Original Research Paper

Cancer Control
Volume 27: 1-12

DOI: 10.1177/1073274820904959
journals.sagepub.com/home/ccx
SAGE

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

Roopa S. Rao, MDS[1], Lizbeth Raju K, MDS[1], Dominic Augustine, MDS[1], and Shankargouda Patil, MDS, PhD, MFDS-RCPS, FICD, FPFA[2]

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

Received March 11, 2019. Received revised May 18, 2019. Accepted for publication January 13, 2020.

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

[1] Department of Oral Pathology and Microbiology, Faculty of Dental Sciences, M.S. Ramaiah University of Applied Sciences, India
[2] Department of Maxillofacial Surgery and Diagnostic Sciences, College of Dentistry, Jazan University, Saudi Arabia

Corresponding Author:
Shankargouda Patil, Department of Maxillofacial Surgery and Diagnostic Sciences, College of Dentistry, Jazan University, Jazan, Saudi Arabia.
Email: dr.ravipatil@gmail.com

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.[3] 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."[4] 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.[5] Cancer stem cells are most often in inactive G0 phase which renders them resistant to radiotherapy and chemotherapy thereby aiding in tumor progression and recurrence even after profound conventional therapy.[6]

Since the origin of CSCs theory, CSCs have been screened upon in various tumors affecting brain, lung, pancreas, prostrate, as well as head and neck.[7-12] However, identification of CSCs from tumor tissue remains to be a challenge. Identification of CSCs has been employed by means of fluorescent-conjugated antibodies to cell surface markers that are specifically enriched in CSCs in combination with flow cytometry. Apart from cell surface markers such as CD133, CD44, nonsurface markers such as aldehyde dehydrogenase (ALDH) enzymes SRY (sex determining region Y)-box 2 (SOX2), B cell-specific Moloney murine leukaemia virus integration site 1 (Bmi1), and octamer-binding protein 4 (OCT4) have been observed in CSCs of HNSCC.[13]

Studies have emphasized metabolic reprogramming in cells as one of the hallmarks of cancer and close relation between metabolic enzymes and CSCs have been noted.[14] Aldehyde dehydrogenase is one such cytosolic enzyme essential for the oxidation of various intracellular aldehydes to carboxylic acids. They play a vital role in physiology of various organs and act as a potent morphogen in embryogenesis.[15] A strong correlation has been observed between ALDH+ cells and stemness of tumor cells, wherein these cells have the ability to form tumor spheroid-like bodies during cell culture.[16] Increase in ALDH expression in various tumors such as gastric, lung, breast, pancreatic, as well as head and neck cancers correlates with poor clinical prognosis.[17-21]

There is increasing evidence that suggests the role of polycomb group (PcG) of proteins in the initiation, progression, and recurrence of cancer apart from its physiologic function of maintaining embryonic and adult stem cell.[22] Bmi1 is one of the members of PcG proteins having important associations with transcription factors such as c-myc, thereby regulating cell proliferation and apoptosis.[23] Studies have shown increased Bmi1 in CSC population of cells from HNSCC samples suggesting it to be a CSC marker.[12,24,25] Bmi1 has also been noted for its role as a predictive marker for malignant transformation of oral leukoplakic lesions.[26]

Wen et al[27] 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.[28] OCT4 has also been noted to be increased in oral potentially malignant disorders (OPMDs) such as oral epithelial dysplasia (OED) including frank carcinomas.[29-31]

However, limited studies have been employed to assess the expression patterns 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.[32] 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 μm 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 nonspecific 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 al[31] 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 ALDHI1 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 gingivobuccal 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 ALDHI1, 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 clinical 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

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

		ALDH1				Bmi-1			OCT4			
		n (%)										
Groups	No. of Cases	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)	.000[a]	24 (60.0)	16 (40.0)	0 (0.0)	0000[a]
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.
[a]Statistically significant $P < .05$.

Table 2. Expression of ALDH1, BMI1, and OCT4 in Metastatic and Nonmetastatic Groups of OSCC.

		ALDH1				Bmi-1			OCT4			
		n (%)										
Groups	No. of Cases	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)	.000[a]	4 (20.0)	16 (80.0)	.661	8 (40.0)	12 (60.0)	0 (0.0)	.000[a]
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.
[a]Statistically significant $P < .05$.

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).

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 nicotinamide adenine dinucleotide phosphate-dependent enzymes

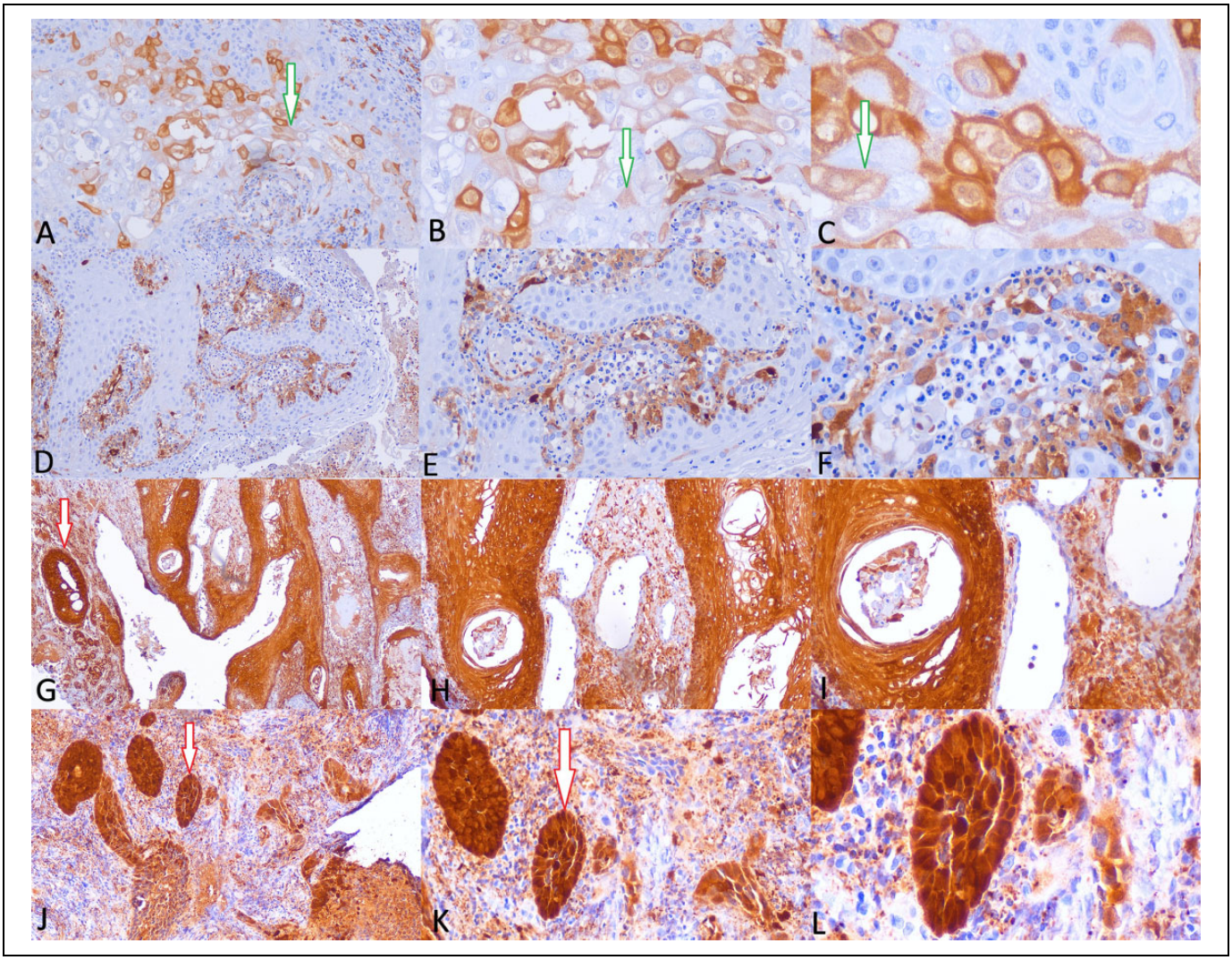

Figure 1. Photomicrographs of epithelial dysplasia and OSCC subjected to staining with antibody to ALDH1 at different magnifications. A, Section of epithelial dysplasia showing low cytoplasmic expression of ALDH1 (IHC, ×100). B, (IHC, ×200). C, (IHC, ×400). D, Section of nonmetastatic OSCC showing low 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.

consisting of 19 human isoenzymes. These enzymes have a broad localization in cytoplasm, mitochondria, and nucleus.[35] 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[36] and Visus et al[37] 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.[36] 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 signalling. 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.[35]

Assessment of ALDH with clinicopathologic parameters showed significant correlation with respect to site, histological grade, tumor size, and tumor staging. Michifuri et al[38] and

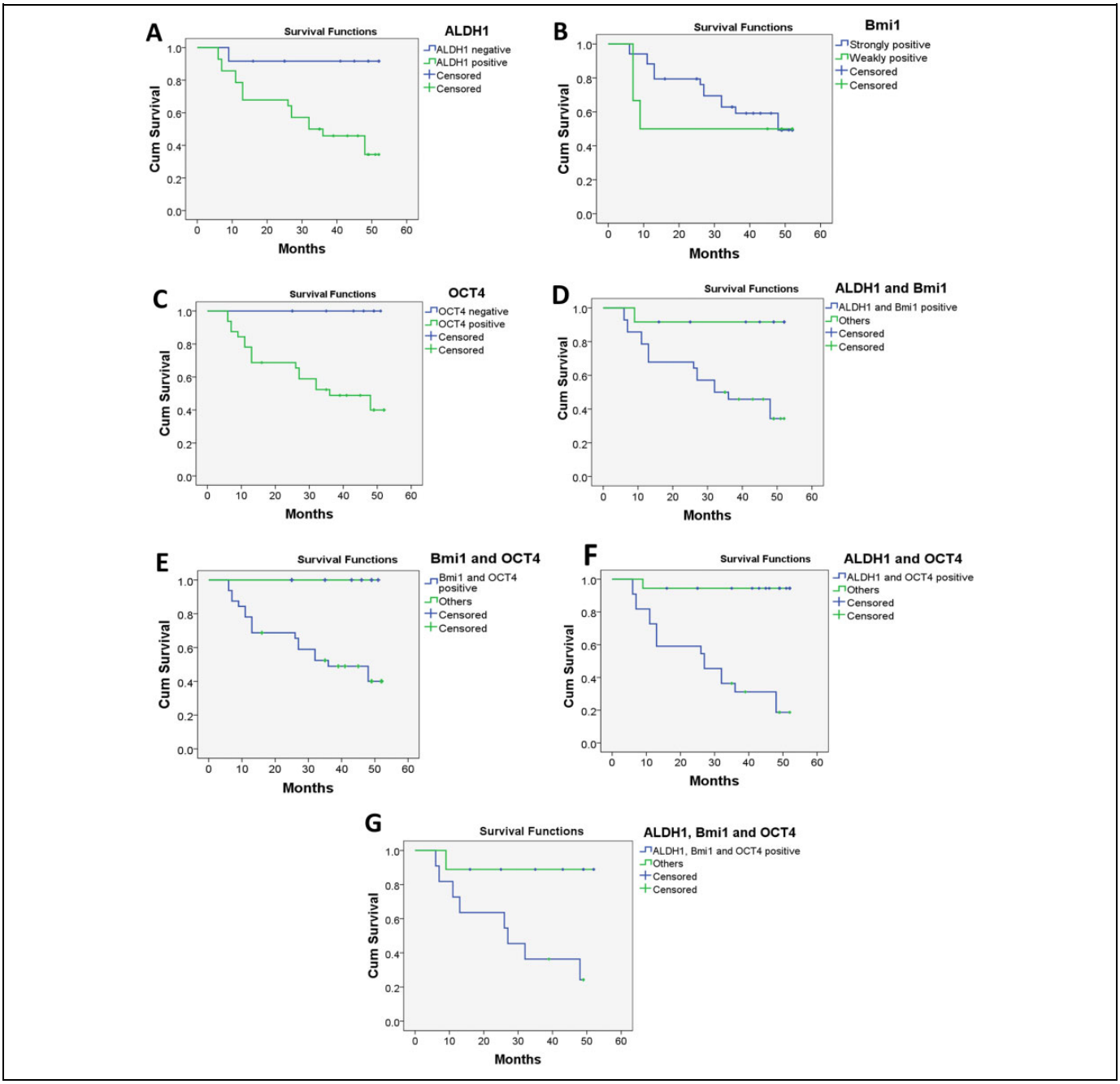

Figure 2. Kaplan-Meier plots comparing the expression of markers and their combinations with cumulative survival. A, ALDH1, (B) Bmi1, (C) OCT4, (D) ALDH1 and Bmi1, (E) ALDH1 and OCT4, (F) Bmi1 and OCT4, and (G) ALDH1, Bmi1, and OCT4. ALDH1 indicates aldehyde dehydrogenase 1; Bmi1, B cell-specific Moloney murine leukaemia virus integration site 1; IHC, immunohistochemistry; OCT4, octamer-binding protein 4.

Tamatani et al[39] 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.[16] Evidence suggests ALDH1 positive cells to have a greater invasive capacity when compared to ALDH1 negative population of cells.[40] 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.[41-44] ALDH1 plays a critical role in metabolizing reactive aldehydes

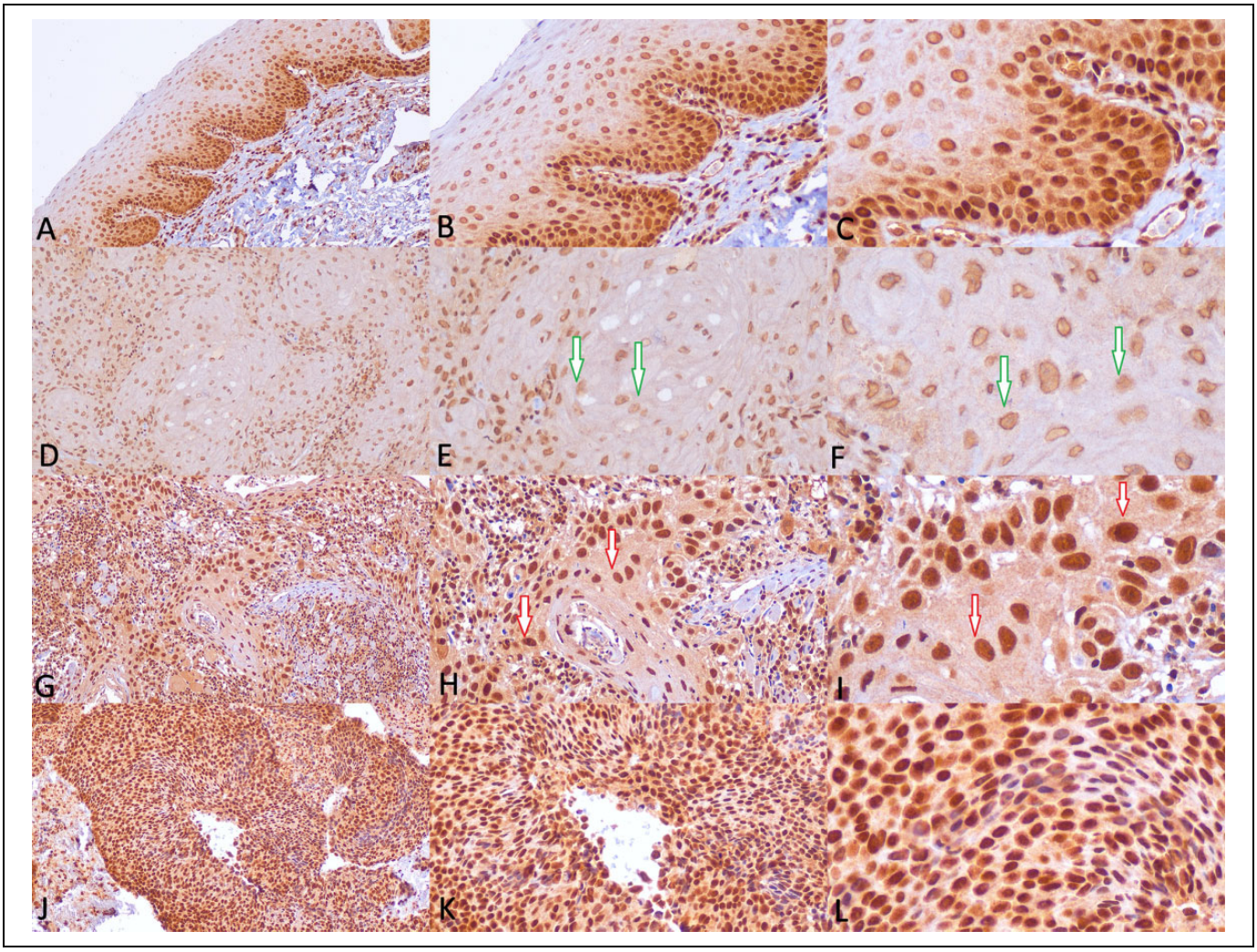

Figure 3. Photomicrographs of epithelial dysplasia and OSCC subjected to staining with antibody to Bmi1 at different magnifications. A, Section of epithelial dysplasia showing weak nuclear expression of Bmi1 (IHC, ×100). B, (IHC, ×200). C, (IHC, ×400). D, Section of nonmetastatic OSCC showing weak nuclear 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.

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.[45] 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.[46] 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.[47-50] Bmi1 expression has been noted to increase in preneoplastic lesions such as oesophageal adenocarcinomas and oral dysplasias.[26,51] 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.[23] 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.[52] Further, this may account for the enhanced gene

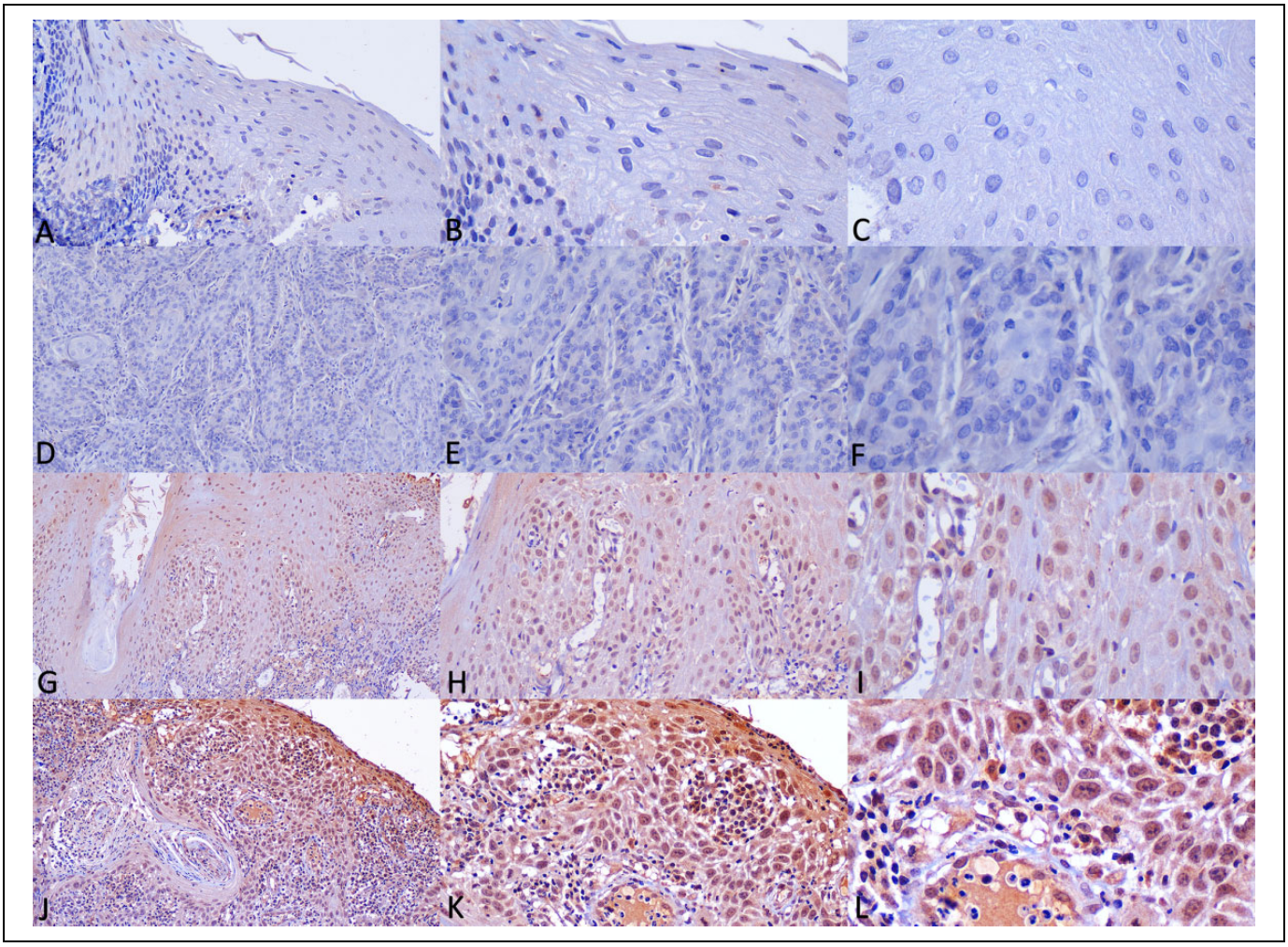

Figure 4. Photomicrographs of epithelial dysplasia and OSCC subjected to staining with antibody to OCT4 at different magnifications. A, Section of epithelial dysplasia showing negative nuclear expression of OCT4 (IHC, ×100). B, (IHC, ×200). C, (IHC, ×400). D, Section of nonmetastatic OSCC showing negative 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.

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[53] 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.[54] Evidence suggests that increased ROS lead to decreased Bmi1 expression.[53] 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,[39] Jayasooriya et al,[30] and Wu et al.[55] However, Hayry et al[56] 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.[57] 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.

Octamer-binding protein 4 is a homeodomain transcription factor belonging to the Pit-Oct-Unc family.[58] 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.[59] The OCT4 expression has also been considered as one of the nonsurface markers of CSCs. Studies by Major et al and Hochedlinger et al[60] 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 Chiou et al,[61] Huang et al,[31] and Jayasooriya et al.[30] 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.[62] 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.[63] 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 ALDHI1 and Bmi1 was also observed in oral and oesophageal carcinoma samples.[55,64] 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.[65]

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.[66,67]

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.[68] 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.[24] These population of cells had greater capacity for foci formation, migration/invasion, and sphere formation in cell cultures. Further these cells also portrayed decreased radio chemosensitivity. 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).

ORCID iD

Shankargouda Patil https://orcid.org/0000-0001-7246-5497

Supplemental Material

Supplemental material for this article is available online.

References

1. Chi AC, Day TA, Neville BW. Oral cavity and oropharyngeal squamous cell carcinoma-an update. *CA Cancer Jclin*. 2015; 65(5):401-421.
2. Moro JS, Maroneze MC, Ardenghi TM, Barin LM, Danesi CC. Oral and oropharyngeal cancer: epidemiology and survival analysis. *Einstein (Sao Paulo)*. 2018;16(2):eAO4248.
3. Satpute PS, Hazarey V, Ahmed R, Yadav L. Cancer stem cells in head and neck squamous cell carcinoma: a review. *Asian Pac J Cancer Prev*. 2013;14(10):5579-5587.
4. Clarke MF, Dick JE, Dirks PB, et al. Cancer stem cells—perspectives on current status and future directions: AACR workshop on cancer stem cells. *Cancer Res*. 2006;66(19):9339-9344.
5. Monroe MM, Anderson EC, Clayburgh DR, Wong MH. Cancer stem cells in head and neck squamous cell carcinoma. *J Oncol*. 2011;2011:1-8.
6. Chen W, Dong J, Haiech J, Kilhoffer MC, Zeniou M. Cancer stem cell quiescence and plasticity as major challenges in cancer therapy. *Stem Cells Int*. 2016;2016:1-16.
7. Singh SK, Clarke ID, Terasaki M, et al. Squire JIdentification of a cancer stem cell in human brain tumors. *Cancer Res*. 2003; 63(18):5821-5828.
8. Ho MM, Ng A V, Lam S, Hung JY. Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. *Cancer Res*. 2007;67(10):4827-4833.
9. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res*. 2005;65(23):10946-10951.
10. Hermann PC, Huber SL, Herrler T, et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell*. 2007;1(3): 313-323.
11. Yang ZF, Ho DW, Ng MN, et al. Ngai PSignificance of CD90+ cancer stem cells in human liver cancer. *Cancer Cell*. 2008;13(2): 153-166.
12. Prince ME, Sivanandan R, Kaczorowski A, et al. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proc Natl Acad Sci*. 2007; 104(3):973-978.
13. Major AG, Pitty LP, Farah CS. Cancer stem cell markers in head and neck squamous cell carcinoma. *Stem Cells Int*. 2013;2013: 1-13.
14. Xu X, Chai S, Wang P, et al. Aldehyde dehydrogenases and cancer stem cells. *Cancer Lett*. 2015;369(1):50-57.
15. Evans RM. The steroid and thyroid hormone receptor superfamily. *Science*. 1988;240(4854):889-895.
16. Chen YC, Chen YW, Hsu HS, et al. Aldehyde dehydrogenase 1 is a putative marker for cancer stem cells in head and neck squamous cancer. *Biochem Biophys Res Commun*. 2009;385(3): 307-313.
17. Li X, Xu Q, Fu X, Luo W. ALDH1A1 overexpression is associated with the progression and prognosis in gastric cancer. *BMC Cancer* 2014;14:705.
18. Jiang F, Qiu Q, Khanna A, et al. Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung cancer. *Mol Cancer Res*. 2009;7(3):330-338.
19. Ohi Y, Umekita Y, Yoshioka T, et al. Aldehyde dehydrogenase 1 expression predicts poor prognosis in triple-negative breast cancer. *Histopathology*. 2011;59(4):776-780.
20. Kahlert C, Bergmann F, Beck J, et al. Low expression of aldehyde dehydrogenase 1A1 (ALDH1A1) is a prognostic marker for poor survival in pancreatic cancer. *BMC Cancer*. 2011;11:275.
21. Zhou C, Sun B. The prognostic role of the cancer stem cell marker aldehyde dehydrogenase 1 in head and neck squamous cell carcinomas: a meta-analysis. *Oral Oncol*. 2014;50(12):1144-1148.
22. Valk-Lingbeek ME, Bruggeman SWM, van Lohuizen M. Stem cells and cancer; the polycomb connection. *Cell*. 2004;118(4): 409-418.
23. Guney I, Wu S, Sedivy JM. Reduced c-Myc signaling triggers telomere-independent senescence by regulating Bmi-1 and p16(INK4a). *Proc Natl Acad Sci U S A*. 2006;103(10):3645-3650.
24. Chen YC, Chang CJ, Hsu HS, et al. Inhibition of tumorigenicity and enhancement of radiochemosensitivity in head and neck squamous cell cancer-derived ALDH1-positive cells by knockdown of Bmi-1. *Oral Oncol*. 2010;46(3):158-165.
25. Yu CC, Lo WL, Chen YW, et al. Bmi-1 regulates snail expression and promotes metastasis ability in head and neck squamous cancer-derived ALDH1 Positive Cells. *J Oncol*. 2011;2011.
26. Liu W, Feng JQ, Shen XM, Wang HY, Liu Y, Zhou ZT. Two stem cell markers, atp-binding cassette, g2 subfamily (abcg2) and bmi-1, predict the transformation of oral leukoplakia to cancer. *Cancer*. 2012;118(6):1693-1700.
27. Wen J, Park JY, Park KH, et al. Oct4 and Nanog expression is associated with early stages of pancreatic carcinogenesis. *Pancreas*. 2010;39(5):622-626.
28. Jerabek S, Merino F, Schöler HR, Cojocaru V. OCT4: dynamic DNA binding pioneers stem cell pluripotency. *Biochim Biophys Acta*. 2014;1839(3):138-154.
29. Ali M, Swasan N, Safa F. Immunohistochemical expression of octamer-binding protein-4, in oral epithelial dysplasia induced in experimental rats. *IOSR J Dent Med Sci*. 2018;17(7):22-46.
30. Jayasooriya P, Fernando C, Suraweera A, Dissanayake U. Stem cell markers as a resource to predict prognosis of betel quid

induced oral squamous cell carcinoma: an immunohistochemical investigation. *Stomatol Dis Sci*. 2017;1(1):29-34.
31. Huang CF, Xu XR, Wu TF, Sun ZJ, Zhang WF. Correlation of ALDH1, CD44, OCT4 and SOX2 in tongue squamous cell carcinoma and their association with disease progression and prognosis. *J Oral Pathol Med*. 2014;43(7):492-498.
32. Barnes L, Eveson JW, Reichart P, Sidransky D. *World Health Organization Classification of Tumours: Pathology & Genetics of Head and Neck Tumours Edited*. Lyon: IARC press; 2005.
33. Ortiz RC, Lopes NM, Amôr NG, et al. CD44 and ALDH1 immunoexpression as prognostic indicators of invasion and metastasis in oral squamous cell carcinoma. *J Oral Pathol Med*. 2018;47(8): 740-747.
34. Li D, Tang H, Fan J, et al. Expression level of Bmi-1 oncoprotein is associated with progression and prognosis in colon cancer. *J Cancer Res Clin Oncol*. 2010;136(7):997-1006.
35. Clark DW, Palle K. Aldehyde dehydrogenases in cancer stem cells: potential as therapeutic targets. *Ann Transl Med*. 2016; 4(24):518-518.
36. Liu W, Wu L, Shen X-M, et al. Expression patterns of cancer stem cell markers ALDH1 and CD133 correlate with a high risk of malignant transformation of oral leukoplakia. *Int J Cancer*. 2013;132(4):868-874.
37. Visus C, Ito D, Amoscato A, et al. Identification of human aldehyde dehydrogenase 1 family member A1 as a novel CD8+ T-cell-defined tumor antigen in squamous cell carcinoma of the head and neck. *Cancer Res*. 2007;67(21):10538-10545.
38. Michifuri Y, Hirohashi Y, Torigoe T, et al. High expression of ALDH1 and SOX2 diffuse staining pattern of oral squamous cell carcinomas correlates to lymph node metastasis. *Pathol Int*. 2012; 62(10):684-689.
39. Tamatani T, Takamaru N, Ohe G, Akita K, Nakagawa T, Miyamoto Y. Expression of CD44, CD44v9, ABCG2, CD24, Bmi-1 and ALDH1 in stage I and II oral squamous cell carcinoma and their association with clinicopathological factors. *Oncol Lett*. 2018;16(1):1133-1140.
40. Nishida S, Hirohashi Y, Torigoe T, et al. Gene expression profiles of prostate cancer stem cells isolated by aldehyde dehydrogenase activity assay. *J Urol*. 2012;188(1):294-299.
41. Zou B, Sun S, Qi X, Ji P. Aldehyde dehydrogenase activity is a cancer stem cell marker of tongue squamous cell carcinoma. *Mol Med Rep*. 2012;5(4):1116-1120.
42. Leinung M, Ernst B, Döring C, et al. Expression of ALDH1A1 and CD44 in primary head and neck squamous cell carcinoma and their value for carcinogenesis, tumor progression and cancer stem cell identification. *Oncol Lett*. 2015; 10(4):2289-2294.
43. Qian X, Wagner S, Ma C, et al. Prognostic significance of ALDH1A1-positive cancer stem cells in patients with locally advanced, metastasized head and neck squamous cell carcinoma. *J Cancer Res Clin Oncol*. 2014;140(7):1151-1158.
44. Kurth I, Hein L, Mäbert K, et al. Cancer stem cell related markers of radioresistance in head and neck squamous cell carcinoma. *Oncotarget* 2015;6(33):34494-34509.
45. Haupt Y, Alexander WS, Barri G, Klinken SP, Adams JM. Novel zinc finger gene implicated as myc collaborator by retrovirally accelerated lymphomagenesis in E mu-myc transgenic mice. *Cell*. 1991;65(5):753-763.
46. Sherr CJ, McCormick F. The RB and p53 pathways in cancer. *Cancer Cell*. 2002;2(2):103-112.
47. Arnes JB, Collett K, Akslen LA. Independent prognostic value of the basal-like phenotype of breast cancer and associations with EGFR and candidate stem cell marker BMI-1. *Histopathology*. 2008;52(3):370-380.
48. Qin Z-K, Yang J-A, Ye Y-L, et al. Expression of Bmi-1 is a prognostic marker in bladder cancer. *BMC Cancer*. 2009;9:61.
49. Song W, Tao K, Li H, Jin C, Song Z, Li J, et al. Bmi-1 is related to proliferation, survival and poor prognosis in pancreatic cancer. *Cancer Sci*. 2010;101(7):1754-1760.
50. Zhang X, Tian T, Sun W, Liu C, Fang X. Bmi-1 overexpression as an efficient prognostic marker in patients with nonsmall cell lung cancer. *Medicine (Baltimore)*. 2017;96(26): e7346.
51. Kang MK, Kim RH, Kim SJ, et al. Elevated Bmi-1 expression is associated with dysplastic cell transformation during oral carcinogenesis and is required for cancer cell replication and survival. *Br J Cancer*. 2007;96(1):126-133.
52. Pai R, Pai S, Lalitha R, et al. Over-expression of c-Myc oncoprotein in oral squamous cell carcinoma in the South Indian population. *Ecancermedicalscience*. 2009;3:128.
53. Cordisco S, Maurelli R, Bondanza S, et al. Bmi-1 reduction plays a key role in physiological and premature aging of primary human keratinocytes. *J Invest Dermatol*. 2010;130(4): 1048-1062.
54. Dröge W, Schipper HM. Oxidative stress and aberrant signaling in aging and cognitive decline. *Aging Cell*. 2007;6(3): 361-370.
55. Wu TF, Li YC, Ma SR, Bing L, Zhang WF, Sun ZJ. Expression and associations of TRAF1, BMI-1, ALDH1, and Lin28B in oral squamous cell carcinoma. *Tumor Biol*. 2017;39(4). doi: 101042831769593.
56. Häyry V, Mäkinen LK, Atula T, et al. Bmi-1 expression predicts prognosis in squamous cell carcinoma of the tongue. *Br J Cancer*. 2010;102(5):892-897.
57. Attner P, Du J, Nãsman A, et al. The role of human papillomavirus in the increased incidence of base of tongue cancer. *Int J Cancer*. 2010;126(12):2879-2884.
58. Khan W, Augustine D, Rao R, Sowmya S, Haragannavar V, Nambiar S. Stem cell markers SOX-2 and OCT-4 enable to resolve the diagnostic dilemma between ameloblastic carcinoma and aggressive solid multicystic ameloblastoma. *Adv Biomed Res*. 2018;7(1):149.
59. Nichols J, Zevnik B, Anastassiadis K, et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell*. 1998;95(3):379-391.
60. Hochedlinger K, Yamada Y, Beard C, Jaenisch R. Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues. *Cell*. 2005;121(3): 465-477.
61. Chiou SH, Yu CC, Huang CY, et al. Positive correlations of Oct-4 and Nanog in oral cancer stem-like cells and high-grade oral

squamous cell carcinoma. *Clin Cancer Res*. 2008;14(13): 4085-4095.
62. Yu CC, Chang YC. Enhancement of cancer stem-like and epithelial-mesenchymal transdifferentiation property in oral epithelial cells with long-term nicotine exposure: reversal by targeting SNAIL. *Toxicol Appl Pharmacol*. 2013;266(3):459-469.
63. Yu MA, Kiang A, Wang-Rodriguez J, et al. Nicotine promotes acquisition of stem cell and epithelial-to-mesenchymal properties in head and neck squamous cell carcinoma. *PLoS One*. 2012; 7(12): e51967.
64. Hwang C-C, Nieh S, Lai C-H, et al. A Retrospective Review of the Prognostic Value of ALDH-1, Bmi-1 and nanog stem cell markers in esophageal squamous cell carcinoma. *PLoS One*. 2014;9(8):e105676.
65. Lakshminarayana S, Augustine D, Rao R, et al. Molecular pathways of oral cancer that predict prognosis and survival: a systematic review. *J Carcinog*. 2018;17(1):7.
66. Majumdar B, Patil S, Sarode SC, Sarode GS, Rao RS. Clinico-pathological prognosticators in oral squamous cell carcinoma. *Transl Res Oral Oncol*. 2017;2 doi:2057178X 1773891.
67. Prasad K, Rao R, Augustine D, et al. Pathway based prognostic gene expression profile of buccal and gingivo-buccal oral squamous cell carcinoma in smokeless tobacco chewers. *Head & Neck*. 2019; 41(2):388-39768.
68. Raju KL, Augustine D, Rao RS, et al. Biomarkers in tumorigenesis using cancer cell lines: a systematic review. *Asian Pac J Cancer Prev*. 2017;18(9):2329-2337.