Original Article

EGFR over-expression in Head and Neck Squamous Carcinoma & it's correlation with etiological factors like alcohol and tobacco

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Abstract

Objectives: The present study was carried out to investigate the possible association of Epidermal Growth Factor Receptor (EGFR) expression with personal habit as possible risk factors for development of Head Neck Squamous cell carcinoma (HNSCC).

Study design: Immunohistochemical analysis was performed on the samples obtained from 103 patients with HNSCC, 25 patients with pre-cancerous condition and 116 healthy controls. The data was analyzed using parametric and non parametric tests. The categorical data was analyzed by chi square test. Correlation analysis was also performed.

Results: In our study in carcinoma group, correlation coefficient for personal habit status of the patient with EGFR expression was -0.031 and p=0.755 which is not significant. The EGFR expression intensity is higher in cancer patients compared to the normal subjects. This difference is statistically significant (p=0.000).

Discussion: EGFR expression was high in patients with oral cancer compared to premalignant and malignant diseases. However, our study does not show any association of risk of development of HNSCC due to the consumption of cancer causing products to personal habits of the subjects.

Introduction

Head and neck cancers are considered among the 10 most common cancers globally [1]. According the International Classification of Diseases (ICD), Head and Neck cancer can occur in oral cavity, pharynx and the larynx (ICD-10, C00.0-14.0). Oral cavity cancers include cancers of the tongue, mouth, gum, floor of the mouth, palate and other unspecified parts of the mouth (ICD-10, C01.0-06.0). In developed countries HNSCC accounts for 6% of all cancers whereas in developing countries it accounts for 30% [2]. 90% cases of H&N cancer are squamous cell carcinoma as they arise from the squamous cell epithelial of the mucosal lining [3]. About one fourth of all cancer diagnosed in men is Head and Neck Squamous cell Carcinoma (HNSCC) while, in case of females, only one tenth of all the cancers diagnosed are HNSCC [1]. Men are affected twice as often as compared to women. Of all the cancers head and neck cancers in India accounted for 30 % in male and 11-16 % mortality in female. Over 200,000 cases of Head and Neck cancers occur each year and nearly 80,000 are diagnosed every year in India. Cancer of the oral cavity is the major site in India whereas cancer of the pharynx is the most common site in France [4]. In India, Dibrugarh (Assam) has reported the highest incidence of head and neck cancers i.e. 49.6%. [2]. More than 90% of oral cancers occur in patients older than 45 years of age [5].

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The overall 5-year-survival rate is still around 50-60% [6]. Despite many advances in the treatment there is no respectable improvement in the survival rate of head and neck squamous cell carcinoma. Patients are presented with advanced stage of the disease [7]. Even after the treatment of current era like surgery, radiation chemotherapy, patients are left with adverse effect of compromised face, speech and swallowing [8]. This ultimately diminished quality of life of the patients.

Etiologic agents involved in the development of Head and neck cancers include tobacco, alcohol consumption, diet, genetic susceptibility, certain chemicals, and radiations in addition to viral infections such as exposure to human papilloma virus (HPV) [9-11]. Tobacco and alcohol usage are the major risk factors, which lead to nutritional deficiencies, and susceptibility to various carcinogens and thus lead to immune suppression. Seventy five percent of head and neck cancers are attributed to tobacco and alcohol usage. Heavy alcohol drinkers are frequently heavy smokers as well [12-15]. The risk for development of oral cancer is 3 to 9 times greater in those who smoke or drink and as much as 100 times greater in those who both smoke and drink heavily than in those who neither smoke nor drink [16]. A wide variety of tobacco habits like bide smoking, tobacco chewing, and cigarette smoking account for a large majority of these cancers [17]. Strategies for prevention of H&N cancer might be much more effective if the individuals with increased risk could be identified before they develop HNSCC.

Over expression of growth factor is an added cause for cancer. These growth factors work as intermediate for several signal pathways. In head & neck cancer epidermal growth factor receptor (EGFR) is the most important growth factor that has been studied. A mature EGFR is a 170 kDa transmembrane glycoprotein. It is composed of a single polypeptide chain of 1186 amino acids residues [18-20].

Proteins dock on phosphorylated residues, leading to the activation of signaling pathways that promote cell growth, proliferation, differentiation, and migration. ErbB family ligands are EGF and transforming growth factor-a (TGF-a). The epidermal growth factor family of receptor tyrosine kinases consists of four receptors, EGFR (ErbB1), ErbB2 (Her2/Neu), ErbB3 (Her 3) and ErbB4 (Her 4). ErbB1 is also known as EGFR and HER. Alteration in the function of EGFR has been linked with oncogenic transformation, autonomous cell growth, invasion, angiogenesis and development of metastasis in several cancers. The present study was carried out to investigate the possible association of EGFR expression with personal habit factors acting as possible risk factors for development of HNSCC. Therefore, we compared the expression of EGFR by immunohistochemistry in normal individual patients with precancerous stage and patients of HNSCC using various parameters.

Material and Methods

The study was carried out in the department of surgical oncology, IMS, BHU from July 2007- Dec 2013. For sample collection of cancerous and premalignant cases, patients attending the O.P.D and those admitted in the Department of Surgical Oncology, Sir Sunder Lal hospital, BHU were selected. For normal subjects samples were taken from the Faculty of Dental Sciences, Sir Sunder Lal Hospital, BHU, Varanasi. The precancer lesions were classified clinically as described in earlier studies [21-23].

Sample collection

A total of 103 patients with HNSCC, 116 samples of subjects with normal oral cavity and 25 samples of subjects with precancer lesions were included in the study. A detailed history was taken and examination was carried out after obtaining the written informed consent from all the patients. The study was approved by the Ethical Committee of the Institute of Medical Sciences. Personal habits like use of tobacco and alcohol were
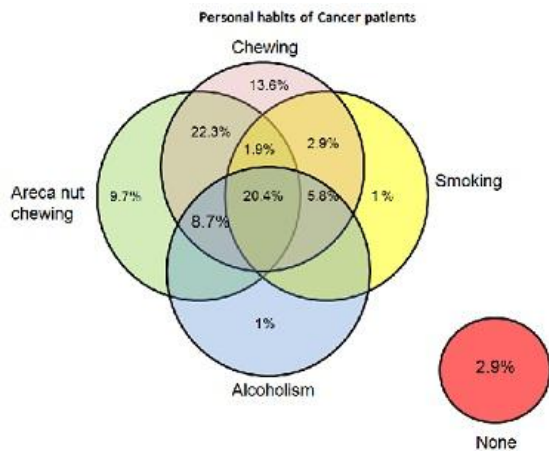
recorded and details of their consumption were also recorded.

Immunohistochemical Analysis of EGFR localization

Tissue processing: Tissues obtained from normal individuals and patients fixed in formalin, paraffin blocks prepared and 4 micron sections cut. The sections were then processed for immunohistochemistry as outlined below

Immunohistochemistry for EGFR

4 μm sections were cut from the blocks and were deparaffinized in xylene followed by hydration in a graded series of alcohols. Sections were left under running water for 15 minutes. Endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 for 30 minutes at room temperature. After rinsing in TBS buffer (Ph7.4, for 30 min.), the sections were incubated with primary antibody against EGFR at 4°C overnight. After 3 washing with tris buffer for 10 min. each, covered the sections with secondary antibody. The details of primary and secondary antibodies used are detailed below. Wash the samples in TBS (3×10'). Incubate the section in ABC solution for 30 min. After 3 TBS washing, sections were counterstained with 3-3'-diaminobenzidine (DAB) followed by hematoxylin. Slides were washed in

running water and were mounted with DPX. EGFR SC-03 Monoclonal antibody (Santa Cruz, CA, USA) was used with Vectastain Elite ABC detection system.

The degree of EGFR was assessed quantitatively in each group by image analysis using the following score:

IHC score 0 = undetectable levels, IHC score (+) = 10-30 % immunoreactivity,

IHC score (++) = 30-60% immunoreactivity, IHC score (+++) = >60% immunoreactivity

Statistical analysis

The variables in the study were correlated with expression levels of EGFR and other patient data. The data have been analyzed using parametric and non parametric tests. The categorical data has been analyzed by chi square test. Correlation analysis has been done.

Results

In cancer group, 13.6% patients have habit of chewing tobacco, 1% has habit of smoking, 1% is involved in drinking alcohol and 9.7% are using areca nut. 20.4% are involved in all the activities. 2.9% patients are using tobacco for chewing as well as for smoking also. 5.8% patients are taking both, tobacco in the form of chewing and alcohol. Highest percentage of the patients i.e. 22.3% are taking tobacco for chewing in combination with areca nut. 3.9% patients have habit of chewing areca nut simultaneously with smoking. 5.8% patients have habit of chewing tobacco, smoking and also drinking alcohol. 1.9% patients are areca nut chewer with habit of taking tobacco in the form of chewing and smoking both. 8.7% are taking alcohol along with chewing tobacco and areca nut. However there are also 2.9% patients of Head and Cancer who do not have any of these habits (Figure 1)

Figure 2 depicts the personal habit of patients in precancerous group. In precancerous group 4%
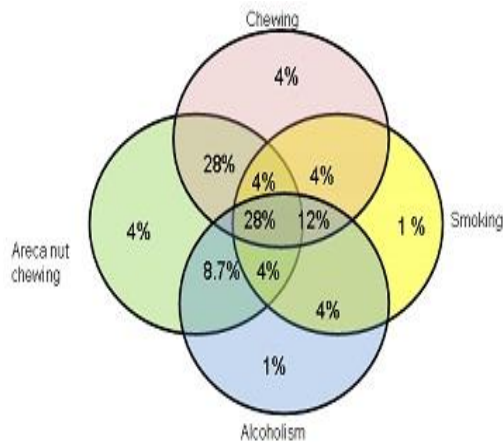
Figure 2: Personal habits of patients of precancerous group

patients are addicted to tobacco chewing, 1% for smoking, 1% for alcohol and 4 % are for areca nut chewing. The number is also higher in patients (28%) who have habits of chewing tobacco and areca nut both. 4% patients are habitual to use tobacco for chewing and smoking, 12% for chewing, smoking and alcohol, 4% for smoking, alcohol and chewing areca nut. 8.7% patients are using alcohol with chewing habit of areca nut. As in cancer group, precancerous patients also have a habit of all chewing tobacco, smoking, drinking alcohol and chewing areca nut and the number of patients with these habits is 28%.

EGFR expression in all three groups

Patients in all the three groups expressed EGFR. 69/116 of the normal subjects, 24/25 patients with premalignant lesions and 100/103 cancer patients expressed EGFR. However the intensity is higher in cancer patients compare to normal subjects. This difference is statistically significant (p=0.000).(Table 1)

Table 2 shows cross tabulation of EGFR expression in Cancer, precancerous and normal groups by IHC with various factors. In cancer group, 89 male patients in carcinoma group shows positive results for EGFR staining whereas only 3 shows negative results. all of the 11 female patients showed EGFR

Table 1: Intensity of staining of EGFR in three study groups.

Intensity of staining	Normal	Premalignant	Malignant
0	47	1	3
1	66	3	15
2	3	8	27
3	0	13	58

expression. For this group p value is 0.543. In precancerous group, 20 male patients are EGFR positive and only 1 is EGFR negative. For female patients, EGFR expression is positive in all 4 patients. p=0.656.In normal group, majority of the male participants (46) are EGFR positive. Out of 72 male patients, 26 are EGFR negative whereas EGFR expression in female subjects shows better results as compare to male. Out of 44 patients, 23 show positive EGFR staining. p value for this group is 0.216. The p value of all the three groups proves that gender has no significant role with EGFR expression by IHC.

EGFR expression is cross tabulated with the marital status of carcinoma and precancerous patients. Out of 101 married patients of carcinoma group, 98 show EGFR expression and only 3 in widowed. p=0.805.In precancerous group, EGFR expression have been shown in 23 married and 1 widowed patient and the p value is 0.835. Marital status of patients has no significant role with EGFR expression by IHC.

In cancer group, 95 Hindu patients have been included. Out of 95, 92 patients are showing EGFR expression and the p value is 0.610.In precancerous group 23 Hindu patients have shown positive results for EGFR by IHC and the p value is 0.001. This p value is mainly because of the higher number of Hindu patients in this group. Out of 25 patients, 23 patients are of Hindu religion.

Out of 103 patients in carcinoma group, only 2 patients have history of Head and Neck cancer in their family and both the patients have EGFR expression by IHC. 98 patients who don't have a

Table 2: EGFR expression analysis by IHC with various factors

		Carcinoma			Precancerous			Normal		
		Ab	+	p value	Ab	+	p value	Ab	+	p value
Gender	Male	3	89	.543	1	20	.656	26	46	.216
	Female	0	11		0	4		21	23	
	Total				1	24		47	69	
Marital statuts	Married	3	98	.805	1	23	.835	-	-	-
	Unmarried	O	2		0	1				
	Total	3	11		1	24				
Religion	Hindu	3	92	.610	0	23	.001	-	-	-
	Others	0	8		1	1				
	Total	3	10		1	24				
Family History H&N	Yes	0	2	.805	0	0		-	-	-
	NO	3	98		1	24				
Family History Any Ca	Yes	0	10	.564	0	2	.763	-	-	-
	NO	3	90		1	22				
Personal Habit Status	Current	1	13	.583	1	13	.366	-	-	-
	Past	2	84		0	11				
	Never	0	3		0	0				
Personal Habit Amount	Daily	3	92	.878	1	23	.835	-	-	-
	Occasional	0	5		0	1				
	Never	0	3							

history of Head and Neck cancer in their family express EGFR by IHC. There is no significance (p=0.805) of family history of Head and Neck Cancer with EGFR expression by IHC. In precancerous group, all the patients included in this study don't have family history of Head and Neck cancer. Except one patient, all have shown EGFR expression. P value can't be calculated for this factor.

EGFR expression has been cross tabulated with history of any type of cancer in patient's family. In Cancer group, 10 patients who have history of any type of cancer in their family Express EGFR and 90 patients who do not have history of any type of cancer express EGFR by IHC P value is 0.564. In precancerous group p value is 0.763. Both the patients with family history of any type of cancer in their family have shown EGFR expression and 22 patients who do not have this type of history

express EGFR by IHC. p values of both the groups show that there is no significance.

In the cross tabulation of EGFR expression with patients habit status, 84 in carcinoma group who left the habit of chewing or smoking tobacco, alcohol and areca nut have shown positive results for EGFR expression. In precancerous group also the highest number of patients for positive EGFR expression is of patients who have left these habits. p value for cancer group is 0.583 and for precancerous group is 0.366 and both values show that there is no significance of habit status with EGFR expression by IHC.

Majority of the patients who take these carcinogenic products daily express EGFR by IHC in carcinoma group and same results are in precancerous group. p value for cancer group is 0.878 and for precancerous is 0.835. EGFR expression has no significance with this factor.

Discussion

EGFR is a transmembrane tyrosine kinase receptor of the erbB-family that is normally expressed at low levels on the surface of most normal cells. EGFR is over expressed in head and neck tumors. Over expression of EGFR has been associated with a more aggressive clinical behavior, resistance to treatment and a poor prognosis. Studies have shown EGFR over expression as an independent prognostic marker of survival in betel quid chewers and a component of a prognostically significant molecular profile. Signal transduction from activated transmembrane receptors like EGFR depends on a variety of downstream mediators that are frequently altered in various cancer types.

Activation of the EGFR results in the initiation of a diverse array of cellular pathway. In response to toxic environmental stimuli, such as ultraviolet irradiation, or to receptor occupation by EGF, the EGFR forms homo- or heterodimers with other family members [24].

Each dimeric receptor complex initiates a distinct signaling pathway by recruiting different Src homology 2 (SH2) -containing effecter proteins. Dimerization results in autophosphorylation initiating a downstream cascade culminating in cellular responses such as cell proliferation or apoptosis. The activated EGF-R dimer complexes with the adaptor protein, Grb, coupled to the guanine nucleotide releasing factor, SOS. The Grb-SOS complex can either bind directly to phosphotyrosine sites in the receptor or indirectly through Shc. These protein interactions bring SOS in close proximity to ras, allowing for ras activation. This subsequently activates the ERK and JNK signalling pathways that, in turn, activate transcription factors, such as c-fos, AP-1, and Elk-1 that promote gene expression and contribute to cell proliferation [25].

Alcohol and smoking also increase the EGFR expression. The role of EGFR in the development of premalignant tissue changes which are probably influenced by chronic toxic irritation.

EGFR2 (Her 2/New) can also be used as a marker in distinguishing normal oral mucosa (NOM)/epithelia l dysplasia (ED) from OSCC. The significant increase of Her 2 makes it a valuable marker for OSCC. Not only EGFR gene's amplification is responsible for squamous cell carcinoma of the head and neck but also the increased EGF binding capacity [26]. EGFR also indirectly helps in tumor formation. EGFR directly interacts with β- catenin which is an E- cadherin-mediated cell adhesion molecule. This interaction includes tyrosine phosphorylation of β- catenin which causes dysfunction of the E- cadherin-mediated cell adhesion in cancer, resulting in increased cell motility, invasion and metastasis [27].

In our study personal habit of patients have not shown any significance with EGFR expression. In carcinoma group, correlation coefficient for personal habit status of the patient was -0.031 and p=0.755. It has been found that individuals of all the three groups expressed EGFR. However the intensity is higher in cancer patients compare to normal subjects. This difference is statistically significant (p=0.000). Few other studies also showed relationships between the above mentioned etiological factors and development of HNSCC and its prognosis [27-36]. However, further studies are necessary to clarify the role of these factors in modulating the EGFR expression and its function.

Competing interests

The author(s) declare that they have no competing interests.

Ethical Considerations

Authors declare that the present study was approved by the Institute Ethics Committee.
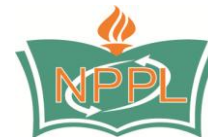
Abbreviations

Head and Neck Squalors cell Carcinoma (HNSCC), epidermal growth factor receptor (EGFR), Head and neck cancer (H&N cancer), Oral Squalors cell carcinoma (OSCC)

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World Journal of Pathology