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Original Article

Pathology Section

COX-2 Expression as a Prognostic Inflammatory Marker in Head and Neck Squamous Cell Carcinoma: A Cross-sectional Study

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ABSTRACT

Introduction: Head and Neck Squamous Cell Carcinoma (HNSCC) ranks as the sixth leading cause of cancer worldwide. HNSCC is an aggressive neoplasm involving multistep carcinogenesis. As it is associated with an increased risk of recurrence, morbidity and mortality, many studies are being undertaken to understand its pathophysiology at the molecular level, identify newer prognostic factors and develop novel therapeutic agents.

Aim: To assess the clinicopathological features of all cases of HNSCC, including age, sex, site and risk factors and to demonstrate the expression of the Cyclooxygenase-2 (COX-2) marker in various grades of the tumour in a subset of cases.

Materials and Methods: A cross-sectional study was conducted on HNSCC at the Institute of Pathology, Rajiv Gandhi Government General Hospital, Chennai, Tamil Nadu, India for a period of one year from July 2014 to June 2015. A total of 50 cases (representing different grades) were randomly selected, 25 each from the oral cavity/oropharynx and upper respiratory tract. Immunohistochemistry (IHC) was performed using the COX-2 marker and the results were tabulated in an MS Excel sheet to assess the incidence among different age groups, genders, various risk factors, and sites. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) 21.0 software, using the Chi-square test to assess the significant expression of COX-2 in varying grades of the tumour. A p-value <0.05 was considered significant.

Results: The study results (total cases=50) showed an increased incidence of HNSCC among males (n=39; 78%) compared to females (n=11; 22%). Additionally, HNSCC most commonly affected patients in the 5th to 6th decade (n=24; 48%) of life. The occurrence was most commonly noted in the tongue (n=8; 16%) and hypopharynx (n=13; 26%) among all Squamous Cell Carcinoma (SCC) cases of the oral cavity and upper respiratory tract, respectively. Out of the 50 cases included to analyse COX-2 expression, 41 cases (82%) of HNSCC showed COX-2 expression, and its association with different grades was evident by a significant p-value of 0.027.

Conclusion: This study demonstrated significant expression of COX-2 in all grades of HNSCC, supporting the role of inflammation in multistep carcinogenesis. Furthermore, molecular targeted therapy against COX-2 could be implemented as an adjunctive therapy with chemoradiation to hinder the progression and aggressive behaviour of the tumour.

Keywords: Cyclooxygenase-2, Immunohistochemistry, Molecular-targeted therapy, Squamous cell carcinoma of head and neck

INTRODUCTION

The SCC of the oral cavity and upper respiratory tract ranks as the sixth leading cause of cancer worldwide [1]. The highest incidence is noted in developing countries like India, Sri Lanka, Pakistan, and Bangladesh [1-3]. HNSCC includes neoplasms from the oral cavity, nasopharynx, oropharynx, hypopharynx, and larynx, excluding neoplasms of salivary glands [4]. The tumour arises from the squamous epithelial lining of the mucosa. Of all the malignancies that arise from the head and neck region, SCC comprises more than 90%. SCC is an aggressive malignant tumour with a higher rate of recurrence, mortality, and morbidity due to the late presenting symptoms leading to a delay in diagnosis and advanced staging of the tumour [5-8].

There are many risk factors associated with HNSCC, such as tobacco, alcohol, betel quid, and Human Papilloma Virus (HPV) infection [3]. The association with these risk factors has been confirmed by the International Agency for Research on Cancer (IARC) [9,10]. The carcinogens like polycyclic hydrocarbons, nitrosamines, acetaldehyde, and benzopyrene present in these risk factors are metabolised by Cytochrome P450 enzymes. This leads to the production of reactive metabolites that are carcinogenic, affecting the Deoxyribonucleic Acid (DNA) of the keratinocyte [11]. The usage of tobacco and alcohol acts synergistically as cancer promoters. In adding to this tobacco has the ability to induce chronic inflammation and increased production of reactive oxygen species [12]. Virchow hypothesised in 1863 that cancer originates from the site of inflammation [13].

Numerous tumour and inflammatory markers are involved in the pathogenesis of HNSCC, such as p53, p16, COX-2, Vascular Endothelial Growth Factor (VEGF) and Epidermal Growth Factor Receptor (EGFR). This study aims to study the clinicopathological features of HNSCC, including age, sex, and risk factors, and to demonstrate the expression of COX-2 in HNSCC in various grades so that selective COX inhibitors can be added along with chemoradiation to improve the prognosis of the patient.

MATERIALS AND METHODS

A cross-sectional study was conducted on SCC arising from the head and neck at the Department of Pathology, Rajiv Gandhi Government General Hospital, Chennai, Tamil Nadu, India. The study period was one year from July 2014 to June 2015. Ethical clearance for the study was obtained from the Institutional Ethical Committee (IEC) (EC Reg.No.ECR/270/Inst./TN/2013).

Inclusion criteria: Infiltrating SCC arising from HNSCC was included in the study. Exclusion criteria: Non-neoplastic, benign, premalignant lesions, and malignancies other than SCC were excluded from the study.

Sample size: A selection of participants for this study conducted in a hospital setting was made using a convenience sampling method, adhering to predefined criteria for inclusion and exclusion.

A total of 50 cases of HNSCC were included in the study, 25 cases from the oral cavity and 25 cases from the upper respiratory tract. Clinicopathological data such as age, sex, site, and associated risk factors like tobacco, alcohol, and smoking were collected for these 50 cases. The tumours were graded according to the World Health Organisation (WHO) criteria [14] into well, moderately, and poorly differentiated SCC based on the degree of differentiation. Fifty cases representing different grades were randomly selected, 25 each from the oral cavity/oropharynx and upper respiratory tract, and their paraffin blocks were retrieved. Immunohistochemistry (IHC) using a COX-2 antibody was performed on these paraffin-embedded tissue samples to assess the expression of COX-2.

Immunohistochemical evaluation (IHC Methodology): Immunohistochemical analysis for COX-2 expression was performed on 4 µm thick paraffin-embedded tissue samples using the "Super-sensitive polymer Horseradish Peroxidase (HRP) system" (Polyexcel HRP/DAB (diaminobenzidine) Detection system-Two-step, PathnSitu Biotechnologies Private Limited, India), which was based on non biotin polymeric technology. The antibody used was a rabbit monoclonal antibody SP21 (PathnSitu Biotechnologies Private Limited, India) against COX-2 antigen, and COX-2 expression was detected by adding a secondary antibody conjugated with HRP-polymer and DAB substrate. Colonic mucosa was used as the positive control for COX-2 expression.

A four-point evaluation scale was used (Hussain N and Bhatt MLB) as described below. Intense brown staining of the cytoplasm was considered positive [15]:

0- No staining

1+- Staining of <5% of tumour cells

2+- Staining of 5-30% of tumour cells

3+- Staining of >30% of tumour cells

The scores were grouped as negative (score 0 and 1+) and positive (score 2+ and 3+).

STATISTICAL ANALYSIS

The results were tabulated in an MS Excel sheet to assess the incidence among different age groups, gender, various risk factors, and sites. Statistical analysis was performed using the Chi-Square test with SPSS 21.0 software to assess the association of COX-2 expression in varying grades of HNSCC. A p-value <0.05 was considered significant.

RESULTS

A total of 50 cases of HNSCC were included in the study, with 25 cases from the oral cavity and 25 cases from the upper respiratory tract. HNSCC had a peak incidence in the 51-60 years age group, comprising around 48% of all patients. This study revealed an interesting trend with an increasing incidence (n=13; 26%) of malignancy in the younger population (<50 years) [Table/Fig-1]. There was increased usage of tobacco chewing (n=8; 27.60%) in oral SCC and smoking usage (n=8; 27.60%%) in upper respiratory tract SCC [Table/Fig-2]. It was also observed that the incidence of HNSCC was more common among males than in females [Table/Fig-3].

Age (years)	N (%)
31-40	5 (10)
41-50	8 (16)
51-60	24 (48)
61-70	9 (18)
71-80	4 (8)
Total	50 (100)

[Table/Fig-1]: Age-wise distribution of HNSCC.

Risk Factor	Frequency	Percentage (%)
Alcohol	5	17.20%
Alcohol+Smoking	3	10.30%
Smoking	8	27.60%
Tobacco	8	27.60%
Tobacco+Alcohol	5	17.20%
Total	29	100.00%

[Table/Fig-2]: Risk factor association of HNSCC.

Gender	Frequency (n)	Percentage (%)
Female	11	22
Male	39	78
Total	50	100

[Table/Fig-3]: Gender-wise distribution in HNSCC.

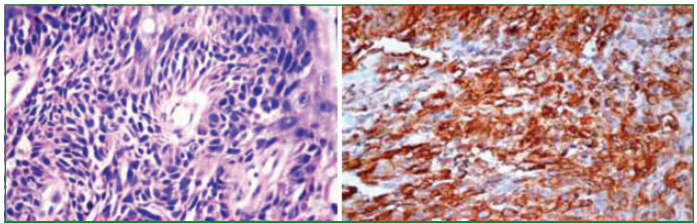
Around 21 patients (42%) were not associated with any of the habits associated with an increased risk of malignancy. Site-specific data for all the SCC analysed showed that there was the highest occurrence of SCC in the tongue (16%) [Table/Fig-4] within the oropharyngeal region and in the hypopharynx (26%) among the upper respiratory tract tumours [Table/Fig-4].

Site	n (%)
Buccal mucosa	3 (6)
Floor of mouth	3 (6)
Gingivobuccal sulcus	1 (2)
Hard palate	3 (6)
Lip	1 (2)
Retromolar trigone	1 (2)
Tongue	8 (16)
Tonsil	4 (8)
Soft palate	1 (2)
Glottis	4 (8)
Hypopharynx	13 (26)
Subglottis	2 (4)
Supraglottis	6 (12)
Total	50 (100)

[Table/Fig-4]: Distribution of the site of HNSCC

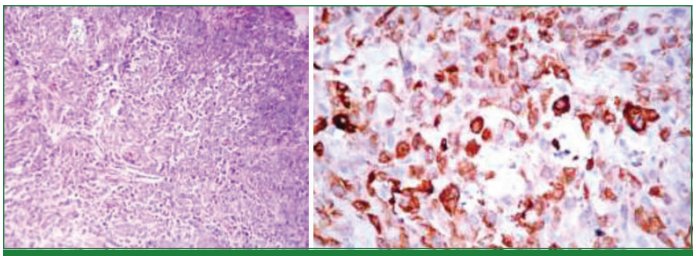
The SCC specimens were graded, according to WHO recommendations, into three groups based on the degree of differentiation. Out of the total 50 cases, 20 cases were well differentiated [Table/Fig-5] (40%), 19 cases were moderately differentiated [Table/Fig-6] (38%), and 11 cases were poorly differentiated [Table/Fig-7] (22%).

COX-2 expression in HNSCC: A total of 41 cases (82%) showed expression of COX-2, and 9 cases (18%) were negative for COX-2 expression. The maximal number of COX-2 expression was noted in well-differentiated tumours (18 cases; 36%), followed by

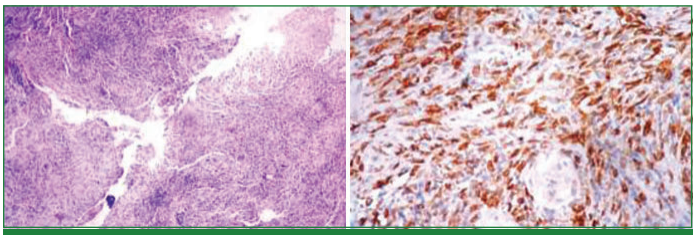


[Table/Fig-5]: Well differentiated SCC-Tonsil: H&E (400X)/IHC COX-2-Positive 3+(400X).

moderately differentiated tumours (17 cases; 34%) [Table/Fig-8,9]. A Chi-square test showed a statistically significant association between the expression of COX-2 in HNSCC and the grades of the tumour (p-value=0.027).



[Table/Fig-6]: Moderately differentiated SCC-Hypopharynx H&E (100X)/IHC COX-2-Positive 2+(400X).



[Table/Fig-7]: Poorly differentiated SCC-Hard palate H&E (100X)/IHC COX-2-Positive 3+(400X).

Grades	COX-2 Expression-Scoring			
	0	1+	2+	3+
Well differentiated (n=20)	1	1	7	11
Moderately differentiated (n=19)	1	1	9	8
Poorly differentiated (n=11)	2	3	3	3

[Table/Fig-8]: Scoring of COX-2 expression.
Four-point evaluation scale: Intense brown staining of the cytoplasm-Positive
0-No staining; 1+→<5% of tumour cells; 2+→ 5-30% of tumour cells; 3+→>30% of tumour cells
Negative- Score 0 & Score 1 Positive- Score 2 & Score 3

Grade	COX-2 Expression (n=50)	
	Positive	Negative
Well differentiated (n=20)	18 (36.00%)	2 (4.00%)
Moderately differentiated (n=19)	17 (34.00%)	2 (4.00%)
Poorly differentiated (n=11)	6 (12.00%)	5 (10.00%)
Total (%)	41(82.00%)	9 (18.00%)

[Table/Fig-9]: COX-2 expression in HNSCC.
Chi-square test=7.204; p-value=0.027* Statistically significant

DISCUSSION

SCC remains the most common malignancy in the head and neck region. These tumours have a significant impact on speech, taste, voice, swallowing, and respiration. Developing countries like India show an increasing trend, constituting 30-50% of all malignancies [16]. About 50% of HNSCC patients reach the advanced stage due to late symptomatic presentation [8]. The 5-year survival rate is 10-40% [17], and the prognosis remains poor for years even after intense therapies including chemoradiation and mutilating surgeries. This may be attributed to a poor understanding at the molecular level.

The concept of chronic inflammation causing carcinogenesis has been studied in many malignancies occurring in the liver, oral cavity, stomach, colon [18], etc. Hence, many studies have focused on inflammatory mediators showing overexpression in malignancies. These inflammatory mediators are associated with genetic alterations and epigenetic changes causing the inactivation of tumour suppressor genes [19]. Chronic inflammation also leads to the production of reactive oxygen and nitrogen species, which can cause DNA damage. One of the most common inflammatory mediators is the enzyme COX-2, which is involved in arachidonic acid metabolism. There are two isoforms of COX- COX-1 and COX-2. COX-1 is a constitutive enzyme found in normal cells, while COX-2 is an inducible enzyme that is increased during inflammation and carcinogenesis. Overexpression of COX-2 inhibits apoptosis, leading to increased survival of DNA damaged cells [20]. It also promotes cell proliferation and angiogenesis/lymphangiogenesis by upregulating VEGF expression [21]. This increases the chances of lymph node metastasis and recurrence [22]. Additionally, COX-2, along with other mediators like matrix metalloproteinases, promotes Epithelial-Mesenchymal Transition (EMT) and increases the risk of invasion [23]. Given the pathophysiology of COX-2, many therapeutic trials have been conducted using selective COX-2 inhibitors like celecoxib in the treatment of colon cancer and oral SCC [24]. Various studies have shown the expression of COX-2 not only in malignant lesions but also in premalignant lesions, supported by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) assays, western blot analysis, and immunohistochemical analysis [25].

In this study, immunohistochemical analysis was performed on 50 cases of head and neck SCC to assess COX-2 expression. The clinicopathological analysis showed an increased occurrence of HNSCC in the 6th decade of life (48%), which was consistent with the findings of Mohammad S et al., (30%) [26]. There was also a significant preponderance of males (78%) compared to females (22%), similar to the study by Patel MM and Pandya AN (male-75%; female-25%) [27]. The most common site of SCC was the tongue, followed by the buccal mucosa, which was consistent with the study by Chan G et al., [28]. However, this observation differs from the study by Dasgupta S et al., which showed a higher incidence in the buccal mucosa (33.33%) among oral cavity tumours [Table/Fig-10] [29]. This variation in expression among different sites may be due to differences in associated risk factors and genetic causes. Among upper respiratory tract tumors in the current study, the most common occurrence of SCC was noted in hypopharynx (52%) followed by supraglottis (24%) which was compared with the study done by Zhang Q et al., [30] [Table/Fig 11].

Tumour location	Current study, 2023	Dasgupta S et al., [29] India, 2019	Chan G et al., [28] New York,1999
Tongue	32%	25%	45.8%
Buccal mucosa	16%	33.33%	33.3%
Tonsil	16%	14.81%	4.2%
Other sites	36%	26.86%	16.7%

[Table/Fig-10]: Comparison of distribution of SCC in different sites of oral cavity.

Site	Current study, 2023	Zhang Q et al., [30] China, 2021
Hypopharynx	52%	3.2%
Supraglottis	24%	50.2%
Glottis	16%	43.6%
Subglottis	8%	2.4%

[Table/Fig-11]: Comparison of distribution of SCC in different sites of upper respiratory tract.

In the present study, well differentiated SCC was the most common grade noted. This finding was comparable to the data shown by Dasgupta S et al., and Jerjes W et al., [Table/Fig-12] [29,31]. COX-2 expression was noted in 41 cases (82%) in the current study, which was similar to the studies conducted by Mohammed S et al., (84%) and Sekimizu M et al., (71%) [Table/Fig-13] [26,32]. Comparative studies have shown higher expression of COX-2 in well-differentiated tumours, followed by poorly differentiated tumours. However, the current study showed increased expression of COX-2 in well-differentiated tumours and minimal expression in poorly differentiated tumours. Additionally, the current study showed intense expression of COX-2 in well-differentiated tumours, contrasting with the study by Thomas N et al., where COX-2 expression intensity was noted to be highest in poorly differentiated tumours [Table/Fig-14] [33].

Grade	Current study, 2023	Dasgupta S et al., [29] India, 2019	Jerjes W et al., [31] UK, 2010
Well differentiated	40%	62.03%	27.8%
Moderately differentiated	38%	35.19%	52.2%
Poorly differentiated	22%	2.78%	20%

[Table/Fig-12]: Comparison of grades of HNSCC [29,31].

COX-2 Expression	Current study, 2023 (n=50)	Mohammed S et al., [26] India (n=44), 2011	Sekimizu M et al., [32] Japan (n=75), 2019
Positive	82% (n=41)	84.09% (n=37)	71% (n=53)
Negative	18% (n=9)	15.91% (n=7)	29% (n=22)

[Table/Fig-13]: Comparison of COX-2 expression in HNSCC [26,32].

This deviation in decreased expression in poorly differentiated tumours may be due to the fact that the tumour cells might have crossed the G1 phase in the cell cycle and may also be due to tumour cell proliferation arrest [34]. This was also supported by the variation in the activity of cell-dependent kinase enzymes [35]. The comparison among grades showed varied results among different studies. Hence, the expression of COX-2 in all grades alone was considered.

Intensity of COX-2 Expression		No staining	+1	+2	+3
Current study (n=50)	Well differentiated (n=20)	1 (5%)	1 (5%)	7 (35%)	11 (55%)
	Moderately differentiated (n=19)	1 (5.3%)	1 (5.3%)	9 (47.3%)	8 (42.1%)
	Poorly differentiated (n=11)	2 (18.1%)	3 (27.3%)	3 (27.3%)	3 (27.3%)
Thomas N et al., [33] Kerala (n=30)	Well differentiated (n=10)	0	7 (70%)	2 (20%)	1 (10%)
	Moderately differentiated (n=10)	0	3 (30%)	5 (50%)	2 (20%)
	Poorly differentiated (n=10)	0	0	2 (20%)	8 (80%)

[Table/Fig-14]: Comparison of intensity of COX-2 expression in HNSCC.

Among the 50 cases, nine cases showed negative results. This may be due to its association with many other factors. Therefore, a combined IHC study including EGFR, p16, ki-67, and p53 will help determine the molecular pathogenesis for the COX-2 negative cases. There have also been studies stating that cases with underexpression of COX-2 respond well to chemoradiation compared to COX-2 overexpressed tumours. Clinical trials [24,36] have supported the fact that the addition of selective COX-2 inhibitors potentiates the effect of chemoradiation, thereby increasing the outcome of aggressive HNSCC tumours.

Limitation(s)

The study was conducted only on 50 patients due to financial constraints. Including a larger number of cases and conducting follow-up studies with COX-2 inhibitors will further support the current study and improve the well-being of HNSCC patients.

CONCLUSION(S)

The concept of inflammation potentiating the risk for tumourigenesis has been widely observed. This study also confirmed the significant expression of COX-2 in all grades of HNSCC. COX-2 overexpression leads to tumour progression and promotes the aggressive course of the tumour. With this concept, selective COX-2 inhibitors can be used in combination with standard treatment protocols like chemoradiation to improve patient outcomes. The use of Celecoxib, a selective COX-2 inhibitor, has already shown favourable outcomes among colon cancer patients.

In conclusion, IHC analysis for COX-2 should be performed in patients with HNSCC so that the addition of targeted therapy against this protein expression can be considered through further therapeutic trials. This will ultimately increase survivability and improve the well-being of the patient.

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- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. Yes

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