Prognostic Significance of ALDH1, Bmi1, and OCT4 Expression in Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma

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Abstract

Background: Increasing evidence suggests the involvement of cancer stem cells (CSCs) in both oral epithelial dysplasia (OED) and oral squamous cell carcinoma (OSCC). Among the various CSC markers, aldehyde dehydrogenase (ALDH) 1, B cell-specific Moloney murine leukaemia virus integration site 1 (Bmi1), and octamer-binding protein 4 (OCT4) have been noted to increase in OSCC. The aim of the study was to analyze ALDH1, Bmi1, and OCT4 expression in OED and OSCC with clinicopathologic correlation and survival analysis.

Methods: A total of 40 cases each of OED and OSCC were retrieved from departmental archives. Expression of ALDH1, Bmi1, and OCT4 was analyzed using immunohistochemistry and was correlated with clinicopathological parameters. A follow-up ranging from 6 to 52 months was considered for Kaplan-Meier survival analysis. The log-rank test was performed to analyze significant difference in survival rates.

Results: The expression levels of ALDH1, Bmi1, and OCT4 increased significantly from OED through OSCC (P < .05). The expression of ALDH1 and OCT4 showed a significant correlation with lymph node metastasis. Positive cases of ALDH1 showed a significantly reduced survival rate compared to cases showing negative expression. Kaplan-Meier survival analysis showed a significant reduction of survival rate (P = .00) in patients showing a positive expression for all the 3 markers.

Conclusion: ALDH1 and OCT4 could be used as individual prognostic markers for assessing prognosis. ALDH1, Bmi1, and OCT4 could be used as a collective panel of markers to enable surgeons in predicting the prognosis of patients and thereby carry out prompt follow-up for such cases.

Keywords
neoplastic stem cells, mouth neoplasms, aldehyde dehydrogenase 1, Bmi1 protein, POU5F1 protein, survival analysis

Introduction

Despite advancements in the field of medicine, head and neck squamous cell carcinoma (HNSCC) remains to be the sixth most common cancer worldwide, with 300 000 cases being diagnosed every year.1 The overall 5-year survival rate for intraoral carcinoma ranges from 40% to 58% with the majority of deaths occurring within the first 5 years.2 The reason for such poor prognosis could be attributed to metastasis to regional lymph nodes, decreased response to current treatment, and the presence of cancer stem cells (CSCs) within the tumor microenvironment.

CSCs are characterized by their ability to self-renew and differentiate into various cell lineages, which is essential for tumor initiation, progression, and drug resistance.3-5 Various CSC markers, such as aldehyde dehydrogenase (ALDH), B cell-specific Moloney murine leukemia virus integration site 1 (Bmi1), and octamer-binding protein 4 (OCT4), have been reported to be overexpressed in OSCC.6-8 ALDH1 is a marker for CSCs, and its expression is associated with poor prognosis and resistance to chemotherapeutics.9,10 Bmi1 is a member of the BMI family of transcriptional regulators, and its overexpression is observed in various malignancies.11 OCT4 is a critical regulator of embryonic stem cell self-renewal and pluripotency and is implicated in cancer stem cell properties.12,13 The aim of the study was to analyze ALDH1, Bmi1, and OCT4 expression in OED and OSCC with clinicopathologic correlation and survival analysis.
therapeutics, and locoregional recurrences. Hence, improved diagnostic and therapeutic strategies have become a necessity in the present-day medical science to improve mortality and morbidity of affected patients.

The possible reasons for the aggressive biological behavior of cancer have been a booming area of research. Once such explanation was the existence of cancer stem cells (CSCs) proposed by Francesco Durante in 1874. As defined by the American Association of Cancer Research “CSCs are a subset of cells with the capability of self-renewal and differentiation into heterogeneous lineages that constitute the tumor mass.” Recently, there has been improving evidence that supports CSC theory in HNSCC. It has also been suggested that survival of CSCs could be held accountable for the aggressiveness and recurrence of HNSCC. Few properties of CSCs that make them diverse from other tumor cells are their self-renewing ability to differentiate into varied phenotype, capacity to initiate tumors even from minute numbers, and increased chemoresistance. Cancer stem cells are most often in inactive G0 phase which renders them resistant to radiotherapy and chemotherapy. CSCs have been noted for its role as a predictive marker for malignant transformation of oral leukoplakic lesions. Wen et al proposed carcinogenesis to be a recapitulation of embryogenesis and that proteins involved in embryogenesis play an important role in carcinogenesis also. Among the various markers involved in embryogenesis, OCT4 is considered to be very crucial in the maintenance and pluripotency of embryonic stem cells. OCT4 has also been noted to be increased in oral potentially malignant disorders (OPMDs) such as oral epithelial dysplasia (OED) including frank carcinomas. However, limited studies have been employed to assess the expression pattern of ALDH1, Bmi1, and OCT4 in OPMDs and OSCC in the Indian population with clinicopathological correlation and survival analysis. The aim of the study was to analyze the expression of the above-mentioned markers in OSCC and OED samples and further note the association of clinicopathologic parameters and survival rates.

Materials and Methods

Selection of Patients and Tissue Samples

The study was approved by the board of Vision Group on Science and Technology, Government of Karnataka, India (No: VGST/GRD-631/2016-17/2017-18/185). Written informed consent was obtained from the patients prior to the biopsy procedure. A total of 80 formalin-fixed paraffin-embedded blocks were retrieved from departmental archives of Department of Oral Pathology & Microbiology, Faculty of Dental Sciences, MS Ramaiah University of Applied sciences, Bengaluru, India. The selected cases were histopathologically confirmed samples of 40 OED and 40 OSCC. Histopathological grading was carried out based on World Health Organization classification and Broder’s criteria for OED and OSCC, respectively. Among the 40 OSCC samples, 20 were nonmetastatic cases and 20 were metastatic. The follow-up for the selected patients ranged from 10 to 53 months with a mean follow-up period of 33.3 months.

Immunohistochemical Procedure

Thick sections of 4 mm were taken onto poly-L-lysine coated glass slides. The sections were deparaffinized and subjected to rehydration through decreasing grades of alcohol. The antigen retrieval was done by immersing slides in citrate buffer (pH 6.0) for 15 minutes using a pressure cooker. Further endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxidase for 10 minutes. Further, blocking of non-specific binding was carried out by bovine serum albumin for 30 minutes. The sections were then incubated overnight at 4°C with primary antibodies ALDH1 (1:100; Medaysis, Livermore, California, USA). Bmi1 (1:200; Medaysis), and OCT-4 (1:200; Medaysis). This was followed by incubation with respective
horseradish peroxidase-conjugated secondary antibody. The chromogen 3,3′-diaminobenzidine was used to localize the antigen–antibody binding. Counterstaining with hematoxylin was done, and sections were then mounted and viewed under a light microscope. Positive and negative controls were stained for each antibody.

**Immunohistochemical Analysis**

The immunostained sections were evaluated based on the criteria given by Ortiz et al.33 Li et al,34 and Huang et al11 for ALDH1, Bmi1, and OCT-4, respectively. The evaluation was done under by 2 independent observers who were blinded about the patient outcome utilizing Olympus Optical Microscope BX53F2 (Tokyo, Japan). Immunohistochemical score for ALDH1 was given based on percentage of cells stained: 0 (<5%), 1 (5%-20%), 2 (21%-50%), and 3(>50%). The score for ALDH1 was further graded as negative (0), low expression (1), and high expression (2-3). Immunohistochemical evaluation of Bmi1 and OCT4 was performed by assessing the intensity of stain and percentage of cells stained. Staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The scoring for percentage of cells was given as 0 (0%), 1 (1%-25%), 2 (26%-50%), 3 (51%-75%), and 4 (>75%). The final immunoreactive score (IRS) was obtained by adding scores of staining intensity and percentage of cells stained. The IRS was then graded for Bmi1 as negative (0-1), weakly positive (2-4), and strongly positive (5-7). The IRS grading for OCT4 was given as negative (0-1), weakly positive (2-4), and strongly positive (5-7). In cases of any disagreement, the slides were re-examined to obtain a consensus. The expression of ALDH1, Bmi1, and OCT4 was dichotomized into ALDH1 negative/positive, Bmi1 weakly positive/strongly positive, and OCT-4 negative/positive for assessing the survival analysis. The photomicrographs were captured with a Jenoptik Progres Gryphax Arktur USB 3.0 microscope camera, Jena, Germany.

**Statistical Analysis**

The difference in the expression of ALDH1, Bmi1, and OCT-4 between OSCC and OED was analyzed using chi-square test and Fisher exact test. The relationship between the clinicopathologic parameters and the expression of each CSC marker was evaluated using Fisher exact test. The correlation among the markers was analyzed using Spearman correlation. Kaplan-Meier survival analysis was done to assess the effect of the markers on the survival of cases with OSCC. Log-rank (Mantel-Cox) test was carried out to evaluate whether the resultant survival curves had any statistically significant difference. The statistical tests were performed using IBM SPSS Statistics for Windows, version 20 (IBM Corp, Armonk, New York, USA). For all statistical tests, a P value of <.05 was considered to be statistically significant.

**Results**

**Demographic Findings**

A total of 40 cases of OED and 40 cases of OSCC were selected after histopathologic confirmation of diagnosis. The 40 cases of OED comprised of 80% (32/40) mild epithelial dysplasia and 20% (8/40) were moderate epithelial dysplasia. OSCC cases comprised of 55% (22/40) well differentiated, 40% (16/40) moderately differentiated, and 5% (2/40) poorly differentiated grades. The age of the selected patients with OED ranged from 31 to 86 with a mean age of 56. 8 years and the age of selected OSCC cases ranged from 27 to 72 with a mean age of 59.6 years. Among cases of OED, 18 (45%) were females and 22 (55%) were males. OSCC cases consisted of 17 (42.5%) females and 23 (57.5%) males. Most of the OED cases selected had occurrence in buccal mucosa (80%) followed by tongue (15%) and lip (5%). The most common site of occurrence in the selected OSCC cases was buccal mucosa (35%) followed by tongue (30%) and gingivo-buccal sulcus (GBS; 10%). Among the 40 cases of OSCC, 35% belonged to the stage I or II and 65% belonged to stage III or IV.

**Immunohistochemistry Findings**

The antibodies to ALDH1, Bmi1, and OCT4 were standardized using negative and positive controls. Aldehyde dehydrogenase 1. Immunostaining in tissue samples exhibited cytoplasmic staining that was considered as positive and graded based on the selected criteria. The expression pattern of ALDH1, Bmi1, and OCT4 has been shown in Tables 1 and 2. A significant difference in the expression pattern was noted between OED and OSCC (P = .000). In OED, 65% (26/40) of cases showed negative expression, 25% (10/40) showed low expression, and only 10% (4/40) showed high expression (Figure 1A-C). Unlike OED, 65% of OSCC exhibited high expression (Figure 1D-L) and 5% and 30% of cases showed low and negative expression, respectively. Further, in OED, correlation of ALDH expression with clinicopathologic parameters showed no significant results with respect to age, gender, habit history, site, and histologic grades. The ALDH expression correlated significantly with clinicopathologic parameters such as site, histological grade, tumor size, and tumor staging in OSCC samples. The ALDH1 expression increased significantly in cases having OSCC with lymph node metastasis compared to nonmetastatic cases (P = .000). However, no significant correlation could be observed with age, gender, and habit history in OSCC. A Kaplan-Meier analysis showed that the survival rates for patients stratified into ALDH1 negative and ALDH1 positive were 91.7% and 34.4%, respectively (P = .01; Figure 2A) (Supplemental material).

**B Cell-specific Moloney murine leukaemia virus integration site 1**

The Bmi1 expression increased significantly from OED to OSCC (P = 00): 85% (34/40) of OSCC cases showed strong
positivity as compared to OED where strong positivity was shown by only 10% (4/40; Table 1 and Figure 3A-C). In OSCC, Bmi1 expression was similar across groups exhibiting lymph node metastasis and nonmetastatic group (Table 2 and Figure 3D-L). Correlation with clinicopathologic parameters in OED showed no correlation with age, site, habit history, and histological grade. However, a correlation was noted among males and females, where all the males showed a weak positivity as compared to females where 76.5% (13/17) showed weak positivity and rest 23.5% (4/17) showed strong positivity. In OSCC, except for age and site, all other clinicopathologic parameters showed no correlation with Bmi1 expression. Kaplan-Meier survival curves did not show any statistically significant difference between Bmi1+ve and Bmi1-ve cases (Figure 2B).

Octamer-binding protein 4. OSCC cases showed significantly increased OCT4 expression as compared to OED (P = .000; Table 1); 60% (24/40) and 40% (16/40) of cases with OED showed negative and weak expression, respectively (Figure 4A-D). In OSCC, 45% (18/40) of cases were weakly positive, 35% (14/40) were strongly positive, and 20% (8/40) were negative for OCT4, (Figure 4D-L). The OCT4 expression showed no correlation with the clinicopathologic parameters in OED samples as well as OSCC samples with respect to age, gender, habit history, and histologic grading. In OSCC cases, a significant correlation existed between OCT4 expression and metastatic OSCC. A strong positive staining was observed in 70% (14/20) of lymph node metastatic cases in contrast to the nonmetastatic cases where none of the cases showed strong positive staining (Table 2). A significant increase in OCT4 expression was associated with site and Tumour, Node, Metastasis (TNM) stage III+IV as compared to stage I+II. Kaplan-Meier survival analysis showed a reduction in the cumulative survival rate of OCT4 positive cases to 40% as opposed to OCT4 negative cases where cumulative survival was 100% (P = .019; Figure 2C).

**Table 1.** Expression of ALDH1, Bmi1, and OCT4 in OED and OSCC Tissue Samples.

| Groups | No. of Cases | ALDH1 | Bmi-1 | OCT4 |
|--------|-------------|--------|--------|------|
|        | n (%)       | Negative | Low Expression | High Expression | P Value | Weakly Positive | Strongly Positive | P Value | Negative | Weakly Positive | Strongly Positive | P Value |
| OED    | 40          | 26 (65.0) | 10 (25.0) | 4 (10.0)       | .000a    | 36 (90.0) | 4 (10.0)       | .000a    | 24 (60.0) | 16 (40.0) | 0 (0.0)      | 0000a     |
| OSCC   | 40          | 12 (30.0) | 2 (5.0)   | 26 (65.0)      |          | 6 (15.0)  | 34 (85.0)      |          | 8 (20.0)  | 18 (45.0) | 14 (35.0)    |          |

Abbreviations: ALDH1, aldehyde dehydrogenase 1; Bmi1, B cell-specific Moloney murine leukaemia virus integration site 1; OCT4, octamer-binding protein 4; OED, oral epithelial dysplasia; OSCC, oral squamous cell carcinoma.

*aStatistically significant P < .05.

**Table 2.** Expression of ALDH1, Bmi1, and OCT4 in Metastatic and Nonmetastatic Groups of OSCC.

| Groups  | No. of Cases | ALDH1 | Bmi-1 | OCT4 |
|---------|--------------|--------|--------|------|
|         | n (%)        | Negative | Low Expression | High Expression | P Value | Weakly Positive | Strongly Positive | P Value | Negative | Weakly Positive | Strongly Positive | P Value |
| Nonmetastatic | 20          | 12 (60.0) | 2 (10.0) | 6 (30.0)       | .000a    | 4 (20.0) | 16 (80.0)      | .661    | 8 (40.0) | 12 (60.0) | 0 (0.0)      | .000a     |
| Metastatic | 20           | 0 (0.0)  | 0 (0.0) | 20 (100.0)     |          | 2 (10.0)  | 18 (90.0)      |          | 0 (0.0)  | 6 (30.0)  | 14 (70.0)    |          |

Abbreviations: ALDH1, aldehyde dehydrogenase 1; Bmi1, B cell-specific Moloney murine leukaemia virus integration site 1; OCT4, octamer-binding protein 4; OSCC, oral squamous cell carcinoma.

*aStatistically significant P < .05.

**Correlation Between ALDH1, Bmi1, and OCT4**

The correlation among the expressions of ALDH1, Bmi1, and OCT4 was tested using Spearman correlation coefficient test. A significant positive correlation was observed between ALDH1 and Bmi1 expression (R = 0.363, P = .001). No correlation could be observed between ALDH1 and OCT4 or Bmi1 and OCT4.

Another intriguing finding in this study was the significantly decreased cumulative survival of double positive cases when compared to cases showing single-marker positivity. The following significant values were obtained by Kaplan-Meier analysis for OSCC groups showing double positive for ALDH1 and Bmi1 (P = .010; Figure 2D); ALDH1 and OCT4 (P = .000; Figure 2E), Bmi1 and OCT4 (P = .019; Figure 2F), and combination of all the 3 markers (P = .020; Figure 2G).

**Discussion**

Aldehyde dehydrogenase comes under the superfamily of nicotine adenine dinucleotide phosphate-dependent enzymes...
consisting of 19 human isoenzymes. These enzymes have a broad localization in cytoplasm, mitochondria, and nucleus. They primarily function to prevent aldehyde toxicity by catalyzing the oxidation of endogenous and exogenous aldehydes into their corresponding carboxylic acids. Increased ALDH1 expression has been frequently noted in various cancers and has been used as a stem cell marker. In the present study, only 35% of cases with OED showed positive ALDH expression, unlike OSCC where 70% cases showed ALDH positivity. Similar findings were observed by Liu et al and Visus et al where 38.3% and 32.5% of patients with OED showed ALDH expression, respectively. Such a finding could suggest the role of ALDH in the stepwise transformation of OED to carcinomas.

Various studies have suggested the expression of ALDH1 to be a predictive marker for malignant transformation of oral leukoplakias. This increase in ALDH from OED to OSCC could be attributed to the role of ALDH in regulating various pathways that contribute to tumorigenesis and stem cell signaling. They are primarily regulated by retinoic acid compounds and other oncogenic pathways such as MUC1-C/ERK and WNT/β-catenin. ALDH oxidizes various aldehydes participating in different signalling mechanisms, minimize reactive oxygen species (ROS) production, prevent DNA damage, and mediate RA signalling cascades. These mechanisms bring about effects on various cellular processes such as cellular proliferation, differentiation, oncogenesis, stemness, and resistance to chemo/radiotherapy.

Assessment of ALDH with clinicopathologic parameters showed significant correlation with respect to site, histological grade, tumor size, and tumor staging. Michifuri et al and

Figure 1. Photomicrographs of epithelial dysplasia and OSCC subjected to staining with antibody to ALDH1 at different magnifications. A, Section of epithelial dysplasia showing low cytoplasmic expression of ALDH1 (IHC, ×100). B, (IHC, ×200). C, (IHC, ×400). D, Section of nonmetastatic OSCC showing low cytoplasmic expression of ALDH1 (IHC, ×100). E, (IHC, ×200). F, (IHC, ×400). G, Section of nonmetastatic OSCC showing high cytoplasmic expression of ALDH1 (IHC, ×100). H, (IHC, ×200). I, (IHC, ×400). J, Section of metastatic OSCC showing high cytoplasmic expression of ALDH1 in tumor islands (IHC, ×100). K, (IHC, ×200). L, (IHC, ×400). ALDH1 indicates aldehyde dehydrogenase 1; IHC, immunohistochemistry; OSCC, oral squamous cell carcinoma.
Tamatani et al also noted ALDH1 to be significantly associated with increasing histologic grades and lymph node metastasis. Chen et al noted ALDH1 expression to be increased in cases with OSCC showing lymph node metastasis. Evidence suggests ALDH1 positive cells to have a greater invasive capacity when compared to ALDH1 negative population of cells. Therefore, high ALDH1 expression could suggest the presence of an increased number of cells with invasive potential in a tumor population leading to increased risk of lymph node metastasis. Survival analysis showed a decrease in the cumulative survival in the ALDH-positive cases of OSCC when compared to the ALDH1-negative cases, suggesting it to be a potent prognostic marker. Similar findings were seen in tongue carcinoma and other HNSCC, where poor survival was noted in cases showing ALDH1 positivity. ALDH1 plays a critical role in metabolizing reactive aldehydes.
thereby reducing oxidative stress in cells. Many chemotherapeutic drugs and radiotherapy generate oxidative stress and lipid peroxidation-derived aldehydes. However, although most tumor cells respond to therapy, there are subpopulation of cells with increased ALDH1 activity rendering them resistant to oxidative damage caused by conventional therapies. This could possibly explain the reason for the correlation of increased ALDH1 activity with poor clinical prognosis as recurrence rates tend to be higher. These observations suggest the potency of ALDH1 to help surgeons predict the prognosis of the patient.

Bmi1 was first isolated as an oncogene that interacted with c-myc in murine lymphomas. It is thought of as a stemness-related gene regulating various biological processes by functioning as a transcriptional repressor. It plays an important role in carcinogenesis and stem cell renewal through chromatin and histone modification and thereby influence the major tumor suppressor genes such as Rb and p53. Bmi1 has been proved to be an efficient predictor for assessing the prognosis in cancers such as breast carcinoma, bladder carcinoma, pancreatic cancer, as well as lung carcinoma. Bmi1 expression has been noted to increase in preneoplastic lesions such as oesophageal adenocarcinomas and oral dysplasias. The results were in accordance with the present study where Bmi1 expression was increased in cases of OED and OSCC. This could imply its role in the malignant transformation of OED. The exact molecular mechanism existing behind the increase in Bmi1 expression is not fully understood. However, Bmi1 has been found to be a direct transcriptional target of c-Myc in human fibroblasts. c-Myc is found to be frequently amplified in cases with OSCC, which in turn could attribute for the increased Bmi1 expression via c-Myc-dependent promoter activation. Further, this may account for the enhanced gene

Figure 3. Photomicrographs of epithelial dysplasia and OSCC subjected to staining with antibody to Bmi1 at different magnifications. A, Section of epithelial dysplasia showing weak nuclear expression of Bmi1 (IHC, ×100). B, (IHC, ×200). C, (IHC, ×400). D, Section of nonmetastatic OSCC showing weak nuclear expression of Bmi1 (IHC, ×100). E, (IHC, ×200). F, (IHC, ×400). G, Section of nonmetastatic OSCC showing strong nuclear expression of Bmi1 (IHC, ×100). H, (IHC, ×200). I, (IHC, ×400). J, Section of metastatic OSCC showing strong nuclear expression of Bmi1 in tumor islands (IHC, ×100). K, (IHC, ×200). L, (IHC, ×400). Bmi1, B cell-specific Moloney murine leukaemia virus integration site 1; IHC, immunohistochemistry; OSCC, oral squamous cell carcinoma.
Bmi1 expression in the event of malignant transformation in oral carcinogenesis. Correlation of Bmi1 expression in cases having OSCC with clinicopathologic parameters such as gender, habit history, histologic grade, tumor size, lymph node metastasis, and TNM staging showed no significant associations. However, there was a significant correlation with site and age in OSCC cases. The older age group (>60 years) showed reduced Bmi1 expression as compared to younger age-group. Cordisco et al. observed downregulation of Bmi1 in primary human keratinocytes obtained from older healthy donors when compared to young donors. This could be attributed to the accumulation ROS associated with mammalian aging. Evidence suggests that increased ROS lead to decreased Bmi1 expression. In elderly patients, increased oxidative stress and ROS production have been noted which could ascribe to the reduced Bmi1 expression. Nevertheless, whether this explanation holds true in patients with OSCC needs to be explored. In the present study, Bmi1 expression did not affect the survival rate of OSCC cases, which was in accordance with other studies by Tamatani et al., Jayasooriya et al., and Wu et al. However, Hayry et al. found Bmi1 expression to favor better prognosis in tongue carcinoma in contrast to our results. This could be due to the varied pathogenesis involved in OSCC in accordance with different etiological factor and different epithelial types in the population groups. In an Indian population, majority of oral cancers occurring are habit (tobacco) related as opposed to those in the Western population. The present study consisted of OSCC cases among which 75% had a habit history of consuming tobacco whereas, in the aforementioned study, the population has not been defined in terms of habit history. Since etiopathogenesis of tobacco-related cancers is different from that of nonhabit related, studies with larger sample size with defined population have to be done for better clarity.

**Figure 4.** Photomicrographs of epithelial dysplasia and OSCC subjected to staining with antibody to OCT4 at different magnifications. A, Section of epithelial dysplasia showing negative nuclear expression of OCT4 (IHC, ×100). B, (IHC, ×200). C, (IHC, ×400). D, Section of nonmetastatic OSCC showing negative nuclear expression of OCT4 in tumor islands (IHC, ×100). E, (IHC, ×200). F, (IHC, ×400). G, Section of nonmetastatic OSCC showing weak nuclear expression of OCT4 (IHC, ×100). H, (IHC, ×200). I, (IHC, ×400). J, Section of metastatic OSCC showing strong nuclear expression of OCT4 in invading tumor cells (IHC, ×100). K, (IHC, ×200). L, (IHC, ×400). IHC indicates immunohistochemistry; OCT4, octamer-binding protein 4; OSCC, oral squamous cell carcinoma.
Octamer-binding protein 4 is a homeodomain transcription factor belonging to the Pit-Oct-Unc family. It has been well established to be one of the most crucial transcription factors that aid in controlling the self-renewal and pluripotency of embryonic stem cells. The OCT4 expression has also been considered as one of the nonsurface markers of CSCs. Studies by Major et al and Hochedlinger et al have reported an increase in the OCT4 expression in OPMDs such as OED which is in accordance with the results of our study. Interestingly, knockdown of OCT4 resulted in the regression of the malignant component. This could suggest the role of OCT4 in the early event of carcinogenesis. The present study showed an increased expression of OCT4 in OSCC when compared to OED. A similar increased OCT4 expression in oral carcinoma was observed in studies by Chiu et al., Huang et al., and Jayasooriya et al. Clinicopathologic correlation showed a significant association with parameters such as site, lymph node metastasis, and TNM staging. Hochedlinger et al also showed a significant correlation between OCT4 and TNM staging, however, results of the current study were not in par their study for parameters such as tumor size, histologic grade, and lymph node metastasis. The disparity could be owed to the small sample size in the current study and thereby further investigations with greater sample size are warranted for validation. Further, a reduced survival rate was observed in OCT4-positive cases with in their study similar to the present study. There appears to be very limited investigations on the role of OCT4 in the etiopathogenesis of OPMDs and OSCC based on our knowledge. Investigation at the molecular level with appropriate validations is required to comment on the same.

Interestingly, the site of occurrence of OSCC seemed to have a marked effect on the expression of ALDH1, Bmi1, and OCT4, wherein all cases of carcinoma of GBS showed strong positivity for all the 3 markers. Majority of the cases are related to the habit of tobacco consumption. Nicotine is one of the major constituents of tobacco. Long-term exposure to nicotine in normal gingival oral epithelial cells and OSCC cell lines upregulated the expression of ALDH1 in a dose-dependent manner. Similarly, nicotine exposure on HNSCC cell lines upregulated the expression of stem cell markers such as OCT4, Nanog, CD44, and Bmi1 and promoted the sphere-forming ability in squamous cell carcinoma cells. The chronic exposure to carcinogens like nicotine due to the placement of smokeless tobacco in GBS could be a plausible explanation for such a find. It should be noted that among the 40 cases with OSCC, only 4 cases of GBS were present. Therefore, the present results need to be validated in a larger sample size.

Spearman correlation among the markers showed a significant positive correlation between ALDH1 and Bmi1 expression in the tissue samples. A similar positive association between ALDH1 and Bmi1 was also observed in oral and oesophageal carcinoma samples. The HNSCC cell culture studies have noted that ALDH1-positive cells exhibited an increased Bmi1 expression and silencing of Bmi1 greatly reduced the tumorigenicity of the cells and made them more prone to radiotherapy. These results infer a relationship between ALDH and Bmi1. However, the underlying molecular mechanism and the related pathways are yet to be elucidated.

The various clinicopathological factors that have been commonly associated with poor prognosis include site of occurrence, tumor size, lymph node metastasis, perineural invasion, vascular invasion, metastasis to distant site, and resistance to conventional treatment modalities such as chemotherapy and radiotherapy.

The fundamentals that govern these pathologic outcome lie on the physiological and metabolic changes occurring in a neoplastic cell at the molecular level. These cellular changes have a cumulative effect on the various processes such as cell proliferation, differentiation, stemness, tumorigenesis, metastatic and invasive potential, DNA repair, as well as resistance to chemotherapy and radiotherapy. The current study showed a reduced survival rate in OSCC groups showing double positive for either of the 2 markers (ALDH1, Bmi1, and OCT4) as well as positivity for all 3 markers. Chen et al observed that cells exhibiting an increased ALDH expression also exhibited an increased expression of Bmi1 and stemness markers such as OCT4. These population of cells had greater capacity for foci formation, migration/invasion, and sphere formation in cell cultures. Further these cells also portrayed decreased radiochemosensitivity. Thereby, it may be postulated that tumors cells in OSCC exhibiting positivity for all the 3 markers could have a more aggressive biologic behavior resulting in poor clinical outcome. Further investigations using more sensitive methods such as polymerase chain reaction-based in situ hybridization are required to validate the results and also assess the cellular co-localization of these markers in the tissue sample for better understanding.

This is a unique study that utilized tobacco-induced cases of OSCC and OED, it evaluated the expression of 3 stem cell markers in the OSCC and OED cases in Indian population with clinicopathologic correlation and survival analysis. In the present study, all the markers increased significantly in OSCC samples as compared to OED. Further, the Kaplan-Meier survival analysis showed ALDH1 and OCT4 to be associated with a poor prognosis, thereby making them potent individual prognostic biomarkers. Interestingly, decreased survival outcomes seen among patients showing positivity for ALDH1, Bmi1, and OCT4 indicate their use as a collective panel of markers that could help assist surgeons in predicting the prognosis of patients with OSCC. Patients with OSCC whose tumor samples show expression of either 2 or all the 3 markers may require careful treatment approach along with prompt follow-up. However, longitudinal studies employing more sensitive methods on a larger sample size are required to draw a definitive inference. The early detection of CSC population in OPMDs or OSCC by use of these markers could help in identifying high-risk OPMD cases as well as aid in pinpointing frank cases of OSCC that need prudent observance.
Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This project was funded by Vision Group on Science and Technology, Government of Karnataka, under the scheme Research Fund for Talented Teachers’ 2016-2017 (Grant Sanction No: VGST/GRD-631/2016-17/2017-18/185).

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Supplemental Material
Supplemental material for this article is available online.

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