Prognostic implication of the loss of TGFBR2 expression in oral carcinoma

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Oral squamous cell carcinoma (OSCC) is a disease that strikes many worldwide, accounting for more than 145,000 deaths annually. This study examined the role of Transforming Growth Factor Beta (TGFβ) signalling alterations in oral carcinogenesis and also its influence on the disease prognosis. In presented study, we evaluated the protein-level alterations of core TGFβ signalling members in 20 potentially malignant oral disorders (PMDs) – leukoplakia & submucous fibrosis and 87 oral cancer samples by Western blotting. Further, we analysed the association between these alterations and prognosis of oral carcinoma. For statistical analyses, univariate test like Student's 't'-test to compare expression level of various genes and logrank test has been used to compare the Kaplan-Meier survival curves. The multivariate model such as Cox's proportional hazard regression was used to verify the independent influence of each variable on the survival endpoints. A gradual decrease in the expression of TGFβ signalling members like SMAD2, SMAD4, TGFBR1 and TGFBR2 have been noted from normal to PMD in oral cancers. The bio-activeforms of SMAD2/3 also showed a similar trend. SMAD3 protein was downregulated significantly to the PMD stage itself. Thus an inverse correlation was observed between expression of TGFβ members and oral cancer progression. Furthermore, oral cancer patients showing TGFBR2 downregulation exhibited poor disease-free survival (p=0.005) and poor overall survival (p=0.012). Thus, assessing the TGFBR2 protein levels can serve as one of the prognostic marker for oral cancer.

Key words: oral cancer,TGFBR2 expression, prognosis, Western immunoblot

Oral cancers comprise a group of malignancies that develop on lips, tongue, gingiva, floor of mouth and other unspecified parts of the mouth namely buccal mucosa, retro-molar and vestibular area [1]. The most common type of oral cancer is squamous cell carcinoma (OSCC) than the verrucous and adeno-squamous cell carcinoma, it accounts for more than 90% of the oral cancers [2]. Annually, oral carcinoma accounts for ~274,000 cases and ~145,000 deaths, of which two-thirds occur in developing countries [3,4]. Tobacco and betel chewing are main cause for such high prevalence of oral cancer in developing countries [5] and HPV infection rate is comparatively low in these populations [6].

Despite the advances in cancer treatment, the five-year survival rate of oral carcinoma is less than 50% throughout the world. Furthermore, this scenario has not improved over the past two decades [3,4]. Local and/or loco-regional recurrences are common in oral cancers, even in those patients who initially showed complete response to the treatment [7].

Thus, the oncologist has to bear many factors in mind while deciding and selecting the treatment protocol for oral cancer, as patients show varied response to each treatment plan which may be due to the differences in the molecular pathways altered during carcinogenesis. This scenario necessitates the development of molecular markers, which can divide patients into different molecular groups such that optimum treatments can be administered to each group.

TGFβ signalling is a well-known tumor suppressor signalling pathway. Binding of the TGFβ family of cytokines to the type II TGFβ Receptor (TGFBR2) can trigger the downstream signalling cascade. On ligand binding mediated activation, TGFBR2 forms heteromeric complex with type I TGFβ receptor (TGFBR1). The TGFBRI/RII receptor complex then conveys downstream signalling through the pathway consisted of SMADs, SMAD2 and SMAD3. They are commonly called receptor regulated SMADs or R-SMADs. The phosphorylated R-SMADs can form heteromeric complex with the common

mediator SMAD (Co-SMAD), SMAD4 and this enables the nuclear translocation of the complex and the expression of various target genes [8].

TGFβ signalling cascade is often altered in epithelial malignancies. Loss of TGFβ tumor suppressor pathway members like SMAD4, TGFBR1 and TGFBR2 accounts for roughly 50% of the solid tumors of pancreas as well as colon [9,10]. In one of the earlier studies, Kim et al. (1996) described the inverse correlation between the loss of TGFBR1 and TGFBR2 expression and the tumor grade in human prostate cancer[11]. Their results indicate that human prostate cancer cells frequently show a gradual loss of TGFβ receptor expression during cancer progression. Hence, it is possible that studies regarding the expression alteration of TGFβ signalling in cancer may provide novel biomarkers for cancer therapy. In addition, studies on the relationship between TGFβ signaling alterations and oral cancer progression are lacking. Therefore, in this work, we have evaluated the association between protein level alterations of TGFβ signaling members such as SMAD2, SMAD3, SMAD4, TGFBR1 and TGFBR2 in PMD and OSCC cases by employing Western-immunoblotting technique. We have also analyzed the bioactive forms of SMAD proteins- pSMAD2 and pSMAD3 levels in these samples [8]. Further, we have assessed the relationship between these alterations and clinicobiological features of oral cancers. The result shows that TGFBR2 expression level has been reduced in 60% of oral cancers samples and downregulation of TGFBR2 is significantly associated with poor treatment response. Hence, assessing the expression pattern of TGFBR2 before treatment may be useful to know the aggressive nature of the tumor and also it could may help in the treatment planning.

Materials and methods

Sample collection, ethics statement, and follow-up. OSCC samples and normal oral mucosa samples were collected from the Head & Neck Cancer Clinic of the Regional Cancer Centre, Thiruvananthapuram, India. The PMD samples and normal oral mucosa were collected from the Department of Oral Medicine and Radiology, Government Dental College, Kozhikode, India. Normal mucosa samples were collected from the buccal mucosa of healthy individuals visited the outpatient clinic for third molar extraction. All samples were collected after obtaining patient's written informed consent. This study was approved by the Institutional Review Board and the Human Ethics Committee. In total, 87 OSCCs, 20 PMDs and 25 normal samples were collected for the present study. The clinical staging of the patients followed the WHO criteria [12]. Standard treatment protocols were administered to the patients according to their tumor stage and individual clinical status, as we have detailed elsewhere [13]. After the treatment, all patients were actively followed-up at respective clinics, at an interval of four to eight weeks for a minimum period of 36 months [13].

Western immuno-blotting. Approximately 50μg of total protein isolated from the tissue samples were separated on 10% SDS-PAGE gel and transferred to polyvinylidene difluoride membrane (Millipore,Billerica, MA, USA). The membrane was blocked using 5% of BSA (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and the membrane was incubated with corresponding primary antibodies for two and a half hours. Primary antibodies against β-Actin, SMAD4, TGFBR1, TGFBR2, pSMAD2/3 and secondary antibodies were purchased from (Santa Cruz Biotechnology, Inc., Dallas, TX, USA. Primary antibodies against SMAD2 and SMAD3 were purchased from (Imgenex India Pvt. Ltd., Bhubaneswar, Odisha, India. Horseradish peroxidase (HRP) conjugated secondary antibody and Super Signal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL, USA) were used for detecting specifically bound primary antibodies. The immunoblots were documented in a Chemiluminescence cum Fluorescence Imaging System, FluorChem M (Protein Simple Inc., Santa Clara, California, USA).

Normalization of the data. The digitally acquired bands were quantified densitometrically using the Image J software (http://rsbweb.nih.gov/ij/) of National Institute of Health (NIH), Bethesda, Maryland, USA. In order to account for the variations arising from differences in protein load, we normalized the protein level expression data using β-Actin as loading control. The ratio between the intensity (densitometric value) of the band of interest (target protein) to loading control gene in the same sample has been calculated. In order to analyze the protein level expression variations of TGFβ members in oral carcinoma, the expression ratio TGFβ members in tumor samples were compared with the mean expression ratio of the same TGFβ member in normal samples. Altogether, 20 normal tissues were used for assessment. To reduce the bias, we considered ±20% expression variations from the mean expression ratio in normal as unaltered category (in order to account for the person to person variations in gene expression).

Statistical analysis. The associations between the expression-level alterations of analysed TGFβ members and various clinico-pathological factors and survival endpoints were examined as we have explained elsewhere [13]. Student's t-test was employed for comparing the expression pattern of various proteins in carcinoma samples with normal mucosa samples. The Kaplan-Meier survival plot with the Log-Rank test was used to assess the association of expression-level alterations of various proteins with survival endpoints such a as disease-free survival (DFS) and overall survival (OS). To assess the independent influence of factors that showed significance in the Kaplan-Meier survival analyses, the multivariate Cox's proportional hazard regression model was used. A two-sided p-value <0.05 was considered statistically significant unless otherwise noted.

Results

Disruption of TGFβ signaling is frequent in OSCCs. The clinico-pathological characteristics of the study cohort

are given in Table 1. The present study identified expression alteration of at least one TGFβ member evaluated in most of the OSCC samples, indicating the importance of TGFβ signal disruption during oral cancer progression. Notably, some of the analyzed OSCC samples showed SMAD3, SMAD4 and TGFBR2 expression as less as 10% of the normal samples

Table 1. Clinico-pathological characteristics of the study cohort

Clinico-pathological features	OSCCs	PMDs
All Cases	**87**	**20**
Sex		
Male	56(64.4%)	10(50.0%)
Female	31(35.6%)	10(50.0%)
Age (Mean age ± SD)	55.2 ±11.9	48.9 ±13.6
Site		
Tongue	40(46.0%)	1(5.0%)
Buccal mucosa	47(54.0%)	19(95.0%)
Habit		
None	12(13.8%)	2(10.0%)
Chewing	28(32.2%)	12(60.0%)
Smoking	3(3.4%)	1(5.0%)
Alcohol	5(5.7%)	2(10.0%)
Chewing+Snuff	10(11.5%)	1(5.0%)
Smoking+Alcohol	12(13.8%)	1(5.0%)
Chewing+Smoking+Alcohol	17(19.5%)	1(5.0%)
Histopathology		
Well differentiated squamous cell carcinoma (WDSCC)	18(20.6%)	
Moderately differentiated squamous cell carcinoma (MDSCC)	61(70.1%)	
Poorly differentiated squamous cell carcinoma (PDSCC)	9(10.3%)	
Early OSF		3(15.0%)
Moderate OSF		3(15.0%)
Advanced OSF		2(10.0%)
Leukoplakia with Mild Dysplasia		5(25.0%)
Leukoplakia with Moderate Dysplasia		4(20.0%)
Leukoplakia with Severe Dysplasia		1(5.0%)
Leukoplakia turning malignant		2(10.0%)
T-Status		
T1	9(10.3%)	
T2	29(33.3%)	
T3	16(18.4%)	
T4	31(35.6%)	
Not Available	2(2.3%)	
N-Status		
N0	28(32.2%)	
N1	29(33.3%)	
N2	27(31.0%)	
N3	1(1.1%)	
Not Available	2(2.3%)	
Disease Stage (OSCCs)		
I	6(6.9%)	
II	13(14.9%)	
III	21(24.1%)	
IV	45(51.7%)	
Not Available	2(2.3%)	

OSCC= Oral Squamous Cell Carcinoma; PMD=Potentially malignant oral disorders; OSF=Oral submucous fibrosis; SD= Standard deviation.

(Figure 1). As compared to normal oral mucosa samples, more than 60% of the OSCC cases showed diminished levels of SMAD2 and TGFBR2 protein, whereas decreased SMAD3 and SMAD4 protein levels were observed in roughly 50% of samples. However, in most of the samples the expression of TGFBR1 is almost same as in normal controls (Figure 1 & Figure 2A). Notably, robust expression of bioactive forms of SMADs- pSMAD2 and pSMAD3 was found only in 44.8% and 36.7% of samples respectively, indicating disabling of TGFβ signalling is a common event in OSCCs (Table 2).

TGFβ members show a gradual decrease in expression from normal to OSCC through potentially malignant oral disorders. In order to get an idea about the role of these alterations in oral carcinogenesis, we evaluated the expression pattern of TGFβ members in premalignant oral lesions like oral submucous fibrosis (OSF) and leukoplakia. The mean protein levels of various TGFβ members in PMDs and OSCC with respect to normal mucosa is given in Table 3. Interestingly, all the analyzed genes except SMAD3 showed a gradual decrease of protein expression from normal to PMD to OSCCs (Figure 2A & 2B), suggesting the possibility for the gradual silencing of TGFβ signaling during oral carcinogenesis. There was a significant difference between SMAD2 and TGFBR2 expression between PMD and OSCC cases(p=0.015 and p=0.014 respectively). No statistically significant difference between PMDs and OSCCs was observed with reference to the expressions of other analyzed genes (Supplementary Figure 1 and Table 3).

The pSMAD/total SMAD ratio in PMDs and OSCCs are similar to normal. Further, we queried whether there is any deviation from the pSMAD to SMAD ratio in PMDs as well as in OSCCs as compared to the normal tissues. Interestingly, we did not observe any significant variation in this ratio (Figure 2C). The PMD cases showed phospho-SMADs to total SMADs ratio was similar to that of normal tissue, whereas oral cancer cases showed slightly higher pSMAD/SMAD ratio. However, these values were not statistically significant.

Correlation between protein level alterations of TGFβ signalling members and clinical outcome in OSCCs. Analysis using Kaplan-Meier estimates revealed that the protein level alterations of TGFBR2 has a significant influ-

Table 2. The protein level expression patterns of TGFβ signaling members in OSCC samples.

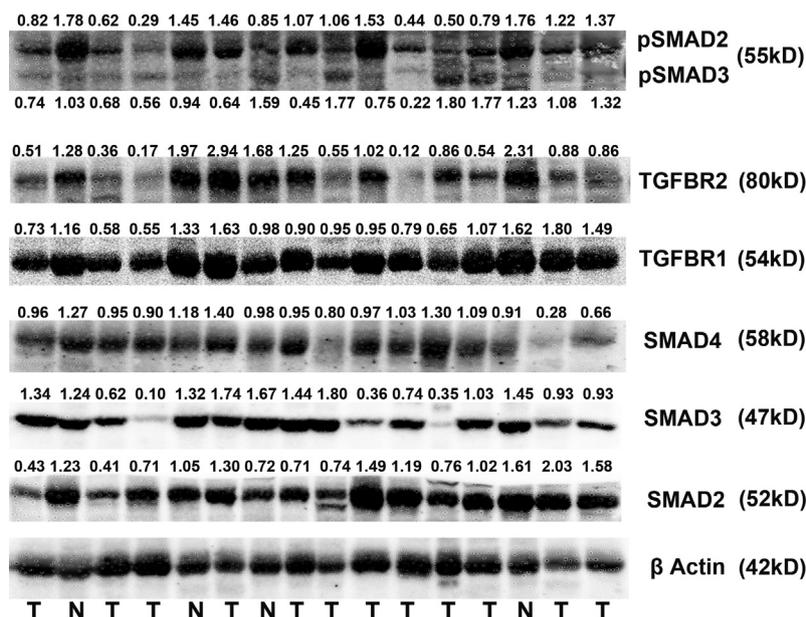| Protein	Protein levels (Compared to Normal)		
	No change	Upregulation	Down regulation
pSMAD2	27(31.0%)	12(13.8%)	48(55.2%)
pSMAD3	11(12.6%)	21(24.1%)	55(63.2%)
SMAD2	20(23.0%)	7(8.04%)	60(68.9%)
SMAD3	30(34.5%)	11(12.6%)	46(52.9%)
SMAD4	20(23.0%)	21(24.1%)	46(52.9%)
TGFBR1	32(36.8%)	14(16.1%)	41(47.1%)
TGFBR2	12(13.8%)	14(16.1%)	61(70.1%)

Figure 1. Western immuno-blot image showing protein level expression patterns of the analysed TGFβ pathway members in OSCCs. The β-Actin protein was used as loading control. The values given in the figure are normalized values using β-Actin. (T=Tumor; N=Normal).

ence on the disease-free survival (p value=0.005; Figure2D) as well as overall survival (p value=0.012; Figure 2E) of oral cancer patients. Interestingly, the protein levels of other genes including SMAD2 (which showed down regulation in a significant percentage of samples) did not show statistically significant association with oral cancer prognosis. However, there was a five-fold increase in the relative risk of reduced overall survival in SMAD2 down regulated cases. Indeed, the well-known clinical variables T-status, N-status and stage of disease were also found to significantly influence the disease-free survival in the univariate analysis (DFS: p values 0.002, 0.004 & 0.001 respectively and OS: p values <0.001 for all three variables). Multivariate analysis using Cox's regression model revealed that TGFBR2 protein levels have an independent influence on both the disease-free survival as well as overall survival of the patients (p values DFS=0.020 & OS= 0.025; Table 4). Among clinical factors, only 'stage of the disease' is found to have an independent prognostic effect (Supplementary Table 1).

Discussion

Loss of TGFβ responsiveness is a hallmark of epithelial malignancies. In addition to mutations, epigenetic alterations of this pathway are reported in a variety of cancers including HNSCCs [9]. Hence, the present study analyzed protein level alterations in TGFβ-SMAD signalling members in oral cancer samples and attempted to delineate the correlation between these alterations and disease progression. We found that, as compared to normal, the expression of TGFβ members is

lower in PMDs and least in OSCCs. This indicates possibility of gradual silencing of this tumor suppressor pathway during the progression from normal to PMDs to OSCCs. Previously, we have shown that mutations form only part of the molecular alterations of this pathway in OSCCs and occur mostly in Stages 3 & 4 OSCCs [14]. This suggests the involvement of some other mechanisms like promoter methylation ormiRNA-mediated silencing of this pathway during oral cancer progression. Notably, many authors have clearly shown that methylation is an important mechanism involved in the silencing of TGFβ signalling in cancers [15-17]. Since we have not studied the mechanism underlying this gradual silencing of TGFβ signaling, it is inappropriate to reach a conclusion without understanding the exact mechanism.

Table 3. The pattern of protein expression of TGFβ members in PMD and OSCC samples. The mean expression is given as the mean log fold change with respect to normal.

Protein	Mean protein expression levels (Compared to Normal)		p-Value (t-test)
	PMDs	OSCCs	
pSMAD2	-0.08059	-0.1795	0.191
pSMAD3	-0.1347	-0.1921	0.543
SMAD2	-0.0587	-0.1840	**0.015**
SMAD3	-0.1240	-0.1268	0.967
SMAD4	-0.0574	-0.1147	0.356
TGFBR1	-0.0472	-0.1205	0.221
TGFBR2	-0.0745	-0.2822	**0.014**

Also, the number of PMD cases used for the present analysis is comparatively low (n=20). This necessitates evaluation in a larger cohort of PMDs.

More than 50% of the analyzed OSCC samples showed reduced expression level of TGFβ-SMAD signaling members. Even though diminished levels of SMAD3, SMAD4 and TGFBR1 were found in a number of samples, the degree of downregulation was less as compared to SMAD2 and TGFBR2. In addition, only around 45% of samples showed robust R-SMAD phosphorylation, indicating disrupted SMAD signalling in OSCCs. Interestingly, we observed that the pSMAD/SMAD ratios are not significantly altered in PMDs and OSCCs. However, this result reflects the possibility of reduced SMAD phosphorylation due to reduction in the total
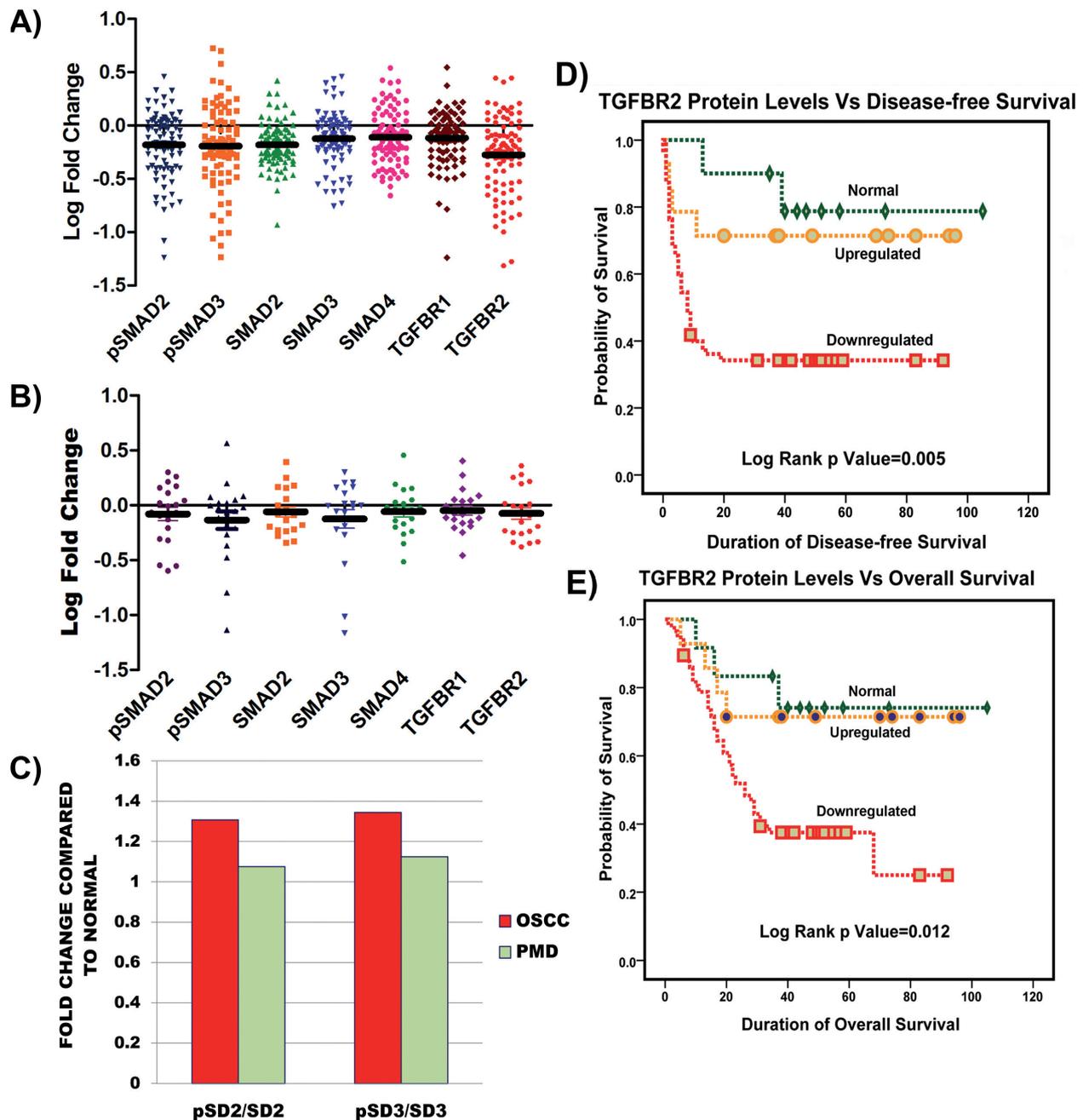

Figure 2. Expression pattern and prognostic association of TGF TGFβ pathway members in OSCCs. A & B) Scatter plot diagram showing the expression pattern of analysed TGFβ pathway members in OSCCs and PMDs, respectively. C) The ratio of pSMAD to total SMAD in OSCCs and PMDs as compared to normal. D & E) Prognostic significance of TGFBR2 protein levels in OSCCs.

SMAD protein available. During the statistical analysis, this scenario can generate a pseudo-normal pSMAD/total SMAD ratio. However, this does not point towards an intact SMAD signalling cascade in malignancy. Our results are in agreement with Paterson's findings regarding the effect of reduced TGFBR2 levels in oral carcinoma [18]. His group showed the functional significance of the decrease in TGFBR2 expression by transfecting a dominant-negative TGFBR2 construct into human oral carcinoma cell lines with normal TGFBR2 profile. Importantly, they found that the dnTGFBR2 causes decreased growth inhibition and SMAD binding activity whereas Fra-1 and collagenase-1 expression remained unchanged. These results show that decreased TGFBR2 protein relative to TGFBR1 leads to selective gene regulation, with loss of growth inhibition but the transcription of AP-1-dependent genes that are involved in the regulation of the extracellular matrix remains unchanged. This study [18] could also explain the present result, because we also found a similar expression profile reduced TGFBR2 levels as compared to TGFBR1 and decreased SMAD phosphorylation status in more than 50% of OSCC cases. However, another study on oral cancer demonstrated that the overexpression of TGF-b1 and IL-6 were significantly correlated with the risk of lymph node involvement, disease recurrence and shorter survival in patients with advanced stage of disease [19]. But they have not looked on the expression status of other important downstream factors of the TGF-b1.

In a notable work, the expression of TGFBR2 was analyzed in the successive oncogenic stages of HNSCCs, from normal epithelium to dysplasia to carcinoma. Normal squamous epithelium and squamous epithelium near the tumors (adjacent normal) showed homogenous receptor expression of TGFBR2. Dysplastic epithelium and carcinoma in situ showed a mild decrease in receptor expression. Well-differentiated to moderately differentiated carcinomas showed heterogeneous expression of TGFBR2 whereas poorly differentiated carcinomas were completely devoid of TGFBR2 [20]. This study had indicated that TGFBR2 expression inversely correlates with disease aggressiveness and suggests that aberrant TGFBR2 expression is a contributing factor to the pathogenesis of HNSCCs. However, we could not find such a correlation between TGFBR2 protein levels and histopathology (p=0.484), even though a marginal association between low TGFBR2 expression and stage of disease was observed (p = 0.063). This may be due to low sample size and has to be studied in a large population to confirm the present observation.

The clinico-pathological significance of SMAD4 and TGFβ1 in squamous cell carcinoma of the esophagus was evaluated in a notable study (21). Interestingly, patients with SMAD4 expression had a higher rate of early-stage carcinoma (p= < 0.01) and fewer lymph node metastases (p= < 0.01) compared to those with reduced SMAD4 expression. However, in multivariate analysis SMAD4 expression was not found to be independent and clinical factors such as patient age, depth of invasion, stage, and venous invasion were independent prognostic factors [21]. Further, a recent study demonstrated that decreased levels of both SMAD4 mRNA and protein in glioma compared to normal (P < 0.001). There was a gradual decrease in SMAD4 expression levels from grade I to grade IV glioma [22]. The survival rate of SMAD4-positive patients was higher than that of SMAD4-negative patients and demonstrated that the loss of SMAD4 was a significant and independent prognostic indicator in glioma.

Similar to above mentioned studies, we also sought to determine the association between protein level TGFβ-SMAD signalling alterations and clinical outcome in OSCCs. In 't'-test analysis, only TGFBR2 protein level was found to evolve as a promising prognostic factor in OSCCs. TGFBR2 downregulation was associated with poor disease-free survival as well as overall survival (p=0.005 and p=0.012 respectively). Even though SMAD2, pSMAD2 and pSMAD3 showed down regulation in a similar fashion, these were not associated with clinical outcome. TGFBR2 protein level was significant in multivariate analysis also for both DFS (p=0.020) and OS p=0.025). Stage of the disease was found to be a significant factor for DFS (p=0.004) and OS (p=0.001) in this analysis. Further, TGFBR2 downregulated samples have more than two-fold increased risk for both poor DFS and OS. Notably, the final analysis of the results indicated the superior nature of TGFBR2 expression levels as compared to traditional clinical variables such as T and N status that are commonly used for predicting prognosis.

Conclusion

Even though the role of new markers in predicting the response and prognosis of head and neck cancer is still under development, it is becoming clear that individual markers are inadequate in constructing a prognostically meaningful tumor profile for each patient. Hence, it is suggested to use a number of well-characterized markers acting in unrelated cellular pathways for successfully seperate prognostic patient categories of greater utility than traditional TNM staging. Our results clearly indicate that those oral cancers that show TGFBR2 downregulation comprise a different group with more aggressiveness. Hence, it could be beneficial for the OSCC patients if TGFBR2 expression pattern is also considered during treatment to define different prognostic groups so as to administer optimum treatment.

Supplementary information is available in the online version of the paper.

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