Original Research

Molecular expression of Forkhead Box C2 gene (FOXC2) and Prospero homeobox gene (PROX-1) in oral squamous carcinoma and their correlation with clinicopathological parameters: A prospective cohort study

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Abstract

Background: Forkhead box C2 gene (FOXC2) acts as an epithelial–mesenchymal transition (EMT) inducer while Prospero homeobox 1 gene (PROX-1) function as a regulator of lymphangiogenesis and angiogenesis in oral squamous cell carcinoma (OSCC). It is presumed that PROX-1 has both tumour-suppressive and oncogenic effects. The main aim of this study is to evaluate the role of PROX-1 and FOXC2 in the invasion and progression of OSCC cases and to correlate their expression with various histopathological parameters.

Materials and Methods: A prospective cohort study was conducted in a total sample size of 52 OSCC tissues and histologically tumour-free margins of 20. mRNA expression and protein levels of FOXC2 and PROX-1 were evaluated using real-time PCR and sandwich enzyme-linked immunosorbent assay techniques. Chi-square analysis and correlation analysis were done. Kaplan–Meier analysis evaluated the survival rate.

Results: Mean Ct values of FOXC2 were 1.915 ± 0.519 and PROX-1 was 0.061 ± 0.173. There was a significant 2-fold increase in the FOXC2 expression and a 0.5-fold decrease in the PROX-1 expression in OSCC tissue. Increased levels of FOXC2 protein and decreased levels of PROX-1 with a mean difference of 1.64 ± 0.73 ng/ml and 1.27 ± 0.33 ng/ml were observed in OSCC compared to histologically tumour-free margins. A significant positive correlation was found between the FOXC2 expression and clinicopathological parameters such as staging, perineural invasion (PNI) and lymphovascular invasion (LVI) whereas PROX-1 showed a significant negative correlation with histopathological parameters such as staging, PNI, LVI and tumour staging. There was a significant positive correlation between the PROX-1 and histologically tumour-free margins in disease-free survival patients (*P*-value = 0.03).

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Conclusion: FOXC2 and PROX-1 expressions were correlated with lymphovascular invasion, OSCC tumour staging and PNI. Thus, FOXC2 and PROX-1 could be possible therapeutic targets in the treatment of OSCC that can inhibit the EMT in OSCC and thereby favouring a better prognosis.

Keywords: Clinicopathological correlation, disease-free survival rate, forkhead box C2 gene (FOXC2), lymphovascular invasion, oral squamous cell carcinoma, perineural invasion, Prospero homeobox 1 gene (PROX-1), survival rate, tumour histotypes, tumour staging

INTRODUCTION

Head and neck cancer, including oral squamous cell carcinoma (OSCC), is the 7th most common leading cancer worldwide. According to GLOBOCAN 2020, the number of new oral and lip cancers reported worldwide is 377,713.[1] In India alone 77,000 new cases and 52,000 deaths were reported annually, accounting for one-fourth of the total mortality rate.[1] Over the past 30 years, the overall 5-year survival rate has not improved and is still less than 56%.[2] The prognosis of OSCC is influenced by various factors which are assessed in terms of clinicopathological characteristics such as TNM staging, histopathological grading, perineural invasion (PNI) and degree and depth of invasion (DOI).[3] Among the vast array of molecular prognostic factors in head and neck squamous cell carcinoma (SCC), angiogenesis and lymphangiogenesis-related factors play a pivotal role in the progression and invasion of OSCC, resulting in poor therapeutic outcomes and reducing the overall patient survival outcome,[4-6] Tumour metastasis requires that tumour cells acquire invasion by activating an epithelial–mesenchymal transition (EMT).[7] EMT, angiogenesis and lymphangiogenesis were known to be pivotal for tumour progression and metastasis in OSCC.[8] Therefore, elucidating the crucial molecular pathways associated with the transcriptional regulation of genes involved in angiogenesis, lymphangiogenesis and EMT in OSCC is essential for identifying potential target therapies and improving treatment outcomes.

The forkhead box C2 (FOXC2) gene, also known as mesenchyme forkhead 1, is a transcription factor that regulates the maturation and development of mesodermal tissue, including vascular tissue and lymphatic tissue.[9] FOXC2 is often mutated in cancer cells, where it has been contributing to several key processes during cancer progression, including EMT, metabolic reprogramming and drug resistance.[10] Upregulation of FOXC2 has been associated with progression and poor prognosis in various malignancies such as lung, colon, gastric, oral tongue SCC and hepatocellular.[11] Over-expression of FOXC2 mediated the local invasion by upregulating the MMP2 and MMP9 in cancer cells.[12] FOXC2 overexpression downregulates the E-cadherin through the regulation of p120 catenin expression in breast carcinoma.[10] Recent studies showed that FOXC2 expression was associated with proliferation and invasion potential in oral cancer cell lines.[13] FOXC2 has been demonstrated to enhance the expression level of PROX-1 and angiogenesis by enhancement of VEGF-A expression in tongue SCC.[14,15] FOXC2 may have functional implications in OSCC progression and could serve as a predictive prognostic marker and therapeutic target in OSCC.

Prospero homeobox 1 (PROX-1) is a mammalian homolog of the Drosophila homeobox protein, Prospero.[16] Prox1 is important for the embryonic development of various tissues.[16] This gene plays a crucial role in lymphangiogenesis by maintaining the lymphatic endothelial cells in the adult and postnatal stages.[17] Aberrations in PROX1 expression have been demonstrated in different human cancers, although it is not clear whether PROX1 exerts oncogenic and tumour-suppressive functions. It was presumed that PROX-1 has both tumour-suppressive and oncogenic effects.[18] In cases of pancreatic cancer, biliary carcinomas, oesophageal cancer, breast cancer and hepatocellular carcinomas, PROX1 suppresses the growth of the tumour.[14,18-20] In hepatocellular carcinoma, upregulation of PROX1 expression inhibits transforming activity and cellular proliferation and is associated with well-differentiated tumours and a better prognosis.[21] Interestingly, a previous microarray study revealed that the PROX-1 transcripts are seen downregulated in OSCC when compared to tumour-free margins.[22] It was observed that the genetic alteration could be a mechanism leading to PROX1 downregulation by reducing cell cycle inhibitors p27 and p57.[23] Studies revealed that overexpression of PROX-1 reduced cell proliferation, downregulated cyclin D1 and reduced expression of CK18.[24] It also alters the expression WISP3, GATA3, Notch 1 and E2F1. Hence, PROX-1 acts as a tumour suppressor gene in the oral carcinogenesis of OSCC.[17,21]

FOXC2 and PROX-1 are nuclear transcription factors that play an important role in lymphangiogenesis and

angiogenesis.[17] However, whether FOXC2 and PROX-1 play an important role in tumorigenesis and the progression of OSCC remains elusive. To our knowledge, there is no study reported in the literature on the co-expression of FOXC2 and PROX-1 in OSCC and its correlation with the clinicopathological parameters in the Indian population. In the present study, we aim to find the pattern of gene expression and protein expression of FOXC2 and PROX-1 in OSCC and correlate the expression pattern with various histopathological parameters.

MATERIALS AND METHODS

The present study was a prospective cohort study performed in the Department of Oral and Maxillofacial Pathology, Saveetha Dental College and Hospital, Saveetha University (SIMATS) Chennai, Tamil Nadu, India, after procuring approval from the Institutional Ethical Committee-Scientific Review Board (SRB/SDC/OPATH-2004/21/TH-04). The sample size was calculated as $n = 52$ using G*power analysis. The study population was selected according to the inclusion criteria, with patients histologically confirmed to have OSCC, with or without any systemic disease and OSCC patients associated with or without any habits, and it excluded the patients with a history of previous surgeries, chemotherapy and radiotherapy for OSCC, having or had any other type of cancers other than OSCC, patients with known immunodeficiency disorders such as AIDS and those with autoimmune disorders. The protocol of the study was approved by the Scientific Review Board, and the samples were collected for two years from November 2020 to October 2022. Samples were collected after getting informed consent from the study subjects.

Collection of OSCC samples

A total of 52 tumour tissue samples were obtained from patients who are histopathologically confirmed cases of OSCC in the incisional biopsy. The tumour margins collected during the tumour resection procedure for intraoperative clearance were histologically assessed using cryosection. The histologically confirmed tumour-free margins were collected ($n = 20$). The tissue specimens were collected in an Eppendorf and stored at −20°C until the molecular examination was performed. The patients were followed up clinically at regular intervals after tumour excision.

Parameters assessed

The patients included in the study were followed prospectively and histopathological parameters of the OSCC patients were retrieved after excisional biopsy from the cancer structured report. All the clinical parameters such as age, gender, site and histopathological parameters such as tumour size, a histopathological subtype of OSCC, pattern of invasion (POI), DOI, PNI, LVI, nodal involvement and the pTNM staging of OSCC (AJCC cancer staging, manual 8th edition, 2018) were tabulated [Figure 1]. The survival details of the patients were obtained from the clinical record and were tabulated in Excel.

Procedure

The expression of FOXC2 and PROX-1 was evaluated in 52 OSCC and 20 histologically tumour-free margins using a CFX96 real-time PCR system (BIO-RAD LABORATORIES INC, HERCULES, CA, USA). Quantitative real-time PCR was performed using the TB GREEN PREMIX Ex Taq II (Thermo Fisher) on the applied biosystems real-time PCR system (Thermofisher Scientific). Glyceraldehyde 3 phosphate dehydrogenase (GAPD) was used as a reference or housekeeping gene. Divergent primers for FOXC2, PROX-1 and GAPDH were procured from Eurofins, India [Table 1]. The levels of FOXC2 and PROX-1 protein in tissue samples were assessed using the standard sandwich ELISA technique according to the protocol provided by the manufacturer of the ELISA kits (Human Forkhead box protein ELISA Kit and PROX-1 ELISA Kit -BT lab- Kit, Catalog number E7260Hu/Catalog number E7260Hu, Japan).

Statistical analysis

Statistical analysis was done using SPSS Software version 23.0. All the data were expressed as mean ± SD. The association and correlation between the expression of FOXC2 and PROX-1 along with clinicopathological parameters are analysed using Chi-square (X^2) analysis and Kendall's tau correlation analysis, respectively. Kaplan–Meier plots and Cox proportional hazard model were used for multivariate analysis and for determining the *P*-value.

RESULTS

Demographic data of OSCC

The study included 52 OSCC tissue samples and 20 histologically tumour-free margins. Among the 52 OSCC tissue samples, 76.9% ($n = 40$) were male patients with a mean age group of 56.06 years. The most common site for the occurrence was buccal mucosa (48.1%), followed by RMT (25%), tongue (17.3%), gingivobuccal sulcus region (7.7%) and floor of the mouth (1.9%). The demographic and histopathological data of the OSCC cases are tabulated in Table 2.

Expression of FOXC2 and PROX-1 in OSCC tissues using real-time PCR

The mRNA expression of FOXC2 was evaluated using RT PCR in OSCC tissue ($n = 52$) and histologically tumour-free margins ($n = 20$). The mean value of FOXC2 and PROX-1

Table 1: Primers used in this study

Gene	Primer sequence (forward primer)	Reverse primer
FOXC2	F: 5'-GCCGACGGATTCCTGCGCTC-3'	R: 5'-CGCTCCTCGCTGGCTCCA-3'
PROX-1	F: 5'-CTCCGTGGAACTCAGCGC-3'	R: 5'-GCCG CTTAAG CTG-3'
GAPDH (housekeeping gene)	F: 5'GTCTCCTCTGACTTCAACAGCG3'	R: ACC ACC CTG CTG CTG TAG CCAA-3'

Table 2: Percentage of the clinical details of OSCC patients included in the study

Clinical parameters	Category	Frequency (*n*=52)	Percentage (%)
Age	<55 years	34	65.4
	56–70 Years	16	30.8
	>70 years	2	3.8
Gender	Male	40	76.9
	Female	12	23.1
Tumour site	Buccal mucosa	25	48.1
	Tongue	9	17.3
	RMT	7	13.5
	GBS	4	7.7
	Floor of the mouth	1	1.9
Tumour size	<2 cm	3	5.7
	2–4 cm	27	51.9
	>4 cm	22	42.4
Histopathological grading	WDSCC	25	49
	MDSCC	27	51%
TNM Staging	I	3	5.8%
	II	12	25%
	III	14	26%
	IV	22	42%
PNI	Present	50	50%
	Absent	50	50
LVI	Present	11	21.2
	Absent	41	78.8
ENE	Present	17	32.7
	Absent	34	65.4
Survival status	Alive	36	69.2
	Recurrence	9	17.3
	Mortality	7	13.5

expression was 1.915 ± 0.519 and 0.062 ± 0.173 compared to the histologically tumour-free margins of 1.000 ± 0.000, respectively. There was a significant 2-fold increase in the FOXC2 expression and a 0.5-fold decrease in the PROX-1 expression in OSCC tissues when compared to the histologically tumour-free margins and with statically significant *P*-value < 0.05 [Figure 2].

Clinicopathological correlation of FOXC2 and PROX-1 expression in OSCC tissue

Clinicopathological correlation of FOXC2 expression in OSCC

mRNA expression of FOXC2 showed an increase in mean ct value in advancing tumour stages, with a mean $2^{-\Delta\Delta C}$ value of 2.15 ± 0.5 in Stage IV, $2^{-\Delta\Delta C}$ value of 1.9 ± 0.5 in Stage III, $2^{-\Delta\Delta C}$1.8. ±0.5 in Stage II and $2^{-\Delta\Delta C}$ value of 1.336 ± 0.43 in Stage I. As the grading advanced, there was increased expression of FOXC2 with a moderate positive correlation with a statistically significant *P*-value = 0.000 [Figure 3]. Correlation analysis showed a positive correlation between the PNI and LVI with the FOXC2 expression, statistically significant *P*-value = 0.000 and 0.002 (r^2 = 0.499, 0.152) [Table 3] No significant correlation was observed between the FOXC2 and other histopathological parameters like tumour size, node invasion, histopathological subtypes, POI and DOI (*P*-value >0.05) [Table 3].

Clinicopathological correlation of PROX-1 expression in OSCC

The expression of PROX-1 showed a decrease in the mean ct value as the tumour stage was advancing, with a mean $2^{-\Delta\Delta C}$ value of 0.07 ± 0.5 in Stage I, $2^{-\Delta\Delta C}$value 0.07. ±0.5 in Stage II, a $2^{-\Delta\Delta C}$ value of 0.06 ± 0.5 in Stage III and a $2^{-\Delta\Delta C}$ value of 0.04 ± 0.5 in Stage IV. Kendall's tau regression analysis showed a strong negative correlation between the pathological staging and PROX-1 expression with a significant *P*-value = 0.000 (r^2= - 0.713) [Figure 4]. A negative correlation between the PNI and FOXC2 expression was noted with a statistically significant *P*-value = 0.049 (r^2= -0.499). PROX-1 expression showed a negative correlation in the patient with LVI with a statistically significant *P*-value = 0.001 (r^2= -0.078). No significant correlation was observed between the PROX-1 and other histopathological parameters like tumour size, node invasion, histopathological subtypes, POI and DOI with a *P*-value < 0.05 [Table 4].

Correlation between the FOXC2 and PROX-1 expression in OSCC tissue

The linear regression correlation analysis was done to correlate the FOXC2 and PROX-1 expressions. The correlation analysis showed a strong negative correlation between FOXC2 and PROX-1 expression with a statistically significant *P*-value = 0.001 (r^2= -0.759) [Figure 5].

Correlation between the FOXC2 and PROX-1 expression with the survival of OSCC

Among the 52 OSCC patients, survival details were collected from the clinical record and tabulated. Of these 69.2% (*n* = 36) cases were disease free and 17.3% (*n* = 9) showed local recurrence. There was a mortality rate of 13.7% (*n* = 7) among the OSCC cases. Survival analysis in the OSCC patients with the FOXC2 expression and PROX-1 expression among the diseased and disease-free individuals was assessed using Kaplan–Meier analysis. No statistically significant *P*-value was observed with log-rank test *P*-value = 0.08 and 0.09, respectively [Figure 6].

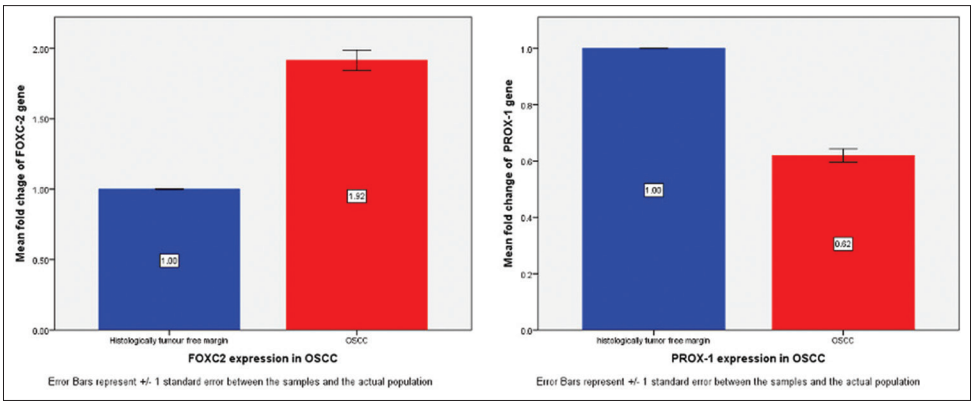

Figure 1: Bar graph represents the mean comparison of FOXC2 and PROX-1 expression in OSCC tissues and histologically tumour-free margins. There was a significant 2.0-fold increase in the FOXC2 expression and 0.5-fold decrease in the PROX-1 expression in OSCC tissues when compared to the histologically tumour-free margins and with a statistically significant *P*-value = 0.000

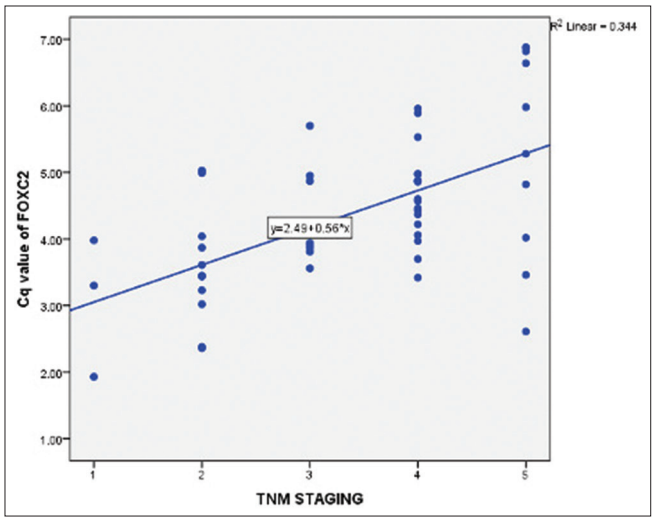

Figure 2: Linear regression graph showing moderate positive correlation between the pathological staging and FOXC2 expression with a statistically significant *P*-value with 0.000 (r2 = 0.452)

Correlation of the FOXC2 and PROX-1 expression between the histological tumour-free margin and with the survival of OSCC patients

The linear regression correlation analysis between the FOXC2 expression in the histologically tumour-free margin with diseased and disease-free individuals showed no significant *P*-value > 0.05. However, when the correlation analysis was done between the PROX-1 expression in the histopathologically tumour-free margin with diseased and disease-free individuals, there was a significant positive correlation in the disease-free individuals with a *P*-value = 0.03 (r = 0.232).

Comparison of FOXC2 and PROX-1 Protein levels in OSCC tissue using ELISA

FOXC2 proteins were analysed using an enzyme-linked immunosorbent assay in 52 OSCC samples. The mean FOXC2 protein in OSCC tissues was 3.712 ± 0.977 ng/ml with a histologically tumour-free margin showing 2.031 ± 0.880 ng/ml. The mean difference of 1.64 ± 0.73 ng/ml increase in the protein levels was observed in OSCC when compared to the histologically tumour-free margin with a statistically significant *P*-value (0.000 > 0.005). Conversely, there was a statistically significant decrease in the protein levels of PROX-1 in OSCC compared to the histologically tumour-free margin with mean values of 5.004 ± 0.822 ng/ml in OSCC and 3.723 ± 1.334 ng/ml in histologically confirmed tumour-free margin The mean difference was 1.27 ± 0.33 ng/ml with a *P* value of 0.000 [Figure 7].

Table 3: Statistical analysis of the correlation between the mean expression FOXC2 in OSCC in association with various clinicopathological parameters

Clinicopathological parameters		Mean SD ($2^{-\Delta\Delta Ct}$)	FOXC2 cq value (Correlation coefficient r^2)	*P*
Tumour size	<2 cm	1.4±0.07	0.236	0.226
	2–4 cm	2.22±0.4		
	>4 cm	2.23±0.3		
TNM staging	I	1.36±0.15	0.452*	0.000*
	II	1.8±0.5		
	III	1.9±0.4		
	IV	2.02±0.5		
Histopathological subtypes	WDSCC	1.4±0.2	0.654	0.236
	MDSCC	2.4±0.1		
pN	N+	1.9±0.5	0.037	0.749
	N-	1.8±0.5		
DOI	< 2 cm	1.6±0.3	0.098	0.342
	2–4 cm	1.8±0.3		
	>4 cm	1.82±0.2		
PNI	PNI+	1.9±0.5	0.499 *	0.000*
	PNI-	1.8±0.5		
LVI	LVI+	1.9±0.5	0.152 *	0.002*
	LVI-	1.7±0.4		
POI	I	1.81±0.5	0.219	0.762
	II	1.8±0.4		
	III	2.1±0.6		
	IV	2.1±0.5		

*Denotes *P* value <0.05, which is statistically significant

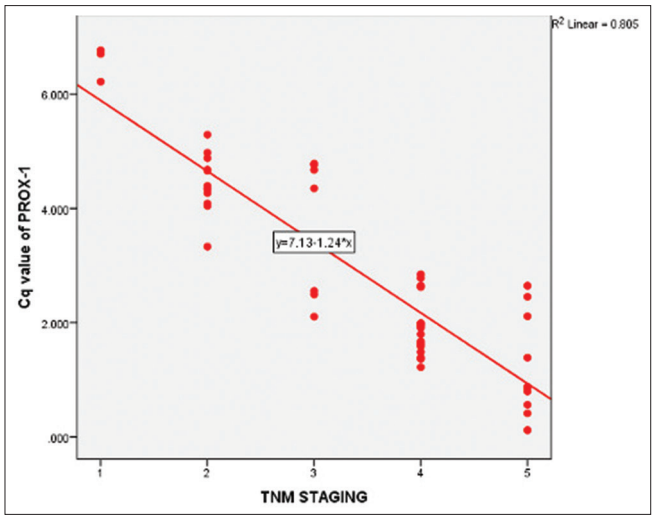

Figure 3: Linear regression graph showing significant strong negative correlation between the pathological staging and PROX-1 expression (*P*-value = 0.000; r2= -0.713*)

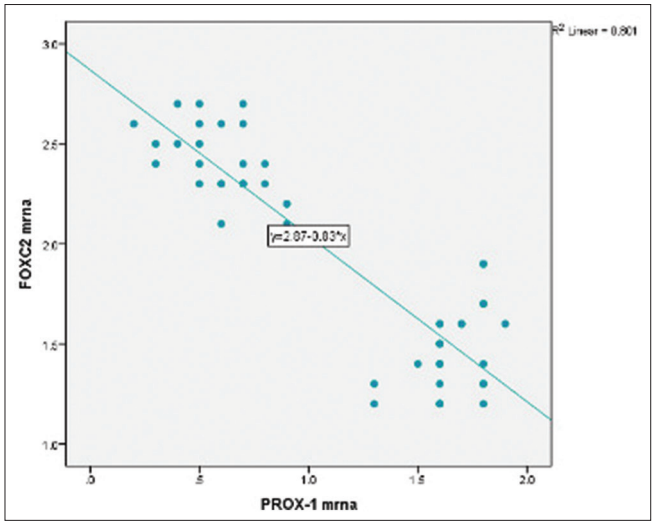

Figure 4: Linear regression plot represents a significant negative correlation between the expression of PROX-1 and FOXC2 expression with a *P*-value = 0.000 (r^2= -0.759)

DISCUSSION

OSCC is a highly prevalent malignancy of the oral cavity.[25-27] The poor prognosis is attributed to various factors like delayed diagnosis, late-stage presentation, involvement of adjacent structures in the oral cavity, lymph node metastasis and distant metastasis.[28-30] Analysing the molecular changes pertaining to the carcinogenesis of OSCC is crucial, as it provides a better understanding of the disease and improves treatment strategies.[30] Even though there are numerous histopathological and clinical prognostic factors, there is a lacunae in predicting the prognosis of OSCC patients. Accurate molecular biomarkers and potent molecular-targeted medicines must be established to improve the overall prognosis and patient survival.

FOXC2 gene is an important regulator of EMT which has been highlighted recently in the discovery of molecular inhibitors to directly target these transcription factors.[31] FOXC2 is an important regulator of EMT, and studies have been done evaluating the expression of FOXC2 in various cancers.[12,32-34] Studies have reported that elevated levels of FOXC2 expression were associated with poor patient survival in tongue SCC.[13] FOXC2 has been shown to be upregulated in various malignancies whereas PROX-1 which plays a key role in cell cycle inhibition has been demonstrated to be downregulated in various malignancies.[32,33] OSCC treatment can be accomplished by focusing on FOXC2, which can further inhibit EMT. The lacuna is that there is no evidence assessing the role of FOXC2 and PROX-1 in OSCC and its adjoining tumour-free margin. Hence, this study is done to decipher the same.

Our study results showed a significant 2-fold upregulation (1.9 ± 0.8) in the ct values of FOXC2

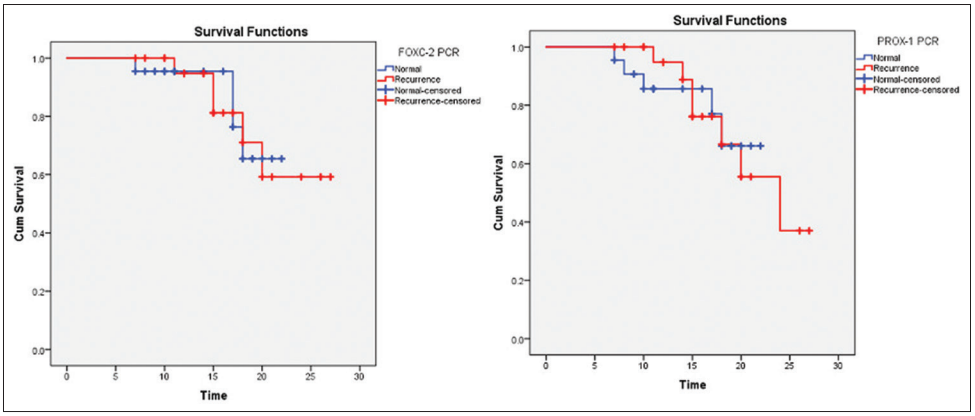

Figure 5: The above Kaplan–Meier curve represents the survival analysis of the patient with increased FOXC2 expression and decreased PROX-1 expression among the diseased and disease-free OSCC patients with no significant *P*-value = 0.08 and 0.09, respectively (log rank test)

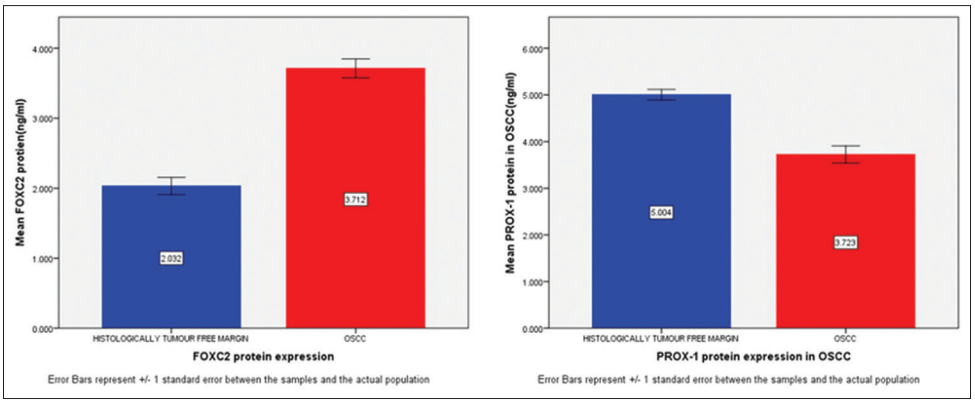

Figure 6: Represents the mean comparison of protein levels of FOXC2 and PROX-1 in OSCC samples when compared to the adjacent histologically confirmed tumour-free margin. Significant increase in the FOXC2 protein levels with a mean difference of 1.64 ± 0.73 ng/ml was observed in OSCC when compared to the histologically tumour-free margin with a *P*-value = 0.000. Conversely, there was a significant decrease in the PROX-1 protein levels with a mean difference of 1.27 ± 0.3 in OSCC when compared to the histologically tumour-free margin with a *P*-value = 0.000

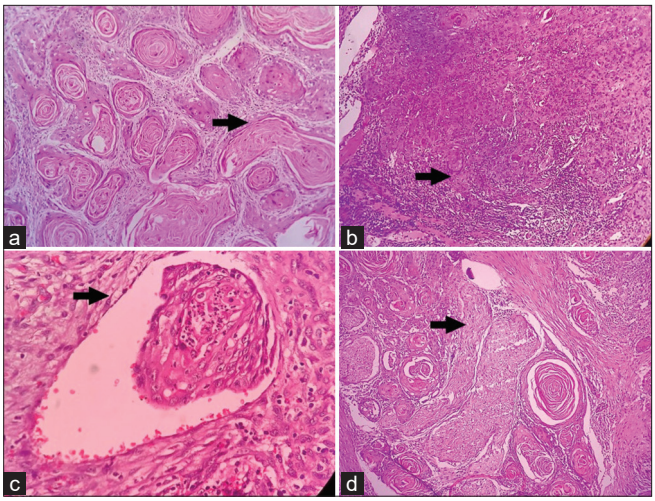

Figure 7: Indicates the H&E image of the parameters assessed: Histological subtypes (a) H&E image (10×): Well-differentiated squamous cell carcinoma (b) H&E image (10×): Moderately differentiated squamous cell carcinoma (c) H&E image (40×): lymphovascular invasion (d) H&E (40×): Perineural invasion

Table 4: Statistical analysis of the correlation between the mean expression PROX-1 in OSCC cases and association with various clinicopathological parameters

Clinicopathological parameters		Mean SD ($2^{-\Delta\Delta Ct}$)	PROX-1 cq value (Correlation coefficient r^2)	*P*
Tumour site	Buccal mucosa	0.6±0.3	-0.09	0.349
	Tongue	0.7±0.1		
	RMT	0.7±0.5		
	Other Sites	0.8±0.6		
Tumour size	<2 cm	0.8±0.3	-0.059	0.573
	2–4 cm	0.7±0.2		
	>4 cm	0.5±0.2		
TNM staging	I	0.7±0.1	-0.713*	0.000*
	II	0.6±0.2		
	III	0.6±0.1		
	IV	0.4±0.4		
Histopathological subtypes	WDSCC	1.4±0.2	0.387	0.268
	MDSCC	2.4±0.1		
Node invasion	N+	0.6±0.3	-0.067	0.564
	N-	0.8±0.4		
DOI	<2 cm	0.9±0.2	-0.087	0.243
	2–4 cm	0.6±0.4		
	>4 cm	0.4±0.3		
PNI	PNI+	0.2±0.1	-0.499*	0.0498*
	PNI-	0.8±0.1		
LVI	LVI+	0.7±0.2	-0.078*	0.001*
	LVI-	0.9±0.2		
POI	I	0.9±0.1	-0.879	0.074
	II	0.8±0.1		
	III	0.7±0.2		

*Denotes *P* value <0.05, which is statistically significant

expression among OSCC tissue samples when compared to histologically tumour-free margin (*P*-value = 0.000). A similar study showed 36.1% increased FOXC2 expression in OSCC compared to normal mucosa.[15] Increased expressions of FOXC2 were also studied in various cancers such as ovarian cancer,[34] breast cancer,[10] colorectal cancer[35] and gastric cancer.[34] Studies have shown FOXC2 to be a regulator protein that stabilises the E-cadherin at the adhesion junction of the epithelial cells by downregulating the p120 catenin.[36] To assess the progression of the disease, the expression of FOXC2 was further correlated with the clinicopathological parameters such as tumour location, size, histological subtype, pathological stage, lymph node invasion and perineural invasion. TNM staging had a significant influence on the prognosis of OSCC patients.[37] A recent study on tongue OSCC showed a significant correlation between the FOXC2 expression in OSCC pathological TNM staging, the pattern of invasion and local recurrence.[13,15] Similarly, increased expression of FOXC2 was associated with advanced tumour stages and poor patient prognosis in gastric carcinoma.[38] Previous studies have reported that FOXC2 can activate TGF-β signalling by directly regulating its expression or with other transcription factors that control TGF-β.[39] Our study results demonstrated a significant 1-fold upregulation in the expression of FOXC2 with advancing stages of OSCC

and a mean ct value of 2.03 (*P*-value = 0.000) (r^2 = 0.452). Activated TGF-β binds to its receptor on the cell surface, initiating the downstream signalling cascade that includes activation of the P13/AKT pathway which can enhance cell survival, proliferation and tumorigenesis.

LVI and PNI are considered to be predictive factors for the prognosis of multiple cancers.[40] In our study, OSCC patients with 21.2% LVI and 50% perineural invasion showed a positive correlation with increased FOXC2 expression (r^2 = 0.152 and 0.499) (*P*-value = 0.000). However, limited data supports this evidence of a correlation between FOXC2 expression and LVI in OSCC. Previous studies done in breast carcinoma showed a correlation with PNI, histotypes, lymph node metastasis and pathological staging.[41,42] Studies have also shown that the knockdown of FOXC2 expression is associated with the inhibition of proliferation, invasion and migration of colon cancer and its mechanism was related to the inhibition of EMT.[41] FOXC2 expression induces EMT and is triggered by various other signals including TGF-β and EMT-inducing factors like snail, twist and goosecoid which contribute to cancer invasion. It has also been reported that FOXC2 induces invasion and migration through the FOXC2-VEGF signalling pathway.[13] This could be due to the migration and accelerated proliferation of lymphatic endothelial cells and vascular endothelial cells which are regulated by FOXC2 through VEGFR-2/VEGFR-3 pathways. Therefore, these correlated parameters can indeed be valuable in determining the invasive ability of the tumour.

PROX-1 is a transcription factor that plays a critical role in various aspects of embryonic development and tissue differentiation.[20,43] Prospero homeobox has been shown to function as both a tumour-suppressor gene and oncogene in various cancers. Rodrigues *et al*.[24] have demonstrated downregulated expression of PROX-1 in OSCC cell lines. Various *in vivo* studies showed decreased expression of PROX-1 in various cancers like hepatocellular carcinoma,[20] gastric carcinoma[44] and haematological malignancies.[42] Consistent with these studies, our results showed a significant 0.5-fold decrease in the expression of PROX-1 in OSCC tissue with a mean ct value of 0.5 compared to the histologically tumour-free margins (*P*-value = 0.000). Consequently, this downregulation can result in increased activity of cyclin D1, a protein involved in the cell cycle progression.

The dysregulation of cyclin D1 in the G1 phases causes abnormal cell proliferation and contributes to tumorigenesis. Considering our results, PROX-1 could play a critical role in OSCC.

The correlation of PROX-1 gene expression with histopathological parameters has not been estimated until now in OSCC. The current study revealed a negative correlation of PROX-1 in patients with PNI, LVI and in pathological stages of OSCC. Previous studies have shown similar results with a gradual decrease in the expression of PROX-1 as the staging advances in colorectal carcinoma and pancreatic carcinoma.[18,45] Our study results showed a negative correlation of PROX-1 expression with tumour stages implying that downregulation of PROX1 expression may promote OSCC progression (r2= -0.713) (*P*-value = 0.000). OSCC cell line studies showed that PROX-1 expression was associated with the regulation of cell differentiation.[24] Downregulation of PROX1 may be an important phenomenon in the progression from normal to precancerous cells by regulating cell differentiation. The current study showed reduced PROX-1 expression correlated with increased LVI (0.7 ± 0.2) and perineural invasion (0.2 ± 0.1) in OSCC patients with a *P*-value < 0.05 (r2= -0.499 and r2= -0.078). This finding is in accordance with previous studies showing downregulated expression of PROX-1 and their association with the LVI in various cancers such as hepatocellular cancer and oesophageal cancer,[12,18,25] Previous studies have reported that PROX-1 along with FOXC2 plays a key role in lymphangiogenesis and angiogenesis. Reduced PROX-1 expression has been associated with LVI in OSCC patients and therefore can be utilised for predicting lymph vascular invasion.[18,24] It has also been studied that the downregulation of PROX-1 is associated with the activation of NOTCH signalling, modulating the NOTCH pathway which can indirectly downregulate the PROX-1 expression.[46]

A recent meta-analysis on FOXC2 expression showed that overexpression of FOXC2 was associated with poor survival in cancer patients.[47] Liu *et al*.[48] in gastric cancer demonstrated that FOXC2 overexpression correlated with the poor prognosis of the patients. Similar studies done in oesophageal carcinoma showed increased FOXC2 expression as an independent prognostic factor for patient's survival.[12] This is the first study to correlate the expression of surgical margin status with survival status. Our study showed no correlation between the expression of FOXC2 and survival rate which could be a result of a minimal follow-up period of 24 months. Future research can be conducted with a longer follow-up period to establish FOXC2 as a potential marker for predicting the prognosis of OSCC. A significant positive correlation of PROX-1 expression was observed when comparing the histologically clear tumour margin of diseased (recurrence) and disease-free survival (*P*-value = 0.004). Our finding

with increased expression of PROX-1 in histologically confirmed tumour-free margins of disease-free patients can be due to PROX-1 acting as a tumour suppressor in the disease-free patient of OSCC. Thus, PROX-1 can be a predictive factor in the prognosis of OSCC patients in disease-free survival.

Our study showed a significant negative correlation between FOXC2 and PROX-1 indicating an increased expression of FOXC2 and downregulation of PROX-1 with a *P*-value = 0.001 (r^2= -0.759). Similar research showed that upregulated FOXC2 expression correlated with increased PROX-1 expression in OSCC tissues.[15] FOXC2 has been demonstrated in the regulation of PROX-1 expression, through the induction of lymphangiogenesis and angiogenesis by activation of VEGFR2/VEGFR3.[15] This shows that the downregulation of PROX1 expression is associated with the malignant phenotype in OSCC.[24] The downregulation of PROX-1 expression, potentially influenced by FOXC2, in OSCC highlights the complexity of molecular interaction involved in cancer progression.

Similarly, when the protein levels of PROX-1 and FOXC2 in OSCC were compared to the histopathological tumour-free margin, there was an increase in FOXC2 protein expression and a decrease in PROX-1 protein levels (*P*-value = 0.00). Consistent with our results, previous studies conducted in serum showed an increase in the protein levels of FOXC2 in hepatocellular carcinoma, attributed to the enhanced proliferation of tumour cells and inhibition of apoptosis by activating the Akt/mTORC1 and ERK/mTORC1 signalling pathways.[49] In accordance with our results, decreased protein levels of PROX-1 have been demonstrated in thyroid carcinoma.[49] These results indicate that PROX-1 protein can possibly control the EMT pathways and potentially inhibit the invasive properties of the OSCC cells. Our novel study evaluated the protein levels of FOXC2 and PROX-1 in OSCC tissues. Future studies could be done in serum in order to establish FOXC2 and PROX-1 as potential non-invasive tumour biomarkers to predict the prognosis of OSCC.

The major limitation of our study is that protein expression was analysed in tissue samples. Another limitation was that the 24-month follow-up period of the patients may not be sufficient to correlate the expression of FOXC2 and PROX-1 with survival rates.

In conclusion, this study presented a novel evaluation of the role of FOXC2 and PROX-1 in OSCC and correlated it with the clinicopathological parameters in the Indian population. Our study revealed an upregulation of FOXC2 expression and downregulation of PROX-1 expression which correlated with OSCC tumour staging, PNI and LVI. In the histologically tumour-free margins, increased PROX-1 expression was observed when compared to OSCC tissues, and no variations in the FOXC2 expression in disease-free patients. FOXC2 could cause tumour invasion through the EMT pathway by downregulating the expression of E-cadherin and p120 catenin. FOXC2 can also promote cell proliferation through the activation of MAPK and AKT pathways, subsequently downregulating p27 and upregulating cyclin D1. Furthermore, the expression of PROX-1 was downregulated in OSCC, and the corresponding correlation with histopathological parameters was observed. Decreased expression of PROX1 could reduce the expression of WISP3, GATA3 and Notch1 leading to proliferation, invasion and metastasis in OSCC. With the identified role of FOXC2 and PROX-1 from the above study, these proteins emerge as a potential possible target for treating OSCC patients. Further investigations are required with long-term follow-up and with more advanced molecular techniques to establish prognostic markers for OSCC.

CONCLUSION

FOXC2 and PROX-1 expressions were correlated with LVI, OSCC tumour staging and PNI. Thus, FOXC2 and PROX-1 could be possible therapeutic targets in the treatment of OSCC that could inhibit the EMT in OSCC and thereby favour a better prognosis. PROX-1 can act as a tumour suppressor in OSCC and amplification of PROX-1 can improve the prognosis of OSCC patients.

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Conflicts of interest
There are no conflicts of interest.

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