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Significance of immunohistochemical overexpression of cyclooxygenase-2 in overall and disease-free survival of oral squamous cell carcinoma patients

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Abstract

Objective. In Pakistan, oral cancer ranks as the most common malignancy in males and the second most common malignancy in females. Cyclooxygenase-2 has been explored as an agent of carcinogenesis in oral and other neoplasms. This study aimed to observe the expression of cyclooxygenase-2 in oral squamous cell carcinoma, and to correlate the expression with patients' clinical features and overall and disease-free survival.

Methods. Immunohistochemistry for cyclooxygenase-2 was performed on a total of 100 oral squamous cell carcinoma formalin-fixed, paraffin-embedded blocks. Expression was correlated with patients' clinicopathological variables and overall and disease-free survival.

Results. Cyclooxygenase-2 was overexpressed in 55 per cent of oral squamous cell carcinoma patients. Overexpression was correlated with overall survival ($p = 0.013$) and disease-free survival ($p = 0.001$) on univariate analysis. However, on multivariate analysis, cyclooxygenase-2 was associated with only disease-free survival ($p = 0.044$) and not overall survival ($p = 0.208$).

Conclusion. Expression of cyclooxygenase-2 is associated with poorer overall survival and higher rates of recurrence in oral squamous cell carcinoma patients.

Introduction

Worldwide, oral cancer is the sixth most commonly diagnosed cancer, accounting for 2.1 per cent of total cancer cases.[1] Higher prevalence is observed in Asia (especially Pakistan, India, Bangladesh, Sri Lanka and Nepal) than in Europe or USA.[2,3] In India, oral cancer is the most common form of cancer among males, while in Pakistan it is the second most common cancer.[4,5] This is attributed to the increased risk for oral cancer development in these regions associated with smoking, and the high consumption of alcohol and smokeless tobacco, paan, gutka, areca nut and betel quid.[6]

Generally, 90 per cent of all oral malignancies exist as oral squamous cell carcinoma (SCC), which globally accounts for an estimated 275 000 new cases per year.[7,8] Despite the relative ease of diagnosis, oral cancer is associated with a low survival rate, of 40–50 per cent.[9] This is because patients often present with advanced disease. In addition, access to medical care is limited in many parts of the world and treatment strategies are often inadequate, resulting in severe cosmetic problems that reduce quality of life. In Pakistan, oral cancer ranks as the most common malignancy in males and second most common malignancy in females,[6,10] which is ascribed to the widespread use of cultural forms of smokeless tobacco, areca nut and betel quid.

Although the precise cause of oral cancer is unknown, it is evident that some substantial changes occur at the molecular level before the development of clinically significant malignant lesions. One such change is thought to occur in the expression of the cyclooxygenase-2 ('COX-2') enzyme.[11] Cyclooxygenase-2 (also known as prostaglandin-endoperoxide synthase 2) is a rate-limiting enzyme involved in arachidonic acid metabolism, which is considered critical to various biological processes, including angiogenesis, inflammation, thrombosis, ovulation and various other pathways of immunological importance. Though in the normal cellular environment cyclooxygenase-2 is expressed in negligible amounts, its expression increases in response to inflammation, growth factors and tumour progression.[12,13]

Cyclooxygenase-2 has been linked to malignant processes such as increased anogiogenesis,[14] apoptosis prevention[12] and metastasis.[14] Cyclooxygenase-2 overexpression has been observed in oesophageal, colorectal, breast, urinary bladder, lung, stomach and pancreatic cancers.[15–21] A study assessing the expression of cyclooxygenase-2 by immunohistochemistry revealed a higher expression in oral SCC as compared to leukoplakia and normal tissues.[11] Pandey *et al.* reported similar findings using reverse transcription polymerase chain reaction, with higher cyclooxygenase-2 expression in malignant tissues as compared to normal tissue or pre-malignant lesions.[22] Increased cyclooxygenase-2 expression has also been related to lymph node metastasis, histological grade and tumour–node–metastasis (TNM)

Main Article

Dr S M Adnan Ali takes responsibility for the integrity of the content of the paper

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stage in oral SCC.[23] Selective inhibition of cyclooxygenase-2 has been achieved by designing cyclooxygenase-2 inhibitors, which resulted in cell proliferation suppression by inhibiting the G_0/G_1 cell cycle phase, leading to cell cycle arrest and apoptosis.[13]

Although the role of cyclooxygenase-2 has been explored in relation to oral SCC, information regarding the Pakistani population is lacking. As Pakistan is a high-risk population for oral cancer, we aimed to: investigate the overexpression of cyclooxygenase-2 in oral SCC patients, correlate it with clinicopathological features, and study its effect on overall and disease-free survival.

Materials and methods

Patient selection

A total of 100 patients who had been diagnosed and treated for oral SCC at Aga Khan University Hospital during the years 1991–2004 were recruited in this study. Inclusion criteria were: complete medical history, clinicopathological data, habit history (i.e. data on the use of high-risk substances such as tobacco, betel quid and areca nut), adequate specimen and follow-up details. Exclusion criteria were: incomplete medical records, lack of follow up (with minimum follow-up time set at 60 months) and those who did not undergo surgery at the Aga Khan University Hospital. Ethical approval was obtained from the Ethical Review Committee of Aga Khan University Hospital. Participating patients were informed about the purpose of the study and were asked to provide written informed consent.

Immunohistochemistry

Four-µm thick sections were sliced from formalin-fixed, paraffin-embedded blocks of surgical specimen onto precoated slides. Dewaxing occurred at 60 °C for 30–40 minutes, followed by deparaffinisation in xylene and rehydration in a graded water-ethanol series. Antigen retrieval was achieved using Dako Antigen Retrieval Solution diluted at 1.5 ml per 50 ml, at 100 °C for 40 minutes. After cooling at room temperature for 20 minutes, the slides were incubated in buffer solution for 10 minutes. Endogenous peroxidase activity was blocked using peroxidase blocking reagent for 10 minutes. This was followed by rinsing and incubation with primary mouse monoclonal anti-human cyclooxygenase-2 antibody (Clone CX-294; Dako, Glostrup, Denmark) diluted at 1:100 for 30 minutes. Subsequently, sections were: washed and treated with secondary antibody horseradish peroxidase/Flex (Dako) for 30 minutes, rinsed and treated with EnVision+ system of horseradish peroxidase with 3,3'-diaminobenzidine (DAB) (Dako) for visualisation. Haematoxylin was applied for 30 seconds to provide a counterstain. Lastly, the slides were: dehydrated in a water-ethanol series, mounted using mounting medium (Dako); and viewed at 20× and 40× magnification. Experimental controls were run alongside each batch. Brain tumour samples were used as the positive control, and incubation with buffer instead of primary antibody served as the negative control.

Slide evaluation

Independent evaluation was conducted by two pathologists who were unaware of the clinical history and blinded to each other's readings. Any discrepancies arising between them were resolved using a conference microscope.

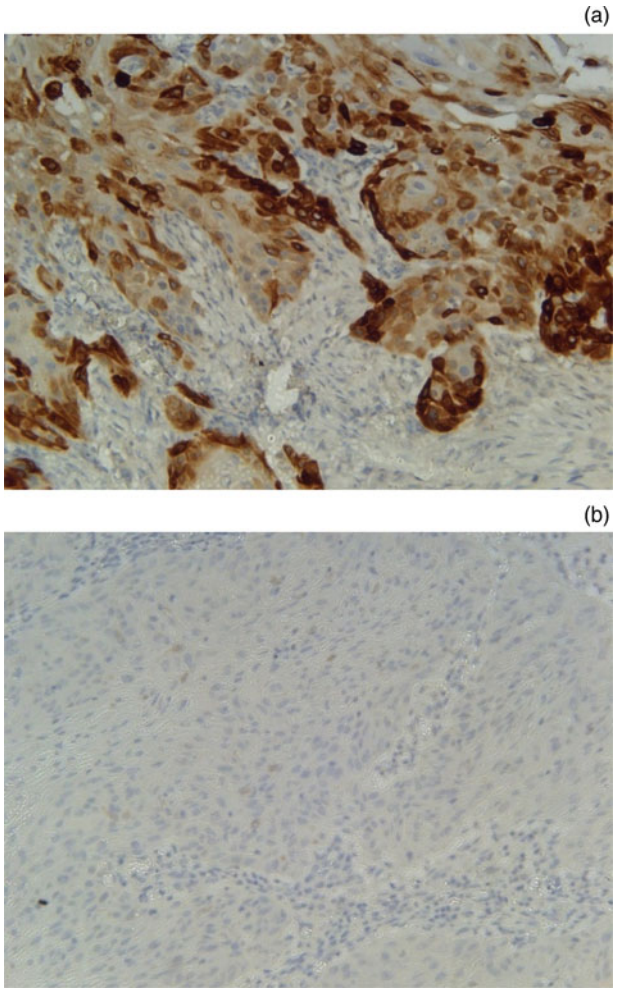

Fig. 1. Photomicrographs of: (a) cyclooxygenase-2 positive oral squamous cell carcinoma (SCC) tissue showing strong brown cytoplasmic staining, and (b) cyclooxygenase-2 negative oral SCC tissue showing haematoxylin counterstain (magnification × 20).

The following criteria were used for scoring cyclooxygenase-2 positivity, according to the percentage of cells stained: less than 10 per cent = negative, 10 to 20 per cent = mild, more than 20 to 40 per cent = moderate, and more than 40 per cent = strong positivity. As existing literature reports various scoring criteria for cyclooxygenase-2 positivity, with little uniformity among them, we used our own criteria. These criteria were adapted from a study with a similar sample size conducted on the Indian population, which can be considered as genetically close.[24]

Statistical analysis

Analysis was performed using SPSS® version 22 statistical software. Descriptive analysis was performed, reporting the mean ± standard deviation (SD) or median (interquartile range) for continuous variables, depending on the normality assumption of the variables; the two groups (i.e. cyclooxygenase protein overexpression and no overexpression) were evaluated using the independent *t*-test or Wilcoxon rank sum (Mann–Whitney test) respectively. For categorical variables, frequency and percentage were reported; the two groups were assessed using the chi-square test of independence if the frequency in each cell was 5 or more, or using the Fisher's exact test if the frequency was less than 5 in any cell.

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Table 1. Clinicopathological characteristics*

Clinical features	Patients (*n*/%)†	COX-2 overexpression (*n* (%))†		*P*-value§
		Positive‡	Negative**	
Age	52.73 ± 12.68	52.16 ± 13.67	53.42 ± 11.476	0.624
Gender				0.282
– Male	57	34 (61.8)	23 (51.1)	
– Female	43	21 (38.2)	22 (48.9)	
High-risk habits?				0.354
– Yes	77	40 (72.7)	37 (82.2)	
– No	17	12 (21.8)	5 (11.1)	
Habit pattern				0.451
– Single	38	21 (38.2)	17 (37.8)	
– Multiple	39	19 (34.5)	20 (44.4)	
– NA	23	15 (27.3)	8 (17.8)	
Tobacco/smoking?				0.401
– Yes	32	15 (27.3)	17 (37.8)	
– No	45	25 (45.5)	20 (44.4)	
– NA	23	15 (27.3)	8 (17.8)	
Paan or supari use?				0.523
– Yes	59	31 (56.4)	28 (62.2)	
– No	18	9 (16.4)	9 (20.0)	
– NA	23	15 (27.3)	8 (17.8)	
Chalia, gutka or niswar use?				0.515
– Yes	28	14 (25.5)	14 (31.1)	
– No	49	26 (47.3)	23 (51.1)	
– NA	23	15 (27.3)	8 (17.8)	
Primary site				0.615
– Cheek	64	34 (61.8)	30 (66.7)	
– Tongue	36	21 (38.2)	15 (33.3)	
Degree of differentiation				0.266
– Well differentiated	41	20 (36.4)	21 (46.7)	
– Moderately differentiated	56	32 (58.2)	24 (53.3)	
– Poorly differentiated	3	3 (5.5)	0 (0.0)	
Surgical margins				0.647
– Clear	65	34 (61.8)	31 (68.9)	
– Near	28	16 (29.1)	12 (26.7)	
– Involved	7	5 (9.1)	2 (4.4)	
Tumour (T) stage				0.554
– T_1	21	11 (20.0)	10 (22.2)	
– T_2	45	22 (40.0)	23 (51.1)	
– T_3	16	10 (18.2)	6 (13.3)	
– T_4	18	12 (21.8)	6 (13.3)	
Nodal (N) stage				0.571
– N_0	75	42 (76.4)	33 (73.3)	
– N_1	17	10 (18.2)	7 (15.6)	
– N_2	8	3 (5.5)	5 (11.1)	

(*Continued*)

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Table 1. (*Continued.*)

Clinical features	Patients (*n*/%)†	COX-2 overexpression (*n* (%))†		*P*-value§
		Positive‡	Negative**	
AJCC stage				0.676
– I	18	9 (16.4)	9 (20)	
– II	31	15 (27.3)	16 (35.6)	
– III	27	17 (30.9)	10 (22.2)	
– IV	24	14 (25.5)	10 (22.2)	
Overall survival				0.01#
– Dead	43	30 (54.5)	13 (28.9)	
– Alive	57	25 (45.5)	32 (71.1)	
Disease-free survival				0.004#
– Recurrence	68	44 (80)	24 (53.3)	
– No recurrence	32	11 (20)	21 (46.7)	

*Total $n = 100$. †Age presented as mean ± standard deviation, in years. ‡$n = 55$; **$n = 45$. §*P*-value calculated using chi-square or Fisher's exact test (as applicable). #$P < 0.05$, indicating significance. COX-2 = cyclooxygenase-2; NA = data not available; AJCC = American Joint Commission on Cancer

Follow-up time for each patient was calculated in months. Disease-free survival time was measured from the date of surgery to the date of recurrence (treatment failure), or to the date of last follow up if no recurrence. Overall survival time was measured from the date of surgery to the date of death (treatment failure), or to the date of last follow up if the patient was alive. Patients who were alive at the time of last contact were considered censored observations in overall survival analysis.

Kaplan–Meier survival curves, log-rank tests and Cox proportional hazards regression analysis were used to compare disease-free survival (recurrence) and overall survival (death). A second proportional hazard regression model, adjusted for the other study covariates, was used to examine the independent effect of treatment. Adjusted hazard ratios with 95 per cent confidence intervals were reported. A *p*-value of less than 0.05 was considered significant.

Results

Patient demographics

The selected 100 patients had all been diagnosed with oral SCC. They had a mean (± SD) age of 52.73 ± 12.68 years, with ages ranging from 20 to 78 years. The majority of patients were male (57 per cent) and aged over 40 years (85 per cent). The most commonly reported primary lesion site was the cheek (64 per cent), followed by the tongue (36 per cent).

The consumption of substances known to be high-risk factors for oral SCC was recorded for our population. The majority of patients (77 per cent) had a positive habit history. Habits included the use of tobacco (smoking and smokeless), areca nut and betel quid in the culturally prevalent forms of paan, supari, chalia, gutka and naswar. Habit pattern assessment showed that 32 per cent of patients were habitual cigarette smokers, and 59 per cent and 28 per cent were betel quid and areca nut chewers respectively. The habit history of 23 patients was either insufficient or not reported. The habitual use of any high-risk substance was not determined to have any effect on cyclooxygenase-2 expression.

Histologically, the most common diagnosis was moderately differentiated tumour (56 per cent) and American Joint Committee on Cancer stage II (31 per cent). According to the TNM staging, stages T_2 and N_0 were most often diagnosed; none of the patients had distant metastasis.

Upon last follow up, 57 per cent of patients were reported alive and 68 per cent had experienced disease recurrence.

Cyclooxygenase-2 and clinicopathological factors

Cyclooxygenase-2 overexpression was positive (as illustrated in Figure 1a) in 55 per cent of patients and negative (Figure 1b) in the remaining 45 per cent. Based on staining intensity, 20 per cent of positive specimens were mild, 29 per cent were moderate and 6 per cent were strong.

The associations of cyclooxygenase-2 overexpression and patients' clinicopathological factors are shown in Table 1. There was no significant relationship between cyclooxygenase-2 positivity and any of the clinicopathological factors tested by chi-square, except for overall survival and disease-free survival. Regarding the latter variables, cyclooxygenase-2 positivity was a significant indicator of lower overall survival ($p = 0.01$) and a higher chance of disease recurrence ($p = 0.004$).

Overall and disease-free survival

Table 2 shows the univariate analysis findings for overall survival and disease-free survival. For our study population, mean overall survival time was 146.88 months and disease-free survival time was 78.86 months. Cyclooxygenase-2 positivity was a statistically significant indicator both for overall survival ($p = 0.013$) and disease-free survival ($p = 0.001$). Other factors that had a notable impact on overall survival were American Joint Committee on Cancer stage ($p = 0.001$), surgical margins ($p = 0.045$), T stage ($p = 0.033$) and N stage ($p < 0.001$). Similarly, disease-free survival was also associated with American Joint Committee on Cancer stage ($p = 0.005$) and N stage ($p < 0.001$).

The Kaplan–Meier analysis also supported these findings, as shown in Figure 2. The mean overall survival time for cyclooxygenase-2 positive patients was 120.76 months, as compared to the much longer survival time of 174.53 months for cyclooxygenase-2 negative patients. This was reflected in the mean disease-free survival times, which were 48.02 months

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Table 2. Univariate analysis for overall and disease-free survival

		Overall survival		Disease-free survival	
Variables	Patients (*n*)	Months	*P*-value*	Months	*P*-value*
Gender			0.957		0.586
– Male	57	142.05		72.29	
– Female	43	148.81		84.58	
High-risk habits?			0.819		0.694
– Yes	77	118.87		64.11	
– No	17	136.41		75.54	
Primary site			0.329		0.289
– Cheek	64	109.75		58.76	
– Tongue	36	161.88		96.60	
Degree of differentiation			0.162		0.199
– Well differentiated	41	171.39		100.99	
– Moderately differentiated	56	129.00		63.72	
– Poorly differentiated	3	48.00		41.33	
Surgical margins			0.045†		0.376
– Clear	65	158.10		83.05	
– Near	28	77.64		56.52	
– Involved	7	47.42		45.71	
Tumour (T) stage			0.033†		0.139
– T_1	21	175.95		80.87	
– T_2	45	149.82		95.22	
– T_3	16	124.56		50.64	
– T_4	18	68.77		37.23	
Nodal (N) stage			<0.001†		<0.001†
– N_0	75	171.84		94.32	
– N_1	17	50.58		28.04	
– N_2	8	27.62		11.25	
AJCC stage			0.001†		0.001†
– I	18	177.50		90.95	
– II	31	179.90		114.30	
– III	27	113.77		47.32	
– IV	24	62.54		34.95	
COX-2 overexpression			0.013†		0.001†
– Positive	55	120.76		48.02	
– Negative	45	174.53		115.46	

*P-value calculated using log-rank analysis. †$P < 0.05$, indicating significance. AJCC = American Joint Commission on Cancer; COX-2 = cyclooxygenase-2

and 115.46 months for cyclooxygenase-2 positive and negative patients respectively.

On multivariate analysis, only cyclooxygenase-2 was a significant independent predictor of prognosis (Table 3). Disease-free survival was significantly affected if the patient was cyclooxygenase-2 positive ($p = 0.044$); however, overall survival was not a significant independent factor on multivariate analysis ($p = 0.208$).

Discussion

Oral cancer is attributed to a multifactorial aetiology involving genetic and environmental factors. Various genetic alterations have been associated with oral SCC. These include alterations in chromosomes 3, 9, 11 and 13, the overexpression of various oncogenes, as well as the inhibition of tumour suppressor genes and other apoptotic factors and DNA repair mechanisms, which together lead to carcinogenesis.[25] Various biomarkers have been correlated with oral SCC incidence and disease progression, suggesting their critical role in tumourigenesis, with one such marker being cyclooxygenase-2. We investigated whether cyclooxygenase-2 was overexpressed in oral SCC patients of Pakistan, and examined the effect this had on patient prognosis. The Reporting Recommendations for Tumour Marker Prognostic Studies ('REMARK') guidelines were used as a standard of reporting for this study.[26]

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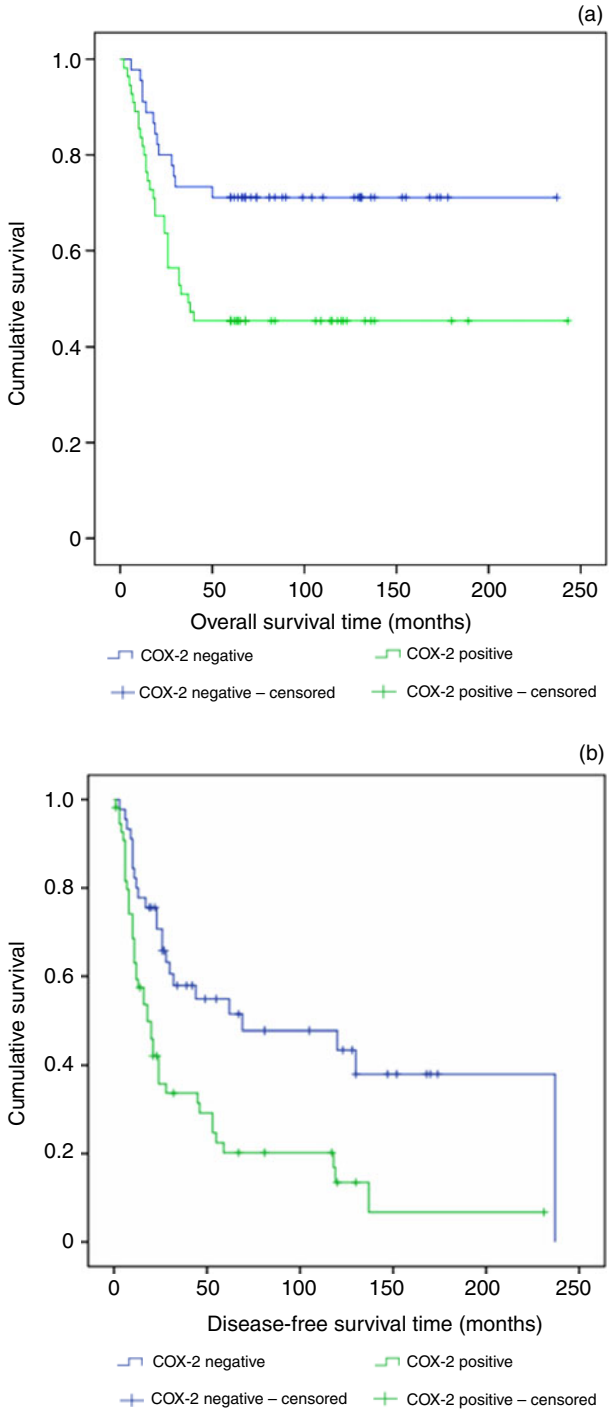

Fig. 2. (a) Overall survival and (b) disease-free survival curves. Cyclooxygenase-2 (COX-2) positive patients had significantly lower overall and disease-free survival rates.

Previous studies have suggested that cyclooxygenase-2 is usually up-regulated in pathological conditions such as oral SCC, oesophageal, lung, breast, colorectal and other forms of cancer.[12,15–19] Cyclooxygenase-2 overexpression has also been reported in oral pre-malignant lesions. Shibata *et al.* investigated the expression of cyclooxygenase-1 and cyclooxygenase-2 in patients with dysplasia and SCC, and revealed that cyclooxygenase-2 expression was strongly associated with higher stages of dysplasia rather than SCC.[27] Another study also demonstrated that cyclooxygenase-2 overexpression influenced progression towards malignancy.[28]

In this study, immunohistochemical analysis revealed cyclooxygenase-2 overexpression in the majority of oral SCC patients (55 per cent), suggesting that this may be common in oral SCC carcinogenesis. The overall demographics of our patients correlate with previous studies on the Pakistani oral SCC population, with buccal mucosa being the most common lesion site and moderately differentiated American Joint Committee on Cancer stage II carcinoma being the most common diagnosis.[7,29] There was a higher number of females in our study as compared to previous reports, and the mean age of our population was lower than the reported average of 64 years.[30] However, there was no significance of age or gender in terms of cyclooxygenase-2 expression in our population.

We found a slightly higher percentage of cyclooxygenase-2 positive patients (55 per cent), as compared to previous studies, in which cyclooxygenase-2 was found overexpressed in 28 per cent, 42 per cent and 14 per cent of oral SCC specimens using immunohistochemistry.[30–32] This relatively high rate of cyclooxygenase-2 in our population may be explained by the heavy consumption of high-risk substances. Continuous abrasion of buccal mucosa associated with chewing tobacco, chalia, gutka and naswar causes inflammation; as cyclooxygenase-2 is one of the key mediators of the process, its overexpression can be attributed to persistent consumption of these factors.

The majority of patients were users of substances that are known oral SCC risk factors, but their habits were not correlated with cyclooxygenase-2 overexpression. There were an almost equal number of cyclooxygenase-2 positive and negative patients when distributed according to their particular habits. Contrary to our results, Moraitis *et al.* reported higher overexpression of cyclooxygenase-2 in smokers, and suggested that this expression was regulated through the production of prostaglandins E2 and tyrosine kinase.[33] In another study, smoking was associated with the induction of cyclooxygenase-2, which led to the increased production of prostaglandin E2 and thromboxane A2; increased levels of these inflammation mediators can lead to tumour development and progression.[34]

Correlating cyclooxygenase-2 expression with any clinicopathological factor did not yield significant results, except for overall survival ($p = 0.01$) and disease-free survival ($p = 0.004$). The impact of cyclooxygenase-2 on survival in combination with other factors was observed on the univariate analysis, as cyclooxygenase-2 positive patients had markedly lower overall survival ($p = 0.013$) and disease-free survival ($p = 0.001$). However, the multivariate analysis showed that cyclooxygenase-2 positivity had an independent effect on disease-free survival only, while overall survival was affected by a combination of factors, one of which was cyclooxygenase-2 positivity. Moreover, the Kaplan–Meier analysis showed that cyclooxygenase-2 positive patients had lower overall survival and higher recurrence rates than their cyclooxygenase-2 negative counterparts.

Similar to our results, Cha *et al.* also evaluated oral SCC by immunohistochemistry to assess cyclooxygenase-2 overexpression, and found that cyclooxygenase-2 expression was statistically significant for overall survival on univariate analysis, but this was not replicated on multivariate analysis.[31] A study conducted by Haffner *et al.*, however, revealed that cyclooxygenase-2 overexpression was independently and significantly associated with poor prognosis.[30] Other studies have linked cyclooxygenase-2 independently with poor overall survival in pancreatic cancer[35] and in combination with other variables in ovarian cancer.[36]

There has been debate regarding the association between the degree of tumour differentiation and cyclooxygenase-2 expression. An increased expression of cyclooxygenase-2 has been observed in well differentiated tumours, as compared to poorly differentiated tumours, in lung, laryngeal and oesophageal cancer.[19,37,38] In contrast, other studies have reported an

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Table 3. Multivariate analysis for overall and disease-free survival

		Overall survival			Disease-free survival		
Variable	Patients (*n*)	*P*-value*	Hazard ratio	95% CI	*P*-value*	Hazard ratio	95% CI
COX-2 over-expression		0.208	1.522	0.792–2.927	0.044†	1.715	1.015–2.899
– Positive	55						
– Negative	45						

*P-value calculated using log-rank analysis. †$P < 0.05$, indicating significance. CI = confidence interval; COX-2 = cyclooxygenase-2

increased expression of cyclooxygenase-2 in poorly differentiated carcinomas of the oesophagus and tongue, rather than in well differentiated tumours.[39,40] In our study, the overexpression of cyclooxygenase-2 was not associated with histological classification, American Joint Committee on Cancer stage, gender or TNM staging, which is similar to the findings of Pandey *et al.*[22] The lack of any statistically significant correlations may be a result of missing patient information or the small sample size, which may not be a true representation of the population.

Having ascertained the role of cyclooxygenase-2 in tumour development and progression, inhibitors of cyclooxygenase-2 have been found to play a critical role in cancer treatment. The inhibition of cyclooxygenase-2 decreases angiogenesis, cell proliferation and invasiveness, G_0/G_1 cell cycle arrest, p21 expression, and prostaglandin E2 production.[30,41,42] Kim *et al.* demonstrated the anticancer effects of celecoxib, which significantly reduced the invasive potential of cancer cell lines by inhibiting cyclooxygenase-2.[17] In addition, various anti-apoptotic proteins have radioresistant properties, and the regulation of such factors is controlled by the nuclear factor kappa B pathway, which is associated with the increased production of cyclooxygenase-2, hence its playing a major role in radioresistance.[43,44] The present evidence suggests a role of cyclooxygenase-2 as a prognostic marker and in targeted therapy of oral SCC patients.

- Oral squamous cell carcinoma (SCC) is one of the most commonly occurring cancers worldwide
- Cyclooxygenase-2 has been tested as a prognostic marker in various cancers
- Immunohistochemistry was used to detect cyclooxygenase-2 expression in 100 oral SCC cases in Pakistan
- Increased cyclooxygenase-2 expression is found in oral SCC patients, leading to poor overall and disease-free survival
- Inhibiting cyclooxygenase-2 and its related pathways can help prevent cell proliferation and invasive potential of tumour-promoting factors

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Competing interests. None declared

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