Immunodetection of Epithelial-Mesenchymal Transition and Tumor Proliferation Markers in GLi-1-positive Oral Squamous Cell Carcinoma

Rebeca C.M. Dantas, PhD, Vanessa S.N. Guimarães, MSc,† Renata O. de Souza, MSc,† Ludmila F. Valverde, PhD,† Manuela T.A. Vidal, PhD,† Raphael L.R. Nogueira, BSc,† Leonardo O.S. da Rocha, BSc,† Giovana T. Araújo, BSc,† Jean N. dos Santos, PhD,† and Clarissa A.G. Rocha, PhD†*

Abstract: In oral squamous cell carcinoma (OSCC), involvement and activation of the Hedgehog pathway (HH) may be related to epithelial-mesenchymal transition and cell proliferation. The present study aimed to evaluate epithelial-mesenchymal transition and proliferative potential in OSCC cases demonstrating activation of the HH pathway. Twenty-three GLi-1-positive OSCC cases were submitted to immunohistochemical detection of Snail, Slug, N-cadherin, E-cadherin, β-catenin, and MCM3 proteins. Clinical-pathologic immunoexpression data were obtained from the invasion front and tumor islets, and then compared. At the invasion front, OSCC cases presented positive Snail, Slug, and MCM3 expression in the nuclei of tumor cells. Loss of membrane and cytoplasmic expression of E-cadherin and β-catenin was also observed. Positive N-cadherin expression was observed in 31.78% of the cases. GLi-1 immunoexpression was associated with loss of membrane E-cadherin ($P < 0.001$), membrane β-catenin ($P < 0.001$), and cytoplasmic β-catenin ($P = 0.02$) expression. In the tumor islets, we observed nuclear expression of GLi-1, Snail, Slug, and MCM3. E-cadherin and β-catenin showed positivity in tumor cell membranes. Statistically significant positive correlations between GLi-1 and Snail ($P = 0.05$), E-cadherin ($P = 0.01$), and cytoplasmic β-catenin ($P = 0.04$) were found. GLi-1 was associated with clinical staging, while membrane β-catenin expression was related to the presence of metastasis in lymph nodes and to clinical staging. The HH pathway may be involved in regulating the expression of the mesenchymal phenotype. The loss of membrane E-cadherin and β-catenin expression was observed at the tumor front region, whereas cell adhesion protein expression was detected in tumor islets regardless of MCM3.

Key Words: squamous cell carcinoma, mouth neoplasms, Hedgehog protein, epithelial-mesenchymal transition, cell proliferation

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Oral cancer is the sixth most common tumor type in the world,[1] and oral squamous cell carcinoma (OSCC) is the most frequent histologic subtype (about 90%). The morbidity and mortality of this disease is high, and the 5-year survival rate of patients is ~50%.[2]

The pathogenesis of malignant tumors is heterogenous, and embryonic signaling pathways, such as the Hedgehog (HH) pathway, have demonstrated importance in the context of tumorigenesis.[3] In normal adult cells, this pathway is generally inactive, and its reactivation can play a significant role in the development of several human tumors.[4] Activation of the HH pathway occurs when one of the SHH, DHH, or IHH ligands binds to its *Patched* (PTCH) receptor, leading to nuclear translocation of *glioma-associated oncogene homolog* transcription factors (GLi-1, GLi-2, and/or GLi-3). This consequently activates different groups of genes involved in cell proliferation and differentiation,[4,5] contributing to important events in tumor progression, such as the epithelial-mesenchymal transition (EMT).[6]

EMT is an important event in tumor progression, as it increases the ability of malignant cells to invade the extracellular matrix and to achieve metastatic niches.[6–8] This process leads to changes in expression, distribution, and/or function of adhesion proteins, such as E-cadherin, N-cadherin, and β-catenin.[8] These alterations are mediated by the activation of transcription factors (Snail, Slug, and Twist) whose expression is regulated by several signaling pathways, and, among them, is the HH pathway,[9] as has already been demonstrated in pancreatic carcinoma,[5] hepatocellular carcinoma,[10] and tumors of the gastrointestinal tract.[11]

Aberrant cell proliferation is also a preponderant event in malignant tumors and has been studied as a prognostic indicator.[12] Despite evidence of HH pathway activation in OSCC,[13] relationships between the HH pathway molecules (such as GLi-1) and others involved in EMT (Snail, Slug, N-cadherin, E-cadherin, and β-catenin) have not been studied.

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From the *Laboratory of Oral Surgical Pathology, School of Dentistry of the Federal University of Bahia; and †Laboratory of Pathology and Molecular Biology, Gonçalo Moniz Institute, Oswaldo Cruz Foundation, Salvador, Bahia, Brazil.
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Reprints: Clarissa A.G. Rocha, PhD, Laboratory of Pathology and Molecular Biology, Gonçalo Moniz Institute, Oswaldo Cruz Foundation, Waldemar Falcão Street, 121, Candeal, Salvador 40296-710, Bahia, Brazil (e-mail: cgurgel@bahia.fiocruz.br).

TABLE 1. Clinical and Histologic Characteristics of Oral Squamous Cell Carcinoma Cases

Clinical Parameters	Total [n (%)]
Sex	
Male	15 (65.22)
Female	8 (34.78)
Tumor size	
T1-T2	14 (60.87)
T3-T4	9 (39.13)
Metastasis—lymph nodes	
N0	6 (26.09)
N1-N3	17 (73.91)
Metastasis—distant	
M0	17 (78.26)
Mx	6 (26.08)
Clinical stage	
I-II	4 (17.39)
III-IV	19 (82.61)
Muscle invasion	
Yes	10 (43.48)
No	13 (56.52)
Bone invasion	
Yes	4 (17.39)
No	19 (82.60)
Vascular invasion	
Yes	2 (8.70)
No	21 (91.30)
Perineural invasion	
Yes	1 (6.67)
No	22 (95.65)
Histologic grade	
Well-differentiated	13 (56.52)
Moderately differentiated	9 (39.13)
Poorly differentiated	1 (4.35)
Anatomic site	
Tongue	12 (52.17)
Floor of mouth	8 (34.78)
Retromolar region	2 (8.70)
Gingiva	1 (4.35)

Accordingly, the present work aims to evaluate associations between GLi-1, the main transcription factor activator in the HH pathway, and other tissue markers of EMT and proliferation in OSCC.

MATERIALS AND METHODS

The present study received approval from the Institutional Review Board for Ethical Human Research of the Gonçalo Moniz Research Institute (IGM-FIOCRUZ) located in Salvador, Bahia, Brazil (protocol no. 2,295,634).

OSCC samples (n = 37) from patients admitted for surgical tumor excision were obtained from a reference hospital for cancer treatment. Of these initial cases, 23 OSCC samples presented immunopositivity for nuclear GLi-1 protein, which is consistent with HH pathway activation.[4,6,14] Next, immunostaining for Snail, Slug, N-cadherin, E-cadherin, β-catenin, and MCM3 was performed. Tumors were classified using the *TNM Classification of Malignant Tumors* criteria[15] and the histologic parameters established by the World Health Organization (WHO) (2017).[16] The clinical and histologic features of the included OSCC cases are described in Table 1.

Immunohistochemical Studies

After performing morphologic analysis (hematoxylin and eosin) of all surgical specimens, the most representative slides were selected from each case. Sections that were 4-μm-thick were obtained, followed by deparaffinization using xylol, alcohol rehydration, and heat-induced epitope retrieval for 45 minutes. Data on antibody manufacturer, clone, dilution, retrieval, and positive controls are described in Table 2.

After endogenous peroxidase blockage (Peroxidase Blocking Solution; Dako, Santa Clara, CA) for 10 minutes in dark conditions, and the blockage of nonspecific proteins (Protein Blocking Solution; Dako) for 20 minutes, the primary antibodies were incubated overnight at 4°C in a humid chamber. Horseradish peroxidase Link and horseradish peroxidase Enzyme reagents (Advance; Dako Corporation, Carpinteria, CA) were then applied to the histologic sections for 20 minutes each. Reactions were developed with 3,3-diaminobenzidine (Dako Corporation) and counterstained with Harris hematoxylin. For negative controls, each primary antibody was replaced with normal serum of the same isotype.

All histologic sections were scanned using a VS110-AS scanner (Olympus Corporation, Hamburg, Germany) coupled to an Olympus BX61VSF microscope under ×200 magnification, followed by visualization using Olyvia 2.4 software (Olympus Corporation). All proteins were analyzed in 2 specific areas of the histologic sections, the invasive tumor front and the tumor islets. In 4 cases, the invasive tumor front analysis was compromised, and therefore only the tumor islets were considered. Semiquantification was performed by 2 examiners, one of whom analyzed only GLi-1, while the other analyzed the other evaluated proteins in the absence of any knowledge as regards the results obtained for GLi-1. Tumor areas proximate to muscle and adipose tissue were not considered for analysis.

Initially, a descriptive analysis of the immunomarkers was performed, considering the localization of immunostaining (membrane, cytoplasm, and/or nucleus) and cell type

TABLE 2. Description of Antibody Clone Manufacturer, Antigen Retrieval, and Dilution

Antibody	Brand	Clone	Control	Retrieval	Dilution
GLi-1	Novus Biologicals	Polyclonal	Placenta	Citrate pH 6.0	1:600
E-cadherin	Dako	NHC38	Oral mucosa	Citrate pH 6.0	1:50
β-catenin	BD	14/β-catenin	Oral mucosa	Citrate pH 6.0	1:100
N-cadherin	Novus Biologicals	EPR1792Y	Prostate cancer	Citrate pH 6.0	1:500
Snail	Abcam	ab53519	Breast cancer	Citrate pH 6.0	1:25
Slug	Novus Biologicals	1A6	Ameloblastoma	EDTA pH 9.0	1:150
MCM3	Dako	101	Breast cancer	EDTA pH 9.0	1:50

(tumor and/or stromal cells). For membrane markers with positivity (E-cadherin, β-catenin, N-cadherin), the continuity/discontinuity of immunostaining was also evaluated. Positivity for Snail and Slug transcription factors was considered only when localized within the nucleus. Marker positivity was classified in each case of OSCC according to the following semiquantitative criteria: score 0, when 0% to 5% immunostained epithelial cells were present; score 1+ for 6% to 25%; score 2+ for 26% to 50%; and score 3+ for $>50\%$ of immunostained epithelial cells.

Statistical Analysis

Data were organized in contingency tables ($r\times c$) and submitted to statistical analysis using the Statistical Package for the Social Science (SPSS), version 17.0 for Windows (SPSS Inc., Chicago, IL). Monte Carlo analysis was used to evaluate Fisher exact test results. To assess correlations between markers, φ coefficients were calculated. A significance level of 5% ($P<0.05$) was adopted for all tests.

RESULTS

Immunoexpression of GLi-1, SNAIL, SLUG, N-cadherin, E-cadherin, β-catenin, and MCM3 Proteins

GLi-1

The immunoexpression of this protein was observed mainly in the nucleus and cytoplasm of tumor parenchyma cells (Fig. 1). At the invasion front, a 3+ score was mostly prevalent (n = 10; 52.63%), which was also the case in tumor islets (n = 16; 69.56%) (Fig. 2, Table 3).

Snail

Snail was positive in all OSCC cases, both at the front (n = 19) and in tumor islets (n = 23). Immunoexpression of this protein was observed in the nucleus and cytoplasm (Fig. 1). For semiquantification purposes, only nuclear staining was considered, resulting in a predominant score of 3+ at the invasion front (n = 12, 63.16%) and tumor islets (n = 22, 95.66%) (Fig. 2, Table 3).

Slug

At the invasion front, positivity for Slug was seen in 11 cases (57.89%) and in 22 cases (95.65%) within the tumor islets, both with localization in the nucleus and cytoplasm (Fig. 1). At the invasion front, a score of 0 (n = 8; 42.11%) predominated. In tumor islets, a score of 3+ (n = 12; 52.17%) was the most prevalent (Fig. 2, Table 3).

N-cadherin

At the invasion front, N-cadherin positivity was found in 6 cases (31.58%) and in 12 cases (52.17%) in the tumor islets. Immunoexpression was observed in the membrane and cytoplasm of tumor cells. Discontinuity in immunostaining in the membrane was observed in neoplastic cells at the invasion front, whereas continuous immunostaining was seen in tumor islets (Fig. 3). Among positive samples, a score of 1+ (n = 4; 21.05%) was the most prevalent at the invasion front and in the tumor islets (n = 6; 26.09%) (Fig. 2, Table 3).

E-cadherin

E-cadherin expression was observed in 5 OSCC cases (26.32%) at the invasion front, and in 20 cases (89.96%) in tumor islets. Immunoexpression was mostly observed in the membrane, and also present in the cytoplasm. Of note, immunostaining was discontinuous in cells at the invasion front, whereas continuous membrane immunostaining was observed in tumor islets (Fig. 3). At the front, a score of 0 (n = 14; 73.68%) predominated, while in tumor islets a 1+ score (n = 9; 39.13%) was more frequent (Fig. 2, Table 3).

β-catenin

In cases positive for β-catenin, localization was observed in the membrane and cytoplasm of tumor cells. At the invasion front, membrane expression was found in 12 OSCC cases (63.16%) and cytoplasmic expression in 11 (57.89%), whereas in tumor islets membrane expression was present in all cases (n = 23) versus 18 cases in the cytoplasm (78.26%). Discontinuous immunostaining was observed in cell membranes at the invasion front, while continuity was seen in the plasma membrane in tumor islets. Nuclear labeling was scarce (< 5% of positive cells) in tumor cells, regardless of localization (front or islets) (Fig. 3). At the front, a score of 1+ was more frequent in cellular membranes (n = 10; 52.63%), while a 3+ membrane score was found in tumor islets, (n = 13; 56.52%) and in the cytoplasm (n = 10; 43.48%) (Fig. 2, Table 3).

MCM3

All cases of OSCC presented positive nuclear immunostaining for MCM3 exclusively in tumor cells, both at the invasion front and in the tumor islets (Fig. 3). At the front, a score of 3+ (n = 8, 42.11%) was the most frequent, followed by 1+ (n = 7; 36.84%) and 2+ (n = 4; 21.05%). In the tumor islets, the following scores were observed: 3+ in 17 cases (73.91%), 2+ in 3 cases (13.04%), and 1+ in 3 cases (13.04%) (Fig. 2, Table 3). Figure 4 illustrates immunostaining scores for all investigated proteins, both at the tumor front and in the islets, in accordance with the histologic degree of OSCC differentiation.

Correlations Between GLi-1, EMT, and MCM3 Marker Expression

At the invasion front, significant negative correlations were found between GLi-1 and N-cadherin ($\varphi=-0.68$; $P<0.001$), E-cadherin ($\varphi=-0.72$; $P<0.001$), membrane β-catenin ($\varphi=-0.39$; $P<0.001$), and cytoplasmic β-catenin ($\varphi=-0.51$; $P=0.02$). Snail was highly positively associated with GLi-1 ($\varphi=0.90$, $P=0.43$), and a correlation between GLi-1 and Slug immunoexpression ($\varphi=0.10$, $P=0.06$) was found, yet without statistical significance (Table 4).

In the tumor islets, positive statistically significant correlations were observed between GLi-1 and Snail ($\varphi=0.97$, $P=0.05$), cytoplasmic β-catenin ($\varphi=0.83$, $P=0.04$), and E-cadherin ($\varphi=0.42$, $P=0.01$). GLi-1 and N-cadherin ($\varphi=-0.90$, $P<0.01$) were negatively

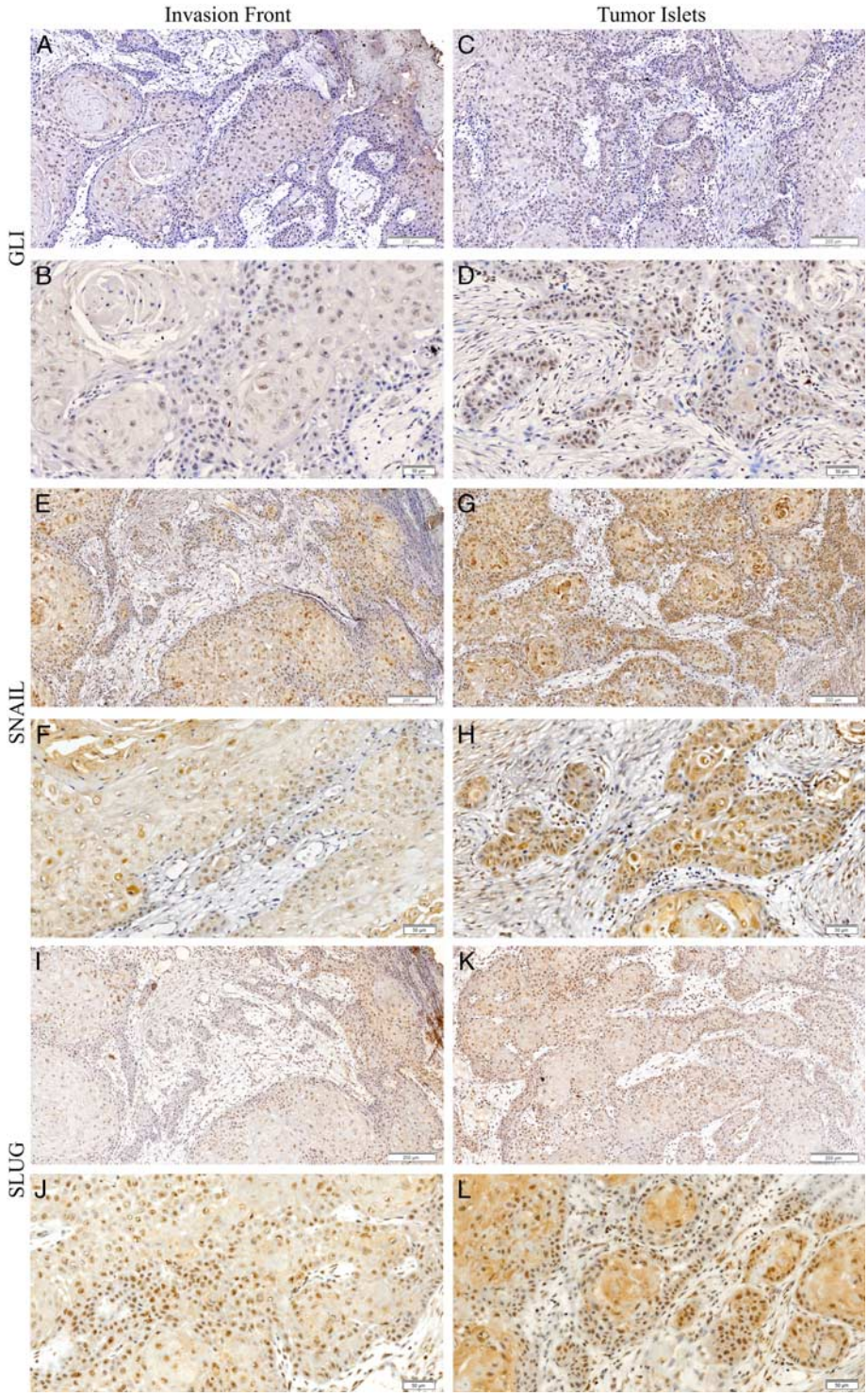

FIGURE 1. Immunoexpression of GLi-1, Snail, and Slug in oral squamous cell carcinomas. A and B, Nuclear GLi-1 expression in the invasion front region at an increase of ×20 and ×40, respectively. C and D, Nuclear GLi-1 expression in the tumor islets region at an increase of ×20 and ×40, respectively. E and F, Nuclear Snail expression in the invasion front region at an increase of ×20 and ×40, respectively. G and H, Nuclear Snail expression in the tumor islets region at ×20 and ×40 magnification, respectively. I and J, Slug nuclear expression in the invasion front region at ×20 and ×40 magnification. K and L, Slug nuclear expression on tumor islets at ×20 and ×40 magnification, respectively.

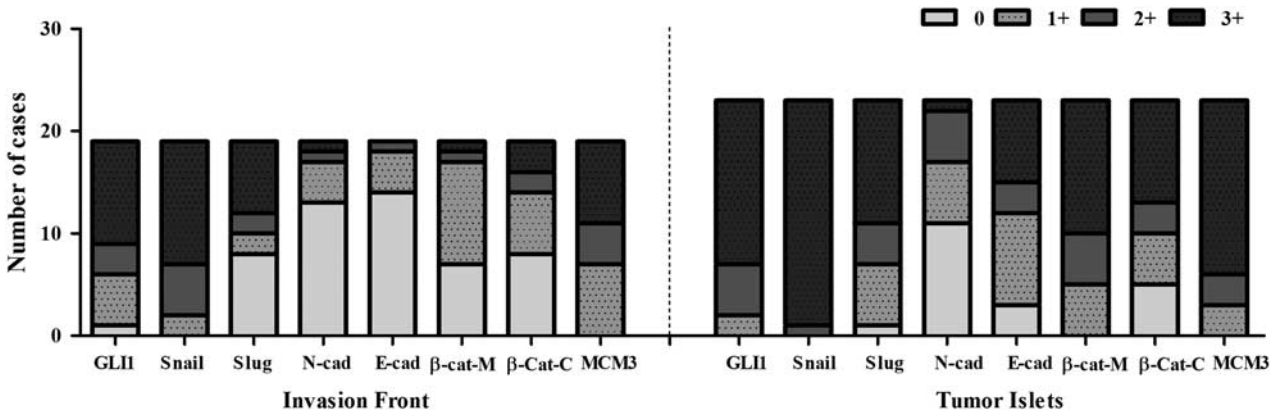

FIGURE 2. Distribution of immunoexpression scores for GLi-1, MCM3, and epithelial-mesenchymal transition markers at the tumor front region and in tumor islets.

correlated. A very strong association was observed between GLi-1 and Slug ($\varphi = 0.92$, $P = 0.33$), as well as with membrane β-catenin ($\varphi = 0.96$; $P = 0.57$), but without statistical significance (Table 4).

At the invasion front, associations between MCM3/GLi-1 ($\varphi = 0.88$; $P = 0.75$) and MCM3/Snail ($\varphi = 0.70$; $P = 0.26$) were very strong, but not statistically significant. MCM3 and Slug ($\varphi = -0.35$; $P = 0.006$) presented a negative statistically significant correlation. N-cadherin ($\varphi = -0.82$, $P < 0.001$) and E-cadherin ($\varphi = -0.84$; $P < 0.001$) were significantly negatively correlated with MCM3, and also very strongly associated. Correlations were also observed between MCM3 and membrane β-catenin ($\varphi = -0.17$; $P < 0.001$) and cytoplasmic β-catenin ($\varphi = -0.55$; $P = 0.007$) (Table 4).

In the tumor islets, correlations between MCM3 and GLi-1 ($\varphi = 0.98$, $P = 0.80$), Snail ($\varphi = 0.99$, $P = 0.16$), Slug ($\varphi = 0.96$, $P = 0.39$), β-catenin ($\varphi = 0.96$, $P = 0.51$), and cytoplasmic β-catenin ($\varphi = 0.90$, $P = 0.06$) were observed, yet without statistical significance. Strong, statistically significant correlations were found between MCM3 and E-cadherin ($\varphi = 0.54$, $P = 0.02$), and between MCM3 and N-cadherin ($\varphi = -0.87$; $P < 0.001$) (Table 4) as well.

The following correlations were observed between the EMT markers at the invasion front: E-cadherin/Snail ($\varphi = -0.77$, $P < 0.001$), E-cadherin/Slug ($\varphi = 0.52$, $P = 0.01$), N-cadherin/E-cadherin ($\varphi = 0.56$, $P = 0.06$), and cytoplasmic β-catenin/E-cadherin ($\varphi = 0.90$, $P = 0.15$). In tumor islets, E-cadherin/Snail ($\varphi = 0.45$; $P < 0.001$), E-cadherin/Slug ($\varphi = 0.73$, $P = 0.48$), N-cadherin/E-cadherin ($\varphi = -0.56$, $P = 0.01$), membrane β-catenin/E-cadherin ($\varphi = 0.62$; $P = 0.14$), and cytoplasmic β-catenin/E-cadherin ($\varphi = 0.58$; $P = 0.61$) were also correlated (Table 4).

No statistical differences were found between the degrees of tumor differentiation and expression levels of the evaluated markers. At the invasion front, clinical staging (III-IV) was found to be positively correlated with the expression of GLi-1 ($P = 0.01$) and membrane β-catenin ($P = 0.02$). The presence of lymph node metastasis ($P = 0.04$) and clinical staging ($P = 0.05$) was observed to be inversely correlated with membrane β-catenin in the tumor islets. In addition, statistical significance was detected in relation to tumor size and N-cadherin immunoexpression, both at the invasion front ($P = 0.03$) and in the tumor islets ($P = 0.04$).

DISCUSSION

This study evaluated EMT (Snail, Slug, N-cadherin, E-cadherin, and β-catenin) and tumor proliferation (MCM3) markers in 23 cases of OSCC positive for nuclear GLi-1, an indicator of HH pathway activity,[17] in 2 histologically distinct tumor areas: the front of invasion and tumor islets. Distinct expression patterns between these 2 tumor microenvironments is important due to heterogeneity—that is, malignant cells exhibit different markers in different tumor areas.

TABLE 3. Immunostaining Score for Front of Invasion and Tumoral Islets

	Immunohistochemical Expression of Proteins [n (%)]							
	Front of Invasion				Tumoral Islets			
	0	1+	2+	3+	0	1+	2+	3+
GLi-1	1 (5.26)	5 (26.32)	3 (15.78)	10 (52.63)	0 (0)	2 (8.69)	5 (21.73)	16 (69.56)
Snail	0 (0)	2 (10.52)	5 (26.32)	12 (63.16)	0 (0)	0 (0)	1 (4.34)	22 (95.66)
Slug	8 (42.11)	2 (10.52)	2 (10.52)	7 (36.84)	1 (4.34)	6 (26.08)	4 (17.39)	12 (52.17)
N-cadherin	13 (68.42)	4 (21.05)	1 (5.26)	1 (5.26)	11 (47.82)	6 (26.09)	5 (21.73)	1 (4.34)
E-cadherin	14 (73.68)	4 (21.05)	1 (5.26)	0 (0)	3 (13.04)	9 (39.13)	3 (13.04)	8 (34.78)
Membrane β-catenin	7 (36.84)	10 (52.63)	1 (5.26)	1 (5.26)	0 (0)	5 (21.73)	5 (21.73)	13 (56.52)
Cytoplasmic β-catenin	8 (42.11)	6 (31.58)	2 (10.52)	3 (15.78)	5 (21.73)	5 (21.73)	3 (13.04)	10 (43.48)
MCM3	0 (0)	7 (36.84)	4 (21.05)	8 (42.11)	0 (0)	3 (13.04)	3 (13.04)	17 (73.91)

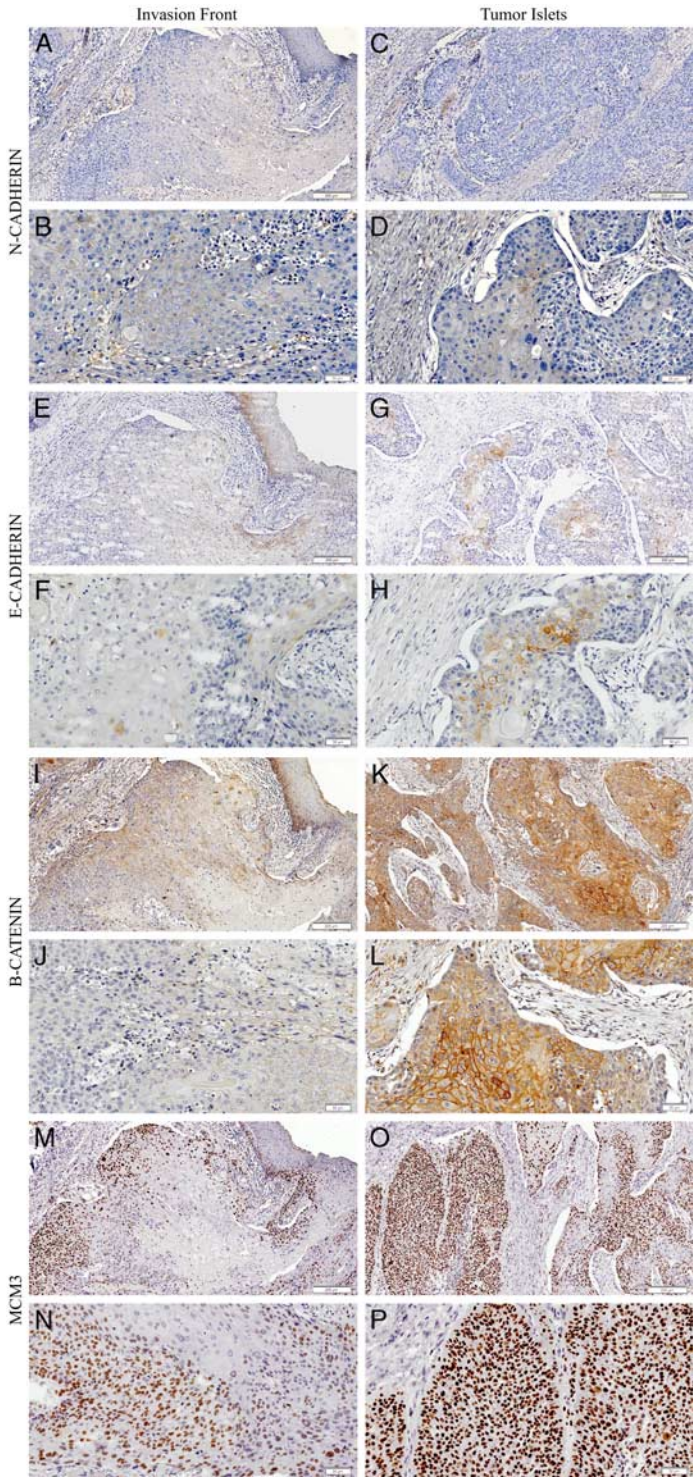

FIGURE 3. Immunoexpression of N-cadherin, E-cadherin, β-catenin, and MCM3 in oral squamous cell carcinomas. A and B, Sparce N-cadherin expression in the invasion front region at the ×20 and ×40 increase, respectively, and the tumor islets (C, D) at the ×20 and ×40 increase, respectively. E and F, Loss of E-cadherin expression in the front region of invasion at an increase of ×20 and ×40, respectively, and membrane and cytoplasmic marking on a tumor islets (G, H), respectively (increase of ×20 and ×40). I and J, Loss of β-catenin expression in the invasion front region and membrane and cytoplasmic expression on tumor islets (K, L), respectively (increase of ×20 and ×40). Nuclear expression of MCM3 in the invasion front region (M, N) and tumor islets (O, P) at ×20 and ×40 magnification. full color online

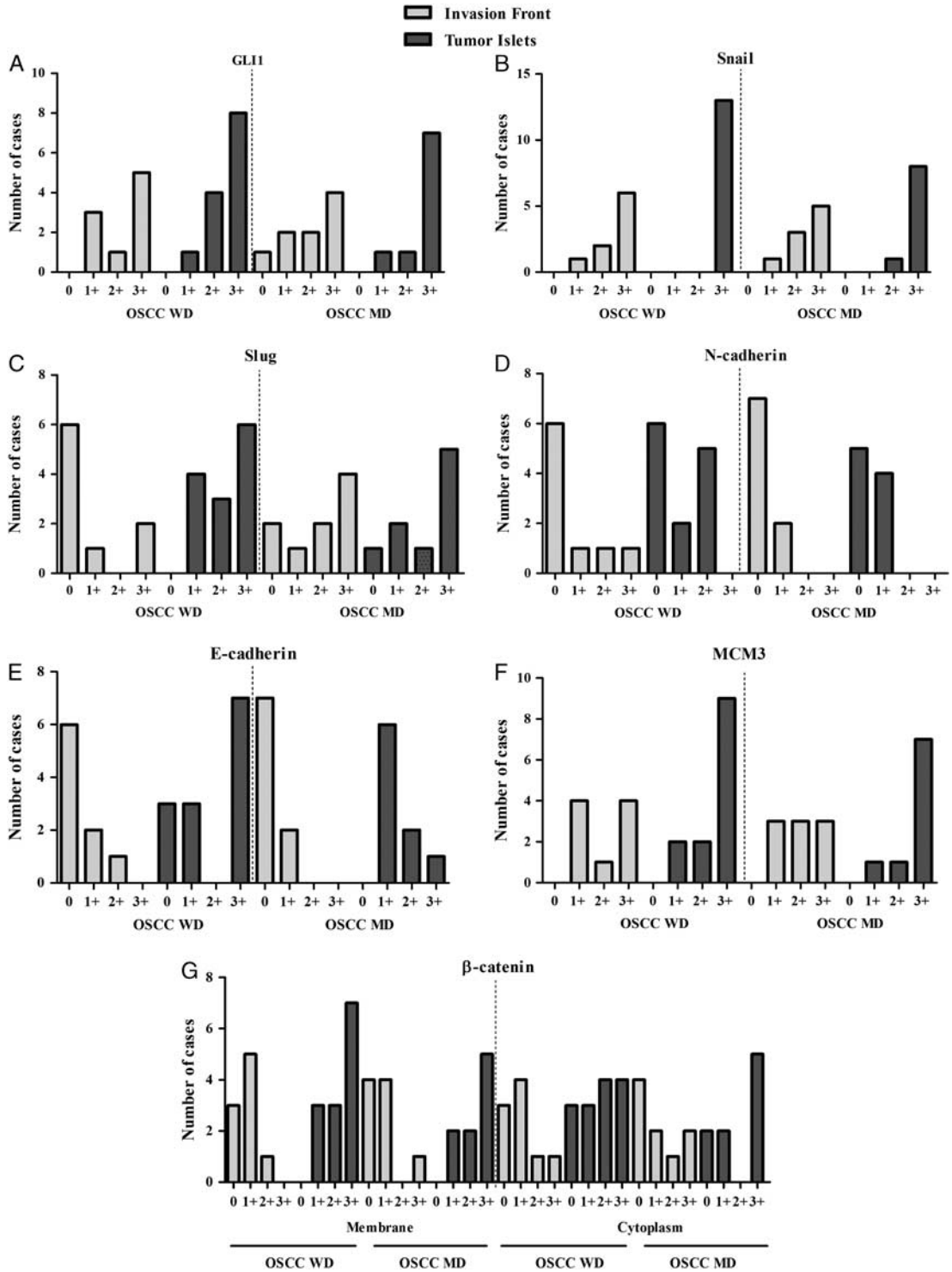

FIGURE 4. Immunoexpression scores for GLi-1, epithelial-mesenchymal transition, and MCM3 markers at the tumor front region and in tumor islets according to the histologic degree of OSCC differentiation. GLi-1 (A), Snail (B), Slug (C), N-cadherin (D), E-cadherin (E), MCM3 (F), and β-catenin, membrane, and cytoplasm (G). MD indicates moderately differentiated; OSCC, oral squamous cell carcinoma; WD, well-differentiated.

Studies of the HH pathway in OSCC available in the literature have demonstrated the participation of this pathway in protumorigenic mechanisms, such as invasion[18] and proliferation.[13] Although studies involving other types of cancer have linked the GLi-1 protein with tumor aggressiveness,[19] this aspect has remained largely

TABLE 4. Correlations Between GLi-1 Expression and Epithelial-Mesenchymal Transition and MCM3 Markers in Patients With Oral Squamous Cell Carcinoma; Association Strength Measured by the φ Coefficient

	Front of Invasion						Tumoral Islets					
	GLi-1		MCM3		E-cadherin		GLi-1		MCM3		E-cadherin	
	φ	*P*	φ	*P*	φ	*P*	φ	*P*	φ	*P*	φ	*P*
GLi-1	—	—	0.88	0.75	—	—	—	—	0.98	0.80	—	—
Snail	0.90	0.43	0.70	0.26	−0.77	**< 0.001**	0.97	**0.05**	0.99	0.16	0.45	**< 0.001**
Slug	0.10	0.06	−0.35	**0.006**	0.52	**0.01**	0.92	0.33	0.96	0.39	0.73	0.48
N-cadherin	−0.67	**< 0.001**	−0.82	**< 0.001**	0.99	1.00	−0.90	**< 0.001**	−0.87	**< 0.001**	−0.56	**0.01**
E-cadherin	−0.72	**< 0.001**	−0.84	**< 0.001**	—	—	0.42	**0.01**	0.54	**0.02**	—	—
Membrane β-catenin	−0.39	**< 0.001**	−0.17	**< 0.001**	0.56	0.06	0.96	0.57	0.96	0.51	0.62	0.14
Cytoplasmic β-catenin	−0.51	**0.02**	−0.55	**0.007**	0.90	0.15	0.83	**0.04**	0.90	0.06	0.58	0.61

Bold values indicate statistical significance ($P < 0.05$).

unexplored in OSCC.[20] Meanwhile, membranous β-catenin has been associated with clinical staging and the presence of lymph node metastasis in the context of oral cancer, consistently with the present results.[21]

Reduced membrane E-cadherin expression at the invasion front was a frequent finding; notably, the loss of immunoexpression of this protein was linked to perceptible discontinuity in membrane immunopositivity. Despite the lower degree of immunostaining observed in tumor islets, the positive expression of this protein was maintained, even when discontinuous. Similar results were reported by Costa et al[22] and Wang et al,[23] who demonstrated that reduced E-cadherin expression at the invasion front may be related to the increased invasion and migration capability of tumor cells.

A strong positive correlation between the immunodetection of GLi-1 and Snail was detected at the invasion front and in tumor islets. This corroborates other studies by Wang et al[23] in gastric cancer and by Min et al[24] in esophageal squamous cell carcinoma, who observed that GLi-1 overexpression positively regulated Snail expression. The expression of Slug, another transcription factor of the SNAIL family, has been related to invasion, resistance to apoptosis, and to the acquisition of typical stem cell characteristics by tumor cells.[25] Our study found a positive association between Slug and E-cadherin at the invasion front, corroborating a study by Joseph et al[26] showing the preservation of E-cadherin levels in Slug-positive oral carcinoma cells. Here, GLi-1 and Slug were found to be weakly positively correlated, regardless of the region evaluated, which consequently necessitates the enhanced understanding surrounding the possible regulation of Slug levels by GLi-1, in addition to the evaluation of this protein in OSCC case series with different histologic gradations.

Cadherin switching is a peculiar event in EMT proteins, and the switch from E-cadherin to N-cadherin refers to a decrease in E-cadherin expression with a resulting increase in N-cadherin expression.[7,27,28] Despite the present observation of an inversely proportional and statistically significant correlation between the expression of N-cadherin and E-cadherin in tumor islets, this aspect was not evidenced at the invasive front. In addition, the literature contains discrepancies in N-cadherin expression in different tumor types, as most studies consider that any level of detection of this protein is important in EMT[22]; this stands in contrast to our study, which focused on grouping cases according to quantitative expression scoring and degree of immunostaining continuity.

Herein, β-catenin expression was evaluated in the membrane, cytoplasm, and nucleus, with positivity rarely observed in the latter cell compartment. Distinct functions are exerted by this protein depending on the compartment in which it is located.[29] For example, cytoplasmic β-catenin expression has been related to lower tumor aggressiveness.[29,30] In murine osteosarcoma cells, immunopositivity in this cell compartment was also related to a loss of metastatic potential.[30] In our study, cytoplasmic β-catenin was more common in the tumor islets and was identified together with more pronounced membrane immunostaining. In addition, none of the included cases exhibited distant metastasis, and the vast majority were classified as well-differentiated. In the OSCCs evaluated, the loss of β-catenin membrane immunostaining at the invasion front was evidenced, while pronounced and continuous expression was seen in the tumor islets, corroborating results reported by other studies involving OSCC.[31] Although nuclear β-catenin is considered significant with regard to EMT,[32] the data deposited in the interactome biorepository with respect to head and neck tumors indicate that nuclear β-catenin is not a frequent finding in OSCC,[33] despite the presence of some results to the contrary.[34]

The correlation between higher levels of GLi-1 and decreased β-catenin and E-cadherin expression at the tumor front, in addition to elevated Snail expression, serve to corroborate the participation of the HH pathway in EMT. Yue et al[35] observed a similar relationship in lung carcinoma. In addition, we observed a high correlation between the cytoplasmic levels of β-catenin and GLi-1. Considering that both molecules are immobilized in the same cytoplasmic protein complex,[36] this further strengthens our hypothesis that nuclear expression of GLi-1 is related to EMT mediated by the HH and WNT pathways in OSCCs.

In the same way that EMT is a peculiar biological event in tumors, cell proliferation represents one of the most important characteristics related to the progression

of malignant neoplasia.[12] In this respect, MCM3 has been one of the markers highlighted in studies on tumor cell growth and proliferation. A study by Valverde et al[12] indicated that MCM3 expression is not influenced by external factors such as inflammation, and it can therefore be considered a reliable marker of cellular proliferation in OSCC. Moreover, this protein is only expressed during cell division.[37] Herein, elevated MCM3 expression was observed in the cell nucleus, specifically in tumor islets, which indicates that this compartment may play a relevant role in tumor growth.

Although MCM3 is considered relevant in tumor growth proliferations, no relationship with GLi-1 was observed in our study. However, other studies have demonstrated that GLi-1 was correlated with immunolabeling for cyclin D1[38] and MYC,[17] the main cell-cycle target genes in the HH pathway. Thus, it has been suggested that, in OSCC, subsequent to the G1 phase in which MCM3 is expressed, GLi-1 can participate in cell-cycle regulation. Notably, this correlation was also not found in studies focused on tumors in salivary glands.[39]

In conclusion, the correlation between GLi-1 and EMT markers (Snail, E-cadherin, and β-catenin) suggests that, in OSCC, the HH pathway may act to regulate mesenchymal phenotype expression, which seems to be more relevant at the tumor front. No associations between GLi-1 and MCM3 were observed. Loss of adhesion protein expression (E-cadherin and β-catenin) predominantly occurred at the front region, whereas adhesion protein expression was detected in tumor islets independent of MCM3 expression. The patterns of expression and localization (front and tumor islets) of N-cadherin and Slug in this tumor type suggest that these markers do not significantly reflect the EMT process in OSCC.

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REFERENCES

1. Yang WE, Ho CC, Yang SF, et al. Cathepsin B expression and the correlation with clinical aspects of oral squamous cell carcinoma. *PLoS ONE*. 2016;11:e0152165.
2. Ramos O, Bernardi L, Lauxen I, et al. Fibronectin modulates cell adhesion and signaling to promote single cell migration of highly invasive oral squamous cell carcinoma. *PLoS One*. 2016;11:e0151338.
3. Zhang J, Tian XJ, Xing J. Signal transduction pathways of EMT induced by TGF-β, SHH, and WNT and their crosstalks. *J Clin Med*. 2016;5:41.
4. Carballo GB, Honorato JR, de Lopes GPF, et al. A highlight on sonic Hedgehog pathway. *Cell Commun Signal*. 2018;16:11.
5. Zhang Y, Xue X, Zhao X, et al. Vasohibin 2 promotes malignant behavior of pancreatic cancer cells by inducing epithelial-mesenchymal transition via Hedgehog signaling pathway. *Cancer Med*. 2018;7:5567–5576.
6. Sharma A, De R, Javed S, et al. Sonic Hedgehog pathway activation regulates cervical cancer stem cell characteristics during epithelial to mesenchymal transition. *J Cell Physiol*. 2019. Doi: 10.1002/jcp.28231.
7. Merzoug-Larabi M, Spasojevic C, Eymard M, et al. Protein kinase C inhibitor Gö6976 but not Gö6983 induces the reversion of E- to N-cadherin switch and metastatic phenotype in melanoma: identification of the role of protein kinase D1. *BMC Cancer*. 2017;17:12.
8. Gloushankova NA, Zhitnyak IY, Rubtsova SN. Role of epithelial-mesenchymal transition in tumor progression. *Biochemistry*. 2018;83:1469–1476.
9. Liu Y, DU F, Zhao Q, et al. Acquisition of 5-fluorouracil resistance induces epithelial-mesenchymal transitions through the Hedgehog signaling pathway in HCT-8 colon cancer cells. *Oncol Lett*. 2015;9:2675–2679.
10. Della Corte CM, Viscardi G, Papaccio F, et al. Implication of the Hedgehog pathway in hepatocellular carcinoma. *World J Gastroenterol*. 2017;23:4330–4340.
11. Yang Z, Cui Y, Ni W, et al. Gli1, a potential regulator of esophageal cancer stem cell, is identified as an independent adverse prognostic factor in esophageal squamous cell carcinoma. *J Cancer Res Clin Oncol*. 2017;143:243–254.
12. Valverde LF, Freitas RD, Pereira TA, et al. MCM3: a novel proliferation marker in oral squamous cell carcinoma. *Appl Immunohistochem Mol Morphol*. 2018;26:120–125.
13. Ivina AA, Syomkin VA, Babichenko II. The role of sonic Hedgehog signaling pathway in the development of oral squamous cell carcinoma. *Stomatologiia (Mosk)*. 2018;97:4–6.
14. Zhang X, Tian Y, Yang Y, et al. Development of anticancer agents targeting the Hedgehog signaling. *Cell Mol Life Sci*. 2017;74:2773–2782.
15. Patriarch S, Ferretti S, Zanetti R. TNM Classification of Malignant Tumors—eighth edition: which news. *Epidemiol Prev*. 2017;41:140–143.
16. El-Naggar AK. What is new in the World Health Organization 2017 Histopathology Classification? *Curr Treat Options Oncol*. 2017;18:43.
17. Rimkus TK, Carpenter RL, Qasem S, et al. Targeting the sonic Hedgehog signaling pathway: review of smoothened and GLI inhibitors. *Cancers (Basel)*. 2016;8:22.
18. Kuroda H, Kurio N, Shimo T, et al. Oral squamous cell carcinoma-derived sonic Hedgehog promotes angiogenesis. *Anticancer Res*. 2017;37:6731–6737.
19. Lu L, Wu M, Zhao F, et al. Prognostic and clinicopathological value of Gli-1 expression in gastric cancer: a meta-analysis. *Oncotarget*. 2016;7:69087–69096.
20. Fan HX, Wang S, Zhao H, et al. Sonic Hedgehog signaling may promote invasion and metastasis of oral squamous cell carcinoma by activating MMP-9 and E-cadherin expression. *Med Oncol*. 2014;31:41.
21. Ayed-Guerfali DB, Hassairi B, Khabir A, et al. Expression of APC, β-catenin and E-cadherin in Tunisian patients with gastric adenocarcinoma: clinical significance. *Tumor Biol*. 2014;35:1775–1783.
22. Costa LC, Leite CF, Cardoso SV, et al. Expression of epithelial-mesenchymal transition markers at the invasive front of oral squamous cell carcinoma. *J Appl Oral Sci*. 2015;23:169–178.
23. Wang ZS, Shen Y, Li X, et al. Significance and prognostic value of Gli-1 and Snail/E-cadherin expression in progressive gastric cancer. *Tumor Biol*. 2014;35:1357–1363.
24. Min S, Xiaoyan X, Fanghui P, et al. The glioma-associated oncogene homolog 1 promotes epithelial-mesenchymal transition in human esophageal squamous cell cancer by inhibiting E-cadherin via Snail. *Cancer Gene Ther*. 2013;20:379–385.
25. Zhao X, Sun B, Sun D, et al. Slug promotes hepatocellular cancer cell progression by increasing sox2 and nanog expression. *Oncol Rep*. 2015;33:149–156.
26. Joseph MJ, Dangi-Garimella S, Shields MA, et al. Slug is a downstream mediator of transforming growth factor β1-induced matrix metalloproteinase-9 expression and invasion of oral cancer cells. *J Cell Biochem*. 2009;108:726–736.
27. Ozaki-Honda Y, Seki S, Fujiwara M, et al. Prognostic prediction of oral squamous cell carcinoma by E-cadherin and N-cadherin expression in overall cells in tumor nests or tumor cells at the invasive front. *Cancer Microenviron*. 2017;10(1–3):87–94.
28. Tang C, Mei L, Pan L, et al. Hedgehog signaling through GLI1 and GLI2 is required for epithelial-mesenchymal transition in human trophoblasts. *Biochim Biophys Acta*. 2015;1850:1438–1448.
29. Kumar R, Bashyam MD. Multiple oncogenic roles of nuclear beta-catenin. *J Biosci*. 2017;42:695–707.

30. Kidani T, Nakamura A, Kamei S, et al. Overexpression of cytoplasmic β-catenin inhibits the metastasis of murine osteosarcoma cell line LM8. *Cancer Cell Int*. 2014;14:31.
31. Sasaya K, Sudo H, Maeda G, et al. Concomitant loss of p120-catenin and β-catenin membrane expression and oral progression carcinoma with E-cadherin reduction. *PLoS One*. 2013;8:e: 69777.
32. Ribatti D. Epithelial-mesenchymal transition in morphogenesis, cancer progression and angiogenesis. *Exp Cell Res*. 2017;353:1–5.
33. Uhlén M, Fagerberg L, Hallström BM, et al. Proteomics. Tissue-based map of the human proteome. *Science*. 2015;347:1260419.
34. Moles MA, Montoya JA, Salvago MD, et al. Implications of differential expression of β-catenin in oral carcinoma. *Anticancer Res*. 2016;36:1599–15604.
35. Yue D, Li H, Che J, et al. Hedgehog/Gli promotes epithelial-mesenchymal transition in lung squamous cell carcinomas. *J Exp Clin Cancer Res*. 2014;33:34.
36. Wils LJ, Bijlsma MF. Epigenetic regulation of the Hedgehog and Wnt pathways in cancer. *Crit Rev Oncol Hematol*. 2018;121:23–44.
37. Nowinska K, Chmielewska M, Piotrowska A, et al. Correlation between levels of expression of minichromosome maintenance proteins, Ki-67 proliferation antigen and metallothionein I/II in laryngeal squamous cell cancer. *Int J Oncol*. 2016;48:635–645.
38. Onishi H, Katano M. Hedgehog signaling pathway as a new therapeutic target in pancreatic cancer. *World J Gastroenterol*. 2014;20:2335–2342.
39. Vidal MT, Lourenço SV, Soares FA, et al. The sonic Hedgehog signaling pathway contributes to the development of salivary gland neoplasms regardless of perineural infiltration. *Tumor Biol*. 2016;37:9587–9601.