Perturbations of Cytokine Interleukin-6 in Correlation with Cancer Stem Cell Markers in Oral Pre-Cancerous and Cancerous subjects

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Abstract

Oral squamous cell carcinoma (OSCC) contributes vastly to oral cancer and Oral submucous fibrosis (OSMF) is an ongoing, silent events related to high risk of malignancy. The poor prognosis of oral lesions is majorly due to late-stage diagnosis. We aimed to evaluate the diagnostic biomarker ability of Interleukin-6 (IL-6) in conjunction with alterations of Cancer stem cell (CSC) markers: CD44 and CD133 in the oral precancerous and cancerous subjects. A total of 135 individuals were enrolled in the study, in which 60 cancer cases (OSCC), 60 precancer controls (OSMF) and 15 healthy controls were included. Analysis of serum inflammatory cytokines IL-6 was evaluated by ELISA. Expression studies of CD133 and CD44 were performed by immunohistochemistry. CD133 and CD44 positive individuals both in the OSMF and OSCC conditions showed enhanced levels of IL-6 as compared to negative individuals. Also, the enhancement of IL-6 levels in OSCC cases was more pronounced as compared to OSMF IL-6 levels. Multivariate analyses considering CSC markers and habits were also performed and showed significant contribution of CD44 and CD133 positivity on IL-6 enhancement, with no significant influence of habits. The regression models were found to be significant in terms of predicting the IL-6 changes both in the OSMF and OSCC groups. We further showed that irrespective of the type of CSC marker (CD44 or CD133) expression, the levels of IL-6 were increased in both the OSMF and OSCC condition. We concluded that the simultaneous parallel perturbations of CSC biomarkers and IL-6 can be used as early indicators both for oral precancerous and cancerous conditions. Present work indicated the relevance of examining inflammatory cytokine alterations in the CSC positive subjects for better diagnosis of oral lesions.

Keywords: Oral Cancer, IL-6, OSCC, OSMF, CD44, CD133, Cancer stem cells

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Introduction

Cancers of the oral cavity, lip, hypo and oropharynx are identified together as mouth and/or oral cancers. The most common amongst these are the cancers of the lip and oral cavity. Worldwide more than 377,700 cases of oral lesions have been reported in 2020 by World Cancer Research Fund International. The highest number of oral lesions, which is about one-third of the total load of oral cancer globally has been reported from India itself [4,5,12]. Around 90% of oral cancers are contributed mainly by Oral squamous cell carcinoma (OSCC) [6,14]. Oral submucous fibrosis (OSMF) are a continuous, generally unnoticed changes which are linked with high risk of malignancy. It has been estimated that 2-8 % of malignant transformation is via OSMF to OSCC [6,14]. Tobacco intake either chewing or through smoking has been identified as the major risk factor of oral cancer [25]. In India the habit of smoking and chewing are recognised as the common forms of tobacco consumption [12]. There are various issues identified for poor prognosis of OSCC such as inability of most current therapeutic approaches, stage identification, recurrence and hostile metastases [6]. This intrigued us to explore the diagnostic options of oral cancer by studying simultaneously the biomarkers of different origins. We have also included the initial pre-cancer stage (OSMF) in the study to get the insight of possible early diagnosis and related tumorigenic factors.

One of the approaches to examine the chances of tumour recurrence and early diagnosis is examining the expression pattern of cancer stem cell (CSC) markers [7,15]. The two well established CSC markers are CD44 and CD133[13]. In few recent studies various cancer related processes such as cell to cell interactions, cell adhesion and/or migration are linked with CSC marker: CD44. It is a glycoprotein present on the surface of cells and thought to be involved in the spread of oral lesions through altering the migration of cells [13]. Another CSC marker: CD133 is a part of the penta-span transmembrane glycoprotein family. It is reported that that CD133 is expressed in a variety of proliferating potential cells such as the hematopoietic stem cells, endothelial progenitor cells, glioblastomas, neuronal and glial stem[17].

Cytokines- a well-known group of small secreted proteins play important immunological roles through modulating inflammation, proliferation and differentiation of normal as well as transformed cells [8]. Inflammatory cytokines in blood serum have been studied as vital biomarkers for early detection of oral cancer. Interleukin-6 (IL-6) is a multifunctional pleiotropic inflammatory cytokine produced predominantly by cancer-associated fibroblasts in the tumour microenvironment, was reported as a direct critical driver of tumour growth

and metastasis [30]. Thus, it is highly relevant to further, elucidate the association of IL-6 with tumour progression, chemoresistance and prognosis in oral lesions [9].

The general practice for studying the oral cancer is examining several clinicopathological parameters and inflammatory markers in response to different types of tobacco intake habits in given subset of individuals [3,26]. However, some researchers focussing on identification of expression changes of CSC markers in different types of oral cancer lesions [7,16] . Interestingly, we found no previous studies which simultaneously assess the CSC expression and proinflammatory cytokine levels in precancer and cancer stage. Therefore, in the present study we aimed to evaluate the CSC markers (CD133 and CD44) expression and simultaneous IL-6 changes in the precancerous and cancerous individuals. Through this study we want to examine the hypothesis that whether the expression of CSC markers and/or tobacco intake habits in the OSMF and OSCC individuals can be associated with serum IL-6 changes.

Materials and Methods

The present investigation was performed at Eras Lucknow Medical College and Hospital, India and Amity Institute of Biotechnology, Amity University Uttar Pradesh, Lucknow campus, Lucknow, India. A total of 135 individuals were enrolled in the study, in which 60 oral cancer patients (OSCC), 60 pre-cancer controls (OSMF) and 15 healthy controls were included. The categorization of groups was according to standard classifications [3,22]. The OSMF individuals were grouped on the basis of indications like burning sensation, dryness of mouth, oral mucosa blanching and/or ulceration [11,22]. The other important features of OSMF used for characterization includes progressive fibrosis of the submucosal tissue, presence of fibrotic bands at the retromolar region, difficulty in the opening of mouth, marked trismus, difficulties with mastication etc.

Briefly, the major basis of categorization of OSCC group includes (i) Patients' history and physical examination (ii) Deep tumour invasion into adjacent tissues of the tongue and other part of oral cavity (iii) Microscopically variable degree of keratinisation, cellular and nuclear pleomorphism, mitotic activity. (iv) Tumour features including- tumour site and site, histologic grade, lympho-vascular invasion and tumour thickness. Information on socio-demography, clinicopathology (TNM staging, histological grade, tumour differentiation, metastasis) and habits (type, duration and frequency) were recorded for each respective participant. All subjects in the OSCC and OSMF and healthy control group had a past event

history of one or the other habits such as tobacco chewing (includes gutkha, khaini etc), smoking and pan masala (non-tobacco plain masala, betel quid etc).

Sample collection

Before collecting the tissue and blood sample a proper written informed consent was obtained from all related subjects as per ethical guidelines. The study was approved by the institutional human ethical committee. The institutional ethics protocol and ethical clearance number was assigned as ELMC/R cell/2018/EC/2034 (Era's Lucknow Medical College /Research Cell/2018/ Ethics Committee/2034). For Staging and grading we followed WHO criteria as per the International Union Against Cancer [19].

Tissue sample:

Formalin fixed; paraffin embedded tissue specimens were obtained from enrolled patients during surgery at the King George Medical University, Lucknow, India. These samples were collected for three years. Subjects were examined two experienced pathologists confirmed the histological diagnosis of each lesion.

Blood sample:

For the estimation of serum IL-6 levels; Under non-stimulatory circumstance, serum samples were obtained from 9:30 AM and 1:00 PM. Subjects who have withdrawn from activities such as cigarette consumption such as chewing tobacco and consuming alcohol at least one hour before sample collection were considered. The patients were provided with instructions during sample collection and were not allowed to eat any food items or medicines before one hour, as these substances might interact with saliva and affect the outcome of the results. Primarily, this was done to maintain the uniformity of process. Before surgical treatment blood sample were collected from OSCC and OSMF categorised individuals. The blood was immediately centrifuged (Remi, C-24BL) at 1500 rpm at 2-8°C for 15 minutes and serum was collected to perform ELISA. The samples were processed at -20°C (Euronova - 20°C) for future use.

Inclusion criteria

1. Participants with the hallmarks of pre-oral cancer (OSMF) were involved in the study. This includes oral pathologies such as burning sensation, dry mouth, blanching oral mucosa, ulceration, difficulties with mastication, mild or severe trismus and diffuse form of submucous fibrosis etc

2. Patients who have shown the hallmarks of oral cancer (OSCC) were included in the study. This includes deep tumour invasion into adjacent tissues of the tongue and other part of oral cavity, variable degree of keratinisation, cellular and nuclear pleomorphism, mitotic activity. Tumour features includes tumour site and site, histologic grade, lympho-vascular invasion and tumour thickness. Subjects who have not received any previous treatment (pre-operative chemotherapy or radiotherapy).

3. For healthy control group, total 15 subjects (n=15) were included in the study with (i) no symptoms of oral pathology, inflammation, pre-oral cancer or cancer like changes or history of any other disease (ii) Habit of Tobacco and smoking less than 10 years (Control individuals with habits but with no disease were considered) (iii) Adults ≥ 70 years (iv) Age-sex matched

Exclusion criteria

1. Subjects with oral cancer along with other malignancies are excluded. Also, if such situation was found in the past or having immune deficiency syndrome/tuberculosis/other chronic disease.

3. Subjects taking hypersalivate medications (causing increased salivation are excluded) such as antihistamines, anticholinergics, beta-adrenergic blockers and antihypertensives.

4. For healthy controls, individuals with any oral pathology, inflammation or any other comorbidly and chronic disease.

Histopathology

The histopathological analysis was performed using a previous protocol with few modifications [10]. Briefly, the expert pathologists visually examined the tissue specimens during grossing step for suspicious pathological area through microscopic examination. The identified regions were prepared as tissue blocks. The embedded tissues were sectioned and these thin slices were then placed on glass slides. A unique barcode was assigned to each slide to retrieve the information related to the staining protocol for that specific section.

Immunohistochemistry (IHC)

We used the IHC protocol with few modifications [10]. Four 4 µm sections were prepared after routine H & E staining to reconfirm the characterization of oral lesions groups by the expert pathologists. Sections prepared from all pathological grades of OSCC were examined through IHC. The primary antibodies: rabbit anti-CD44 (Dako, Denmark) and polyclonal, anti-CD133 (Proteintech, USA) with dilution of 1:100 and 1:50 were used respectively.

Microslides coated with ploy-l-lysine were carefully fixed on sections. Fresh xylene was used to deparaffinization by giving two dips of 10 min each. The tissue was rehydrated by three exposures of alcohols (90, 80, and 70%) for 5 min each. After further preparation of samples, the tissue specimen was then covered with primary antibodies. They were incubated for 1 h at room temperature. Then washed two times with PBS. Similarly secondary antibody was incubated for 30 min at room temperature. Then washed three times with PBS. The substrate chromogen solution prepared by mixing 1 ml of substrate buffer and a drop of diaminobenzidine was incubated for 5 min at room temperature. Later gently rinsed with PBS. The slides were counterstained by haematoxylin bath for 2–5 min and washed under tap water for 5 min.

Microscopic examination

Microscopic examination was performed according to the previous protocols with few modifications [3,23]. CSC markers expressions was quantitative examined and their density was evaluated by identifying most reactive five hot spot areas on the respective slide at a 10X objective. This was then counting with a magnification of 200-fold using (Model number: ULH1003, Tokyo, Japan). CSC density was defined as the number of labelled CSCs for each of the two antibodies (CD133 and CD44) per optical field (an optical field corresponding to a rectangular examination area of 0.7386 mm^2). The counting was done twice for the respective five hot spot areas and averaged for every tumour section. In the inner tumour area and at the invading tumour edges this practice was repeated for each sample. Light brown stain in the cell membrane was marked as the positive CD44 & CD133 expression. All stained areas demonstrating positivity were identified at a magnification of 20X, and the number of positively stained cells was counted on representative areas of the section, in a minimum of 100 cells per field. For the assessment of the expression of CD133 and CD44 the extent of positive tumour cells was visually estimated under microscope. The IHC immunoreactivity score, is a semi quantitative based method based on the proportion of positive cells with membranous staining and the intensity of staining of particular cell: Immunoreactivity score

= proportion positive score x intensity score. In this study we consider the CD44 and CD133 positivity of all patients who showed positivity above 20% (i;e 20% to 75% positivity).

Estimation of Inflammatory cytokine IL-6

Evaluation of inflammatory cytokine IL-6 was done according to a previously reported method [20] with minor modifications. Concentrations of serum inflammatory cytokine IL-6 was quantified using commercially available ELISA kit (IL-6 Elabscience Cat no. E-EL-H0102). The assay for IL-6 was carried out according to the given protocol. The kit was based on sandwich ELISA method. The absorbance was taken using ELISA reader (BIORAD ELISA plate reader at 450 nm and within 30 minutes of stopping reaction. The results of serum IL were expressed as pg/ml.

Statistical analysis

Data were evaluated by One-Way analysis of variance (ANOVA) with post-hoc test Newman-Keuls Multiple Comparison Test by GraphPad Prism (version 5.01) and Microsoft excel (2016). The multivariate analysis was performed through the method of multiple regression using the software SPSS (Version 23). The coding for CSC markers were recorded as 1 for CD44 or CD133 positive and 0 for CD44 or CD133 negative. Similarly for presence and absence of habits 1 and 0 were used respectively. Separate regression models were created for examining the IL-6 changes each for CSC marker (CD44 and CD133) and habits in the OSMF and OSCC groups respectively. Significance was set at $p<0.05$. All the data are presented as means ± standard error of the means.

Results

Expression of CSC markers (CD133 and CD44) in the OSMF and OSCC groups

Our IHC analysis showed fifty-one (85%) and forty-nine (82%) subjects were positive for CD44 and CD133 respectively in the OSCC cases, while forty-three subjects (72%) were positive for CD44 and forty-six participants (76%) for CD133 in the OSMF group (figure 1).

Figure 1: Expression of CSC markers in the OSMF and OSCC groups

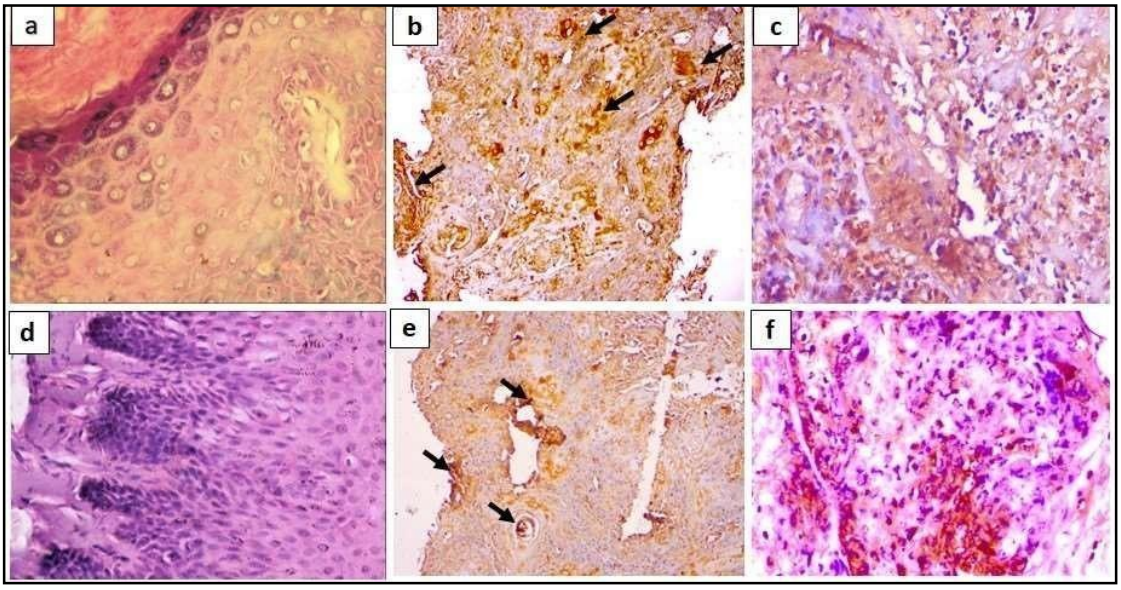

Figure 1: Expression of the CSC markers CD133 and CD44 was evaluated by immunohistochemistry in a series of OSCC and OSMF. Microscopic presentation of immuno- histochemical staining of normal oral epithelium tissue expressed Strong immunostaining of inflammatory areas with CSC markers in oral epithelial cells with magnification 200x (a). staining pattern of tumour tissue of oral epithelium for CD133 showing Moderate cytoplasmic staining in immunoreactivity in (OSCC) with original magnification 200x (b). Immunostaining confirms that inflammatory cells are distributed among various layers of oral epithelium showing advanced stage of OSMF (c). Normal buccal mucosa under expression of CD44 immunohistochemical staining expressed in most of cells of epithelial origin, including fibroblasts (d). Membranous immunohistochemical sections, the positive expression of CD44 is detected of well-differentiated OSCC with magnification 200x (e). Advanced OSMF indicates presence of active cells seen in juxta-epithelial connective tissue of oral submucosa with original magnification 200x (f). Arrows indicates inner tumour area and the invading tumour edges in the OSCC group

IL-6 level in the OSMF and OSCC groups

Initially, we studied the serum level of IL-6 in both the OSMF and OSCC groups which includes all patients (without considering CSC marker expression) in comparison to healthy control subjects (Figure 2). It was observed that the expression level of IL-6 in the OSCC group showed maximum expression (198.2±8.9 pg/ml), while OSMF showed lower (150.4±7.9 pg/ml) expression. One way ANOVA and Newman-Keuls Multiple Comparison test exhibited a significant increase ($P<0.001$) in the levels of IL-6 both in the OSMF and OSCC individuals as compared to control group. Interestingly, we further observed significantly ($P<0.001$) higher levels of IL-6 in the OSCC group as compared to the values in the OSMF group (Figure 2).

Figure 2: IL-6 level in the OSMF and OSCC groups

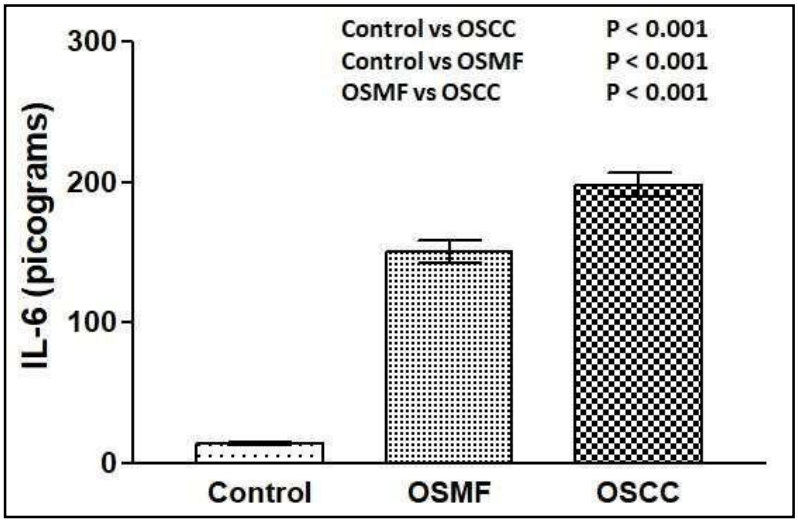

Figure 2: Histogram representing the levels of IL-6 in the control, OSMF and OSCC groups. Results are represented as mean ± S.E.M. One way ANOVA and Newman-Keuls Multiple Comparison Test is used for statistical analysis and comparison.

IL-6 level in CD44 positive vs negative individuals

When comparison was made between CD44 positive and negative individuals in the OSMF group in terms of the expression level of IL-6, the level of IL-6 in the CD44 positive and negative individuals was found to be 146.0± 8.7pg/ml and 41.94±5.8pg/ml respectively. One way ANOVA and Newman-Keuls Multiple Comparison Test showed a significant enhancement in the levels of IL-6 in the CD44 positive ($P<0.001$) individuals, while insignificant increase in CD44 negative individuals as compared to the control group. Also, the IL-6 levels are significantly ($P<0.001$) higher in CD44 positive individuals when compared with CD44 negative in the OSMF group (Figure 3).

When expression level of IL-6 was compared between CD44 positive and negative individuals in the OSCC group, the level of IL-6 in CD44 positive and negative individuals was found to be 198.9± 7.5 pg/ml and 71.47±7.5 pg/ml respectively. One way ANOVA showed a significant increase in the levels of IL-6 both in the CD44 positive ($P<0.001$) and negative ($P<0.05$) individuals of the OSCC group when compared with control group. However, the extent of significance was more in the CD44 positive individuals. The IL-6 levels are significantly ($P<0.001$) higher in CD44 positive individuals when compared with CD44 negative in the OSCC group (Figure 3).

Another important result observed when the CD44 positive individuals of OSCC showed significantly (P<0.001) higher levels of IL-6 as compared to CD44 positive individuals of OSMF (Figure 3).

Figure 3: IL-6 level in CD44 positive vs negative individuals

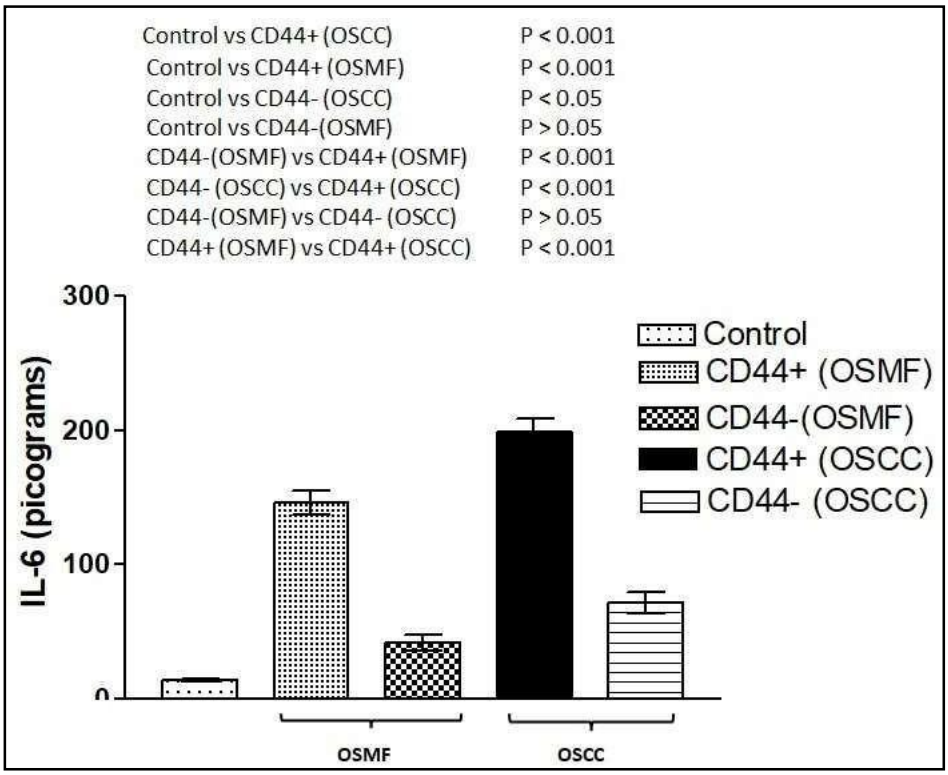

Figure 3: Histogram representing the levels of IL-6 in the control, OSMF (CD44+ & CD44-) and OSCC (CD44+ & CD44-) groups. One way ANOVA and Newman-Keuls Multiple Comparison Test is used for statistical analysis and comparison

IL-6 changes in relation to CD44 expression and habits in the OSMF and OSCC groups

Multiple regression analysis evaluated the effect of habits (smoking, tobacco chewing pan masala), CSC marker (CD44) expression on IL-6 levels in the OSMF and OSCC groups.

The multiple regression analysis (Table 1) showed that in the OSMF group when the individuals crossing the threshold of being CD44 positive then the increment in the IL-6 value is 66.73. This increment is significantly contributing to IL-6 enhancement. On the other hand, the effect of habits (tobacco chewing, smoking and pan masala) are not significantly contributing to the IL-6 value. The model is significant (P<0.002) in terms of predicting the IL-6 changes in the OSMF group.

The multiple regression analysis (Table 1) showed that in the cancer group (OSCC) when the individuals crossing the threshold of being CD44 positive then the increment in the IL-6 value is 140.62, indicating that this increment is significantly contributing to IL-6 enhancement. On the other hand, the effect of habits (tobacco chewing, smoking and pan masala) are not significantly contributing to the IL-6 value. The model is significant ($P<0.001$) in terms of predicting the IL-6 changes in the OSCC group.

Table 1: Regression equations of IL-6 with CSC markers and habits

Variables	Groups	Regression equations	F value (4,55)	R^2	P Value
CD44 Habits	OSMF	IL-6= 39.12 (29.53) + 66.73**(16.67) CD44 + 17.61 (14.58) Tobacco chewing + 7.24 (16.12) Smoking + 32.917 (32.92) Pan masala	4.71	0.25	0.002
	OSCC	IL-6= 36.38 (32.33) + 140.62**(24.76) CD44 + 11.26 (20.85) Tobacco chewing + 16.68 (17.11) Smoking + 13.15 (17.93) Pan masala	9.69	0.41	0.00001
CD133 Habits	OSMF	IL-6= 33.37 (27.65) + 83.72** (16.72) CD133 + 12.81 (17.29) Tobacco chewing - 0.73 (16.12) Smoking + 29.11 (16.23) Pan masala	7.06	0.34	0.0001
	OSCC	IL-6= 39.98 (28.78) + 148.71** (22.31) CD133 + 11.23 (19.73) Tobacco chewing + 10.39 (16.15) Smoking + 25.11 (17.31) Pan masala	12.96	0.48	0.00001

Table 1: Regression equations showing relationship of IL-6 with CSC markers and habits (tobacco chewing, smoking and pan masala). Values in the parenthesis are standard error of the coefficients and ** means $P<0.001$.

OSMF vs OSCC

It can be observed from table 1 that the increment of IL-6 due to CD44 is around two times in OSCC than OSMF. It appears that there is more pronounced effect of CD44 expression on IL-6 enhancement in the OSCC group than OSMF.

IL-6 level in CD133 positive vs negative individuals

When comparison was made between CD133 positive and negative individuals in the OSMF group in terms of the expression level of IL-6, the level of IL-6 in the CD133 positive and negative individuals was found to be 144.7± 8.4 pg/ml and 68.42±4.1 pg/ml respectively shown in figure 4. One way ANOVA showed a significant increase in the levels of IL-6 both in the CD133 positive ($P<0.001$) and negative ($P<0.05$) individuals of the OSCC group. However, the extent of significance was more in the CD133 positive individuals. The IL-6

levels are significantly (P<0.001) higher in CD133 positive individuals as compared to CD133 negative (P<0.05) in the OSMF group (Figure 4).

When expression level of IL-6 was compared between CD133 positive and negative individuals in the OSCC group, the level of IL-6 in CD133 positive and negative individuals was found to be 201.6± 9.5 pg/ml and 67.65±7.4 pg/ml respectively shown in figure 4. One way ANOVA showed a significant increase in the levels of IL-6 in the CD133 positive (P<0.001) individuals, while insignificant increase in CD133 negative individuals as compared to the control group. Also, the IL-6 levels are significantly (P<0.001) higher in CD133 positive individuals when compared with CD133 negative in the OSCC group (Figure 2c). When the CD133 positive individuals of OSCC compared to CD133 positive individuals of OSMF a significantly (P<0.001) higher level of IL-6 was observed (Figure 4).

Figure 4: IL-6 level in CD133 positive vs negative individuals

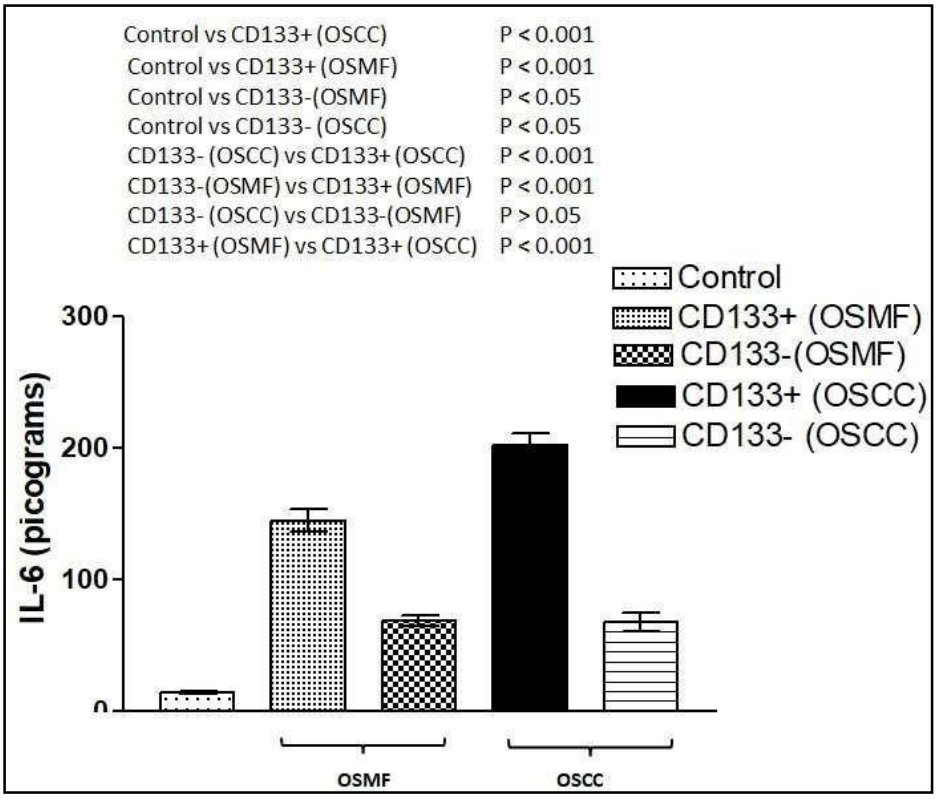

Figure 4: Histogram representing the levels of IL-6 in the control, OSMF (CD133+ & CD133-) and OSCC (CD133+ & CD133-) groups. One way ANOVA and Newman-Keuls Multiple Comparison Test is used for statistical analysis and comparison

IL-6 changes in relation to CD133 expression and habits in the OSMF and OSCC groups

Multiple regression analysis evaluated the effect of habits (smoking, tobacco chewing pan masala), CSC marker (CD133) expression on IL-6 levels in the OSMF and OSCC groups.

The multiple regression analysis (Table 1) showed that in the OSMF group when the individuals crossing the threshold of being CD133 positive then the increment in the IL-6 value is 83.72. This increment is significantly contributing to IL-6 enhancement. On the other hand, the effect of habits (tobacco chewing, smoking and pan masala) are not significantly contributing to the IL-6 value. The model is significant ($P<0.0031$) in terms of predicting the IL-6 changes in the OSMF group.

The multiple regression analysis (Table 1) showed that in the cancer group (OSCC) when the individuals crossing the threshold of being CD133 positive then the increment in the IL-6 value is 148.71, indicating that this increment is significantly contributing to IL-6 enhancement. On the other hand, the effect of habits (tobacco chewing, smoking and pan masala) are not significantly contributing to the IL-6 value. The model is significant ($P<0.001$) in terms of predicting the IL-6 changes in the OSCC group.

OSMF vs OSCC

It can be observed from table 1 that the increment of IL-6 due to CD133 is more than 1.7 times in OSCC than OSMF. It appears that there is more pronounced effect of CD133 expression on IL-6 enhancement in the OSCC group than OSMF.

IL-6 level in CD44 positive vs CD133 positive in the OSMF and OSCC groups

In order to examine the effect of response of CSC marker (CD44 and CD133) expression on the levels of IL-6 in both the OSMF and OSCC groups, we further compared the IL-6 levels of CD44 and CD133 positive individuals in the OSMF group and interestingly no significant ($P>0.05$) variation was observed between the groups (Figure 5). Similar observations were identified when the IL-6 levels of CD44 and CD133 positive individuals were compared ($P>0.05$) in the OSCC group. These results clearly indicated that irrespective of the type of CSC marker (CD44 or CD133) expression, the levels of IL-6 are increased in both the cases.

Figure 5: IL-6 level in CD44 positive vs CD133 positive in the OSMF and OSCC groups

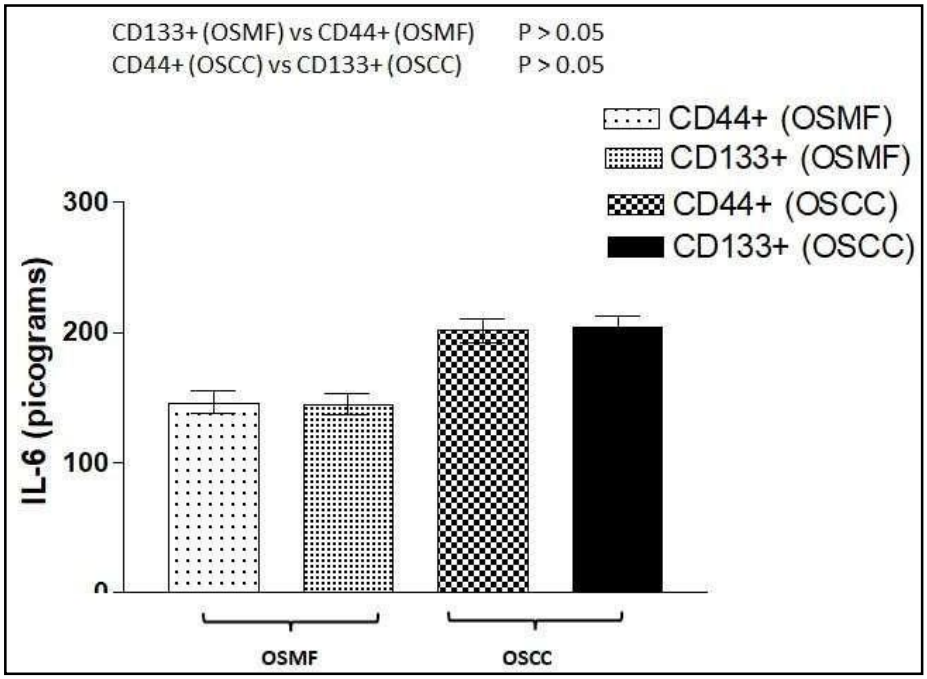

Figure 5: Histogram representing the levels of IL-6 in the OSMF (CD44+ & CD133+) and OSCC (CD44+ & CD133+) groups. One way ANOVA and Newman-Keuls Multiple Comparison Test is used for statistical analysis and comparison.

Effect on IL-6 Levels considering the expression of both CD44 and CD133 in the OSMF and OSCC individuals

While analysing the combined effect of both CD44 and CD133 positivity on IL-6 levels, we observed multicollinearity effect between CD44 and CD133 as the correlation between the two CSC markers is 0.88 in the OSMF group and 0.94 in the OSCC group. There was a multicollinear effect in regressing with both CSC markers therefore, individual effect of the markers was studied. Both markers have significant relationship with IL-6. Also, when Both CD44+ and CD133+ were expressed, no combined effect was observed in terms of enhancing the IL-6 levels by regression analysis.

Discussion

Exploring novel biomarkers that could provide information related to early tumorigenic changes is the aim of researchers these days. In this study we have showed IL-6 changes in the precancer (OSMF) and cancerous (OSCC) conditions and simultaneously examined its

correlation with CSC marker (CD133 and CD44) expression in these individuals. This study is unique and novel in a sense that we have evaluated the biomarker ability of IL-6 in these precancerous and cancerous individuals and explore association with CSC alterations to clear out the picture of early diagnosis in oral cancer. When the biomarkers of different origins (cytokine and CSCs) are concurrently examined the changes provides us a more specific diagnostics inputs as compared to evaluation of only one biomarker at a time.

The present study clearly showed that there is an increase levels of IL-6 in both the OSMF (precancerous) and OSCC (cancerous) individuals. Although the extent of increase was higher in OSCC condition. This is in agreement with previous studies where IL-6 is observed as an important biomarker of cancer-related pathologies [9,11]. Amongst the cytokines: IL-6, IL-8, and TNF-α have been investigated in various conditions of oral lesions [24]. The probable mechanisms suggested are related with increase inflammation and immunoregulatory responses [11]. Generally, the cytokines modulate tissue repair and growth-induced healing. Whereas, cytokines may also induce DNA damage and inhibition in the cancerous cells. Cytokines also modulate DNA repair and functioning of tumour suppressor genes, vascular permeability, tumour cell migration, extravasation of fibrin, leukocyte infiltration, tissue remodelling, alteration of cell-cell adhesion molecules, decreasing of immune response, and angiogenesis [9,11,24,26]. The synthesized cytokines from the tumour may possibly modulate cascade of reaction that could sooner or later acts for the advancement of the carcinogenesis progression [26]. The formation of IL-6 in elevated magnitudes is recognized to be an existing in pathological procedures, and is established as a significant diagnostic biological marker [26].

We have also showed an enhanced expression of CSC markers: CD133 and CD44 in both the OSMF (precancerous) and OSCC (cancerous) conditions. These observations are in backing with few previous reports where expression profile of similar CSC markers was differentially altered in diverse cases of oral cancer [7,13,16]. Recently, CD44 and CD133 have been studied as surface markers of CSCs [13]. CD44 is a hyaluronic acid receptor. It stimulates numerous receptor tyrosine kinases in different type of cancers. The probable biological basis is enhanced tumour cell proliferation and survival rates via activating the MAPK and PI3K/AKT pathways [1,16]. Various studies detected CD133 in normal as well as cancer stem cells in the OSCC and animal models of oral cancer [19].

The most interesting and unique results we reported are the alteration of IL-6 levels in the precancer (OSMF) and cancerous (OSCC) conditions of CD133 and CD44 positive and

negative individuals. Our study presented a general trend that CD133 and CD44 positive individuals both in the precancer (OSMF) and cancerous (OSCC) conditions showed enhanced levels of IL-6 as compared to CD133 and CD44 negative individuals. Although the level of IL-6 also increased in these individuals but clearly the extent is lower. Also, the enhancement of IL-6 levels in OSCC cases irrespective of the type of CSC markers was more pronounced as compared to OSMF IL-6 levels. There was a multicollinear effect in regressing with both CSC markers therefore, individual effect of the markers was studied. Both markers have significant relationship with IL-6. Also, when Both CD44+ and CD133+ were expressed, no combined effect was observed in terms of enhancing the IL-6 levels by regression analysis.

The multiple regression analysis aimed to evaluate IL-6 perturbations and possible contribution of CSC expression and/or habits. We reported that in the OSMF and OSCC group when the individuals crossing the threshold of being CD44 and CD133 positive then the increment in the IL-6 value was significantly enhanced. On the other hand, the effect of habits (tobacco chewing, smoking and pan masala) were not significantly contributing to the IL-6 value. These differential effects of habit might be an indication of smaller data and/or overlapping of habits amongst the individuals in the groups. In order to exactly understand the effect of each habit on IL-6 changes larger data set of each individual habit may be required which can be studied in future. The habits might play role in the development of oral lesions as reported by various studies [12], however their direct correlation with IL-6 has not been established in the present study. Nevertheless, we are not ruling out the possibility that these habits may not alter cytokine levels, we are just saying that in comparison to CSCs expression the contribution of habits seems lesser influential in the present data size.

It can be observed from separate regression models that the increment of IL-6 due to CD44 and CD133 is 2 and 1.5 times more in OSCC than OSMF respectively. This indicates that there is more pronounced effect of CSCs expression on IL-6 enhancement in the OSCC group than OSMF.

IL-6 is a vital chronic inflammatory mediator, enhanced levels of which have been detected in numerous types of cancers [30]. Various major pro-tumour events, including progress, invasion, and angiogenesis are linked with IL-6 perturbations [27]. Additionally, the blockade of type 1 immune response, elevation of Treg cells, and activation of stromal fibroblasts could also be influenced by IL-6, thus contributory to tumour development [27]. IL-6 is also

linked with aging, vascular hypertrophy and fibrosis [28]. The proposed mechanism is associated with reductions in NO bioavailability. This reduction can be related to decrease in eNOS expression and activity followed by an enhancement in NADPH oxidase-derived superoxide. Together this alleviates oxidative stress, endothelial dysfunction in addition to vascular hypertrophy and fibrosis [28].

The simultaneous increase in the expression of CSC markers (CD44 and CD133) with IL-6 can be related to fibroblast activation. CSCs are known to alter tumour progression and chemoresistance [2]. Fibroblasts surrounding a tumour also help in the progression and fibroblast "activation" as an autonomous prognostic marker in oral cancer [2]. CSCs may therefore promote tumorigenesis through communication with stromal fibroblasts via IL-6 perturbations as observed in our study.

IL-6 along with other proinflammatory cytokines such IL-1β facilitates cancer-associated fibroblasts and promotes ECM remodelling possibly through NF-κ B and JAK-ROCK-STAT3 signalling pathways [18]. Recently few studies indicated that certain therapeutic agents modulating Cancer stemness properties are associated with IL-6/Stat3 signalling [21,29]. Thus, indicating the correlation of IL-6 enhancement in CSC positive individuals of OSMF and OSCC conditions.

Conclusion

It was concluded that the CSC biomarkers and pro-inflammatory cytokine IL-6 can be simultaneously used as a potential biomarker for OSCC and OSMF conditions. Multivariate analysis also indicates that there is more pronounced effect of CSCs expression on IL-6 enhancement than habits in oral lesions.

The biomarkers of different origins (cytokine and CSCs) are concurrently examined the changes and hence provide us a more specific diagnostics inputs as compared to evaluation of only one biomarker at a time. These results provide insights related to the carcinogenesis progression intensification. Simultaneous perturbations in the expression of CSC markers and IL-6 could be related to the adverse prognosis of OSCC in the patients. A study of these cytokines in correlation with CSC-induced tumorigenesis will heave further light on its practicality in early detection of oral cancer with pre-cancerous conditions and its potential as a therapeutic target in cancer therapy. The study may deliver better instinct to develop improved methodologies for early identification as well as better prediction of the

aggressiveness of cancers. Present work highlighted the importance of examining inflammatory cytokine perturbations in relation to the CSCs expression in oral lesions.

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Competing interest

The authors declare no conflict of interest, financial or otherwise.

Ethics Approval

All procedures performed in studies involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Research Ethics Committee of Eras Lucknow Medical University Ref no ELMC/R Cell/2018/EC/2034 (Era's Lucknow Medical College /Research Cell/2018/ Ethics Committee/2034). A proper written informed consent was obtained from all related subjects as per ethical guidelines.

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