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Journal of Cancer Biology & Research

Research Article

Expression Analysis of COX-2 in Patients Suffering from Esophageal Squamous Cell Carcinoma

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Submitted: 23 April 2018

Accepted: 23 May 2018

Published: 25 May 2018

Keywords

- Esophageal squamous cell carcinoma
- COX-2
- Immunohistochemistry
- Naswar

Abstract

Objective: To investigate the expression of COX-2 gene in patients suffering from esophageal squamous cell carcinoma

Study design: Descriptive

Place and duration of study: Institute of Basic Medical Sciences, Khyber Medical University

Patients and methods: A total of 69 patients, of esophageal squamous cell carcinoma (ESCC), comprising of Pakistanis and Afghans were enrolled. Various risk factors associated with ESCC were recorded. Immunohistochemical (IHC) expression of COX-2 was determined in normal esophageal mucosa, Carcinoma in situ (CIS) and invasive ESCC. Differences of mean were computed with ANOVA test followed by Post Hoc test. Patients were categorized as positive with high expression or negative with low to nil expression.

Results: Annova test showed large differences in expression of COX-2 in normal healthy mucosa compared to CIS tissue and ESCC with the mean difference of -9.529 and -7.370 respectively, p-value <.05 at 95% confidence interval (CI). No significant difference was noticed in expression of COX-2 in CIS compared to ESCC with the p-value >.05 at 95% CI. Mean age at diagnosis was 55 years. Out of 69 patients of ESCC in our sample, 46 (67%) were users of Naswar.

Conclusion: The cohort, 23-85 years shows statistically significant difference in expression of COX-2 gene in ESCC and CIS tissue sample compared to normal healthy mucosa. Over-expression of COX-2 is positively associated with ESCC.

ABBREVIATIONS

EC: Esophageal Carcinoma; ESCC: Esophageal Squamous Cell Carcinoma; CIS: Carcinoma *in Situ*; COX-2: Cyclooxygenase 2; EAC: Esophageal Adenocarcinoma; US: United States; AJCC: American Joint Committee on Cancer; UICC: Union for International Cancer Control; KP: Khyber Pakhtunkhwa; pTNM: Pathological Staging

INTRODUCTION

Esophageal cancer (EC) is being a global health concern, ranks eighth among the most common types of cancers and is the 6th most fatal disease worldwide [1-3]. Though significant advances have been made in the treatment of ESCC, it is still unresponsive to the treatment strategy and has an adverse prognosis, with a five year survival rate of only 10-15% [2,4]. EC show marked geographical, ethnic, and gender variation. The most common histological subtype of EC in the high incidence regions such as the greater Iran is ESCC [5,6]. While in low incidence areas like the US, Esophageal Adenocarcinoma is the most common subtype [7,8]. In China the incidence of EC accounts for half of the cases all over the world [9]. The male to female ratio of EC mortality is 2:1 [10].

In Pakistan, EC is quite common. Highest incidence zone of EC in Pakistan is the Baluchistan plateau in the Northwest of Pakistan where it is the most common malignancy in both males and females [5,9,11]. In Karachi, the largest city of Pakistan, EC is ranked 7th and 6th most common cancer in men and women respectively. The predominant type in Karachi, Pakistan is ESCC, in contrast, adenocarcinoma is the most common variety in Western countries [1]. The predominance of ESCC in Karachi needs to be viewed in the context that the large number of people

moved to Karachi from Baluchistan and Khyber Pakhtunkhwa (KP) [9]. According to the statistics provided by the Shaukat Khanum Memorial Cancer Hospital and Research Center (SKMCH and RC), cancer of the esophagus is almost 1.9 percent of the total malignancies in Pakistan. Majority of patients who were treated in this tertiary care facility belonged to Punjab and KP [1].

Major risk factors of EC include poor socioeconomic status, use of tobacco, naswar (Snuff or nass), use of alcohol, hot beverages and infrequent consumption of raw fruits and vegetables [3]. Globally, different types of smokeless tobacco like chewing tobacco, wet snuff and dried snuff are used. Fire and air cured tobacco is also used in the form of moist snuff. (It is also called snus). All these are used by inserting the substance inside the lips between the buccal mucosa and gingiva [12,13]. Chewing nass, a smokeless tobacco product, is a mixture of tobacco lime, oil, flavoring and coloring reagent and is used in central parts of Asia and India [12,14]. An addictive habit like oral and nasal use of naswar is a common practice in Pushtoons of Baluchistan and KP, which many studies have found to be a risk factor of EC [9,13].

Precise pathogenic factors and processes leading to EC are still not clear. Molecular studies of ESCC have revealed that genetic alterations such as mutation of T*p*53, loss of *p*16 and an increased expression of CDKN2A play a role in causing ESCC [15].

The classification of College of American Pathologist classifies ESCC in different grades; well, moderately and poorly differentiated carcinoma. The Pathological staging (pTNM) is done according to American Joint Committee on Cancer (AJCC)/ Union for International Cancer Control (UICC) [16], from stage 0- stage IV, depending upon the status of the primary tumor (T), lymph node invasion (N), metastasis (M), grade and location of the tumor.

Cyclooxygenase system plays a crucial role in the progression of esophageal carcinoma from esophagitis to dysplasia (mild, moderate and high) and invasive carcinoma (ESCC) [17]. High grade dysplasia is called carcinoma *in-situ* (CIS). Two isoforms of cyclooxygenase system are characterized as cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). COX-1 is constitutively expressed in most tissues and mediates the synthesis of prostaglandins to control normal physiological functions. COX-2 is an enzyme that mediates the synthesis of prostaglandins and thromboxanes which are the regulators of biological processes like inflammation, proliferation, angiogenesis, tumor growth and transformation [15,17]. Over expression of COX-2 has been reported in many premalignant and malignant tissues but its mRNA and proteins were either absent orat a low level in ordinary tissues. Overexpression of COX-2 was associated with aggressive nature of the tumor and showed reduced survival in many studies [17-19]. A study involving Chinese patients reports that the levels of COX-2 are sufficiently high in ESCC. This raises the possibility that selective inhibitors of COX-2 may be useful in the prevention of this ailment [20,21]. Overexpression of COX-2 was also seen in other epithelial malignancies especially in the gastrointestinal tract like stomach & colon and other viscera e.g. lung, bladder and head & neck [17,18] Prognostic significance is also mentioned in most of the tissues.

This study seeks to investigate the risk factors of EC in high risk population of Pakistan and neighboring Afghanistan. Expression of COX-2 in ESCC is also analyzed and its correlation with clinic-pathological parameters investigated, which has not been explored in depth so far.

MATERIAL AND METHODS

The study has been approved by the ethical review committee of the Institute of Basic Medical Sciences, Khyber Medical University. A total of 69 cases were collected from the cardiothoracic surgical units of Lady Reading Hospital (LRH) Peshawar and Rehman Medical Institute (RMI) Peshawar from March 2013 to March 2015. The Patients included 37 males and 32 females with age of 55.3 ± 13.73 years. All the patients who had been diagnosed with ESCC on endoscopic biopsy and had undergone esophageal resection for malignancy were included in the study. Patients who had, history of EC other than ESCC, had irresistible tumors and those who already had received chemotherapy or radiotherapy were excluded from the study. The patients included in the study were interviewed, after obtaining consent to seek information regarding patient's age, sex, occupation, address, education and personal habits like use of naswar, smoking, using alcohol, intake of hot beverages, raw fruits, vegetables and the level of proteins in typical diet. For the purpose of staging, CT scan reports of the patients enrolled in the study were collected. In the resected esophagus, site and size of the tumor was recorded. Surgical specimens were collected in 10% buffered formalin. Specimens were grossed and cut into sections which included macroscopically normal esophageal mucosa (n=41) (at a distance of around 5 cm from the tumor), adjacent dysplastic mucosa (n=19), tumor tissue (n=69) and any lymph nodes present in the resected specimen. Tissue processing was done for further histopathological evaluation with Haematoxylin and Eosin (H & E) staining to determine the type of tumor, grade, depth of invasion and relevant metastatic lesion in the retrieved lymph nodes, to stage the disease according to CAP protocol 2016 (AJCC staging) [16]. The tumor tissue, dysplastic tissue and normal healthy mucosawere further processed for immunohistochemical staining with a COX-2 biomarker. Clinico-histopathological results were correlated with COX-2 immuno-expression profile.

Immuno-staining with COX-2 antibody

Immunohistochemical analysis was performed on representative blocks of each ESCC patient including the normal mucosa, dysplastic mucosa (where available) and corresponding tumor tissueshowing maximum cellularity. 3-4µm thick sections of formalin fixed paraffin embedded tissue were placed on salinized slides. The sections were de-paraffinized with xylene and rehydrated with descending series of alcohol and subsequently washed with distilled water. For antigen retrieval, deparaffinized slides were incubated in citrate buffer with pH 6 in a microwave for 20 minutes. Endogenous peroxidase activity was blocked by placing 3% Hydrogen peroxidase on the slides after rinsing the slides with phosphate buffer saline (PBS). Sections were then incubated with commercially available mouse monoclonal antibody to COX-2, clone CX 294 (Dako, Denmark) for 30 minutes, and then treated with secondary antibody i.e. Flex HRP. After further washing, Diaminobenzidine Tetrachloride

(DAB) chromogen was added. Slides were counterstained with Haematoxylin and subsequently washed. The immunostained slides were assessed by two Histopathalogists independently. In case of conflicting opinion of the two histopathologist, the cases were reviewed again by a third histopathologist. Appropriate positive control (Smooth muscles of the same tissue) and negative control of the normal healthy mucosa of the same patient (where available) were run with each batch.

Scoring of tumor tissue (ESCC), corresponding CIS and normal healthy mucosa

In this study samples were scored semi-quantitatively by light microscopy with 40x magnification, considering the proportion of stained cells and intensity of staining. In all the cases, 5 High Power Fields of representative areas of neoplastic squamous epithelial cells were selected and a minimum of 800-900 cells were counted manually (Chakhachiro et al., 2013). Proportion of stained cells were scored as 0 (0%), 1(1%), 2(1-10%), 3(11-33%) 4(34-66%), 5(>66%) respectively (Majidi et al., 2014) [22]. Staining intensity was assessed on a scale 0 (negative), 1+ (weak), 2+ (moderate) and 3+ (strong) as reported in other studies [15,18,23]. Immunohistochemical total score was determined with the product of intensity and proportion score. This histoscore has a possible range of 0-15. As in the study of Binghua Li, in this study median was used as the cutoff value for classification of patients in to low and high expression [24]. Median of the score was generated independently in tumor tissue, CIS and normal corresponding tissue using SPSS version 20. The histoscore above cutoff value of 6 was categorized as strong expression and equal to or below it as low expression or as negative. The ESCC was compared independently with the corresponding CIS and normal tissue.

Statistical analysis

SPSS version 20 was used for data entry and statistical analysis. To generate the confidence interval of ESCC, corresponding dysplastic (CIS) and normal healthy mucosa with the biomarker COX-2 expression, ANOVA Test was applied. Pearson's Chi-square test was applied to assess the correlation of the clinicopathological variables like age, Pakistani and Afghan population, unhealthy habits i.e smoking and using naswar, intake of hot beverages and dietary habits, tumor size, pathologic grade, depth of invasion, lymph node metastasis and (AJCC) staging of the disease with COX-2 immunoexpression. Differences were found to bestatistically significant with ap-value of <0.05 as reported in other studies [18].

RESULTS

Clinicopathological and immunohistochemical analysis was carried out in ESCC patients to evaluate impact of risk factors in ESCC and COX-2 expression in different stages of cancer development: normal squamous epithelium of 40 patients, dysplastic tissue (CIS) of 19 patients and invasive ESCC of 69 corresponding patients. Out of 69 patients of ESCC in our sample, 46 (67%) were users of Naswar. Moreover it was bit more in Afghan patients (Figure 1). Out of 69 patients, 67% were Pakistani and 33% were Afghans. Mean age of patients at the time of diagnosis was 55.3 $\pm$ 13.73 years (range 26-85years), 41% were $\leq$ 50 years of age and 59% were more than 50 years of age, consisting of 54% males and 46% females (1.7:1). In this study we find that dysphagiais the most common complaint in all the patients both for solid and liquid food. Low socioeconomic status was observed in 99% of the patients. All the patients belonged to labor class and were mostly consuming vegetables and low protein diet regularly. Rest of the risk factors like smoking, taking alcohol and consumption of hot beverages was studied which showed no significant correlation with clincopathological parameters.

In this study out of 69 ESCC patients, strong COX-2 expression was observed in 67% of the tumor tissue of ESCC patients whereas negative COX-2 expression was observed in 33% of patients including both the 80% true negative and 20% weakly positive (Figure 2). In neoplastic epithelial cells immunostaining was predominant in the peripheral region of the tumor nest compared to the inner cells. High grade dysplasia was found only in 28% of patients; macroscopically the tissue CIS turned out to be mostly tumor which was expected. Among these, 79% showed intense staining with COX-2 in upper 2/3rd of dysplastic epithelium while the basal layer was faintly stained (Figure 3A, Figure 3B) and negative expression was observed in 21% of the patients.The high expression of COX-2 in CIS tissue (n=19/69) may be due to relatively small no. of paired samples of dysplastic change (CIS) compared to total no. of samples of ESCC tissue. Normal healthy mucosa in 40 (100%) of these patients was negative for COX-2 expression.

The correlation of COX-2 expression in normal healthy mucosa, corresponding CIS and ESCC was generated with Annova test, the comparison of mean difference, significant p-Value and confidence interval among all the three tissues is as shown in Table 2A and Table 2B. The COX-2 Immuno expression in normal healthy mucosa compared to CIS tissue was significantly different with the mean difference of -9.529, p-value .000 and 95% (-9.38 to -5.36) confidence interval (CI). Same significant differences were observed when normal was compared with the ESCC with the mean difference of -7.370, p-value .000, 95% CI of (-9.05 -5.69). In comparison of CIS with ESCC, the mean difference was -2.159 with the p-value .128and CI (-4.6to -4.78), the difference is insignificant (Table 2B).

Strong immunoexpression of COX-2 was observed in 71% of patients in the age group $\leq$ 50 years as compared to 63% of the patients in the age group >50 years. However with the p-value $\geq$ 0.05, the result is statistically insignificant. High expression of COX-2 was seen in 54% user of naswar and 61% among non-users though the result is statistically insignificant (Table 3). The tumor size of 1-3 cm, 3-7 cm and >7 cm showed strong immunoexpression in 73%, 63% and 69% of the patients respectively with insignificant p-value. No significant correlation was noted with varying grades of disease, depth of invasion and lymph node metastasis. Mild increase in expression of COX-2 is observed with increasing stage of the disease albeit insignificant (Table 3).

DISCUSSION

ESCC is one of most lethal malignancies with dismal prognosis. Studies are required to develop the specific marker for targeted

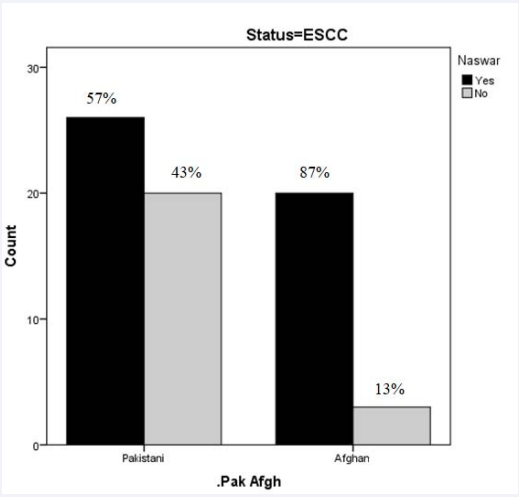

Figure 1 Use of naswar in Pakistani and Afghan patients suffering from esophageal squamous cell carcinoma (ESCC).

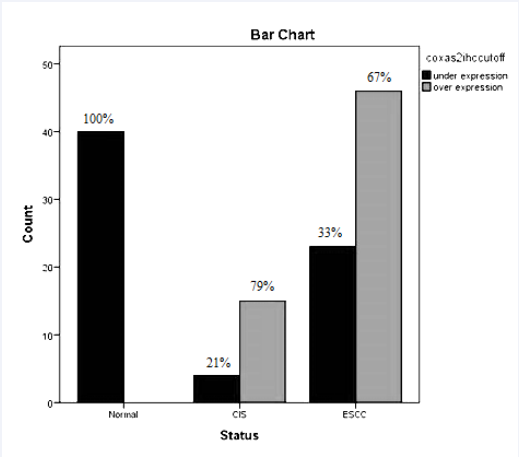

Figure 2 Expression of COX-2 in normal healthy mucosa, Carcinoma in situ (CIS) and esophageal squamous cell carcinoma (ESCC).

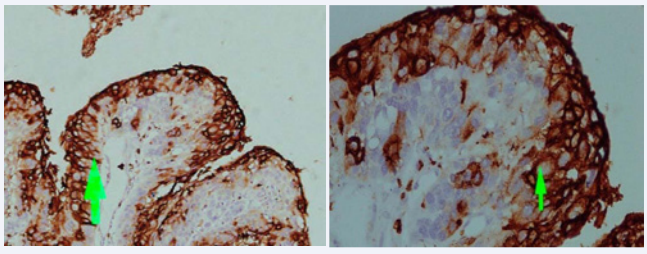

Figure 3 Immunohistochemical expression of COX-2 in Carcinoma *in situ*

Figure 3A: Immunohistochemical expression of COX-2 antibody in Carcinoma *in situ* (CIS) showing staining (membranous and cytoplasm) in upper 2/3rd of mucosa (green arrow head) at low power magnification (10x).

Figure 3B: Immunohistochemical expression of COX-2 antibody in Carcinoma *in situ* (CIS) showing staining (membranous and cytoplasm) in upper 2/3rd of mucosa (green arrow head) at high power magnification (40x).

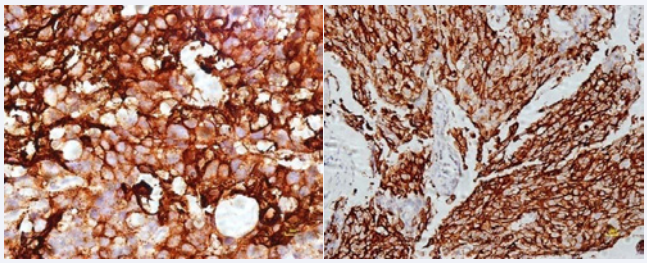

Figure 4 Immunohistochemical expression of COX-2 in esophageal squamous cell carcinoma

Figure 4A: Immunohistochemical expression of COX-2 antibody in esophageal squamous cell carcinoma (ESCC) at low power magnification (10x).

Figure 4B: Immunohistochemical expression of COX-2 antibody (membranous & cytoplasm staining) in Esophageal squamous cell carcinoma (ESCC) at high power magnification [40x].

Table 1: Frequency of Naswar Use in Esophageal squamous cell carcinoma in Khyber Pakhtunkhwa region of Pakistan.

Status	Naswar Use		Total
ESCC	User: No (%)	Nonuser: No (%)	No (%)
	46 (67)	23 (33)	69

Abbreviations: ESCC: Esophageal Squamous Cell Carcinoma;

personalized therapy of ESCC. Increased prostanoid activity is a well-recognized characteristic of gastrointestinal, breast and lung malignancies [22,25]. COX-2 catalyzes the conversion of free arachidonic acid into prostaglandin H_2, the precursor of other prostaglandins and thromboxanes and regulates the cellular processes including angiogenesis, proliferation and apoptosis. COX-2 enzyme is inducible gene in inflammation and carcinogenesis. However expression of COX-2 has not been explored in ESCC and its association with clinicopathological parameter is still not elucidated. Very few relevant studies have investigated the biomarker expression of COX-2 in ESSC. These have reported increased expression of COX-2 in ESCC, deduced through immuno-histochemical staining [26]. Study of the expression pattern of COX-2 in ESCC is important to evaluate the role of this gene in the development of cancer and to determine whether COX-2 selective inhibitors would be beneficial for the targeted therapy.

We conducted this study to observe the impact of risk factors in ESCC and to analyze the expression of COX-2 biomarker in ESCC. .

Mean age at the time of diagnosis in this study was 55 years while a study from Scotland showed median age at the time of diagnosis as 72 years [27]. This indicates the incidence of ESCC in younger patients for the sample used in this study, may have an association with the worse prognosis [27]. Samples from large number of patients of ESCC were collected in 2 years from two tertiary care hospitals of KP province. Those with irresectable esophagus at advanced stage of the disease, EC other than ESCC and those who had neoadjuvant chemotherapy or radiotherapy were excluded. Though no tumor registry exists in Pakistan, we observed high prevalence of ESCC both in local Pushtoons of KP

Table 2: Multiple comparison of COX-2 expression in normal healthy mucosa, CIS and tumor tissue of ESCC
Table 2A: ANOVA.

COX-2 IHC Score					
	Sum of Squares	df	Mean Square	F	p-value
Between Groups	1758.157	2	879.078	48.294	.000

Table 2B: Multiple Comparisons.

Dependent Variable: COX-2 IHC Score Tukey HSD					
(I) Status	(J) Status	Mean Difference (I-J)	p-value	95% Confidence Interval	
				Lower Bound	Upper Bound
Normal	CIS	-9.529*	.000	-12.35	-6.71
	ESCC	-7.370*	.000	-9.38	-5.36
CIS	Normal	9.529*	.000	6.71	12.35
	ESCC	2.159	.128	-.46	4.78
ESCC	Normal	7.370*	.000	5.36	9.38
	CIS	-2.159	.128	-4.78	.46

*. The mean difference is significant at the 0.05 level
Abbreviations: CIS: Carcinoma *in situ*, ESCC: Esophageal Squamous Cell Carcinoma

Table 3: Correlation of COX-2 immunohisto expression with clinicopatological parameters:

Clinicopatological parameters			COX-2 immunohistoexpression		Total N (%)	p-value
			Negative expression N (%)	Strong expression N (%)		
ESCC	Naswar	User	16 (35)	30 (65)	46 (100)	.718
		Nonuser	7 (30)	16 (70)	23(100)	
					69(100)	
ESCC	Age	≤50	8 (29)	20 (71)	28(100)	.488
		>50	15 (37)	26 (63)	41(100)	
					69(100)	
ESCC	Origin	Pakistan	13 22)	33 (78)	46(100)	.206
		Afghan	10 (25)	13 (75)	23(100)	
					69(100)	
ESCC	Smoking	Yes	7 (27)	19 (73)	26(100)	.380
		No	16 (37)	27 (63)	43(100)	
					69(100)	
ESCC	Tumor size (cm)	1-3	3 (25)	9 (75)	12(100)	.740
		3.1-7	15 (37)	26 (63)	41(100)	
		>7	5 (31)	11 (69)	16(100)	
					69(100)	
ESCC	Tumor Grade	I	9 (35)	17 (65)	26(100)	.588
		II	10 (38)	16 (62)	26(100)	
		III	4 (24)	13 (76)	17(100)	
					69(100)	
ESCC	Stage	1	8 (36)	14 (64)	22(100)	.541
		2	9(39)	14 (61)	23 (100)	
		3	6(29)	15(71)	21(100)	
		4	0 (00)	3 (100)	3(100)	
					69 (100)	

ESCC	L.N invasion	NX	8(40)	12 (60)	20(100)	.547
		N0	10 (38.5)	16 (61.5)	26 (100)	
		N1(1-2)	4 (21)	15(79)	19(100)	
		N2 (3-6)	1 (25)	3 (75)	4 (100)	
					69(100)	
ESCC	Depth of invasion	T1	0(0)	2 (100)	2(100)	.583
		T2	14 (33)	28 (66)	42(100)	
		T3, T4	9(36)	16 (54)	25(100)	
					69(100)	

Abbreviations: ESCC: Esophageal Squamous Cell Carcinoma; LN: Lymph Node; NX: Cannot Be Asscessed; N0: No Regional Lymph Node Metastasis; N1: Regional Lymph Node Metastasis Involving 1-2 Lymph Node; N2: Regional Lymph Node Metastasis Involving 3-6 Lymph Node; N: Number; T1: Tumor Invades Lamina Propria or Submucosa; T2: Tumor Invades Muscularispropria; T3: Tumor Invades Adventitia; T4: Tumor Invades Adjacent Structures; Tumor grade: (I: well differentiated, II: moderately differentiated, III: Poorly differentiated). (According to AJCC Staging

and Afghans in relation to various demographic characteristics. At other places within Pakistan even higher the prevalence has been reported, only Karachi accounts for 5% of all the cancers in Pakistan. Quetta is a city in Northern Pakistan where ESCC is the third most common cancer in men. It is noteworthy that the province of KP and the Quetta city are in close proximity to Afghanistan and Iran where this disease is endemic [27].

In our study all male patients were either farmers or laborers while female patients were mostly housewives. Most of the patients are from socioeconomic deprived areas, the rural areas of KP and Afghanistan. Other studies have also reported that ESCC is higher in deprived localities [8].

There is limited published data that have investigated the impact of naswar in ESCC in Pakistan especially in KP [28]. This study highlights the frequency of naswar in high risk population of KP. Majority of our patients use tobacco in different forms like dipping snuff (naswar) as pinch which is placed under the lip between the gingival and buccal mucosa, snuff inhalation and eating naswar. All naswar users were using naswar as pinch, 8-10 times/day for a long period of time since the age of adolescence. Out of 69 patients of ESCC in our sample, 46 (67%) were users of Naswar (Table 1), which is consistent with other published data [12-29]. Although use of naswar was greater in patients suffering from ESCC but we could not identify it as risk factor because normal individuals (non ESCC) were not included in this study for comparison. Major carcinogenic constituents of naswar/snuff are N-nitrosamine and nitrites. Others include cadmium, lead, polonium, formaldehyde and phenolic compounds [13,14]. ESCC may have an association with intensity, duaration and cumulative amount of snuff use. The concentrations of N-nitrosamine and nitrites are increased in fire and air cured processing of loose tobacco leaf. Method of processing of snuff has not been elucidated in this study. Our study deduced that naswar use by the patients in KP and Afghan may be a major risk factor of ESCC.

Drinking alcohol is typically not practiced in this part of the world, which is one of the major risk factor in other high risk regions. Almost all of the patients in our study were primarily vegetarians and were not taking hot beverages or spicy food, rather they consumed animal protein, or fruits occasionally. This finding is different from the results reported in relevant literature, showing a low intake of vegetables (fiber diet) as high risk factor for ESCC [1].

The concept of cancerization in epithelial malignancies was first proposed by Slaughter in 1953. It was proposed that genetic alteration in the progenitor/stem cells results in monoclonal unit of altered cells in the field of lesion. Expansion of these genetically transformed cells acquires further alteration in the contiguous field replacing the normal mucosa in the preneoplastic (dysplastic) lesion field [30,31]. COX-2 enzyme over expression has been noticed in the early preneoplastic field of ESCC [32].

We analyzed precise pattern of COX-2 expression in normal esophageal mucosa, dysplasia, CIS (High grade dysplasia) and invasive ESCC, its correlation with clinicopathological parameters was also investigated. Staining pattern of COX-2 in CIS (Figure 3A & B) and ESCC (Figure 4A & B) was both cytoplasmic and membranous. In some of the cases, staining was seen in the cells at the periphery of the tumor cell nests, suggesting that COX-2 may be associated with the proliferation of the tumor cells. In some studies, it was revealed that a high expression of COX-2 was associated with tumor proliferation and carcinogenesis [26,33].

This study using intensity of staining and percentage of stained tumor cells in all the tissue types, finds variance of expression in ESCC, CIS and normal healthy mucosa (Table 2). Correlation of gene expression distinguished clearly the tumor and CIS tissue from the non tumor tissue. Strong COX-2 over expression was found in 79% of dysplastic mucosa (CIS) and 67% of corresponding invasive ESCC patients compared to negative expression in normal esophageal mucosa (Figure 2). This suggests that induction of COX-2 may have a pivotal role in the progression of epithelial cell transformation to dysplastic epithelium and invasive cancer in esophageal mucosa. Our finding supports the study of Naoki Hashimoto and Zimmermann [20,21], who also noticed immunoexpression of COX-2 in ESCC in 91% of the cases and suggested that COX-2 derived prostaglandin plays an important role in the regulation and proliferation of tumor cells [20,21]. Similarly in the study of Cui Y COX-2 immunoexpression was noticed in 67% of the ESCC patients. However the study finds that COX-2 expression was significantly associated with

lymph node metastasis and a poorly differentiated degree [34]. While in another study of Hu et al. [18], strong expression was found in 3/4th of the ESCC individuals. These values are quite high than our finding. The variance in the findings could be due to a different sample size and a different ethnicity background of the patients. Out of 46 positive cases of COX-2 expression, corresponding dysplastic epithelium was seen in 19 patients. Out of these 19 patients, in 80% the upper 2/3rd showed 3+ intensity of staining (Figure 1A). These results of dysplastic epithelium are different from other studies, in which mostly basal cells or all the dysplastic cells show COX-2 over expression [22]. Another interesting finding that high grade dysplasia/carcinoma *in-situ* (CIS) associated with invasive tumor was intensely stained in 80% of the dysplastic epithelium. This suggests that COX-2 over expression can also be detected in the early stage of the disease. COX-2 over expression was also noticed in dysplastic and ESCC tissue in the study of Yu HP 2004 [32] compared to normal mucosa, supports the view of preneoplastic field of cancerization introduced by slaughter in 1953. These results are concordant with the studies carried out by Zhi et al. (2005) [26], and Majidi et al., (2014) [22]. We deduced from our findings that COX-2 expression plays an important role in the progression of the disease and could help the patients to be diagnosed and treated in the early stages of the disease.

A large number of patients below the age of 50 years were found with a high expression of COX-2 which may be associated with the prognosis of the disease. Alidina [27] finds that ESCC at the age ≤ 55 years influence survival. This contrasts with Hu et al. [18], who find a high expression of COX-2 in patients aged 50 years or more. Patients of ESCC enrolled in this study showed increase frequency of naswar use. This may be due to a difference in the personal and cultural habits as well as ethnic differences. No significant difference of COX-2 expression was noticed among naswar user and non users.Though consumption of alcohol is considered as a risk factor in some studies [1], however, our patients had no history of consumption of alcohol. Similarly, smoking history was also correlated but *p*-values were not significant. Relevant literature also suggests that in less developed countries, smoking does not play a role in prevalence of high ESCC [17].

There was no remarkable statistical difference in patients with increasing tumor size, varying grades, lymph node invasion, and depth of invasion, stage of the disease and their expression of COX-2 (Table 3). The lack of over expression of COX-2 with statistically insignificant clinico pathological variables could be due to lesser number of cases at our disposal. Our results are similar to the Kuo et al. [33], who find that COX-2 over expression was associated with fewer metastasis and less advanced malignancy. Whereas our results are not consistent with the findings of other studies [26,35-38], showing COX-2 over expression was significantly associated with the tumor invasion and disease stage.

In several studies it was noticed that COX-2 promotes proliferation, angiogenesis and inhibits apoptosis [39]. In the current study overexpression of COX-2 in CIS and ESCC tissue may be associated with the development and progression of the disease. Our finding suggests that COX-2 can be a potential predictive biomarker, correlating with the other studies. Selective inhibitors of COX-2 like non-steroidal anti-inflammatory drugs could possibly play a role in the prevention of such malignancies, as reported for its beneficial effect in different malignancies [17].

Two limitations of the study are noteworthy. First, the sample size of this study being relatively small, to assess the correlation of COX-2 expression with the clinicopathological parameters, a larger scale study could be beneficial. Second, as normal individuals i.e. those not suffering from cancer, which use naswar were not the subject of the study, we could not establish whether or not naswar is a risk factor even though a large number of patients were found to be using naswar.

CONCLUSION

Naswar use is more common in patients of ESCC in the KP province of Pakistan and is even more prevalent in the neighboring Afghanistan. We observed high prevalence of ESCC despite the high use of vegetable diet and non-use of spicy food and hot beverages. The high occurrence of this disease demands aggressive measures to prevent the incidence of this disease. It also highlights the need for improved public health practices to reduce the addiction to tobacco and snuffing. The cohort 23-85 years shows statistically significant difference in expression of COX-2 gene in ESCC and CIS tissue sample compared to normal healthy mucosa. Over-expression of COX-2 is positively associated with ESCC.

Further studies on a larger scale are needed to confirm the prognostic and predictive value of COX-2 in this high risk population. High COX-2 expression in ESCC may potentially be beneficial using COX-2 inhibitors.

ACKNOWLEDGEMENT

We are thankful to Higher Education Commission of Pakistan (HEC) for funding this project (HEC-NRPU-4222) and providing salary to Shahida Tasneem.

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Cite this article

Tasneem S, Sarwar MT, Bashir MR, Hussain H, Ahmed J, et al. (2018) Expression Analysis of COX-2 in Patients Suffering from Esophageal Squamous Cell Carcinoma. J Cancer Biol Res 6(2): 1116.