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ORIGINAL PAPER

Immunolocalization of Cancer Stem Cells Marker ALDH1 and its Association with Tumor Budding in Oral Squamous Cell Carcinoma

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Abstract

Tumor budding is a prognostic marker for oral squamous cell carcinoma (OSCC) characterized by the presence of isolated or small clusters of neoplastic cells at the tumor invasive front. Aldehyde dehydrogenase-1 (ALDH1) is associated with tumorigenesis, linked to treatment resistance and shown to identify cancer stem cells (CSC)-like cells. This study aimed to evaluate the expression of ALDH1 and its association with tumor budding in OSCC. Immunohistochemistry was employed in 163 OSCC samples to identify pancytokeratin (AE1/AE3) and ALDH1. While pancytokeratin (AE1/AE3) identified squamous tumor buds, the CSC-like cells were identified using ALDH1. A Chi square test was used to evaluate association between ALDH1 expression and tumor budding, while McNemar's test was used to identify differences in ALDH1 expression between the budding area and the area outside the budding. A positive expression of ALDH1 was observed in 47.24% of the samples and in 70% of anatomic locations affected. No association was observed between ALDH1 expression and tumor budding ($p > 0.05$). In tumors with high-intensity tumor budding, ALDH1 expression was higher in the budding area than in the area outside the budding ($p < 0.05$). The finding that tumor bud cells in OSCC show phenotypic characteristics of CSC-like cells reinforces the relevance of tumor budding in determining the biological behavior of this malignant neoplasm. Moreover, the presence of CSC-like cells in nearly half of evaluated samples of OSCC and in most of the affected anatomic locations is in accordance with the CSC model of oral carcinogenesis.

Keywords Oral cancer · Squamous cell carcinoma · Cancer stem cells · Aldehyde dehydrogenase-1 · Tumor budding

Introduction

The most common malignant tumor of the oral cavity, squamous cell carcinoma poses great morbidity and mortality [1]. Occurring primarily in adults exposed to tobacco and alcohol, oral squamous cell carcinoma (OSCC) is a malignant epithelial tumor with varying degrees of differentiation and an early propensity to lymph node metastasis [1, 2]. Despite considerable advances in treatment, early stage diseases have a risk about 30% of regional metastasis [3]. Specific molecular markers related to tumor invasion and metastasis could provide new insights for the identification of innovative treatments [4].

Malignant cells from epithelial neoplasms show great heterogeneity. Only few are responsible for the origination and biological behavior of tumors, especially their growth and capacity for local invasion and metastasis. Cells belonging to this subpopulation are called *cancer stem cells* (CSC),

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given their competence for self-renewal, their capability to differentiate into heterogeneous lines of tumor cells, and their phenotypic characteristics of stem cells [5, 6]. Identifying CSC-like cells has involved first detecting specific cell surface markers [4, 5]. Among that cell population's common features is the expression of the enzyme aldehyde dehydrogenase [7]. Aldehyde dehydrogenase-1 (ALDH1) is a cytosolic enzyme that promotes the intracellular oxidation of aldehydes, contributing to the oxidation of retinol to retinoic acid in initial stages of stem cell differentiation [5]. Retinoic acid is directly implicated in the management of cell proliferation [8]. In addition to being a specific CSC-like cell marker, ALDH1 expression was linked to treatment resistance and poor prognosis for OSCC [4].

Tumor budding refers to the presence of isolated neoplastic cells or small clusters of neoplastic cells located at the tumor invasive front. The phenomenon indicates two significant aggressive features of malignant tumors: cell adhesion loss and local invasion [9, 10]. As such, tumor budding is a morphological marker of tumor invasion which has become a simple and consistent independent prognostic factor for OSCC [11]. Cancer cells located in the tumor–host interface of OSCC has long been considered more aggressive in terms of metastatic potential. However, the role of CSC-like cells at the tumor budding area has not yet been investigated.

The aim of this study was to evaluate the expression of ALDH1 as a molecular marker of CSC-like cells and its association with the morphological marker of prognosis tumor budding in OSCC. The principal null hypotheses to be tested were: (i) there is no association between the intensity of tumor budding and the ALDH1 expression; (ii) in OSCC samples showing high-intensity tumor budding, there are no differences in the ALDH1 expression between the budding area and the area outside the budding.

Materials and Methods

Samples

This study was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee from Pontifical Catholic University of Minas Gerais - PUC Minas (Protocol CAAE 45330715.7.0000.5137).

In order to perform a preliminary evaluation, 232 archived formalin-fixed, paraffin-embedded samples of OSCC, obtained by incisional biopsies, were retrospectively chosen from the archives of the PUC Minas' Oral Pathology Laboratory. Histological subtypes of OSCC were not included. H&E slides of all the specimens were assessed by a qualified pathologist (MCRH) who excluded 69 samples with insufficient tissue for suitable evaluation of tumor budding or assessment of ALDH-1 immunostaining. Therefore, a total of 163 OSCC samples were selected to be used in the study.

Male patients correspond to 77.3% of the 163 OSCC samples evaluated, whereas 22.7% of the patients were females. The patients' mean age was 58.24 years. The 163 tumors were located across the oral cavity (Table 1).

Immunohistochemistry

Immunohistochemical techniques were employed to identify pancytokeratin (AE1/AE3) and ALDH1. While pancytokeratin (AE1/AE3) identified squamous tumor buds, the CSC-like cells were identified using immunohistochemical stain for ALDH1. Sections of 3-µm thickness were obtained from the paraffin blocks followed by deparaffinization, rehydration, and antigen retrieval that used Trilogy (Cell Marque, Rocklin, CA, USA) (2 washes of 30 min at 98 °C). Endogenous peroxidase and endogenous protein blockade were performed using Hydrogen Peroxide Block and Protein Block solutions (Spring Bioscience, Pleasanton, CA, USA). Sections were washed in TRIS buffer for 5 min and subsequently incubated in a humid chamber for 60 min with anti-pancytokeratin antibody (clones AE1/AE3, diluted 1:50 in TRIS buffer pH 7.4; Leica Biosystems, Newcastle, UK). Separate sections were stained with anti-ALDH1 monoclonal primary antibody (clone 44, diluted 1:50 in TRIS buffer pH 7.4; BD Bioscience, San Diego, CA, USA). Three 5-min washes in TRIS buffer of each specimen were performed, followed by revelation with Reveal biotin-free amplification system (Spring Bioscience). The revelation was carried out using the diaminobenzidine revelation kit (Spring Bioscience). Counterstaining was performed with Mayer's hematoxylin for 1 min, after which the sections were dehydrated

Table 1 Aldehyde dehydrogenase-1 (ALDH1) expression in oral squamous cell carcinoma by anatomic location

Location	*n* (%)[a]	ALDH1 negative	ALDH1 positive
Tongue	86 (35.83%)	41	45
Floor of the mouth	41 (17.08%)	17	24
Alveolar ridge	29 (12.08%)	19	10
Palate	27 (11.25%)	15	12
Lip	19 (7.92%)	17	2
Buccal mucosa	17 (7.08%)	10	7
Retromolar triangle	12 (5.00%)	7	5
Vestibular fornix	6 (2.50%)	6	0
Gingiva	2 (0.84%)	2	0
Maxillary tuber	1 (0.42%)	1	0
Total	240 (100%)	135 (56.25%)	105 (43.75%)

[a]A total of 163 OSCC samples were evaluated in the study. Since numerous tumors involved several sites, the 163 tumors were located in 240 distinct anatomic locations

in one wash in 70% ethanol for 2 min, another in 90% ethanol for 5 min, and three more in absolute ethanol for 5 min each. Sections were diaphanized in three 5-min xylol washes at room temperature. Lastly, the slides were mounted using Permount (Fisher Scientific, Fair Lawn, NJ, USA). The positive control for ALDH1 was the immunostaining of normal salivary gland specimens, whereas the positive controls for pancytokeratin (AE1/AE3) were normal oral mucosa specimens. Negative control was achieved by omitting primary antibodies and by using monoclonal antibodies with identical isotype and different specificities of the primary antibodies.

Tumor Budding Evaluation

The evaluation of tumor budding in 163 samples submitted to pancytokeratin (AE1/AE3) immunostaining method was performed by three independent examiners, all of whom used the same light microscope, with ×10 ocular lens and field number 22 (field number is the diameter of the view-field in the ocular lens). Analysis was performed following criteria proposed by Wang et al. [10]. Tumor budding was defined as the presence of isolated neoplastic cells or in small clusters with less than 5 cells at the invasive front. Initially, the specimens were assessed using the lowest power objective lens to choose areas with the highest tumor budding intensity. Subsequently, using a ×20 objective lens, the number of tumor buds (number of isolated neoplastic cells or small clusters of up to four cells) was counted in just one ×200 power field (the field showing the highest number of tumor buds). The intensity of tumor budding was than categorized as either high-intensity tumor budding (as shown in Fig. 1a, b, five or more tumor buds in one ×200 power field), or low-intensity or no tumor budding (less than five tumor buds or no tumor bud detectable in one ×200 power field). It is important to highlight that the term high-intensity tumor budding does not refer to the intensity of the immunostaining but to the number of tumor buds (five or more in one ×200 power field). Evaluations that generated disagreement among the three examiners were classified according to the results of the majority of examiners.

Interexaminer agreement was assessed by Fleiss' Kappa, whereas the intra-examiner agreement was calculated by Cohen's Kappa. In this last analysis, each examiner independently performed estimations on 40 samples at two times: T1 (initial evaluation) and T2 (30 days later). The sample calculation table published by Sim and Wright [12] was used to calculate the amount of 40 samples for reanalysis, assuming the null hypothesis value of Kappa of zero, a minimum Kappa to detect of 0.40, and a power of 80%. The interval of 30 days among T1 and T2 was considered to be adequate for decreasing bias from the previous analysis of the specimens in T1. Concordance analyses were achieved by StatsToDo statistical programs on the website http://www.statstodo.com (StatsToDo Trading Pty Ltd, Brisbane, QLD, Australia). Compared to the strength of agreement associated with Kappa statistics proposed by Landis and Koch [13], interexaminer agreement was substantial (Kappa = 0.79, 95% CI 0.71–0.88), while intra-examiner agreement was almost perfect for all three examiners: Examiner 1 (Kappa = 0.94, 95% CI 0.85–1.04), Examiner 2 (Kappa = 0.94; 95% CI 0.84–1.04), and Examiner 3 (Kappa = 0.95, 95% CI 0.85–1.04).

Evaluation of ALDH1 Expression

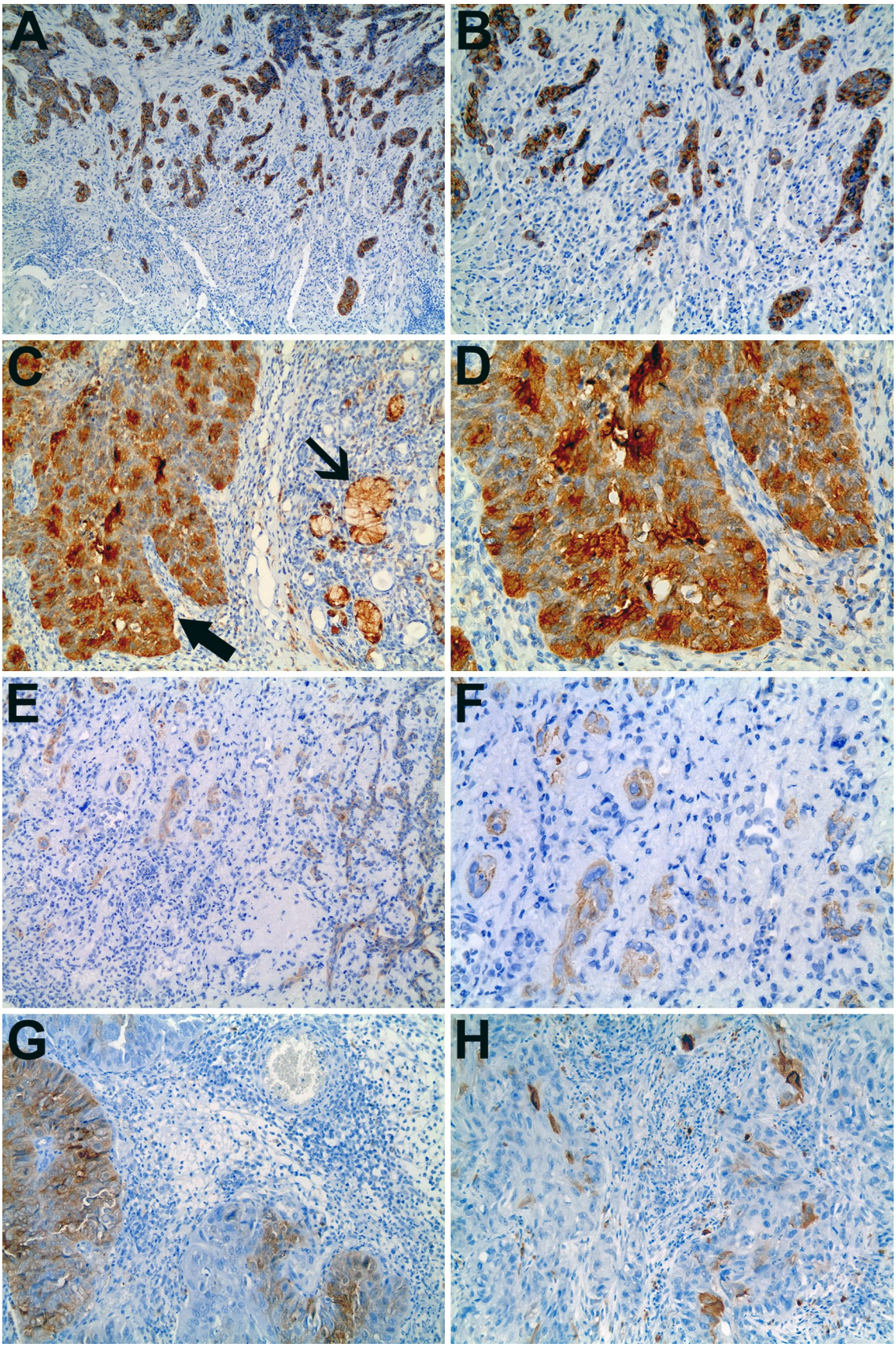
The evaluation of ALDH1 expression was performed using the same light microscope of the tumor budding evaluation.

In samples showing high-intensity tumor budding, evaluation was performed in two different areas: in one single ×200 power field with the highest tumor budding intensity (*the budding area*) and in one single ×200 power field outside the area with the highest tumor budding intensity and showing the highest ALDH1 expression (*the area outside the budding*). In samples showing low-intensity or no tumor budding, analysis was performed in one single ×200 power field showing the highest ALDH1 expression. ALDH1 expression was evaluated by a single examiner and categorized as negative (ALDH1 immunostaining in up to 5% of tumor cells) or positive (ALDH1 immunostaining in more than 5% of tumor cells), according to Hildebrand et al. [14].

In samples showing high-intensity tumor budding, in which the ALDH1 expression was evaluated in two different areas ("*the budding area"* and "*the area outside the budding")*, the scores obtained independently in these two areas were used to evaluate differences in ALDH1 expression between them. Moreover, in samples showing high-intensity tumor budding, the score obtained in the area showing the highest ALDH1 expression ("*the budding area"* or "*the area outside the budding")* was used for: (I) the evaluation of the ALDH1 expression among the different tumor anatomic locations; (II) the assessment of association between ALDH1 expression and tumor budding. Finally, in samples showing low-intensity or no tumor budding, the ALDH1 expression was evaluated in only one area (the area showing the highest ALDH1 expression), from which the score for the two analyzes described above was obtained.

Statistical Analysis

The Chi square test was used to evaluate the association between ALDH1 expression and tumor budding. In the samples with high-intensity tumor budding, McNemar's test was used to identify differences in ALDH1 expression between the budding area and area outside the budding. The level of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◂**Fig. 1** **a** and **b**: Immunostaining for pancytokeratin (AE1/AE3) in a sample with high-intensity tumor budding (i.e., 5 or more tumor buds in one ×200 power field) (**a**: ×100; **b**: ×200). **c** and **d**: Positive aldehyde dehydrogenase-1 (ALDH1) expression (i.e., immunostaining in more than 5% of tumor cells) in a sample with low-intensity or no tumor budding. The thick arrow indicates the neoplastic cells and the thin arrow indicates normal salivary glands showing ALDH1 immunostaining (positive control) (**c**: ×200; **d**: ×400). **e** and **f**: Positive ALDH1 expression in the budding area of a sample with high-intensity tumor budding (**e**: ×200; **f**: ×400). **g** and **h**: ALDH1 immunostaining in clusters of cells (g) and in sporadic cells (h) of the tumor nests (**g**: ×200; **h**: ×200)

significance was set at 5%. Analyses were performed using GraphPad Prism software (San Diego, CA, USA).

The study design and the statistical analyses were demonstrated in the Fig. 2.

Results

A total of 163 OSCC samples were evaluated in the study. Since numerous tumors involved several sites, the 163 tumors were located in 240 distinct anatomic locations. Table 1 illustrates ALDH1 expression according to the anatomic locations. Considering the 240 anatomic locations, ALDH1 expression was negative in 135 (56.25%) and positive in 105 (43.75%) (Table 1). Considering the 163 tumors, ALDH1 expression was negative in 86 (52.76%) and positive in 77 (47.24%) (Table 2). Cytoplasmic ALDH1 immunostaining was observed in isolated tumor cells or in tumor sheets, cords or nests (Fig. 1c–h). Within these tumor sheets, cords or nests it was possible to observe immunostaining in sporadic cells (Fig. 1h), in clusters of cells (Fig. 1g), in most cells or even in almost all of them (Fig. 1c, d), characterizing a heterogeneous pattern.

High-intensity tumor budding was detected in 65 samples (39.88%). Low-intensity or no tumor budding was found in 98 samples (60.12%) (Table 2).

No association was observed between ALDH1 expression and tumor budding ($p > 0.05$; Table 2; Fig. 3).

As presented in Fig. 3, in the 65 samples with high-intensity tumor budding, the following results were observed when the ALDH1 expression was independently evaluated in two different areas *("the budding area"* and *"the area outside the budding"*): 34 samples were ALDH1 negative in the area outside the budding and ALDH1 negative in the budding area; 1 sample was ALDH1 positive in the area outside the budding and ALDH1 positive in the budding area; 3 samples were ALDH1 positive in the area outside the budding and ALDH1 negative in the budding area; 27 samples were ALDH1 negative in the area outside the budding and ALDH1 positive in the budding area. The statistical analysis of these data demonstrated that, in samples with high-intensity tumor budding, ALDH1 expression was higher in the budding area than in the area outside the budding ($p < 0.05$; Fig. 3).

Discussion

CSC-like cells are tumor cells with ability of self-renewal and to generate heterogeneous lineages of malignant cells [15]. It has been demonstrated that CSC-like cells play a role in tumor initiation, metastasis, immune evasion and resistance to current therapies [16]. They are characterized by multiple markers such as CD44, CD24, CD133, OCT4, NANOG, SOX2 and ALDH1 [15, 17]. ALDH1 has been widely used to identify CSC-like cells and showed be relevant to the prognosis in head and neck squamous cell carcinomas (HNSCC) [18, 19]. Higher levels of ALDH1 expression were linked to treatment resistance, higher circulating myeloid-derived suppressor cells and poor prognosis for OSCC [4]. Nevertheless, the role of CSC-like cells positive for ALDH1 at the tumor invasive front has been investigated in some few studies [20, 21].

The present study showed that, in OSCC samples with high-intensity tumor budding, ALDH1 expression was higher in the budding area than in the area outside the budding ($p < 0.05$; Fig. 3). Such an interesting result highlights the capacity of tumor bud cells in OSCC to present invasive, metastatic properties of CSC-like cells. In fact, ALDH1 positive CSC-like cells are supposed to have a considerable ability to invade [22], and the prognostic value of ALDH1 expression has been demonstrated in nasopharyngeal carcinoma [20], HNSCC [14, 18, 19, 23], and OSCC [22]. Moreover, the presence of a subpopulation of CSC-like cells with migratory phenotypic characteristics at the tumor invasive front has been shown in a colon cancer model [6]. Furthermore, our research group has shown, for the first time, a higher cell proliferation index in the budding area when compared to the area outside the budding in OSCC [24].

Moreover, in the present study, no association emerged between ALDH1 expression and intensity of tumor budding ($p > 0.05$; Table 2; Fig. 3). Nevertheless, Luo et al. [20] identified an association between high-intensity tumor budding and higher ALDH1 expression in nasopharyngeal carcinomas, both related to the lower survival of patients. To the best of our knowledge, only one report in the English-language literature has examined that association in OSCC; it also detected no association [21]. Nonetheless, evaluations considering the budding area and the area outside the budding had not yet been investigated. The findings of the present study suggest that, in OSCC with high-intensity tumor budding, the expression of CSC-like cell marker ALDH1 in the budding area as compared to the rest of the tumor may indicate a role in the tumoral differentiation and progression.

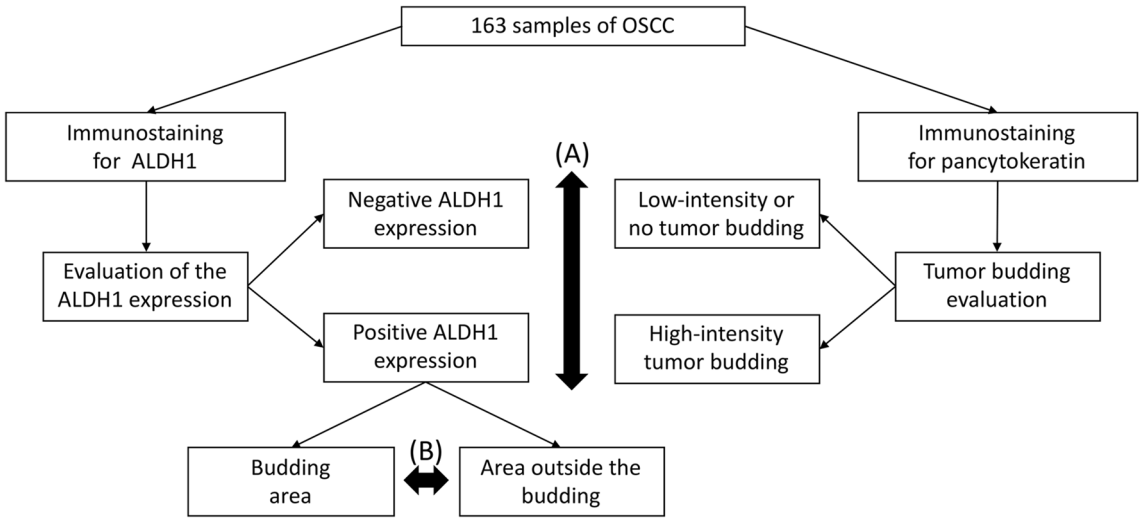

Fig. 2 Study design and statistical analyses performed. **a**: A Chi square test was used to evaluate the association of aldehyde dehydrogenase-1 (ALDH1) expression and tumor budding. **b**: In samples with high-intensity tumor budding, McNemar's test was used to identify differences in ALDH1 expression between the budding area and area outside the budding

Table 2 Aldehyde dehydrogenase-1 (ALDH1) expression and intensity of tumor budding in oral squamous cell carcinoma

ALDH1 expression	Intensity of tumor budding		*p* value
	Low-intensity or no tumor budding	High-intensity tumor budding	
Negative	52	34	n.s
Positive	46	31	

The *p* value was obtained with a Chi square test

n.s. not significant ($p > 0.05$)

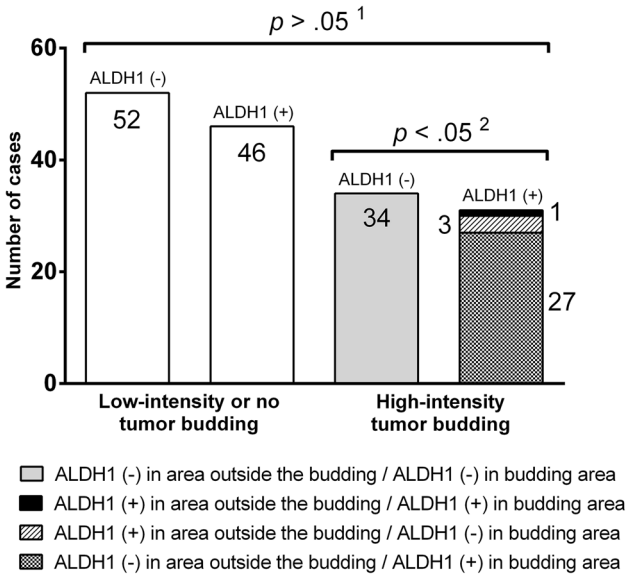

Fig. 3 Aldehyde dehydrogenase-1 (ALDH1) expression and intensity of tumor budding in oral squamous cell carcinoma. A Chi square test showed no association between ALDH1 expression and tumor budding ($p > 0.05$ [1]). A McNemar's test showed that, in tumors with high-intensity tumor budding, ALDH1 expression was higher in the budding area than in the area outside the budding ($p < 0.05$ [2]). ALDH1 (−): negative ALDH1 expression. ALDH1 (+): positive ALDH1 expression

The dissemination of tumor cells is a requirement of the metastatic phenomenon associated with transformation to a migratory phenotype. Tumor budding is a morphological marker of tumor invasion, characterized by isolated neoplastic cells or small clusters of neoplastic cells at the tumor invasive front. This phenomenon represents two important cellular events that denote the aggressiveness of tumor cells: cell adhesion loss and local invasion [9, 10]. Studies have demonstrated the prognostic value of tumor budding evaluation in OSCC [10, 11, 21, 25, 26] and other malignant tumors, including colorectal [9] and nasopharyngeal [20] carcinomas.

In the present study, almost half of cases were positive for ALDH1 expression. Immunostaining revealed that ALDH1 expression was cytoplasmic and heterogeneous, as earlier reported in OSCC [22]. In regard to anatomic locations, positive expression of ALDH1 was observed in seven of the 10 affected sites; exceptions included the vestibular fornix, the gingiva, and the maxillary tuber (Table 1). Those same anatomic locations also had fewer samples (i.e., six, two, and one, respectively). Most of the tumors showing positive ALDH-1 expression were located in the tongue and floor of the mouth (Table 1). Since OSCC in these sites may show a worse prognosis [27], upcoming studies concerning the impact of this finding should be encouraged.

ALDH1 positive CSC-like cells show a marked cancer-initiating ability [22] and ALDH1 expression was detected in several malignant tumors [20–23, 28, 29]. The presence of CSC-like cells in nearly half of the OSCC evaluated samples and in 70% of the affected anatomic locations, as observed in our results, is in accordance with the alternative CSC model of oral carcinogenesis. The traditional clonal evolution model (stochastic model) proposes that genetic and epigenetic changes can randomly occur in oral mucosal cells, thereby creating a neoplastic clone with proliferative capacity and ability to invade and metastasize. Nevertheless, scientific evidence shows that not all cells of the oral mucosa epithelium can generate tumors, even in the presence of genetic and epigenetic changes. Accordingly, only cells of the oral mucosa epithelium with phenotypic characteristics of stem cells - for example, a capacity for self-renewal and survival - can undergo the genetic and epigenetic changes that prompt tumor development. In this new alternative model of oral carcinogenesis, oncogenically transformed stem cells, now called CSC, are responsible for the genesis, sustainable growth, and metastatic dissemination of OSCC [30–33].

A limitation of this study was the analysis of only one marker of CSC-like cells, even though the ALDH-1 is one of the most widely used and reliable CSC-like cells markers [7, 9, 20, 22, 23, 28, 29, 34, 35]. Nevertheless, future studies should evaluate other CSC-like cells markers in tumor budding areas, such as CD44, CD24, CD133, OCT4, NANOG and SOX2 [15, 17]. The use of OSCC from diverse anatomic locations is a further limitation, due to different etiopathogenesis. Finally, the use of samples from incisional biopsies in order to evaluate tumor budding should also be considered a limitation. Nevertheless, Almangush et al. [36] demonstrated that tumor budding scores of OSCC samples obtained by incisional biopsies were statistically related to the scores of tumor resection samples (scores from incisional biopsy samples showed good sensitivity and high specificity in predicting the scores of tumor resection samples). In fact, several reports have adequately assessed tumor budding in OSCC using samples obtained by incisional biopsies [24, 26, 36–38].

In conclusion, in OSCC samples with high-intensity tumor budding, ALDH1 expression was higher in the budding area than in the area outside the budding. The finding that tumor bud cells in OSCC show phenotypic characteristics of CSC-like cells should be related with transformation to a migratory phenotype, reinforcing the relevance of tumor budding in determining the biological behavior of this malignant neoplasm. However, no association was observed between ALDH1 expression and intensity of tumor budding in OSCC. This lack of association should be related to the use of incisional biopsy specimens, described as one of the limitations of this study. Even though tumor budding scores of incisional biopsy samples are frequently correlated to the scores of the tumor resection specimens [36], it is possible that some OSCC samples classified in our study as low-intensity or no tumor budding are, in fact, tumors with high-intensity budding in which the budding area was not removed by the incisional biopsy. Moreover, as CSC-like cells also play a role in tumor initiation [16], the positive ALDH1 expression in samples with low-intensity or no tumor budding should be more related to tumorigenesis than tumor progression. Finally, the presence of CSC-like cells in nearly half of evaluated samples of OSCC and in most of the affected anatomic locations is in accordance with the alternative CSC model of oral carcinogenesis.

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