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SALIVARY EGFR AS BIOMARKER FOR ORAL CANCER & PRE-CANCER :A CASE CONTROL STUDY

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ABSTRACT

Oral cancer is one of the leading cause of cancer in India, new diagnostic modalities for early diagnosis and treatment will increase the survival of the patients. The present study was carried out with an aim to evaluate salivary levels of EGFR in oral cancer and pre-cancer as tumor marker. For this purpose an observational case-control study was carried out in which a total of 72 subjects were enrolled.Of these 24 (33.3%) were patients of premalignant oral lesions and 24 (33.3%) were patients of malignant oral lesions& 24 (33.3%) subjects were normal healthy controls. Demographic information and clinical data was obtained, estimation for EGFR was performed in saliva. In premalignant cases, buccal mucosa was the most common site involved (87.50%) whereas in malignant cases tongue was the most common site involved (n=10;41.67%). Mean salivary EGFR levels were higher in malignant cases (0.23±0.17 pg/ml) and low in controls (0.10±0.19 pg/ml). Mean EGFR levels in premalignant cases were 0.12±0.22 pg/ml. statistically, this difference was not significant (p=0.052). Statistically, no significant difference in mean EGFR levels among different TNM stages could be seen (p=0.145). EGFR levels showed a potential to discriminate between malignant and premalignanat cases but this difference was statistical insignificant due to lower sample size.

KEYWORD: Epidermal Growth Factor, Squamous Cell Carcinoma, Salivary Biomarker.

INTRODUCTION

Oral cancer is a major health problem in the Indian subcontinent where it ranks among the top three types of cancer in the country (Elango*et al.*, 2006)(1). Age-adjusted rates of oral cancer in India arehigh that is, 20 per 100,000 population and accounts for over 30% of all cancers in the country (Sankaranarayanan*et al.*, 2005)(2). Increase in prevalence of oral cancer is observed due to cultural practices such as betel-quid chewing, and varying patterns of tobacco and alcohol use are risk factors that predispose to cancer of the oral cavity(Mamta Agarwal *et al*.2012,Sree Vidya Krishna Rao*et al.*,2013)(3). Smoking and smokeless tobacco usage are identified as the major risk factors(Sadeq Ali Al-Mawer*iet al*.,2014)(4). Infection with high risk HPV genotypes 16 & 18 is also a risk factor for the development of Oral squamous cell carcinomas (Suyamindra S Kulkarni *et al.,*2011)(5).Oral cancer is often preceded by premalignant lesions such as oral submucous fibrosis, leukoplakia and lichen planus. An early recognition and timely intervention is the key to successful management of oral cancer and its prevention from attaining a malignant stage.Although, histopathology is the gold standard yet a number of diagnostic tools including clinical assessment, biochemical and laboratory assessments are performed with variable accuracy.

Saliva as a biofluid is historically well studied biochemically & physiologically, has entered the postgenomic era where its proteomics, genomics &microbiomic constituents have been comprehensively deciphered. An increasing number of systemic diseases and conditions, amongst them oral cancer, have been shown to be reflected diagnostically in saliva. Moreover, using saliva as a diagnostic fluid meets the demands for inexpensive, noninvasive, and accessible diagnostic methodology (Markopoulos *et al.*, KMK Masthan*et al.*,2010)6,7.

Significant difference in level of various proteins ,mRNA, enzymes is seen in saliva of OSCC and control patients these could help in early detection of disease,(8).

Saliva has been found to contain constituents that reflect the diseased or physiological state of the human body, and hence could be utilized for diagnostic purposes (Wong, 2006; Wong, 2006; Castagnola*et al.*, 2011)(9-11), Salivary biomarkers, whether produced by healthy individuals or by individuals affected by specific disease, are sentinel molecules that could be used to scrutinize health and disease surveillance.

Saliva has been long proposed and used as a diagnostic medium (Malamud, 1992; Strekfus and Bigler, 2002)(12,13), because it is easily accessible and its collection is non-invasive, not time-consuming and inexpensive, requires minimal training and can be used for the mass screening of large population samples.

Various biomarkers have been associated with the pathway included with oral cancer like IL-6, IL-8, TNF-α, MMP, Cyclin D1, Ki-67, Maspin, Endothelin-1, EGFR, Her-2, EGF (Balicki *et al.*, 2005; Pickering *et al.*, 2007; Shpitzer*et al.*, 2009; Bernardes*et al.*, 2010; Cheng *et al.*, 2011; Cheng *et al.*, 2014;Rajkumar Krishnan *et al.*,2014)(14,-19). Epidermal growth factor receptor (EGFR) is a 170-kilodalton (kD) trans membrane cell-surface receptor. It is a tyrosine kinase (TK) receptorthat is commonly altered in epithelial tumors.

The present study was planned with an aim to evaluate salivary levels of EGFR in oral cancer and pre-cancer as tumor markers.

MATERIAL AND METHODS

The present study was undertaken at the Department of Pathology Era's Lucknow Medical College, Lucknow. A total of 72 subjects were enrolled for the study. 24 cases of premalignant oral lesions, 24 cases of squamous cell carcinoma & 24 healthy age and gender matched controls. The samples taken were saliva from cases and controls. Tissue biopsy from cases for confirmation of diagnosis. With the consent of the patient's whole, unstimulated saliva was collected from cases and controls (Salimetrics, LLC; 2009),(20).

The subjects were asked to accumulate saliva in the floor of the mouth before spitting in a vial kept on ice every 60 seconds. Approximately 5ml of saliva was collected for analysis.Following collection, saliva samples were centrifuged at 2600g for 15 minutes at 4 degree Celsius. Proteinase inhibitor cocktail (P2714 Sigma Aldrich) was added to each milliliter of the supernatant to prevent protein degradation. All samples were stored at -80 degree Celsius until further used.

Tissue biopsy was also taken from the same patient after collection of saliva. Once the biopsy was taken it was further subjected to routine H & E staining procedures by taking appropriate steps on formalin fixed paraffin embedded tissue. Once the procedure was carried out, their results were compared and noted.

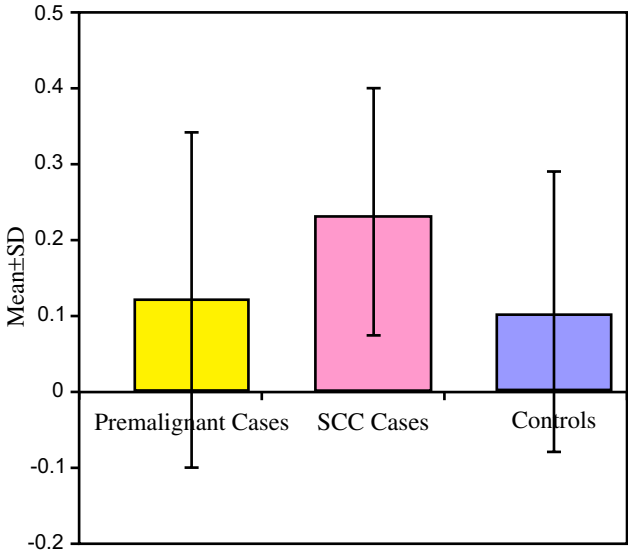
Salivary EGFR was analyzed with ELISA using kit (SEA757Hu) Uscn life Sciences Inc .Results were tabulated and subjected to analysis by SPSS 15 software.

RESULTS

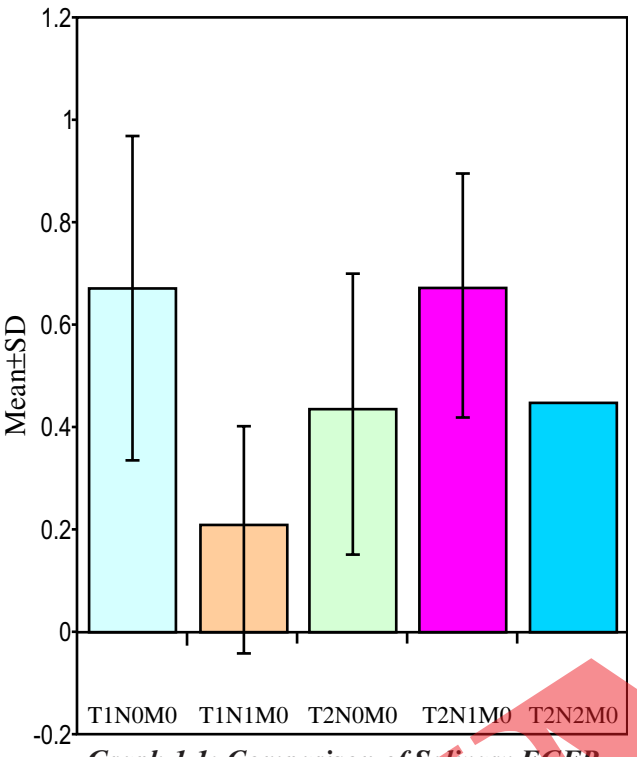
Age of cases ranged from 13 to 70 years. Majority of cases were aged 40 years and above (n=27; 56.3%).Buccal mucosa was found to be the affected site in higher proportion of premalignant cases (87.50%). Tongue was affected in higher proportion of SCC cases (41.67%)

Expression of EGFR(Table 1.0,Graph 1.0) was relatively much lower in control group and ranged between 0 and 0.64pg/ml. EGFR values of premalignant cases ranged between 0 and 0.66 pg/ml while range of EGFR in SCC cases was between 0 and 0.88 pg/ml. Mean EGFR values of controls (0.10±0.19 pg/ml) and premalignant cases (0.11±0.23 pg/ml) was found to be lower than that of SCC cases (0.23±0.17 pg/ml) and difference in EGFR values of controls, premalignant and SCC cases was not found to be statistically significant (p=0.052).An overlap of EGFR values of premalignant and controls was found. Extreme and outlier values were also found SCC cases and outlier values were observed in premalignant and Controls.

Among 24 cases of SCC, Salivary EGFR values was found to be lowest for T1N1M0 stage (0.17±0.21 pg/ml) and maximum for T1N0M0 (0.66±0.31 pg/ml) and T2N1M0 (0.66±0.24 pg/ml), (Table 1.1,Graph 1.1)difference in Salivary EGFR values of SCC cases of different TNM stages was not found to be statistically significant (p=0.145).



Graph 1.0: Comparison of Salivary EGFR levels in Premalignant Cases, Squamous Cell Carcinoma Cases and Controls



Graph 1.1: Comparison of Salivary EGFR values in different TNM Stage of Squamous Cell Carcinoma Cases

DISCUSSION

Of various biomarkers available, we evaluated and compared the expression of salivary EGFR with an objective to differentiate normal, precancerous and cancerous lesions. For this purpose a total of 72 salivary and tissue specimen were obtained from 24 cases of premalignant oral lesions, 24 cases of squamous cell carcinoma and 24 healthy volunteers.

Age of cases ranged from 13 to 70 years. Majority of cases were aged 40 years and above (n=27; 56.3%). There were only 2 (4.17%) cases in age group ≤20 years. Mean age of cases was 40.83±11.85 years. Similar to results in present study, Gambhir*et al.* (2011)21, found 63.0% of their patients to be above 40 years of age. However, Mathew *et al.* (2008)22, in their series, also found majority of oral cancerous and precancerous lesions in subjects aged >40 years. Oral lesions most commonly occurs in middle-aged and older individuals, although a disturbing number of these malignancies is also being documented in younger adults in recent years (Chen *et al.*, 1990; Llewellyn *et al.,* 2001; Schantz and Yu, 2002)23, 24, 25. Oral lesions in young adolescents and children have also been reported in literature. Mathew *et al.* (2008)21,in their study found nearly 9% of their cases to be below 20 years of age. In the series of Gambhir*et al.* (2011)20, 18.4% of patients were aged <20 years. In present study, only 1 (2.1%) case was below 20 years of age. The increasing prevalence of oral lesions in younger age groups might be attributed to increasing prevalence of adverse oral and dietary habits (poor oral hygiene, pan masala, tobacco, smoking, spicy food consumption) (Rana*et al.*, 2009; Jha and Parmar, 2011)26, 27.

In present study, all the malignant cases had oral squamous cell carcinoma. Oral squamous cell carcinoma is the most common type of oral cancer and generally has involvement of tongue (Mirboud and Ahing, 2000; Epstein *et al.*, 2008)28, 29. In a retrospective as well as prospective study of changing pattern of oral lesions carried out at Allahabad (India), Misra*et al.* (2009)30, also reported tongue to be more commonly involved in squamous cell carcinoma cases whereas buccal mucosa to be more commonly involved in premalignant cases in their prospective series. Similar observations have been reported in other studies too (Neville *et al.*, 2009; Shivakumar*et al.*, 2010)31, 32.

Yi Fan*et al.* did a study to analyze the survival rate in oral cancer patients and found out that disease free survival rate were significantly higher in patients with early-stage disease than with advanced stage(Yi Fan*et al.*,2014)(33**).**

In present study, it was observed that mean EGFR levels were minimum in Control (0.10±0.19 pg/ml) and premalignant groups (0.12±0.22 pg/ml) and maximum in SCC cases (0.23±0.17 pg/ml). None of the between group differences were significant (p>0.05). The relationship between EGFR levels and oral malignancy has yielded mixed results. Ino*et al.* (1993)34, in their study showed that salivary EGF levels were markedly low in patients with oral inflammations (stomatitis aphthous, or peritonsillar abscess) or head and neck tumors (squamous cell carcinoma of the tongue, oral cavity, hypopharynx or larynx). Bernards *et al.* (2010)16, in their study did not find a significant difference in salivary EGFR levels between OSCC cases and healthy controls before the surgical procedure. However, in their study they found mean EGFR levels of OSCC cases to be significantly increased by showing a multifold increase after the surgery. The finding thus suggests that post-surgery EGFR levels were influenced by removal of tumor. However, the authors could not provide a justifiable explanation for such fall. IHC expression of salivary EGFR has been shown to be significantly increased between pre-operative OSCC and healthy controls and showed a significant difference. Similar findings were made in our study using different estimation criteria but we could not

achieve a statistically significant association.

We also attempted to evaluate the association of TNM staging with EGFR levels and found a random but significant relationship between tumor staging/nodal involvement and EGFR levels (p=0.003). Although this relationship was statistically significant yet owing to fewer numbers of cases with each TNM stage, it would be hastier to generalize these results. For trend, the lone case of T2N2M0 stage had EGFR expression of 22.98 pg/ml while the range in other TNM stages ranged from 2.57 to 15.43 and mean values in different TNM stages ranged from 5.42±2.48 pg/ml to 7.96±2.29 pg/ml. Given the stage sample sizes ranging from 1 to 8 samples, it is difficult to find out any linear relationship.

With respect to salivary EGFR levels there are limited studies using the technique and methodology like ours. The study closest to our study was that of Ino*et al.* (1993)(33), and Bernardes *et al.* (2010)(16), but both had different end points. Serum EGFR levels have shown to have mixed outcome as far as significance of differences between SCC patients and controls is considered. Feng *et al.* (2010)(35), reported a significant difference while Gokhale*et al.* (2005)(36),did not find a significant difference between two. YasamanSardari*et al* (2012)(37), did a study to evaluate the salivary level and tissue expression of HER2/neu (a member of EGFR family) in patients with head and neck squamous cell carcinoma and observed that there was no overexpression of HER2/neu.

One of the reasons for such discrepancies is the fact that the level of research on this issue is in preliminary phase and the samples included in the study are fewer in number, lack a uniform estimation method and are exploratory in nature rather than being conclusive.

With respect to EGFR levels, the trends obtained in present study indicates that malignant cases generally had higher mean values as compared to premalignant and controls yet the association could not be proven statistically. High variability in range of EGFR levels was observed within each group, thus indicating that the EGFR levels are also affected by some confounding variables, however, the impact of removal of confounding effect by strict criteria could not result in exclusion of confounding effect.

On discussing the issue of high within group variability and possible impact of time of collection, posture at which collection was made, comorbidities and processing conditions.Multiple sampling might be a resolution for some of these issues. It is noteworthy to mention here that for markers that have a high within group variation in expression, the strategy of averaging the levels obtained for multiple specimens could be a better alteration that could be explored. However, in present study this proposition could not be tested and hence it is one of the recommendations of the present study for such estimations in future

The findings in present study, thus indicate a possible role of EGFR levels in differentiation of normal, premalignant and malignant oral lesions which is based on a sound theoretical basis but has been addressed using different methodologies and has limited human studies. The present study adds to the pool of knowledge on the issue and makes some recommendations based on the observations in the present study. The findings of the present study substantiate the observation that salivary EGFR has the potential to discriminate between oral tumors of different types. However, it is disappointing to see that there are limited studies on the issue and as such the work done so far is of pilot level, pinpointing the deficiencies in each study and providing stepping stones for further assessment. In view of high within group variability and exploration of the potential factors responsible for led us to assume that in order to neutralize effect of these potential factors multiple specimen taken at a suitable time apart could be the option. In view of the observations made in the present study, we recommend further studies on improved methodology and on a larger sample size.

CONCLUSION

EGFR levels showed a potential to discriminate between malignant& premalignant oral lesions but were not corroborated statistically. Standardization of estimation assays and multiple sampling is suggested as a possible improvement to yield more consistent and discriminatory outcomes. Further studies on these recommendations are suggested.

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