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ORIGINAL ARTICLE

Quantitative Evaluation of Tumour - Associated Tissue Eosinophilia and Cyclo-oxegenase-2 Gene in Oral Cancer Patients with Assessment of Long Term Outcomes

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Abstract Various histopathological parameters have been extensively studied for prognostication of oral cancer but the focus is now getting diverted towards the role of inflammatory mediators in cancer progression. The present study was undertaken to evaluate two such components of the inflammatory milieu, tumor-associated tissue eosinophilia (TATE) as well as Cyclo-oxygenase-2 (COX-2) gene expression, quantitatively in oral squamous cell carcinoma (OSCC) patients in relation to treatment outcomes and patterns of recurrence. A total of forty five patients with primary OSCC matching our inclusion criteria were selected for the study and followed up over a five year period. TATE was evaluated from the invasive front of the tumor using Haematoxylin and eosin (H & E) stained sections of histopathological specimens and graded as mild, moderate or intense. COX-2 gene expression was obtained from specimens using the reverse transcriptase - polymerase chain reaction (RT-PCR) method. A statistically significant association was observed between degree of TATE and locoregional recurrence ($P < 0.001$). The expression of COX-2 gene ranged from 0.4326 to 0.9998 and a higher mean COX-2 score was recorded in samples with intense degree of TATE followed by moderate and mild TATE. ($P < 0.001$). Using the t-test, the difference in mean COX-2 was found to be statistically significant ($P < 0.001$) between patients who developed locoregional recurrence and those who did not. The analysis of TATE may provide an indication of future recurrence at the time of diagnosis of OSCC. Also, the increased expression of COX-2 gene in OSCC strongly suggests its possible use as a chemopreventive/chemotherapeutic target.

Keywords Oral squamous cell carcinoma · Cyclooxygenase-2 · COX-2 · Tumour associated tissue eosinophilia · TATE · Locoregional recurrence

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Introduction

Oral cancer has an estimated incidence of around 2,75,000 new cases per year and is recognized worldwide as a serious health concern [1]. The easy accessibility of the oral cavity and the fact that oral cancer is usually preceded by visible 'suspicious' mucosal changes, would ideally confer it a theoretical advantage in terms of early diagnosis and a subsequent reduction in patient morbidity [2, 3]. Ironically, however, upto 50 % of oral cancer patients are not diagnosed till they reach an advanced stage of the disease. The estimated five-year survival rate is below 50 % for patients with late stage oral squamous cell carcinoma (OSCC) [3]. Apart from late stage presentation, high recurrence rates and poor quality of life of patients post-treatment play key roles in worsening the survival rates and prognosis of this disease [4–6]. A recent systematic review highlighted the importance of early detection and screening programmes in developing countries as the detection of oral potentially malignant disorders in such countries was often delayed due to various socio-economic factors [7].

Despite the progress made in early detection and therapy, early predictors of cancer recurrence and disease prognosis at the time of diagnosis are still missing for oral cancer. Therefore,

there is a tendency to generalize treatment protocols for patients grouped under similar stages of the disease that may result in under or over-treatment with debilitating consequences [8]. To overcome these difficulties, a vast body of research is being conducted, targeted at elucidating methods of early detection, accurate staging and prognostication with a common aim to improve the quality of life of oral cancer patients.

Histopathology has always been considered the gold standard for diagnosis of oral cancer and as an aid to the assessment of tumor biology and behaviour. Some of the histopathological features of OSCC that are established criteria for predicting prognosis include, the degree of differentiation, lymphovascular/perineural or bone invasion as well as identification of histological subtypes [9]. However, a lesser explored aspect of the tumor population in this regard is its inflammatory component, also a prominent feature in oral potentially malignant disorders (OPMDs). The presence, number and nature of the inflammatory infiltrate may reflect the biological behaviour of the lesion and its response to treatment [10, 11] There is enough evidence now to suggest that the inflammatory cells and cytokines found in peri-tumoral stroma are more likely to contribute to tumor development and progression than to mount an effective host anti-tumor response [10, 11]. One such entity is the *tumor associated tissue eosinophilia* (TATE) which has been reported in diverse sites [12–19] including the head and neck region [20–32]. While its presence has been associated with a good prognosis in some studies [20, 28, 30], others claim it is an indicator of poor prognosis in head and neck cancer [21, 25, 26]. Recently there is increasing evidence for association of TATE with locoregional recurrence, metastasis and a poor prognosis in oral cancer patients [29, 32].

Another pro-inflammatory molecule, *cyclooxygenase-2* (COX-2) was found to be over-expressed in lung, oesophageal and breast carcinomas and some studies of head and neck cancers. It is associated with increased prostaglandin E_2 (PGE_2) production, known to be one among the factors responsible for the invasive nature of OSCC. Thus, infiltration of inflammatory cells and mediators around the tumor, is a significant event, and may play a role in perpetuating tumor progression [33–35]. COX-2 gene expression seems to have sparked much interest regarding its potential use as a chemopreventive target in OSCC.

Therefore our aim was to evaluate TATE as well as COX-2 gene expression quantitatively along the invasive front of the tumor in histopathological specimens of OSCC patients in a long term follow up study in relation to treatment outcomes and patterns of recurrence.

Materials and methods

Patients

A total number of one hundred and fifty two patients undergoing surgical resection for primary OSCC between January 2009 and July 2010 were selected for the study. A detailed case history was recorded, clinical examination and necessary investigations were performed and clinical TNM staging for the tumors was documented.

Patients with primary OSCC confirmed by biopsy who had not undergone radiotherapy, chemotherapy, or any other treatment prior to surgery were included in the study. Meanwhile patients with other primary tumors and unresectable tumors were excluded from the study. Tumors with an expansive growth pattern were also excluded from this study. A major requirement was the availability of tumor tissue for microscopic analysis obtained from the invasive tumor front (ITF). Thus, incisional biopsies were excluded from the study. Hundred and seven of the one hundred and fifty two patients studied were lost to follow up over the years and only the remaining forty five patients with complete five year follow up data to the present day were included in the study.

TATE analysis [32]

Paraffin embedded blocks of the tumor tissue were obtained and the block representing the invasive front of the tumor was first identified for each patient by routine histopathology using Haematoxylin and Eosin (H & E) stain. 3 μ sections were obtained and stained routinely with H & E stain for TATE analysis. Eosinophils at the invasive front were counted under a high power objective (× 400) for 10 continuous high power fields (HPF) using a light microscope (OLYMPUS C-20 i). The mean values obtained per HPF were then graded according to the criteria given by Goldsmith et al. [25]. In this criteria, the score '0' was given for no eosinophils, '1+' was given for presence of 5 to 10 eosinophils/HPF, '2+' for 10 to 20 eosinophils/HPF, '3+' for 20 to 30 eosinophils/HPF and '4+' for more than 30 eosinophils/HPF. We considered scores '0' and '1+' as absent/mild eosinophilia (Fig. 1), scores '2+' and '3+' as moderate eosinophilia (Fig. 2) and '4+' as intense eosinophilia (Fig. 3).

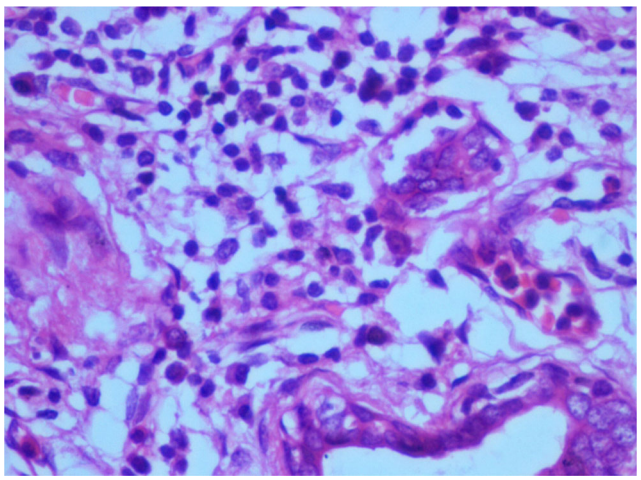

Fig. 1 Mild eosinophilia (under 400× magnification)

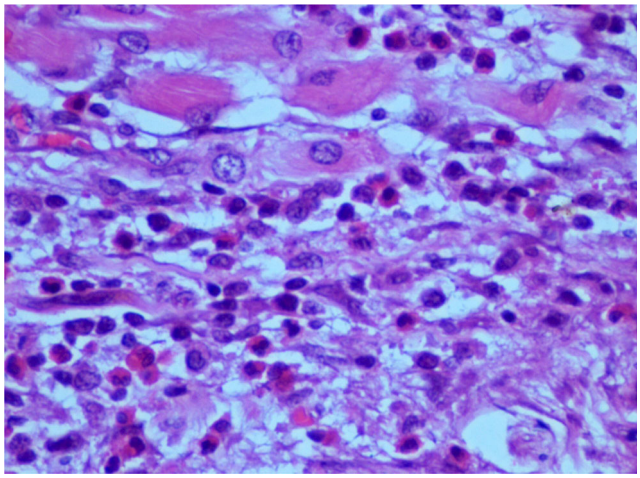

Fig. 2 Moderate eosinophilia (under 400× magnification)

Expression of COX-2 gene [36]

Tumor tissue that was collected from the resected specimens during curative surgery in RNAse-free plastic vials was transported to the laboratory in ice where RNA was extracted immediately. Tissue identified from the margins of the resected tumor specimen was used to carry out the following procedure.

RNA extraction

RNA was extracted using TRIzol reagent (Life Technologies Inc.) from the given sample as per the manufacturer's protocol. All plastic and glassware was made RNAse free by treatment with diethyl pyrocarbonate (DEPC). After adding DEPC, all glassware was incubated overnight at 120 °C for 1 h after which it was baked at 200 °C for 4 h. All reagents were prepared in DEPC-treated water. The tissue was homogenised in 1 ml TRIzol reagent and mixed well. RNA was extracted with 0.2 ml of chloroform. The RNA present in the aqueous layer was precipitated with 0.5 ml of isopropanol

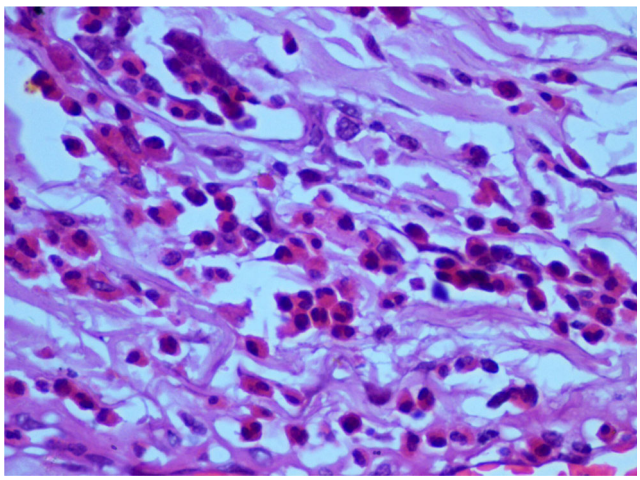

Fig. 3 Intense eosinophilia (under 400× magnification)

washed with 75 % ethanol and was dried and dissolved in DEPC-treated water.

Reverse transcriptase polymerase chain reaction

10 μg of the RNA thus isolated was reverse transcribed to cDNA in a 25 μl reaction mix containing 200 U of Moloney murine leukaemia virus reverse transcriptase (Life technologies Inc.) in IX reaction buffer with 2 μg of random hexamer (New England Bio Labs), 6 U of RNA guard (Amersham) and 100 μM of dNTP mix. The reaction mix without the enzyme was kept at 60 °C for 10 min for removal of secondary structures in mRNA. RT was performed at 37 °C for 1 h. The enzyme was inactivated at 90 °C for 4 min and quick chilled, and 3 μl of the cDNA mix was used for PCR amplification.

PCR

All the PCR amplifications were done in a 50 μl reaction mix containing 2.5 U Taq DNA polymerase (Promega) in 1 X reaction buffer, 1.5 mM $MgCl_2$, 150 μM of dNTP mix, 5–25 pmol of sense and antisense primers ((Life technologies Inc., Genosys, Sigma), and 100 ng of template DNA. The PCR conditions included initial denaturation at 94 °C for 3 min, and 30 cycles of denaturation for 30 s and annealing at 55 °C for 1 min followed by extension at 72 °C for 1.5 min. Final extension of PCR products was carried out at 72 °C for 7 min. The annealing temperature of C-amplification was 50 °C and that of G-amplification was 60 °C. The products were resolved by electrophoresis using a 1.2 % agarose gel buffered with 0.5 x TBE. Bands were visualised by ethidium bromide (0.5 μg/ml) and products of expected size were confirmed with a 100-bp ladder (Sigma).

Statistical analysis

Data was transferred to an excel sheet followed by statistical analysis using chi square test, student's t test and analysis of variance (ANOVA) using SPSS software. In order to find out among which pair of groups there existed a significant difference, multiple comparisons were carried out using Bonferroni method. A value of $P < 0.05$ was considered statistically significant.

Results

A summary of the results is given in Table 1. Out of the 45 patients studied, 25 (56 %) were males and 20 (44 %) females with an age range of 45–76 years. 11 patients belonged to TNM stage I, 10 patients belonged to TNM stage II and 12 patients each belonged to TNM stage III and IV respectively. 23 (51 %) patients developed locoregional recurrence while the remaining 22 (49 %) patients did not. No significant association was observed between gender and locoregional

Table 1 Summary of clinical data and study parameters

Sl. no.	Age in years	Sex	TNM stage	Eosinophils/HPF(mean)	Degree of TATE	COX-2 levels (RT-PCR)	Locoregional recurrence
1.	72	M	II	7	MILD	0.7546	NO
2.	60	F	II	1	MILD	0.7654	NO
3.	55	F	I	19	MODERATE	0.5668	NO
4.	62	F	III	45	INTENSE	0.8918	YES
5.	55	F	I	5	MILD	0.4554	NO
6.	58	F	II	2	MILD	0.8657	NO
7.	55	F	IV	6	MILD	0.9878	YES
8.	65	M	I	4	MILD	0.5643	NO
9.	50	F	III	18	MODERATE	0.8796	YES
10.	67	M	IV	56	INTENSE	0.9874	YES
11.	49	M	II	21	MODERATE	0.7768	NO
12.	76	F	IV	25	MODERATE	0.9987	YES
13.	55	F	IV	20	MODERATE	0.9998	YES
14.	50	M	III	3	MILD	0.7865	YES
15.	66	M	IV	38	INTENSE	0.8862	YES
16.	62	F	IV	52	INTENSE	0.9876	YES
17.	63	M	II	19	MODERATE	0.4968	NO
18.	57	M	I	5	MILD	0.5459	NO
19.	60	M	II	12	MODERATE	0.7224	NO
20.	72	M	III	31	INTENSE	0.8586	YES
21.	66	F	III	17	MODERATE	0.6654	NO
22.	51	M	I	3	MILD	0.4566	NO
23.	58	F	II	7	MILD	0.5117	NO
24.	58	F	III	46	INTENSE	0.9989	YES
25.	72	M	IV	66	INTENSE	0.9996	YES
26.	55	M	I	7	MILD	0.4454	NO
27.	60	M	II	11	MODERATE	0.5216	NO
28.	59	F	IV	49	INTENSE	0.9888	YES
29.	70	M	II	8	MILD	0.6228	NO
30.	53	F	I	3	MILD	0.4326	NO
31.	59	M	III	31	INTENSE	0.8472	YES
32.	45	M	IV	44	INTENSE	0.9898	YES
33.	56	M	IV	58	INTENSE	0.9662	YES
34.	68	F	III	33	INTENSE	0.8722	YES
35.	50	M	I	2	MILD	0.4546	NO
36.	51	F	I	4	MILD	0.4641	NO
37.	62	M	III	55	INTENSE	0.9894	YES
38.	70	M	IV	41	INTENSE	0.9363	YES
39.	58	M	III	33	INTENSE	0.8528	YES
40.	62	F	III	40	INTENSE	0.929	YES
41.	62	M	III	91	INTENSE	0.9786	YES
42.	60	M	I	6	MILD	0.4652	NO
43.	56	M	I	2	MILD	0.4544	NO
44.	61	F	IV	59	INTENSE	0.9988	YES
45.	58	F	II	15	MODERATE	0.6034	NO

Legend: Sr. No.: Serial number; Sex: M- Male, F- Female; TNM stage: TNM stage; HPF: High power field; TATE: Tumor associated tissue eosinophilia; COX-2: Cyclo-oxygenase 2; RT-PCR: Real time- Polymerase chain reaction

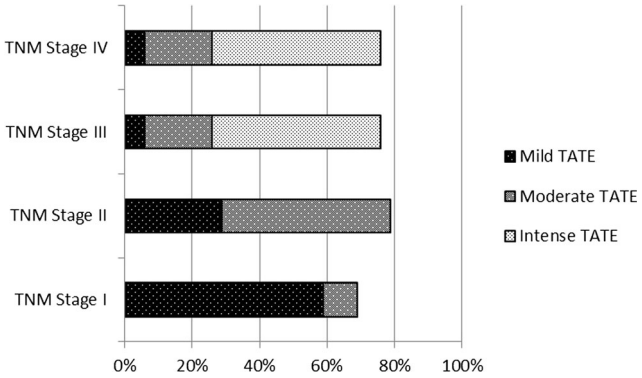

Fig. 4 Association between TNM Stage and Degree of TATE

recurrence ($P > 0.05$). A statistically significant association was observed between TNM Stage and degree of TATE ($P < 0.001$) using the χ^2 test. (Fig. 4) Therefore, more samples in TNM Stage III & IV showed intense degree of TATE and those in Stage I & II showed mild to moderate degrees of TATE. A statistically significant association was also observed between degree of TATE and locoregional recurrence ($P < 0.001$). Samples with intense degree of TATE eventually developed locoregional recurrence whereas very few samples with mild & moderate degree of TATE developed it. (Fig. 5).

The expression of COX-2 gene ranged from 0.4326 to 0.9998 in these patients and using the ANOVA test, comparison of COX-2 values among different degrees of TATE was performed as seen in Table 2. Higher mean COX-2 score was recorded in intense degree of TATE followed by moderate and mild TATE. The difference in mean COX-2 score between them was found to be statistically significant ($P < 0.001$). The difference in mean COX-2 score was statistically significant between mild and intense TATE ($P < 0.001$) as well as between moderate and intense TATE ($P < 0.01$).

Using the t-test, the difference in mean COX-2 was found to be statistically significant ($P < 0.001$) between patients who developed locoregional recurrence and those who did not. (Table 3) Samples with locoregional recurrence had a higher mean COX-2 value compared to those samples without locoregional recurrence. These patients were later re-evaluated for TATE after surgical removal of the recurrent lesion. All of the 23 specimens showed higher degrees of TATE than the corresponding primary OSCC specimens.

Discussion

The dynamics of the tumor-host microenvironment are highly complex. This bio-interface is known to be a hub for markedly increased inflammatory reaction, enhanced vasculature, proliferative fibroblastic activity and microinvasion among other events which help to understand the biologic nature and consequently the clinical behaviour of the tumor tissue [25]. More specifically, this interface is studied and known as the *invasive tumor front* (ITF) where such interactions are readily demonstrated. The ITF is also invaluable in that it is a zone which is usually less differentiated than the bulk of the tumor mass and contains the most aggressive cells of the tumor population [37]. Most of the chronic inflammatory response of the host to tumor progression is also concentrated in this zone. Thus, selection of the appropriate biopsy site is of prime importance for evaluation of TATE. Therefore, we ensured that the assessment of TATE was completed along the ITF for each sample. This was ensured by using the paraffin embedded block of the specimen housing the ITF, as confirmed by histopathology using H & E stain. COX-2 on the other hand is expressed more or less uniformly throughout intra and peri-tumoral tissue. However, COX-2

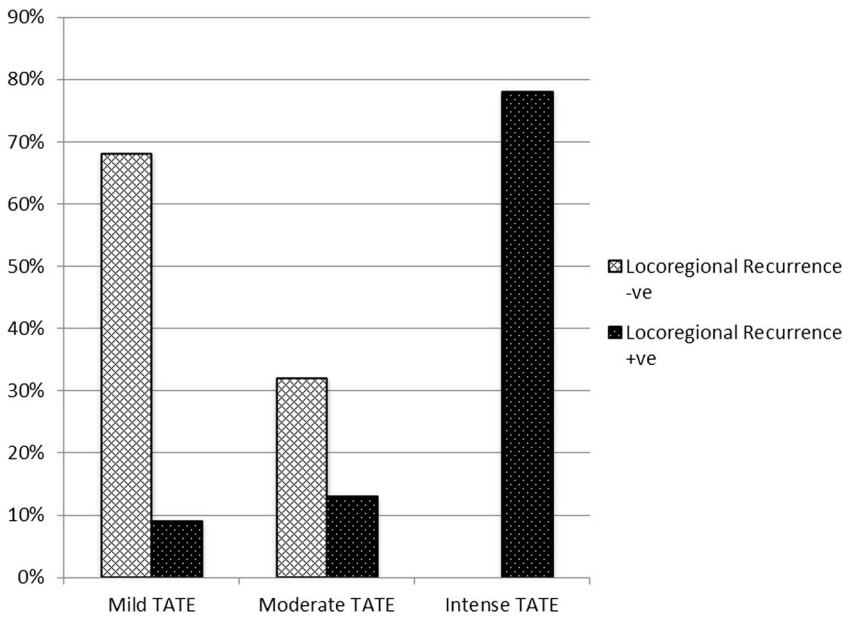

Fig. 5 Association between Degree of TATE and Locoregional Recurrence

Table 2 Comparison of COX-2 values among different Degree of TATE: (ANOVA followed by Bonferroni Multiple Comparison)

Degree of TATE	n	Mean COX-2 values	Std Dev.	SE of Mean	95 % CI for Mean		Min	Max
					Lower Bound	Upper Bound		
Mild	17	0.5902	0.1751	0.0425	0.5002	0.6802	0.4326	0.9878
Moderate	10	0.7231	0.1867	0.0590	0.5896	0.8567	0.4968	0.9998
Intense	18	0.9422	0.0580	0.0137	0.9133	0.9710	0.8472	0.9996

Legend: TATE: Tumor associated tissue eosinophilia; n = Sample size; COX-2: Cyclo-oxygenase 2; Std Dev.: Standard deviation; SE of mean: Standard error of mean; CI: Confidence interval; Min: Minimum; Max: Maximum

being an important mediator for angiogenesis is known to be upregulated at tumor margins where neovascularization is a prominent event. Since tumor tissue for COX-2 analysis was obtained immediately after curative surgery, tissue from the macroscopic margins of the resected tumor specimen was used in the analysis of COX-2 gene expression.

It must be noted here that tumors with an expansive growth pattern were excluded from this study as such a pattern is rarely associated with oral squamous cell carcinoma and their inclusion would have compromised standardization in the study.

Eosinophils are granulocytes which are bone marrow derivatives found transiently in the blood circulation as they move chemotactically towards inflammatory sites in tissue where they tend to reside. Eosinophil count is raised in parasitic infections and allergic diseases but are also play a role in tissue remodelling and modulation of the host immune response [31]. Their presence in intra and peri-tumoral sites has been established in many sites such as larynx, pharynx, oesophagus, skin, breast, lung, intestine, genitourinary tract and also the oral cavity by a number of studies [21]. Usually the inflammatory cell population comprises mainly mononuclear cells and rarely neutrophils, eosinophils when present tend to dominate in number over the other cell populations [31].

Many have attributed their tumor protective role to the secretion of certain cytotoxic mediators such as major basic protein, eosinophil cationic protein and eosinophil peroxidase and their role in facilitating increased permeability of tumor killing cytokines into tumor cells [29]. On the other hand, those who believe TATE to promote tumor growth have suggested the mechanism to be the active release of 92- kd gelatinase (of the matrix metalloproteinases family) which is involved in the breakdown of the basement membrane and the extracellular matrix [25]. A recent observation also states that the prostaglandin $PGE_{2,}$ which is expressed by OSCC and associated with its invasive nature, shares its precursor with another prostaglandin, PGD_2, a potent eosinophil chemotactic molecule [25, 38].

COX enzyme is present in three forms in the body, COX-1 which performs 'housekeeping' functions, COX-2 which is normally absent in cells, but is inducible in inflammatory reactions and COX-3, a novel COX-1 splice variant not functional in humans [39]. Upregulation of COX-2 increases prostaglandin synthesis (particularly PGE_2) with consequent enhancement in the proliferative activity of neoplastic cells, neoangiogenesis, increased invasiveness, greater metastatic potential and inhibition of immune surveillance and apoptosis. With increase in tumor size, an increase in expression of COX-2 gene has been observed [34].

Locoregional recurrence develops much earlier than metachronous disease and carries the worst prognosis. Patients who survive a first encounter with this disease have up to a 20 fold increased risk of developing a second cancer. In developing countries like India, OSCC is more prevalent among low socioeconomic status groups. Such patients are often lost to follow up and do not comply with lifestyle modifications. Thus, early identification of recurrence at the time of diagnosis will be useful in identifying patients at risk and employing aggressive treatment strategies and follow up policies for them.

Previous reports have found significant association between the presence of high degrees of TATE and stromal invasion in OSCC [25, 38]. An increased possibility of locoregional recurrence was also reported in such patients by Alrawi et al. [25]. A recent study has even established the reliability of TATE as a reliable predictor of occult lymph node metastasis in clinically N_0 OSCC patients [32]. On the

Table 3 Comparison of mean COX-2 values between the two groups (t-test)

Locoregional Recurrence	n	Mean COX- 2 values	Std Dev	SE of Mean	Mean Difference	t	P-Value
No	22	0.5733	0.1308	0.0279	−0.36637	−11.966	<0.001*
Yes	23	0.9396	0.0653	0.0136			

Legend: TATE: Tumor associated tissue eosinophilia; n = Sample size; COX-2: Cyclo-oxygenase 2; Std Dev.: Standard deviation; SE of mean: Standard error of mean; * denotes significant difference

contrary, Dorta et al. [21]. and Lorena at al [39]. did not find such an association.

In this study, we observed a significant increase in the intensity of TATE with increasing levels of COX-2 gene expression in OSCC patients. Incidentally most patients who demonstrated upregulation of COX-2 gene and moderate/intense TATE belonged to TNM stages III/IV while the ones with relatively lower values and mild TATE belonged to TNM stage I/II. These observations were in accordance with previous reports by Itoh et al. [35]. Shadab et al. and others [34].

In our study, most of the patients who demonstrated moderate to intense TATE and overexpression of COX-2 gene later developed locoregional recurrence. For the patients that did develop recurrence, the lesion was surgically excised and also analysed for TATE. Interestingly, all of these specimens showed a definite increase in the mean TATE/HPF than that observed in the corresponding primary OSCC tissues. Only one of the patients who demonstrated a relatively high COX-2 gene expression, has not yet developed recurrence but is currently on regular follow up. Thus both COX-2 expression and TATE were found to be significantly raised in later stages of OSCC (TNM stage III/IV) and in patients that developed locoregional recurrence.

One of the factors responsible for specific chemotaxis of eosinophils towards OSCC are believed to be the prostaglandins (PGs). PGD_2 in particular is a potent eosinophil chemotactic molecule and shares a common precursor with PGE_2, a PG commonly overexpressed in OSCC [25]. The generation of PGs is dependent on Cyclooxygenase-2 (COX-2), an inducible enzyme which is upregulated in various neoplasms such as colorectal, lung, breast, stomach, pancreas, urinary bladder and oesophagus and strongly associated with the invasive nature of cancer [34, 35]. Despite initial controversy, researchers more recently have been able to consistently demonstrate COX-2 overexpression in head and neck cancers as well.

Due to the ability of COX-2 inhibitors to effect the arrest of G0/G1 cell cycle and apoptosis, inhibit PGE_2 production, angiogenesis, vascular endothelial cell growth and cyclin-dependent kinase inhibitor p21 expression, their chemopreventive role in OSCC has often been advocated [36]. Tortora et al. suggested the use of COX-2 inhibitors in combination with epidermal growth factor receptor and tyrosine kinase inhibitors to improve their efficacy as chemopreventive agents [40]. COX-2 inhibitors have been suggested to improve response to radiation therapy as it enhances the radiosensitivity of tumor cells [34]. Kao et al. have described concurrent erlotinib, COX-2, and reirradiation as a well-tolerated and active regimen for patients with recurrent head and neck cancer [41]. Furthermore, recent studies have suggested fluorinated derivatives of COX-2 inhibitors such as Celecoxib as attractive targets for molecular imaging in cancer [42].

In conclusion, the inferences from our study are two-fold. Firstly, the analysis of TATE in OSCC specimens along the ITF provide a valuable indication of future recurrence at the time of diagnosis. This is a simple, cost-effective technique which can be performed routinely and can be used to formulate patient-specific treatment plans that yield greater survival rates and better prognosis. Secondly, there is an upregulation of COX-2 gene expression in patients who develop locoregional recurrence. Thus COX-2 gene may play an important role as a target for chemoprevention, particularly in cases identified to be at risk for recurrence.

All these measures will ultimately improve the quality of life of these patients. The findings of this study should encourage more studies evaluating the effect of topical COX-2 inhibitors in OPMDs as chemopreventors, over long term follow up periods, and the effect on the radiosensitivity of oral cancer cells on treatment with COX-2 inhibitors prior to irradiation. To our knowledge, this is the first study that evaluates both TATE and COX-2 simultaneously in OSCC specimens and attempts to explore their association.

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