KLF4 expression in the surgical cut margin is associated with disease relapse of oral squamous cell carcinoma

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Objective. The presence of cancer stem-like cells (CSCs) in the majority of tumors is one of the factors responsible for disease relapse in oral squamous cell carcinoma (OSCC). In this study, we investigated the role of octamer-binding transcription factor 4 (OCT4) and Kruppel-like factor 4 (KLF4) in OSCC progression and disease relapse.

Study Design. In this study, 102 patients with OSCC were included. The expression of β-catenin and CSC markers (KLF4 and OCT4) in surgical cut margin and tumor were investigated through Western blot analysis, immunohistochemistry, and quantitative polymerase chain reaction analysis. The χ^2 test was used to evaluate the association of β-catenin, OCT4, and KLF4 expression with clinicopathologic characteristics. Kaplan-Meier and Cox regression analyses were performed to correlate different clinical factors with the prognoses of patients with OSCC.

Results. We observed increased expression of OCT4, KLF4, and β-catenin in the cut margins (CMs) in recurrent OSCC. The χ^2 test exhibited recurrence as one of the key factors associated with high expression of these markers. Kaplan-Meier and COX regression analyses demonstrated that increased expression of KLF4 in the CM region of recurrent patients was independently associated with a poor prognosis.

Conclusions. Our findings indicated that expression of KLF4 can be used for monitoring disease progression and may serve as prognostic marker to predict recurrence. (Oral Surg Oral Med Oral Pathol Oral Radiol 2019;128:154−165)

Oral squamous cell carcinoma (OSCC) is one of the most common malignancies in the head and neck region. Head and neck squamous cell carcinoma (HNSCC) including within the oral cavity, have a high incidence rate in Southeast Asia.[1,2] In India, oral cancer is one of the major health problems resulting from addiction to tobacco. In India as well as in the other countries on the Indian subcontinent, people consume tobacco in different forms, such as betel quid, tobacco with lime, *bidi*, cigarette, *gutkha*, and *hookah*.[3,4] Apart from poor oral hygiene, human papilloma virus infection and dietary deficiencies significantly contribute toward disease progression.[5−7] Despite recent advances in the treatment of OSCC, persisting major challenges in the treatment of OSCC are disease relapse and resistance to therapy.[8]

The presence of cancer stem-like cells (CSCs) in the bulk of tumor is one of the factors associated with resistance to therapy.[9] It has been reported that CSCs exhibited enhanced tumorigenicity and intrinsic drug resistance, which are subsequently implicated in treatment failure in many cancers.[10] Disease relapse and unfavorable prognosis are attributed primarily to the drug resistance of the CSCs present in these tumors.[11] Several studies have reported that this subpopulation of cells inside the tumor microenvironment expresses different stem cell marker genes, such as *OCT4, KLF4, Sox-2, c-kit, NANOG, CD44, ALDH1*, and *ABCG2*.[12−16] The aberrant expression of these CSC markers has been associated with progression of different types of cancer, such as breast cancer, prostate cancer, and colorectal cancer, as well as HNSCC.[11,17−20]

One of the important CSC markers, OCT4 (octamer-binding transcription factor 4) is expressed in the inner cell mass of blastocysts and germ cells.[21] It acts as a transcription factor and maintains the stemness in embryonic stem cells and is essential for the proper development of the embryo.[22] Recently, OCT4 has been identified as a biomarker in several cancers, including breast, colon, lung, and oral cancer.[23,24] It is responsible for the maintenance of the CSC phenotype by promoting self-renewal and the regulation of the differentiation process.[25] Previous studies have also suggested that increased expression of OCT4 is associated with poor prognosis and resistance to cisplatin in OSCC.[26]

Kruppel-like factor 4 (KLF4), another CSC marker, is a zinc finger transcription factor exhibiting diverse and cell-specific functions.[27] This gene is involved in a wide range of cellular processes, such as cell proliferation, apoptosis, migration, inflammation, differentiation, and tissue homeostasis.[27,28] Previous reports have

Statement of Clinical Relevance

The increased expression of KLF4 marker in the surgical margin was associated with disease recurrence. Thus, it can be used for monitoring disease progression and may serve as a prognostic marker to predict recurrence.

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also suggested its role in reprogramming somatic cells into inducible pluripotent stem cells, highlighting that KLF4 is essential for the maintenance of stem cells and can be regarded as a stem cell marker.[29] Apart from that, a paradox exists regarding the role of KLF4 in cancer. It acts as either an oncogene or a tumor suppressor gene in different cancers. In gastrointestinal tract related malignancies, such as colon and gastric cancers, it acts as a tumor suppressor gene,[30,31] and in breast, skin, and head and neck cancers increased expression of KLF4 has been reported, corroborating its oncogenic role.[32,33]

Several signaling pathways contribute to therapeutic resistance in OSCC. Among them, the Wnt/β-catenin signaling pathway has been reported to be involved in the maintenance and survival of CSCs.[34,35] It was reported in earlier studies that Wnt/ β-catenin signaling contributes to the aggressiveness of tumors and therapy resistance in OSCC.[36−38] In our previous study, we also found that β-catenin plays an important role in cisplatin resistance, recurrence, and prognosis in HNSCC, based on its expression level.[39] It was reported earlier that increased expression of CSC markers, such as OCT4 and KLF4, were associated with aberrant regulation of Wnt/ β-catenin signaling and subsequently promoted neoplastic progression.[40,41]

Therefore, in this study we aimed to investigate the expression of CSC markers (OCT4, KLF4) along with β-catenin in surgical cut margins (CMs) and tumors in patients with OSCC and to evaluate the correlation between the expressions of these markers. We further evaluated the association of different clinicopathologic factors with the prognoses of patients based on expression level of OCT4, KLF4, and β-catenin in surgical CMs and tumors in OSCC.

MATERIAL AND METHODS

Patient sample collection and ethics statement

In this study, tumor tissues from 102 patients with OSCC, along with CMs, were collected at the time of surgery for the period 2010−2013 and were stored appropriately. The patients were further segregated into 2 cohorts, namely, recurrent and nonrecurrent groups. Among the 102 patients with OSCC, 29 patients had recurrence. The specimens from the remaining 73 patients with OSCC indicated primary tumors. Recurrence was considered in patients who had undergone radiotherapy and chemotherapy after primary surgery and later presented with the same disease. All of the patients underwent the same treatment protocol—that is, surgery followed by chemotherapy and radiotherapy. With regard to CMs, the surgeon usually removed 2 to 4 cm from the peripheral boundary of the tumor, that is visually observed as tumor free normal tissue. This is sometimes followed by a frozen section biopsy, and if histopathologically observed to be devoid of tumor, the sections were deemed normal. The study was approved by institutional ethical committee of the School of Biotechnology and Kalinga Institute of Medical Sciences, the Kalinga Institute of Industrial Technology, and conducted according to the tenets of the Helsinki Declaration. Voluntary consent forms were duly signed by patients or their nominees before participation in the study. Data on patients' clinicodemographic features were obtained from their medical records. Histopathologic analysis of each tumor was performed, and histologic grade was evaluated by a qualified pathologist. Each patient was followed up every 3 months after surgery and the prognosis monitored. Disease-free survival (DFS) was calculated from the date of initial surgery to disease relapse and the overall survival (OS) period was calculated from the date of the initial surgery to death of the patient.

Western blot analysis

Western blot (WB) analysis was performed on the surgical CMs and tumors of 102 OSCC samples to investigate the expression of KLF4, OCT4, and β-catenin. Briefly, patient tissue samples were homogenized by using liquid nitrogen and resuspended in radioimmunoprecipitation assay lysis buffer (50 mM Tris-Cl pH 7.4; 150 Mm sodium chloride; 1% NP-40; 0.25% sodium deoxycholate; 1% Triton-X-100; 1 mM ethylenediaminetetraacetic acid; Milli-Q water) to obtain protein lysate. Protein estimation was done by using the Bradford assay. Next, 50 μg of protein lysates were separated on 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane, and WB analyses were performed as reported earlier.[26,29] The membrane was blocked with 5% skimmed milk in phosphate-buffered saline (PBS) with 0.25% Tween 20 for 1 hour at room temperature and then probed with the primary antibody (1:2000) at 4˚C overnight. The membrane was washed with PBS with 0.25% Tween 20 and probed with secondary antibody (1:4000) for 2 hours at room temperature. The blots were visualized by enhanced chemiluminescence by using x-ray film (Kodak, India). The expressions of different proteins were normalized against glyceraldehyde 3-phosphate dehydrogenase which served as loading control. The area density was calculated by using Image J software for expression of each protein against glyceraldehyde 3-phosphate dehydrogenase as the baseline. The normalized protein expression of different markers (β-catenin, OCT4, and KLF4) in the OSCC tumor specimens were represented as a heat map by using the Web-based application "Heatmapper."[30]

Immunohistochemistry analysis

Immunohistochemical analysis of formalin-fixed, paraffin-embedded tissue blocks from patients with

OSCC was performed for β-catenin and CSC markers (OCT4, KLF4) as reported earlier.[29,31] Briefly, formalin-fixed, paraffin-embedded blocks were cut into 3-μm sections and dried at 60°C for 3 hours, deparaffinized, and dehydrated. Antigen retrieval was done by using a microwave oven in 10 mM sodium citrate buffer (pH 6.0) for 20 minutes, followed by peroxide blocking for 30 minutes in the dark. The sections were then blocked with 5% bovine serum albumin in PBS followed by addition of primary antibody (1:1000) at 4°C overnight. Next day, sections were washed with PBS and then incubated with secondary antibody (1:1000) conjugated with horse radish peroxide, followed by washing and treatment with 3,3′-diaminobenzidine chromogen. Then, the sections were washed and counterstained with hematoxylin. Leica (DM 2000) bright-field microscopy was used for imaging. The expression of the marker was represented in terms of Histoscore.[42] Histoscore was calculated by using the following formula:

$$[1 \times (\%\ \text{cells}\ 1+) + 2 \times (\%\ \text{cells}\ 2+) + 3 \times (\%\ \text{cells}\ 3+)]$$

where percentage of positive cells (0%−100%) were multiplied by intensity (weak = 1; moderate = 2; and strong = 3) to obtain a maximum score of 300. Scale bars were calculated by using Image J software. With regard to KLF4 and OCT4, positive markings were considered for nuclear staining, and for β-catenin, both nuclear staining and cytoplasmic staining were considered.

RNA extraction and quantitative real-time polymerase chain reaction

Patient tissue samples were used for total RNA extraction with TRIZOL reagent (Invitrogen) and cDNA synthesis as reported before.[29] Quantitative real-time polymerase chain reaction analyses was

Table I. Association of expression of β-catenin and clinicopathologic characteristics in patients with oral squamous cell carcinoma (OSCC) by χ^2 test (n = 102)

Clinicopathologic characteristics	*No. of patients*	*High expression of β-catenin*		*P value*
		Cut margin	*Tumor*	
Age				
≥60 years	61	32	29	.716
<60 years	41	20	21	
Gender				
Female	15	10	5	.188
Male	87	42	45	
Histologic grade				
Moderate	53	34	19	.006 (*)
Well	49	18	31	
Site of tumor				
Tongue	36	20	16	.495
Buccal mucosa	66	32	34	
Recurrence				
Nonrecurrent	73	30	43	.002 (*)
Recurrent	29	22	7	
Lymph node metastasis				
No	72	34	38	.239
Yes	30	18	12	
Lymphovascular invasion (LVI)				
No	95	47	48	.262
Yes	7	5	2	
Perineural invasion (PNI)				
No	75	40	35	.428
Yes	27	12	15	
Bone metastasis				
No	91	48	43	.305
Yes	11	4	7	
OCT4 expression				
Cut margin (CM) high	58	39	19	
Tumor high	44	13	31	.000 (*)
KLF4 expression				
CM high	55	37	18	.000 (*)
Tumor high	47	15	32	

*Statistically significant.

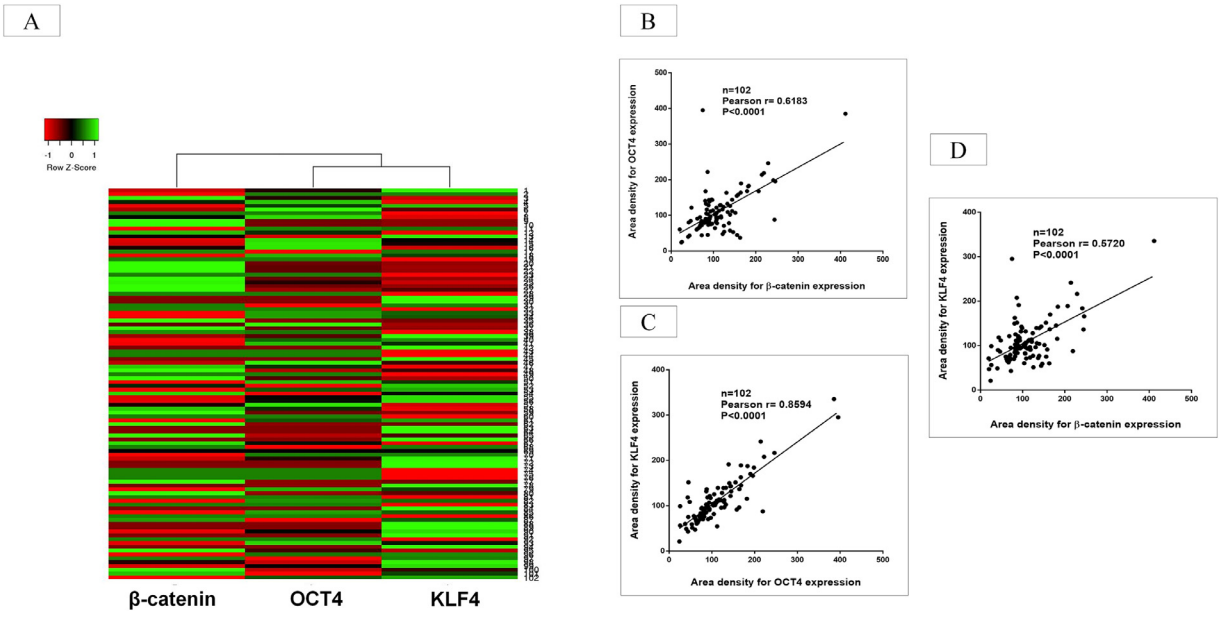

Fig. 1. Expression of β-catenin, KLF4, and OCT4 in oral squamous cell carcinoma (OSCC). **(A)** Heat map of fold change in protein expression of β-catenin, KLF4, and OCT4 in tumor with respect to the cut margin (CM) of 102 patients with OSCC. **(B–D)** Pearson correlation analysis of expression of β-catenin, KLF4, and OCT4 in 102 patients with OSCC.

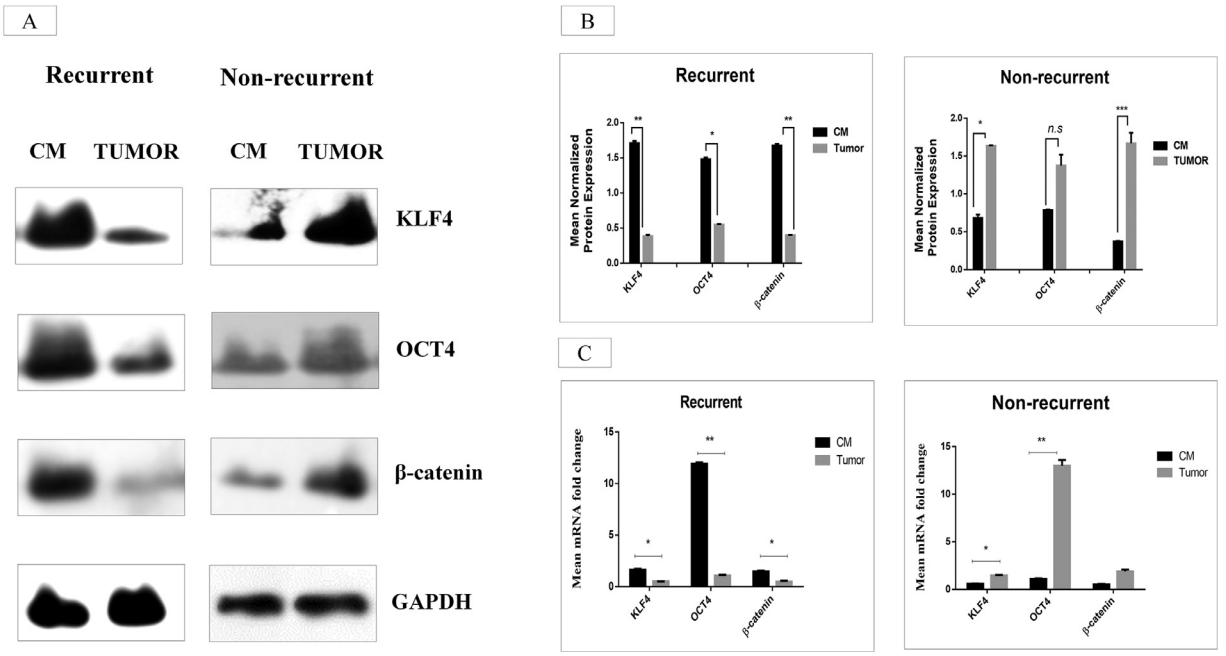

Fig. 2. Western blot and gene expression analysis of cancer stem-like cell (CSC) markers (KLF4, OCT4) and β-catenin in OSCC. **(A)** Representative images of Western blots showing differential expression of β-catenin, OCT4, and KLF4 in the cut margin (CM) and Tumor areas of recurrent and nonrecurrent oral squamous cell carcinoma (OSCC). **(B)** Quantitative representation of β-catenin, OCT4, and KLF4 protein expression in CM and tumor areas of recurrent and nonrecurrent OSCC. **(C)** Graphical representation of gene expression of β-catenin, OCT4, and KLF4 in CM and tumor areas of recurrent and nonrecurrent OSCC.

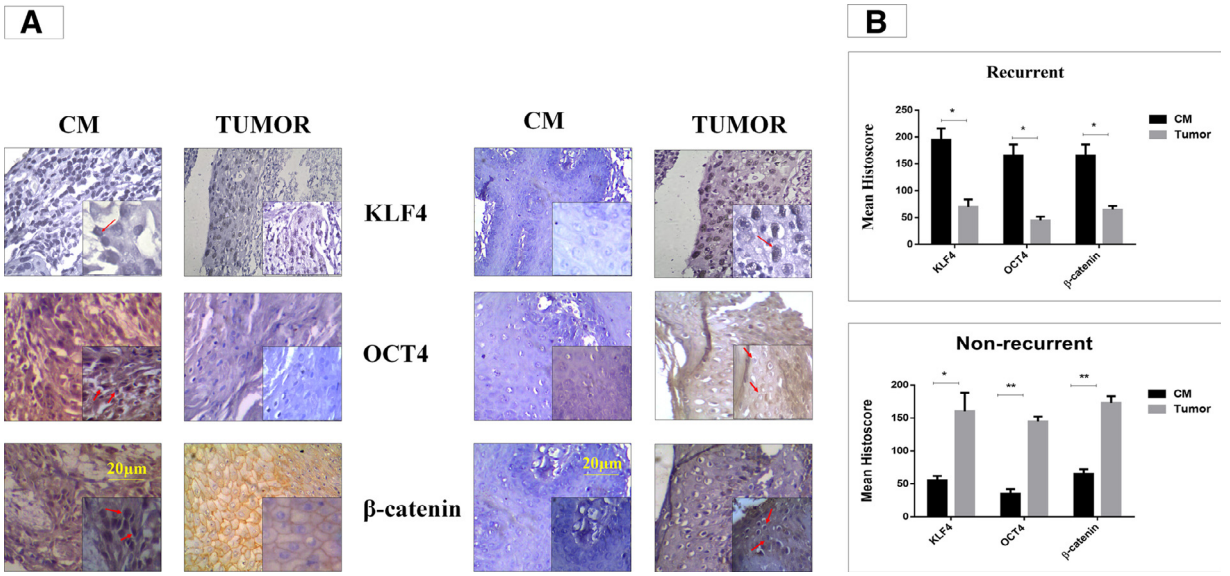

Fig. 3. Immunohistochemical analysis of cancer stem-like cell (CSC) markers (KLF4, OCT4) and β-catenin in OSCC. **(A)** Comparative immunohistochemical analysis of expression of β-catenin, OCT4, and KLF4 in CM and tumor areas of recurrent and nonrecurrent OSCC. **(B)** Graphical representation of mean Histoscore of β-catenin, OCT4, and KLF4 expression in CM and tumor areas of recurrent and nonrecurrent OSCC.

performed for CSC markers (OCT4, KLF4) and β-catenin with Power Up SYBR Green (× 2) (Invitrogen). β-actin was used as the housekeeping gene, and mRNA fold change was calculated by using the $2^{-\Delta\Delta CT}$ method. The primer sequences are provided in Supplementary Table I.

Statistical analysis

Statistical analysis was performed by using the GraphPad Prism 6 software. Pearson correlation analysis was performed by using GraphPad Prism 6 to evaluate the correlation between expression of CSC markers (OCT4 and KLF4) and β-catenin. The χ^2 test was used to evaluate the association of CSC markers expression with clinicopathologic characteristics with the SPSS software version 24(SPSS Inc.). The Kaplan-Meier (KM) analysis and the log-rank test were used to determine the DFS and OS of patients by using the SPSS software. Univariate and multivariate Cox regression analyses were performed for different prognostic factors in SPSS software. *P* value of = .05 or less was considered as statistically significant. Two-way analysis of variance and *t* tests were performed to assess statistical significance.

RESULTS

Expression profiling of CSC markers and β-catenin in surgical CMs and tumor areas in OSCC

In this study, 102 patients with OSCC were included. WB analysis was performed to check for the expression of CSC markers (KLF4 and OCT4) and β-catenin in each OSCC patient's surgically removed tumor tissue and the respective CM counterparts. The fold change in protein expression of CSC markers (OCT4 and KLF4) and β-catenin in tumor specimens with respect to CMs were represented as a heat map for all 102 patients with OSCC (Figure 1A). Pearson correlation analysis also revealed that expression of β-catenin and CSC markers in OSCC were positively correlated with each other (Figures 1B−1D).

Increased expression of CSC markers and β-catenin in surgical CMs of patients with recurrent OSCC

Patients with OSCC were grouped into 2 cohorts, namely, recurrent and nonrecurrent. WB and gene expression analyses demonstrated that patients with recurrent OSCC exhibited increased expression of CSC markers (KLF4 and OCT4) along with β-catenin in the surgical CM region compared with the tumor counterpart. In contrast, in nonrecurrent OSCC, increased expression of KLF4, OCT4, and β-catenin were observed in the tumor region as compared with the CM region (Figures 2A−2C).

Immunohistochemical analysis also showed increased nuclear localization of β-catenin, KLF4, and OCT4 in the epithelial tissues of the surgical CM region in recurrent OSCC compared with the tumor region. In contrast, strong expression of CSC markers (OCT4 and KLF4) and β-catenin were observed in both the nucleus and the cytoplasm of tumor tissues in nonrecurrent OSCC (Figures 3A and 3B). Increased

Table II. Association of expression of OCT4 and clinicopathologic characteristics in patients with oral squamous cell carcinoma (OSCC) by χ^2 test (n = 102)

Clinicopathologic characteristics	*No. of patients*	*High expression of OCT4*		*P value*
		Cut margin	*Tumor*	
Age				
≥60 years	61	34	27	.780
<60 years	41	24	17	
Gender				
Female	15	7	8	.388
Male	87	51	36	
Histologic grade				
Moderate	53	36	17	.019 (*)
Well	49	22	27	
Site of tumor				
Tongue	36	22	14	0.522
Buccal mucosa	66	36	30	
Recurrence				
Nonrecurrent	73	36	37	.015 (*)
Recurrent	29	22	7	
Lymph node metastasis				
No	72	43	29	.366
Yes	30	15	15	
Lymphovascular invasion (LVI)				
No	95	52	43	.110
Yes	7	6	1	
Perineural invasion (PNI)				
No	75	45	30	.286
Yes	27	13	14	
Bone metastasis				
No	91	50	41	.261
Yes	11	8	3	
β-catenin expression				
Cut margin (CM) high	52	39	13	.000 (*)
Tumor high	50	19	31	
KLF4 expression				
CM high	55	41	14	.000 (*)
Tumor high	47	17	30	

*Statistically significant.

nuclear localization of β-catenin demonstrated the transcriptionally active form of β-catenin in the nucleus, along with cytoplasmic expression in the surgical CMs in the recurrent OSCC cases.

Association of different clinicopathologic factors with the expression of CSC markers and β-catenin

To evaluate the association of the expression of CSC markers (OCT4 and KLF4) and β-catenin with different clinicopathologic factors, the χ^2 test was performed (Tables I–III). The χ^2 test with different clinicopathologic factors revealed a significant correlation between histologic grade and disease recurrence with the expression of β-catenin and OCT4 (see Tables I and II). Likewise, a significant association was observed between disease recurrence and the expression of KLF4 (see Table III). We further observed that the expression of β-catenin, OCT4, and KLF4 were also significantly associated with each other (see Tables I–III).

Increased expression of KLF4 in the surgical CM is independently associated with prognosis

Furthermore, we performed univariate Cox regression analysis to evaluate for the association between different clinicopathologic factors with the DFS and OS of patients with OSCC (Table IV). We observed that recurrence was the only factor that was significantly associated with the DFS and OS of patients with OSCC. No significant association was observed between the DFS and OS of the 102 patients with OSCC and the expression of β-catenin and CSC markers. KM analysis also revealed that patients with recurrent OSCC had reduced DFS and OS compared with those with nonrecurrent OSCC (Figures 4A and 4B). We performed univariate Cox regression analysis with different prognostic factors in subcohort of patients with recurrent OSCC and

Table III. Association of expression of KLF4 and clinicopathologic characteristics in oral squamous cell carcinoma (with oral squamous cell carcinoma (OSCC) patients by χ^2 test (n = 102)

Clinicopathologic characteristics	*No. of patients*	*High expression of KLF4*		*P value*
		Cut margin	*Tumor*	
Age				
≥60 years	61	37	24	.096
<60 years	41	18	23	
Gender				
Female	15	10	5	.284
Male	87	45	42	
Histologic grade				
Moderate	53	30	23	.572
Well	49	25	24	
Site of tumor				
Tongue	36	22	14	.282
Buccal mucosa	66	33	33	
Recurrence				
Non-Recurrent	73	33	40	.005 (*)
Recurrent	29	22	7	
Lymph node metastasis				
No	72	40	32	.608
Yes	30	15	15	
Lymphovascular invasion (LVI)				
No	95	50	45	.336
Yes	7	5	2	
Perineural invasion (PNI)				
No	75	44	31	.109
Yes	27	11	16	
Bone metastasis				
No	91	49	42	.965
Yes	11	6	5	
β-catenin expression				
Cut margin (CM) high	52	37	15	.000 (*)
Tumor high	50	18	32	
OCT4 expression				
CM high	58	41	17	.000 (*)
Tumor high	44	14	30	

*Statistically significant.

observed that expression of KLF4, OCT4, and β-catenin exhibited significant association with the DFS and OS of these patients (Table V). KM analysis for the recurrent OSCC cohort revealed that high expression of β-catenin, OCT4, and KLF4 in CMs was associated with a poor prognosis (Figures 4C−4H). In addition, multivariate analysis with each significant variable obtained from univariate analysis demonstrated that KLF4 expression was independently associated with the prognosis of patients with recurrent OSCC (Table VI).

DISCUSSION

The primary treatment regime for oral cancer is surgery, followed by radiotherapy and chemotherapy. Disease relapse in patients with OSCC has been attributed to acquired resistance to chemotherapy and radiotherapy.[43] Chronic insult by chemotherapeutic drugs triggers an adaptive response and promotes cancer cells acquiring a stem cell−like phenotype.[9] In this study, we selected a cohort of patients with OSCC to investigate the expression pattern of CSC markers, along with β-catenin, in CMs and tumor tissue samples. We observed a significant positive correlation between the expression of β-catenin and CSC markers (OCT4 and KLF4) in OSCC tumors. We further divided the patients with OSCC into 2 cohorts, namely, recurrent and nonrecurrent. Increased expression of β-catenin and CSC markers (KLF4 and OCT4) was observed in the surgical CMs of patients with recurrent OSCC compared with the tumor counterpart through WB analysis, gene expression, and immunohistochemistry analysis, whereas nonrecurrent OSCC exhibited increased expression of β-catenin, KLF4, and OCT4 in the tumor region (see Figure 2).

In the majority of HNSCC cases, oral cavity is the primary tumor site. It was reported in earlier studies that Wnt/ β-catenin signaling contributes to aggressiveness of tumor and therapy resistance in OSCC.[36−38] Wnt/ β-catenin signaling pathway was reported as a major pathway that contributes to the maintenance of CSC

Table IV. Univariate Cox regression analysis of prognostic factors in patients with oral squamous cell carcinoma (OSCC) (n = 102)

Variables	*Disease-free survival*			*Overall survival*		
	Hazard ratio	*95% confidence interval*	*P value*	*Hazard ratio*	*95% confidence interval*	*P value*
Age						
<60 years vs ≥60 years	1.505	0.731−3.099	.268	1.593	0.773−3.283	.207
Gender						
Male vs Female	1.306	0.455−3.751	.620	1.291	0.450−3.704	.635
Grade						
Well vs Moderate	0.623	0.294−1.320	.217	0.665	0.314−1.408	.287
Site of tumor						
Buccal mucosa vs Tongue	2.312	0.945−5.659	.066	2.374	0.969−5.815	.059
Recurrence						
Recurrent vs nonrecurrent	5.461	2.520−11.836	.000 (*)	4.243	2.041−8.822	.000 (*)
Lymphovascular invasion						
Yes vs No	0.982	0.232−4.151	.981	0.955	0.227−4.027	.950
Perineural invasion						
Yes vs No	0.857	0.394−2.170	.925	0.850	0.363−1.988	.708
Lymph node metastasis						
Yes vs No	0.521	0.210−1.289	.158	0.516	0.210−1.272	.151
Bone metastasis						
Yes vs No	0.716	0.170−3.020	.649	0.757	0.179−3.198	.705
Expression of β-catenin						
High in cut margin (CM) vs High in tumor	1.934	0.915−4.089	.084	1.910	0.905−4.029	.089
Expression of KLF4						
High in CM vs High in tumor	1.637	0.777−3.448	.194	1.626	0.772−3.421	.201
Expression of OCT4						
High in CM vs High in tumor	1.189	0.570−2.477	.644	1.166	0.561−2.424	.681

*Statistically significant.

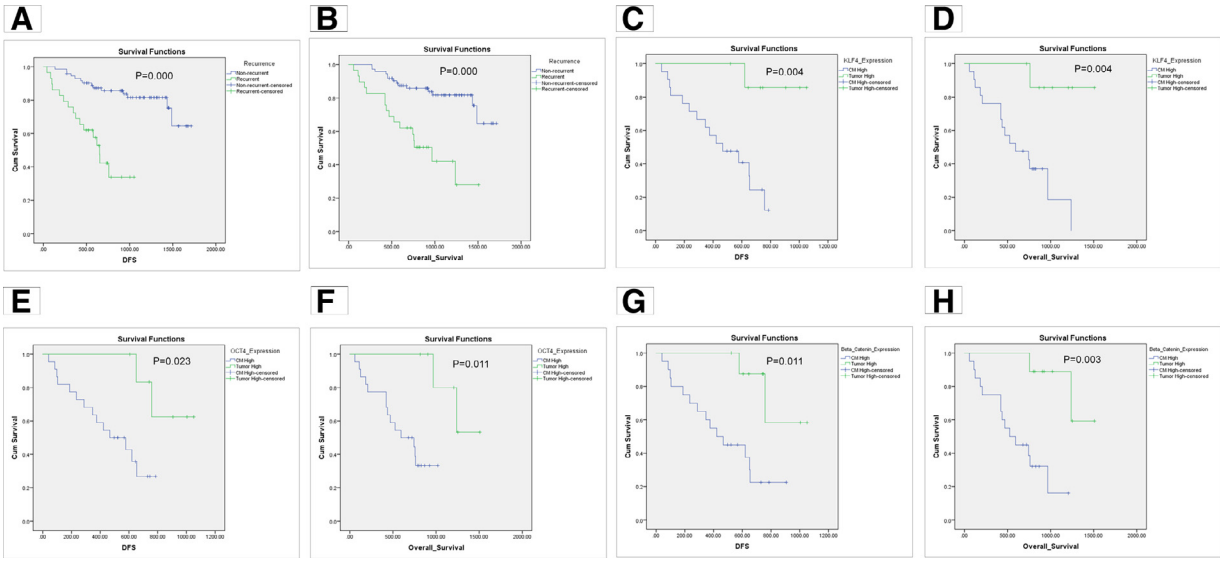

Fig. 4. Kaplan-Meier survival analysis. **(A, B)** Kaplan-Meier curves of disease-free survival (DFS) and overall survival (OS) in recurrent and nonrecurrent OSCC. **(C, D)** Kaplan-Meier curves of DFS and OS on the basis of expression of KLF4 in cut margin and tumor areas in recurrent OSCC. **(E, F)** Kaplan-Meier curves for DFS and OS of patients with recurrent OSCC exhibiting higher expression of OCT4 in cut margins compared with tumor areas. **(G, H)** Kaplan-Meier curves of DFS and OS on the basis of expression of β-catenin in cut margin and tumor areas of recurrent OSCC patients. $P < .05$ is considered statistically significant.

phenotype and survival.[34] In our previous study, we found a correlation between increased expression of β-catenin in the CM region in recurrent HNSCC associated with a poor prognosis.[39] Therefore, we suggest that increased nuclear expression of β-catenin promotes the expression of KLF4 and OCT4.

The understanding of resistance to chemotherapy is one of the major steps in improving treatment efficacy and outcomes in patients with OSCC. Recent studies have reported that CSCs have a possible association with disease relapse and prognosis in different solid tumors.[44] Cancer cells can be induced to acquire the resistance phenotype under chronic exposure to chemotherapeutic stress. The enrichment of CSCs in the preclinical model is an alternative approach to testing potential drugs that can inhibit chemoresistant populations.[45] Three different pathways, namely, the Wnt, Notch, and Hedgehog pathways, are mainly responsible for the regulation and maintenance of CSC characteristics.[46] Previous studies have demonstrated that the presence of CSCs leads to chemoresistance to cisplatin in hepatocellular carcinoma.[47] The Wnt/β-catenin signaling pathway also maintains the self-renewal of CSCs and contributes in tumorigenicity by activating OCT4 in HNSCC.[40]

Furthermore, χ^2 analysis also revealed a significant association between high expression levels of CSC markers (KLF4 and OCT4) and β-catenin in the CM region of patients with disease recurrence. Univariate Cox regression analysis performed on the 102 patients with OSCC also demonstrated that recurrence is a single contributing factor among all of the clinicopathologic characteristics associated with prognosis in patients with OSCC. Univariate Cox regression analysis and KM analysis in the cohort of patients with recurrent OSCC demonstrated that high expression of β-catenin, KLF4, and OCT4 in the CM region of the recurrent group was associated with a poor prognosis. Multivariate Cox regression analysis revealed that increased expression of KLF4 in the CM region of recurrent OSCC was independently associated with a poor prognosis. It was reported earlier that persistent expression of KLF4, a CSC marker, is associated with a poor prognosis in HNSCC.[48] In accordance with the previous studies, we also observed increased expression of KLF4, along with β-catenin and OCT4, in the surgical CMs in recurrent OSCC. These findings corroborated the presence of CSCs in the surgical CM, which may further contribute towards disease relapse.

CONCLUSIONS

The findings from the present study highlight the presence of a CSC population in the CMs, characterized by high expression of β-catenin and CSC markers (KLF4 and OCT4), further contributing to recurrence. We also observed that increased expression of KLF4 in the surgical CM was independently associated with a poor prognosis in patients with OSCC. Thus, KLF4 can be

Table V. Univariate Cox regression analysis of different prognostic factors in patients with recurrent oral squamous cell carcinoma (OSCC) (n = 29)

Variables	*Disease-free survival*			*Overall survival*		
	Hazard ratio	*95% confidence interval*	*P value*	*Hazard ratio*	*95% confidence interval*	*P value*
Age						
<60 years vs ≥60 years	1.992	0.737−5.379	.174	2.146	0.791−5.819	.133
Gender						
Male vs Female	2.332	0.305−17.816	.415	1.945	0.255−14.850	.521
Grade						
Well vs Moderate	0.566	0.194−1.654	.298	0.692	0.240−1.997	.496
Site of tumor						
Buccal mucosa vs Tongue	2.145	0.609−7.551	.235	2.160	0.608−7.670	.234
Lymphovascular invasion						
Yes vs No	1.225	0.156−9.604	.847	1.155	0.147−9.045	.891
Perineural invasion						
Yes vs No	0.428	0.136−1.346	.147	0.421	0.133−1.332	.141
Lymph node metastasis						
Yes vs No	0.863	0.277−2.688	.799	0.896	0.288−2.789	.850
Bone metastasis						
Yes vs No	1.254	0.282−5.578	.766	1.425	0.317−6.408	.644
Expression of β-catenin						
High in cut margin (CM) vs High in tumor	5.583	1.254−24.861	.024 (*)	11.923	1.543−92.121	.018 (*)
Expression of KLF4						
High in CM vs High in tumor	10.978	1.430−84.248	.021 (*)	11.295	1.459−87.461	.020 (*)
Expression of OCT4						
High in CM vs High in tumor	5.224	1.114−24.497	.036 (*)	10.284	1.237−85.524	.031 (*)

*Statistically significant.

Table VI. Multivariate Cox regression analysis with respect to expression of β-catenin, OCT4 and KLF4 in patients with recurrent oral squamous cell carcinoma (OSCC) (n = 29)

Variables	*Disease-free survival*			*Overall survival*		
	Hazard ratio	*95% confidence interval*	*P value*	*Hazard ratio*	*95% confidence interval*	*P value*
Expression of β-catenin						
High in cut margin (CM) vs High in tumor	2.885	0.535–15.562	.218	6.711	0.779–57.853	.083
Expression of KLF4						
High in CM vs High in tumor	8.427	1.084–65.526	.042 (*)	10.754	1.223–94.554	.032 (*)
Expression of OCT4						
High in CM vs High in tumor	2.446	0.443–13.515	0.305	19.590	0.529–725.145	0.106

*Statistically significant.

used as predictive biomarker for monitoring disease progression and may serve as an independent prognostic marker based on its expression level. However, the specificity and sensitivity of KLF4, which were indicated to be highly correlated with recurrence in the current cohort, must be confirmed in larger cohorts for further validation.

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DISCLOSURE

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.oooo.2019.02.021.

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