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Spatial distribution of cancer stem cells in head and neck squamous cell carcinomas

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BACKGROUND: CD44 and aldehyde dehydrogenase 1 (ALDH1) are considered putative markers of highly tumorigenic cells (i.e., cancer stem-like cells) in head and neck squamous cell carcinomas. This small subset of cells is believed to be the primary responsible for tumor initiation and progression. The objectives of this study were (i) to evaluate the patterns of CD44 and ALDH1 expression in the tumor center and in the invasive front, as well as in adjacent non-tumor epithelium, and (ii) to correlate these findings with clinical parameters.

MATERIALS AND METHODS: The sample comprised 44 patients with primary head and neck squamous cell carcinomas. Hematoxylin and eosin (HE) staining was used for histopathological tumor grading and for morphological analysis of adjacent non-tumor epithelium. Semiquantitative analysis was performed in histological sections immunostained for CD44 and ALDH1.

RESULTS: ALDH1 immunostaining in the invasive front showed positive association with tumor size, regional metastasis, tumor histopathological grading, and disease progression. Moreover, expression of this marker in both tumor invasive front and adjacent non-tumor epithelium was related with more aggressive tumors. CD44 immunostaining was heterogeneous in all areas evaluated and did not show association with clinical data.

CONCLUSION: Collectively, these data suggest that ALDH1 immunostaining in the invasive front and in adjacent non-tumor epithelium may help identify tumors with a more aggressive behavior, potentially contributing to improving treatment customization and the monitoring of patients with head and neck cancer.

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Introduction

Emerging evidence suggests that a small subset of cells in head and neck squamous cell carcinomas (HNSCCs) may be primarily responsible for tumor initiation and progression. This cell population has been shown to have an aggressive biological behavior, with the ability to self-renew and to generate heterogeneous lineages of cancer cells (1–15). These cells have characteristics that are similar to those of stem cells and have therefore been referred to as cancer stem-like cells (14–19).

The identification of highly tumorigenic cell populations could contribute to the development of novel therapeutic strategies and thus help mitigate the morbidity and mortality associated with cancer (3). The combination of markers that allow the identification of these cells is known to be tumor dependent. In HNSCCs, the markers most commonly used to identify highly tumorigenic cells are CD44 and aldehyde dehydrogenase 1 (ALDH1; 2, 8, 12, 14, 20, 21).

CD44 cells are a group of transmembrane glycoproteins that act on cell–cell and cell–matrix interactions (9, 22, 23), having hyaluronic acid (a widely abundant component of the extracellular matrix) as the main ligand (10, 23). The interaction between CD44 and hyaluronic acid activates essential characteristics of tumor progression, e.g., cell proliferation, survival, migration, and invasion (1, 10, 24–26). Some studies have reported a relationship between CD44 immunostaining results and tumor behavior (9, 11, 24).

Aldehyde dehydrogenase 1, in turn, is a cytosolic enzyme whose main function is to convert retinaldehyde into retinoic acid in retinol metabolism (27). The by-products of this metabolic process increase the self-renewal ability of mesenchymal stem cells (27, 28). ALDH1 activity is found in neoplastic cells, which show proliferative ability similar

to that observed in stem cells and tumors with aggressive clinical behavior (5, 9, 12, 17, 21, 27–31).

While many studies have demonstrated that ALDH+ CD44+ cells are highly tumorigenic, it remains unknown whether the spatial distribution of these cells within human tumors can be correlated with clinical outcomes. This finding could be very useful in planning the postoperative clinical management of patients with HNSCC.

Therefore, the primary objective of this study was to assess the spatial distribution of CD44+ and ALDH1+ cells in two different areas of HNSCCs (tumor center and invasive front) and also in adjacent non-tumor epithelium in specimens obtained from surgically treated patients. Possible associations between immunostaining data and clinical parameters were also investigated.

Materials and methods

Patients

Our sample comprised 44 patients treated at the Head and Neck Surgery Outpatient Clinic of Hospital de Clínicas de Porto Alegre (HCPA). All patients presented with primary HNSCC and had not undergone chemotherapy or radiotherapy before surgery. Patient data, including gender, age, ethnic origin, and exposure to risk factors, were collected in an interview. Tumor location, size, regional and distant metastasis (pTNM; 32), treatment protocol, and 3–4-year follow-up data were also recorded. Outcome was classified as failure (local, regional, or distant metastasis and death due to tumor) and disease-specific survival. This study was approved by the Research Ethics Committee of the institution of origin (protocol no. 09-315).

Immunohistochemistry

Paraffin blocks containing tumor center, invasive front, and non-tumor epithelial specimens were selected for analysis. Three 3-μm-thick sections were serially cut, of which two were used for CD44 and ALDH1 immunostaining and one for hematoxylin and eosin (HE) staining. Antigenic recovery was performed using Dako retrieval solution, citrate buffer pH 6.0 (Dako, Carpinteria, CA, USA), in water bath. Anti-CD44 antibody (clone EPR1013Y, rabbit, Abcam, Washington, DC, USA) was diluted at 1:100, and anti-ALDH1 antibody (clone 44, mouse, BD Transduction Laboratories, Franklin Lakes, NJ, USA) at 1:50. Non-specific, isotype-matched IgG was used as a negative control.

Histological analysis

Hematoxylin and eosin-stained slides were graded according to the criteria set forth by Bryne et al. (33), into good, moderate, and poor prognosis. Non-tumor epithelial tissue located adjacent to HNSCC was classified according to morphological characteristics into unaltered tissue, epithelial hyperplasia, hyperkeratosis, acanthosis, and epithelial dysplasia. Epithelial dysplasia was defined as the presence of structural and cytological abnormalities confined to epithelial layers, without invasion of other tissues. CD44 and ALDH1 immunostaining was assessed in the entire area available, in both tumor and non-tumor epithelia. Cells presenting brown staining in the membrane and cytoplasm/nucleus were considered positive for CD44 (Fig. 1) and ALDH1 (Fig. 2), respectively.

Immunohistochemical analysis was performed on both the invasive front and tumor center. The invasive front was defined as the deepest cancer cell islands (Figs 3d and m). Tumor center analysis focused on cancer cell islands located in the central region of the lesion, discarding areas of necrosis (Figs 3g and p). Adjacent non-tumor epithelium was considered normal when CD44 expression was observed in basal and parabasal layers, but not in superficial layers (Fig. 3L; 34).

Tumor markers CD44 and ALDH1 were semiquantitatively scored according to the proportion of cells stained in the whole sample as follows: 0, negative; 1, <5% of positive cells; 2, more than 5%; 3, 10–90%; and 4, more than 90% of positive cells (35). As the statistical analysis did not show differences in comparisons using four vs. two scores, CD44 and ALDH1 results were further classified into two groups, namely positive (more than 5% of cells stained; Fig. 3B,C, E,F,H,I) and negative (less than 5% of cells stained; Fig. 3K,L,N,O,Q,R; 36).

Data analysis

All analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 18. Results were tested for normality and revealed a normal distribution of data. Associations between CD44/ALDH1 staining results in the three regions assessed and clinical parameters were assessed using Pearson's chi-square test and Fisher's exact test (significance level set at $P < 0.05$). Result reproducibility was confirmed over the study period by selecting one of every 20 slides (HE, CD44, and ALDH1) for reassessment after a 7-day interval ($\kappa > 0.7$). Examiners were blind to the site of origin of each specimen.

Results

Mean patient age was 58 years (range: 37–78 years), and there was a male predominance of 86.4%. Most patients were currently or formerly exposed to tobacco and alcohol. Detailed clinical data are summarized in Table 1. Of the 44

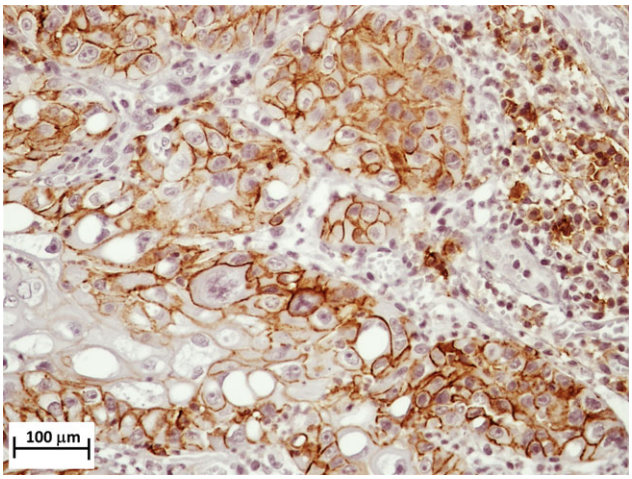

Figure 1 CD44-positive immunostaining in tumor cells. 400× magnification.

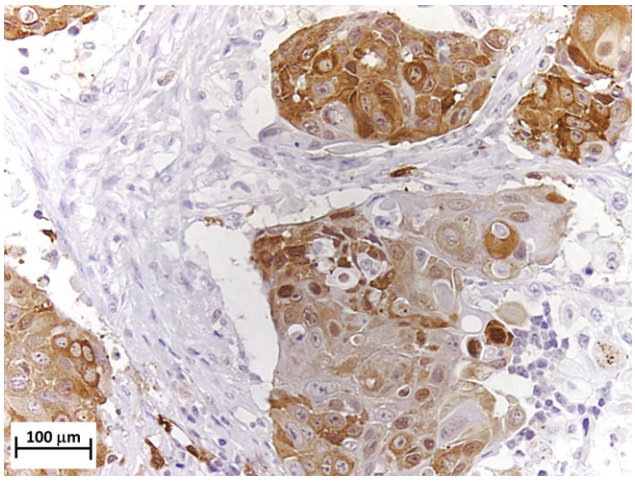

Figure 2 Aldehyde dehydrogenase 1-positive immunostaining in tumor cells. 400× magnification.

tumors assessed, 65.9% were 4 cm in diameter or smaller (T1 and T2), and 68.2% did not present regional metastases at the moment of assessment. None of the patients presented distant metastases. Most tumors were located in the oral cavity, most frequently affecting the tongue, lower lip, and floor of the mouth (Table 1).

Histopathological analysis revealed that 50% of the tumors were considered to be of good prognosis, 38.6% moderate, and 11.4% of poor prognosis. Most of the adjacent epithelium samples were classified as epithelial hyperplasia and hyperkeratosis (31.8% each), followed by epithelial dysplasia (18.2%). Normal epithelium was found in 13.6% of the samples (Table 1). Over the 3–4-year follow-up period, 68.2% of the patients were free from disease, whereas 25% showed disease recurrence/metastasis or died (Table 1).

An association was found between ALDH1 immunostaining results in tumor areas and in adjacent non-tumor epithelium, suggesting that ALDH1-positive tumors also tend to show ALDH1-positive non-tumor epithelium (Table 2). This association was not observed for CD44.

Table 3 shows CD44 and ALDH1 immunostaining results in tumor and non-tumor epithelium in association with clinical parameters and follow-up data. In the invasive front, associations were observed between ALDH1 immunostaining and tumor size (T3 and T4), presence of regional metastases (N1, N2, N3), and histopathological grading (good/moderate). Conversely, ALDH1 immunostaining in the tumor center did not show any association with the parameters assessed. In adjacent non-tumor epithelium areas, an association was observed between ALDH1 immunostaining and histopathological grading and follow-up data: 93.8% of the patients free from disease over the 3–4-year follow-up period showed ALDH1-negative cells in adjacent non-tumor epithelium ($P = 0.019$). These associations were not observed for CD44.

Discussion

Highly tumorigenic cancer stem-like cells present in HNSCC have been considered responsible for the aggressive biological behavior of this tumor (1–14 , 20, 21). Most studies available in the literature describe the use of cell culture techniques, flow cytometry, or transplantation assays in animal models to detect highly tumorigenic cells and thus assess the pathobiology of HNSCC (1, 2, 5, 6, 37). Our sample, in turn, was comprised of surgically removed human primary HNSCC specimens, and highly tumorigenic cells were identified in three different areas of the specimens using the immunostaining method. Sample characteristics, including mean age, gender, and exposure to risk factors (tobacco and alcohol use), were in line with the typical profile described for patients diagnosed with HNSCC (30, 38, 39).

Head and neck squamous cell carcinomas cells are heterogeneous within the same lesion, i.e., their cell hierarchy allows us to find subpopulations with exclusive biological behaviors, e.g., tumor growth and metastatic potential (1, 3, 19, 33). Our results showed positive CD44 and ALDH1 expression in both basal and suprabasal cells, which may indicate that the immunohistochemical technique here employed was not appropriate to identify cancer stem cells in HNSCCs based on these markers, as also reported by other authors (31, 34). Moreover, no specific markers have been established for the identification of cancer stem cell subpopulations in HNSCCs, as is the case with other tumors (37). Notwithstanding, immunohistochemistry did allow us to identify the location of positive cells in different tumoral areas (tumor center and invasive front), as well as in the different layers of adjacent non-tumor epithelium. Also, our analysis revealed a positive correlation between the spatial localization of ALDH1-positive cells and clinical outcomes, especially in the identification of tumors with a more aggressive behavior, despite the method's failure identifying cancer stem cells.

Immunostaining results obtained for the two tumor areas and non-tumor epithelium showed that tumors with ALDH1-positive cells also presented positive surrounding epithelial tissues. The concept of field cancerization, according to which carcinogen-induced changes would be present in HNSCC-related non-tumor tissues before morphological alterations can be found, was first proposed by Slaughter et al. (40) and later examined by Califano et al. (41). We believe that our finding of ALDH1-positive cells in adjacent non-tumor epithelium suggests that changes were already underway, as this enzyme tends to be present in cells with a high tumorigenic potential (12, 27, 29).

Conversely, immunostaining results for CD44 in the two tumor areas and in adjacent non-tumor epithelium did not reveal any significant associations. No association was found between CD44 expression and clinical parameters. Notwithstanding, different results are reported in the literature with the use of other CD44-positive cell detection methods, e.g., flow cytometry (2, 37). The value of CD44 as a marker for highly tumorigenic cells has been questioned previously (26), and some studies have suggested that it may not be relevant in early diagnosis or as a prognostic marker in HNSCCs (42, 43).

Indeed, previous studies have shown conflicting results on the significance of CD44 expression in head and neck tumors (9, 24, 44–48). Studies employing immunochemistry have shown that under-expression of CD44 standard form is

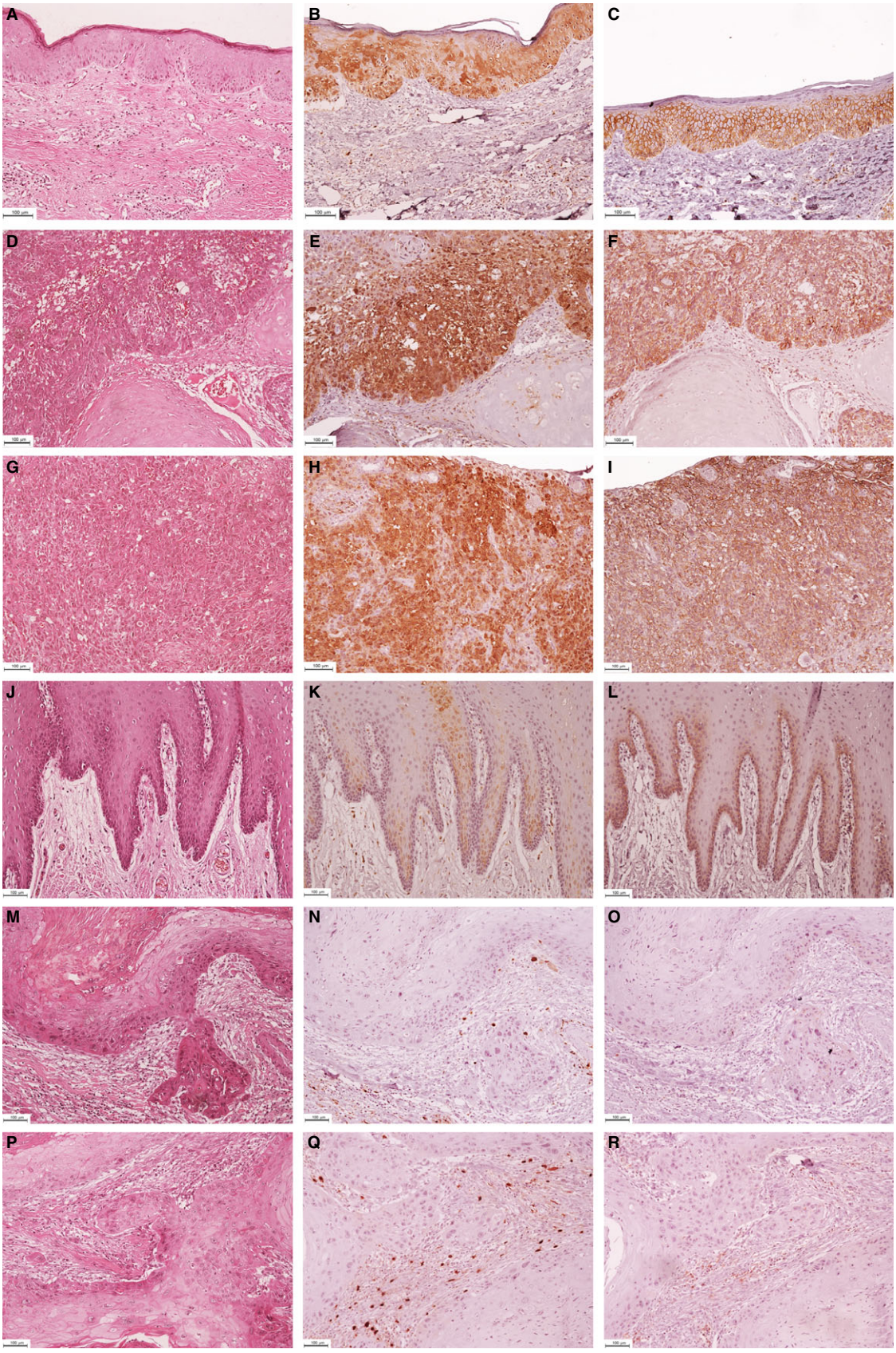

Figure 3 Positive immunostaining in non-tumor epithelium (a: HE; b: ALDH1; c: CD44), tumor invasive front (d: HE; e: ALDH1; f: CD44), and tumor center (g: HE; H: ALDH1; i: CD44). Negative immunostaining in non-tumor epithelium (j: HE; k: ALDH1; l: CD44), tumor invasive front (m: HE; n: ALDH1; o: CD44), and tumor center (p: HE; q: ALDH1; r: CD44). 200× magnification. ALDH1 = aldehyde dehydrogenase 1; HE = hematoxylin and eosin.

a predictor of reduced survival (44, 45, 47). In contrast, others have shown CD44 to be highly expressed in normal, dysplastic, and tumoral head and neck epithelium (11, 24, 26). The expression of specific CD44 variants may be reduced in tumors with a worse prognosis, but overall CD44 expression is not (46–48). Even though these varying results may be due to the use of different antibodies and assessment methods, CD44 immunostaining seems to be ineffective in detecting highly tumorigenic cells in HNSCCs.

CD44 appears to be present at different stages of carcinogenesis (24, 35). At first, CD44 binding to hyaluronic acid in the extracellular matrix stabilizes neoplastic cells in the invasive front, stimulating cell proliferation and consequently the growth of cancer cell islands (24, 25). Single-cell invasion shows a strongly positive expression of CD44 around the entire plasma membrane (35). Subsequently, the loss of CD44 expression suggests a second stage of tumor progression, in which cells lose their cell–cell adhesion, facilitating mobility and consequently the invasion and migration of neoplastic cells (22–25, 44).

Aldehyde dehydrogenase 1 staining in the tumor invasive front and non-tumor tissues showed positive associations with both clinical parameters (tumor size and presence of regional metastasis) and histopathological grading results. The lack of the same associations in the tumor center may be due the biological behavior of neoplastic cells in this region, i.e., less aggressive than cells of the invasive front (33). The association of ALDH1 staining with lymph node metastasis in HNSCC has been demonstrated previously (30). ALDH1-positive tumor cells have high tumor-initiating ability and also high invasion ability, properties that may be related to regional metastasis. In this sense, ALDH1 immunostaining may help identify tumor cells with an undifferentiated phenotype, suggestive of tumors with a stronger ability to invade surrounding tissues and develop metastases (13, 21, 27–30).

In our sample, ALDH1 immunostaining results were similar in tumor tissues and in non-tumor epithelium, suggesting that this marker could be used as an instrument to predict higher or lower risk of disease recurrence. Moreover, an association was observed between ALDH1 immunostaining in non-tumor epithelium and follow-up data: Of all adjacent epithelium specimens testing positive for ALDH1, 35.7% showed metastases, local recurrence, or death within 3–4 years, whereas 93.8% of disease-free patients stained negative for ALDH1. This finding indicates the presence of cells with a highly tumorigenic potential even in cancer-free zones.

Table 1 Patient characteristics and tumor features

Clinical parameters	n	%
Age		
Mean	58	–
Range	37–78	–
Gender		
Male	38	86.4
Female	6	13.6
Tobacco		
Current	19	43.2
Former	24	54.5
Never	1	2.3
Alcohol		
Current	22	50
Former	20	45.5
Never	2	4.5
Follow-up (3–4 years)		
Disease free	30	68.2
Metastasis/recurrence/ death	11	25
No data available	3	6.8
Histopathological grading		
Good	22	50
Moderate	17	38.6
Poor	5	11.4
Size		
T1 and T2	29	65.9
T3 and T4	15	34.1
Regional metastases		
N0	30	68.2
N1, N2, and N3	14	31.8
Localization		
Tongue	9	20.5
Lower lip	7	15.9
Floor of the mouth	6	13.6
Palate	4	9.1
Buccal mucosa	3	6.8
Neck	15	34.1
Non-tumor epithelium		
Normal oral epithelium	6	13.6
Epithelial hyperplasia	14	31.8
Hyperkeratosis	14	31.8
Acanthosis	2	4.6
Epithelial dysplasia	8	18.2
Total	44	100

Table 2 Association between (aldehyde dehydrogenase 1) ALDH1 and CD44 expression in tumor center, invasive front, and non-tumor epithelium

	Non-tumor epithelium						*Non-tumor epithelium*				
	Positive		*Negative*				*Positive*		*Negative*		
ALDH1	n	%	n	%	P	*CD44*	n	%	n	%	P
Tumor						Tumor					
Invasive front						Invasive front					
Positive	**19**	**67.9**	3	18.8	**0.005**	Positive	19	54.3	4	44.4	0.716
Negative	9	32.1	**13**	**81.3**		Negative	16	45.7	5	55.6	
Tumor Center						Tumor Center					
Positive	**18**	**64.3**	3	18.8	**0.009**	Positive	20	57.1	5	55.6	0.999
Negative	10	35.7	**13**	**81.3**		Negative	15	42.9	4	44.4	
Total	44/100%					44/100%					

Pearson's chi-square test and Fisher's exact test, $P < 0.05$.
Bold values are statistically significance of P value.

Table 3 Association between (aldehyde dehydrogenase 1) ALDH1 and CD44 expression in tumor center, invasive front, non-tumor epithelium, and clinical parameters

	ALDH1					*CD44*				
	Positive		*Negative*			*Positive*		*Negative*		
	n	%	n	%	P	n	%	n	%	P
Invasive front										
Size										
T1 and T2	10	45.5	**19**	**86.4**	**0.011**	17	73.9	12	57.1	0.393
T3 and T4	**12**	**54.5**	3	13.6		6	26.1	9	42.9	
Regional metastases										
N0	10	45.5	**20**	**90.9**	**0.004**	17	73.9	13	61.9	0.596
N1, N2, and N3	**12**	**54.5**	2	9.1		6	26.1	8	38.1	
Histopathological grading										
Good	6	27.3	**16**	**72.7**	**0.008**	13	56.5	9	42.9	0.630
Moderate	**12**	**54.5**	5	22.7		8	34.8	9	42.9	
Poor	4	18.2	1	4.5		2	8.7	3	14.3	
Tumor Center										
Size										
T1 and T2	11	52.4	18	78.3	0.136	17	68	12	63.2	0.988
T3 and T4	10	47.6	5	21.7		8	32	7	36.8	
Regional metastases										
N0	11	52.4	19	82.6	0.068	17	68	13	68.4	0.999
N1, N2, and N3	10	47.6	4	17.4		8	32	6	31.6	
Histopathological grading										
Good	7	33.3	15	65.2	0.129	12	48	10	52.6	0.693
Moderate	11	52.4	6	26.1		11	44	6	31.6	
Poor	3	14.3	2	8.7		2	8	3	15.8	
Non-tumor epithelium										
Size										
T1 and T2	15	53.6	14	87.5	0.051	25	71.4	4	44.4	0.235
T3 and T4	13	46.4	2	12.5		10	28.6	5	55.6	
Regional metastases										
N0	17	60.7	13	81.3	0.284	26	74.3	4	44.4	0.117
N1, N2, and N3	11	39.3	3	18.8		9	25.7	5	55.6	
Histopathological grading										
Good	10	35.7	**12**	**75**	**0.026**	18	51.4	4	44.4	0.382
Moderate	**13**	**46.4**	4	25		12	34.3	5	55.6	
Poor	5	17.9	0	0		5	14.3	0	0	
Follow-up										
Disease free	15	53.6	**15**	**93.8**	**0.019**	25	71.4	5	55.6	0.281
Metastasis/recurrence/death	**10**	**35.7**	1	6.3		7	20	4	44.4	
No data available	3	10.7	0	0		3	8.6	0	0	
Total			44/100%					44/100%		

Pearson's chi-square test and Fisher's exact test, $p < 0.05$.
Bold values are statistically significance of *P* value.

In conclusion, CD44 and ALDH1 expression did not show a distinct spatial distribution pattern when invasive front and tumor center specimens were compared. Furthermore, while CD44 expression did not show a clear correlation with clinical outcomes, ALDH1 staining correlated well with tumor size, presence of regional metastasis, and histopathological grading. These results demonstrate that ALDH1 is predominantly found in more aggressive tumors, in both tumor areas and adjacent non-tumor epithelium, and may therefore be useful in improving treatment customization and the postoperative clinical management of patients with head and neck cancer.

References

1. Okamoto A, Chikamatsu K, Sakakura K, Hatsushika K, Takahashi G, Masuyama K. Expansion and characterization of cancer stem-like cells in carcinoma of the head and neck. *Oral Oncol* 2009; **45**: 633–9.
2. 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 USA* 2007; **104**: 973–8.
3. Bhaijee F, Pepper DJ, Pitman KT, Bell D. Cancer stem cells in head and neck squamous cell carcinoma: a review of current knowledge and future applications. *Head Neck* 2012; **34**: 894–9.
4. Sayed SI, Dwivedi RC, Katna R, Garg A, Pathak KA, Nutting CM. Implications of understanding cancer stem cell (CSC) biology in head and neck squamous cell cancer. *Oral Oncol* 2011; **47**: 237–43.
5. Harper LJ, Piper K, Common J, Fortune F, Mackenzie IC. Stem cell patterns in cell lines derived from head and neck squamous cell carcinoma. *J Oral Pathol Med* 2007; **36**: 594–603.
6. Chen C, Wei Y, Hummel M, et al. Evidence for epithelial-mesenchymal transition in cancer stem cells of head and neck squamous cell carcinoma. *PLoS ONE* 2011; **6**: e16466.
7. Oliveira LR, Oliveira-Costa JP, Araujo IM, Soave DF, Zanetti JS, Soares FA. Cancer stem cell immunophenotypes in oral squamous cell carcinoma. *J Oral Pathol Med* 2011; **40**: 135–42.

8. Krishnamurthy S, Dong Z, Vodopyanov D, et al. Endothelial cell-initiated signaling promotes the survival and self-renewal of cancer stem cells. *Cancer Res* 2010; **70**: 9969–78.
9. Kosunen A, Pirinen R, Ropponen K, et al. CD44 expression and its relationship with MMP-9, clinicopathological factors and survival in oral squamous cell carcinoma. *Oral Oncol* 2007; **43**: 51–9.
10. Wang SJ, Bourguignon LYW. Role of hyaluronan-mediated CD44 signaling in head and neck squamous cell carcinoma progression and chemoresistance. *Am J Pathol* 2011; **178**: 956–63.
11. Kokko LL, Hurme S, Maula SM, et al. Significance of site-specific prognosis of cancer stem cell marker CD44 in head and neck squamous cell carcinoma. *Oral Oncol* 2011; **47**: 510–6.
12. 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**: 307–13.
13. Clay MR, Tabor M, Owen JR, et al. Single-marker identification of head and neck squamous cell carcinoma cancer stem cells with aldehyde dehydrogenase. *Head Neck* 2010; **32**: 1195–201.
14. Krishnamurthy S, Nör JE. Head and neck cancer stem cells. *J Dent Res* 2012; **91**: 334–40.
15. Avoranta ST, Korkeila EA, Ristamäki RH, et al. ALDH1 expression indicates chemotherapy resistance and poor outcome in node-negative rectal cancer. *Hum Pathol* 2013; **44**: 966–74.
16. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001; **414**: 105–11.
17. Douville J, Beaulieu R, Balicki D. ALDH1 as a functional marker of cancer stem and progenitor cells. *Stem Cells Dev* 2009; **18**: 17–25.
18. Wicha MS, Liu S, Dontu G. Cancer stem cells: an old idea-a paradigm shift. *Cancer Res* 2006; **66**: 1883–90.
19. Driessens G, Beck B, Caauwe A, Simons BD, Blanpain C. Defining the mode of tumour growth by clonal analysis. *Nature* 2012; **488**: 527–30.
20. Zhang Z, Sant'Ana Filho M, Nör JE. The biology of head and neck cancer stem cells. *Oral Oncol* 2012; **48**: 1–9.
21. Minato T, Yamamoto Y, Seike J, et al. Aldehyde dehydrogenase 1 expression is associated with poor prognosis in patients with esophageal squamous cell carcinoma. *Ann Surg Oncol* 2013; **20**: 209–17.
22. Naor D, Wallach-Dayan SB, Zahalka MA, Sionov RV. Involvement of CD44, a molecule with a thousand faces, in a cancer dissemination. *Semin Cancer Biol* 2008; **18**: 260–7.
23. Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B. CD44 is the principal cell surface receptor for hyaluronate. *Cell* 1990; **61**: 1301–13.
24. Bánkfalvi A, Krabort M, Buchwalow IB, Végh A, Felszeghy E, Piffkó J. Gains and losses of adhesion molecules (CD44, E-cadherin and β-catenin) during oral carcinogenesis and tumor progression. *J Pathol* 2002; **198**: 343–51.
25. Gergolios A, Batistatou A, Charalabopoulos A, Manolopoulos L, Charalabopoulos K. The role of CD44 adhesion molecule in oral cavity cancer. *Exp Oncol* 2006; **28**: 94–8.
26. Mack B, Gires O. CD44s and CD44v6 expression in head and neck epithelia. *PLoS ONE* 2008; **3**: e3360.
27. Duester G. Retinoic acid synthesis and signaling during early organogenesis. *Cell* 2008; **134**: 921–31.
28. Duong V, Rochette-Egly C. The molecular physiology of nuclear retinoic acid receptors. From health to disease. *Biochim Biophys Acta* 2011; **1812**: 1023–31.
29. Ginestier C, Hur MH, Charafe-Jauffret E, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* 2007; **1**: 555–67.
30. 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**: 684–9.
31. Deng S, Yang X, Lassus H, et al. Distinct expression levels and patterns of stem cell marker, aldehyde dehydrogenase isoform 1 (ALDH1), in human epithelial cancers. *PLoS ONE* 2010; **5**: e10277.
32. Takes RP, Rinaldo A, Silver CE, et al. Future of the TNM classification and staging system in head and neck cancer. *Head Neck* 2010; **32**: 1693–711.
33. Bryne M, Koppang H, Lilleng R, Kjaerheim A. Malignancy grading of the deep invasive margins of oral squamous cell carcinomas has high prognostic value. *J Pathol* 1992; **166**: 375–81.
34. Oliveira DT, Odell EW. Expression of CD44 variant exons by normal oral epithelia. *Oral Oncol* 1997; **33**: 260–2.
35. Oliveira DT, Sherriff M, Odell EW. Expression of CD44 variant exons by primary and metastatic oral squamous cell carcinomas. *J Oral Pathol Med* 1998; **27**: 303–7.
36. Visus C, Ito D, Amoscato A, Maciejewska-Franczak M, Abdelsalem A, Dhir R. 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**: 10538–45.
37. Joshua B, Kaplan MJ, Doweck I, et al. Frequency of cells expressing CD44, a head and neck cancer stem cell marker: correlation with tumor aggressiveness. *Head Neck* 2012; **34**: 42–9.
38. Warnakulasuriya S. Global epidemiology of oral and oropharyngeal cancer. *Oral Oncol* 2009; **45**: 309–16.
39. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69–90.
40. Slaughter DP, Southwick HW, Smejkal W. "Field cancerization" in oral stratified squamous epithelium: clinical implications of multicentric origin. *Cancer* 1953; **6**: 963–8.
41. Califano J, Van der Riet P, Westra W, et al. Genetic progression model for head and neck cancer: implications for field cancerization. *Cancer Res* 1996; **56**: 2488–92.
42. Bloor BK, Rajarajan A, Jaafary-Haghighat K, Odell EW. Transcription and expression of CD44 variant exons by oropharyngeal squamous cell carcinomas. *Int J Oncol* 2002; **21**: 907–13.
43. Bloor BK, Jelvagharan M, White KN, Odell EW. Characterization of CD44 splicing patterns in normal keratinocytes, dysplastic and squamous carcinoma cell lines. *Int J Oncol* 2001; **18**: 1053–9.
44. Carinci F, Stabellini G, Calvitti M, et al. CD44 as prognostic factor in oral and ororpharyngeal squamous cell carcinoma. *J Craniofac Surg* 2002; **13**: 85–9.
45. Gonzalez-Moles MA, Bravo M, Ruiz-Avila I, et al. Adhesion molecule CD44 as a prognostic factor in tongue cancer. *Anticancer Res* 2003; **23**: 5197–202.
46. Sato S, Miyauchi M, Takekoshi T, et al. Reduced expression of CD44 variant 9 is related to lymph node metastasis and poor survival in squamous cell carcinoma of tongue. *Oral Oncol* 2000; **36**: 545–9.
47. Stoll C, Baretton G, Soost F, Terpe HJ, Domide P, Löhrs U. Prognostic importance of the expression of CD44 splice variants in oral squamous cell carcinomas. *Oral Oncol* 1999; **35**: 484–9.
48. Wang SJ, Wong G, de Heer AM, Xia W, Bourguignon LY. CD44 variant isoforms in head and neck squamous cell carcinoma progression. *Laryngoscope* 2009; **119**: 1518–30.

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Conflict of interest

The authors have no conflicts of interest to disclose.