incidence of cancer would increase from 1 million in 2012 to more than 1.7 million in 2035.1 Oral cancer is ranked among the top three types of cancer in India and 90 to 95% of all oral cancers are squamous cell carcinoma (OSCC). Most oral cancers result from tobacco smoking or using tobacco in other forms such as gutka, khaini (locally available forms of tobacco), etc. The foremost harmful effect of long-term smoking is increased inflammation which secondarily leads to lipid peroxidation. These events are mediated by family of inflammatory mediators primarily released by adipocytes known as adipokines. Adipokines modulate a variety of metabolic functions within the fat tissue and additional organs such as liver, brain, and muscle to regulate the immune system.2 Adipokines that regulate inflammation have been associated with cancer risks or with treatment outcomes, and there have been suggestions to use these inflammatory mediators as biomarkers for early diagnosis of oral cancer.3 These include adiponectin which has been reported to suppress mature macrophage function as well as leptin that was identified as a T cell stimulatory factor.4 Leptin hormone, an adipokine, participating in hunger regulation via action on hypothalamus has recently been found to be altered in various inflammatory and neoplastic conditions. Reseland et al evaluated the plasma leptin concentration among smokers and nonsmokers and found a lower plasma level in smokers.5 Another harmful effect of smoking is lipid peroxidation, which is a chain reaction that provides a continuous supply of free radicals that initiate further peroxidation forming many damaging aldehydes particularly malondialdehyde (MDA) and propanediol.6 MDA serves as a reliable marker of free radical-mediated lipid peroxidation. It is one of the important indicators of free radical-mediated tissue injury, causes oxidative stress which leads to cancer.7

Leptin and adiponectin have been acknowledged as major endocrine signals in the homeostatic control of body weight.8,9 It is well proven that cigarette smoking is in inverse relation with body weight and body mass index (BMI) of a person.2 Smoking is said to increase adrenergic activity, which could directly increase thermogenesis and thereby reduce weight. Studies on the relationship between smoking status with leptin and adiponectin have reported very mixed findings leading to conflicting reports on the effects of smoking on serum leptin and adiponectin levels.10,11 While there are individual studies that concentrate on the effect of leptin, adiponectin, and MDA in various cancers such as oral, breast, and liver and correlation with BMI, literature search revealed no such study which measured the combined effect of these three serum biomarkers in tobacco users or OSCC. Thus, this study was conceived with the aim to explore the difference if any, in inflammatory biomarkers such as serum leptin and adiponectin. Quantification of MDA levels was done to correlate lipid peroxidation with oxidative stress in tobacco-induced OSCC as compared with tobacco users with no lesion (TOL). Additionally, levels of all the three markers were also correlated with BMI, tumor nodes metastasis (TNM) clinical staging, and histopathological grading in tobacco-induced OSCC.

Material and Methods

The present observational and cross-sectional study was conducted in the Department of Oral Maxillofacial Pathology and Microbiology, Post Graduate Institute of Dental Sciences (PGIDS), Rohtak in collaboration with the Department of Microbiology, Post Graduate Institute of Medical Sciences (PGIMS), Rohtak post clearance from Biomedical and Health Research Ethics Committee (PGIDS/BHRC/22/05) and support from the Multi-disciplinary Research Unit, PGIMS, Rohtak.

A total of 84 subjects were chosen (all males and 28 in each group) and recruited from the outpatient department of our college which were inclusive of healthy individuals, those consuming tobacco but with no lesion, and cases of oral cancer. Males without any systemic disease aged 35 to 65 years were included in the study after obtaining informed consent. Healthy individuals were those who neither consumed tobacco nor had any underlying systemic disease and had BMI within normal range. Second group comprised of individuals consuming tobacco and related product from > 5 years with > 5 intakes per day without any clinical oral lesion or systemic disease. Oral cancer cases were those who were clinically and histopathologically diagnosed with OSCC with history of tobacco and related product consumption from > 5 years with > 5 intakes per day. Patients with history of chemotherapy, radiotherapy, oncological surgery, malignancy, obesity, systemic diseases, bronchial asthma, drug allergies, as well as alcoholics, smokers, and tobacco chewers or had BMI < 18 or > 25 kg/m² were excluded from the study.

After filling the case history pro forma of each case in detail, height and body weight were measured and BMI was calculated with the formula BMI = [weight(kg)/[height(m)]². BMI of healthy individuals is said to be between 18.5 and 24.9 kg/m². Then blood sample was collected and serum was stored for the estimation of leptin, adiponectin, and MDA with enzyme-linked immunosorbent assay (ELISA).

Procedure

Blood samples were collected from all subjects after overnight fasting of 8 to 10 hours. Under aseptic conditions, 4 to 5 mL blood was aspirated from antecubital vein using 20-gauge needle with single-use disposable sterile syringes and transferred to sterile serum collection vactainers for processing.

Processing of Blood

Collected samples of blood were allowed to clot at room temperature and after 1 hour, serum was separated from blood by centrifuging at 3,000 revolutions per minute for 5 minutes and stored at –65°C in sterile vials. Finally, supernatant fluid was collected and transferred into a clean plastic screw-cap vial with an attached label. Serum leptin,
adiponectin, and MDA levels were quantified from the stored vial using sandwich ELISA (Lisawash 4000) using commercial kits (Leptin serum ELISA, Diagnostic Biochem Canada; Adiponectin serum ELISA, Diagnostic Biochem Canada; Human Malondialdehyde GENLISA, Krishgen Biosystems).

Statistical Analysis
Along with calculations of arithmetic means and standard deviations, chi-square test was used to compare mean age and BMI. Pearson’s correlation was used for comparing the adiponectin, leptin, and MDA levels with BMI in all the three groups. Analysis of variance (ANOVA) was used to compare adiponectin, leptin, and MDA mean serum levels and mean BMI in various histopathological grading and TNM staging.

Results
Control group (group I) comprised 28 healthy males with ages ranging from 35 to 65 years (mean age 43.8 ± 9.36 years) while study groups (group II) comprised 28 individuals each who were tobacco users but had no oral lesion (mean age 46.5 ± 10.18 years) and OSCC group (group III) with mean age of 52.6 ± 8.58 years (Table 1). The mean BMI in groups I, II, and III was 21.82 ± 1.93, 21.78 ± 1.94, and 18.13 ± 1.97 kg/m², respectively, which on comparison was highly significant (p = 0.000) (Table 1). Upon quantitative analysis of the serum markers (Table 1), the mean serum adiponectin levels decreased from group I to III with the values 18.71 ± 2.06, 15.24 ± 2.46, and 8.85 ± 3.06 ng/mL, respectively, and the intergroup comparison was highly significant (p = 0.000). Mean serum leptin in group I was 9.13 ± 2.96 ng/mL, in group II it was 6.27 ± 2.36 ng/mL, and in group III it was 3.62 ± 1.11 ng/mL, which also significantly decreased toward group III (p = 0.000). However, the mean serum MDA significantly increased in group III as compared with control and values of group II was almost comparable to that of the control group. Serum MDA values were 1.73 ± 0.831, 1.71 ± 0.90, and 4.57 ± 1.19 nM/L, respectively, in all the three groups (p = 0.000). Pearson’s chi-square test was applied to compare mean age and BMI among the groups which revealed significant results (p = 0.000) (Table 1). The correlation between serum adiponectin, leptin, and MDA levels with BMI also was highly significant (Pearson’s correlation test; p = 0.000) (Table 2). A comparison was also made among the clinical staging and histopathological grades of OSCC with the serum markers and BMI. Upon application of ANOVA between serum levels of the markers and BMI in the similar clinical TNM stages revealed no significant findings. However, upon comparison of BMI with serum markers and histopathological grades of OSCC, significant difference was observed (p = 0.000) in both well and moderately differentiated OSCC; no case of poorly differentiated formed part of the study (Table 3).

Discussion
The primary aim of this pilot study was to compare and correlate the serum leptin, adiponectin, and MDA levels in tobacco-induced OSCC, tobacco consumers with no lesion, and healthy individuals. An attempt was also made to correlate lipid peroxidation with oxidative stress in the study.

Table 1 Intergroup comparison of various parameters among control and study groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I (control) Mean ± SD</th>
<th>Group II (TOL) Mean ± SD</th>
<th>Group III (OSCC) Mean ± SD</th>
<th>p-Valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (y)</td>
<td>43.86 ± 9.36</td>
<td>46.50 ± 10.18</td>
<td>52.64 ± 8.58</td>
<td>0.03</td>
</tr>
<tr>
<td>Mean BMI (kg/m²)</td>
<td>21.82 ± 1.93</td>
<td>21.78 ± 1.94</td>
<td>18.13 ± 1.97</td>
<td>0.000</td>
</tr>
<tr>
<td>Mean serum adiponectin levels (ng/mL)</td>
<td>18.71 ± 2.06</td>
<td>15.24 ± 2.46</td>
<td>8.85 ± 3.06</td>
<td>0.000</td>
</tr>
<tr>
<td>Mean serum leptin levels (ng/mL)</td>
<td>9.13 ± 2.96</td>
<td>6.27 ± 2.36</td>
<td>3.62 ± 1.11</td>
<td>0.000</td>
</tr>
<tr>
<td>Mean serum malondialdehyde levels (nM/L)</td>
<td>1.73 ± 0.831</td>
<td>1.71 ± 0.90</td>
<td>4.57 ± 1.19</td>
<td>0.000</td>
</tr>
<tr>
<td>Comparison of age vs. BMI p-valuea</td>
<td>0.014</td>
<td>0.007</td>
<td>0.003</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ANOVA, analysis of variance; BMI, body mass index; OSCC, oral squamous cell carcinoma; SD, standard deviation; TOL, tobacco users with no lesion.

aPearson’s chi-square test.

bANOVA; p-value: > 0.05, nonsignificant; < 0.05, significant.

Table 2 Correlation between BMI and serum markers adiponectin, leptin, and malondialdehyde among groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>r</th>
<th>p-Valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean BMI vs. serum adiponectin</td>
<td>I</td>
<td>0.622</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0.833</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>0.878</td>
<td>0.000</td>
</tr>
<tr>
<td>Mean BMI vs. serum leptin</td>
<td>I</td>
<td>0.933</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0.965</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>0.977</td>
<td>0.000</td>
</tr>
<tr>
<td>Mean BMI vs. serum malondialdehyde</td>
<td>I</td>
<td>−0.7</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>−0.74</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>−0.9</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Abbreviation: BMI, body mass index.

aPearson’s correlation test; p-value: > 0.05, nonsignificant; < 0.05, significant.
groups and the three markers were also correlated with BMI, TNM staging, and histopathological grading of tobacco-induced OSCC. Only males were recruited in the study keeping in mind the high prevalence of tobacco consumption in males (52.4%) compared with 21.5% in females. Also, hormonal variations in females could influence the serum levels of these biomarkers. In our study TOLs were introduced as a study group to intercept the early changes in serum levels of adiponectin, leptin, and MDA due to continuous consumption of tobacco. At this no lesional stage, alteration in levels of these biomarkers might influence the serum levels of adiponectin in normal individuals as well, so the subjects recruited for the study were checked for BMI to minimize this confounding factor. Epidemiological studies have indicated that the association between BMI and all-cause mortality is dependent upon age. In our study, significant correlation was found between age and BMI among the study groups.

In the present study it was conclusively demonstrated that serum adiponectin and leptin levels were significantly decreased in the OSCC group when compared with TOLs and healthy persons, a finding which is supported by previous individual studies. The possible reason behind the reduction of both adipokines could be due to decreased body fat mass secondary to weight loss in cancer patients. Reduction in serum leptin levels in cancer is an established fact and has been reported in patients of oral cancer, head and neck cancer before induction chemotherapy, advanced gastrointestinal carcinoma, ovarian cancer, hematological malignancies, breast cancer, and prostatic cancer. Bolukbas et al reported significantly lower serum leptin in advanced gastrointestinal carcinoma patients. Similar findings were also observed by Mor et al in their study on ovarian cancer patients. In hematological malignancies found significant decrease in serum leptin. In cases of tobacco consumers with no lesions the serum leptin levels were in between that of OSCC and controls probably due to the constant exposure of these persons to nicotine via tobacco smoking which may dysregulate leptin release. The significant differences between the three groups could be attributed to the amount and duration of nicotine exposure which is most in oral cancer followed by tobacco consumers with no lesions and practically nil in healthy individuals. It could be inferred that decreased leptin levels in OSCC cases corresponds to weight loss due to reduced BMI in our study.

The reduction in serum adiponectin in TOL and oral cancer group as compared with healthy control in the present study was consistent with previous studies conducted in various cancers of breast and liver which states that decreased adiponectin level is related to increased risk of cancer reason being adiponectin receptor dysregulation. Conflicting results also exist in clinical studies of pancreatic cancer that both higher and lower adiponectin levels are reported to be associated with cancer risk. Similar findings in another study by Guo et al on tongue cancer revealed decreased adiponectin levels which stated that despite the inverse correlation between adiponectin and oral cancer, the underlying mechanisms of adiponectin is not fully elucidated. Reduction in levels of adiponectin in tobacco users could be due to oxidative stress provoked by smoking, which reduces adiponectin secretion and expression by inhibiting the function of phosphatidylinositol 3-kinase in adipocytes. Another possible reason could be that circulating adiponectin levels are affected by various factors, including inflammatory, dietary, hormonal, genetic, and medicine. Decreased adiponectin levels in malignancies could also be attributed to sustained inflammatory status of cancer patients that leads to increased proinflammatory cytokines such as tumor necrosis factor-α and interleukin-6, which are all reported to suppress adiponectin levels.

Upon comparison of the oxidative stress biomarker, MDA, findings were comparable in control and tobacco consumers with no lesions groups but serum MDA levels were significantly higher in OSCC cases ($p < 0.000$). The present study results of the OSCC group were in accordance with studies in different cancers such as the study by Sahin et al in which MDA levels increased significantly in lung cancer as compared with healthy controls and tobacco smokers.

### Table 3: Comparison between mean BMI, serum adiponectin, leptin, and malondialdehyde levels with TNM clinical staging and histopathological grades of group III (oral squamous cell carcinoma)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Oral squamous cell carcinoma (group III)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNM clinical staging</td>
</tr>
<tr>
<td></td>
<td>Stage II</td>
</tr>
<tr>
<td>Body mass index</td>
<td>17.7 ± 1.7</td>
</tr>
<tr>
<td>Mean adiponectin levels (ng/mL)</td>
<td>9.2 ± 3.1</td>
</tr>
<tr>
<td>Mean leptin levels (ng/mL)</td>
<td>3.7 ± 1.2</td>
</tr>
<tr>
<td>Mean malondialdehyde levels (nM/L)</td>
<td>4.56 ± 1.23</td>
</tr>
<tr>
<td>$p$-Value</td>
<td>0.667</td>
</tr>
</tbody>
</table>

Abbreviations: ANOVA, analysis of variance; BMI, body mass index; MDSCC, moderately differentiated SCC; SCC, squamous cell carcinoma; TNM, tumor nodes metastasis; WDSCC, well-differentiated SCC.

$^a$ANOVA; $p$-value: > 0.05, nonsignificant; < 0.05, significant.
compared with healthy controls (p < 0.001). Bak et al also stated that the levels of plasma MDA were significantly higher in gastric cancer cases as when compared with healthy controls. Gitanjali et al stated in their study on cervical carcinoma that there was no statistically significant difference in the mean serum MDA levels of healthy controls (3.40 ± 0.75 ηmol/mL) and patients with cervical carcinoma (3.54 ± 1.89 ηmol/mL). Apart from cancer, studies have also reported increased MDA levels in chronic obstructive pulmonary disease and during hemodialysis. This was also in accordance with the study by Chole et al who also found highest MDA levels in OSCC than oral precancer and healthy individuals. But the findings of TOLs did not follow a similar trend as precancer. Morrow et al in their study further elaborated that plasma levels of lipid peroxidation products (F2-isoprostanes) were significantly higher in smokers than in nonsmokers. Smoking enhances lipid peroxidation which is a chain reaction providing a continuous supply of free radicals that initiates further peroxidation in which many damaging aldehydes are formed particularly MDA, propanediol, and 4-hydroxynonenal. Reaction of the Reactive Oxygen Species (ROS) with cellular deoxyribonucleic acid results in oxidative damage which is considered to be crucial in cancer development and thus provides an additional possible mechanism for the apparent association between smoking and mouth, lung, pharynx, esophagus, bladder, and cervical cancers. It is also stated that smoking even a single cigarette leads to a sharp drop of oral peroxidase activity in both smokers (42.5%) and in nonsmokers (58.5%). After 30 minutes, the level of activity returned to 90 to 100% of the presmoking level, presumably due to the secretion of new saliva into the oral cavity. Increased carbonylation of the salivary proteins is seen after loss of oral peroxidase activity, an indicator of the oxidative damage to proteins. Result of study by Craig et al was in contrast with the above said studies, which stated that there was no strong relation between cigarette smoke exposure and lipid peroxide level, suggesting that certain interactions related to oxidation status are not measurable in the serum compartment. Similarly in our study too, comparable levels of MDA in the control and TOL group could be due to same fact that these interactions related to oxidation status are not measurable as peroxidase activity drops down to presmoking levels after certain time and in the TOL group nicotine exposure duration is less as compared with oral cancer cases. When the clinical and histopathological grades of OSCC were compared it was observed that the TNM clinical staging did not show significant difference with the serum markers and BMI index. However, serum adiponectin and leptin levels were slightly decreased in moderately differentiated as compared with well-differentiated OSCC whereas MDA levels were raised. This probably could be due to adiponectin and leptin receptor dysregulation and increased lipid peroxidation with increasing levels of differentiation. This being a pioneer attempt to measure the markers in various subtypes of OSCC, the present study findings should be corroborated with further studies.

Conclusion
This was a maiden attempt to shed light on possible association and mechanism of tobacco which causes alteration in serum levels of leptin, adiponectin, and MDA in TOL and oral cancer cases. The present data could be used as baseline findings and compared with future studies which focus on the same groups but with higher sample size, females, and multicentricity to shed more light into the significance of these parameters with oral cancer.

Clinical Relevance
The present study observations suggest that reduced adiponectin, leptin, and elevated serum MDA levels do indicate a change from normal to a high risk yet noncancerous state. Thus, these changes if intercepted early could serve as valuable markers for both preventive and clinical intervention, and thus, deserve further investigation for the early diagnosis, treatment, and prognosis of OSCC. Free radical mediated lipid peroxidation is an important causative mechanism in oral cancer and antioxidants are considered to be scavengers of these free radicals. Studies on OSCC cell lines have exhibited cancer cell destruction by apoptosis, cytokine stimulation, altered gene expression, cellular differentiation, and prevention of tumor angiogenesis by use of antioxidants. Thus, these antioxidants could be effectively used as adjuncts in the treatment of oral cancer.

Ethical Approval
The study was approved by the Biomedical and Health Research Ethics Committee (PGIDS/BHRC/22/05).

Informed Consent
Informed consent was taken from each participant.

Authors’ Contributions

Funding
All authors have no relevant financial or non-financial interest to disclose.

Conflict of Interest
All authors have no conflict of interest to declare that is relevant to the contents of the article.

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