

Immunohistochemical Expression of CD₅₆ and ALDH₁ in Common Salivary Gland Tumors

Safoura Seifi¹, Maryam Seyedmajidi¹, Jahanshah Salehinejad², Hemmat Gholinia³,
*Fatemeh Aliakbarpour¹

Abstract

Introduction:

Natural killer (NK) cells, of which CD₅₆ is a specific marker, play an important role in host defense against tumors. Cancer stem cells, of which aldehyde dehydrogenase isoform 1 (ALDH₁) is an immunohistochemical marker, are a group of tumorigenic cells which are involved in migration and tumor recurrences. We aimed to evaluate the expression of ALDH₁ and CD₅₆ in common salivary gland tumors, as well as their relationship with each other and with a number of clinicopathologic factors.

Materials and Methods:

Forty-five paraffin blocks of salivary gland tumors (pleomorphic adenoma, mucoepidermoid carcinoma and adenoid cystic carcinoma, 15 samples each) were selected. Malignant tumors were classified into two groups: low-grade (including mucoepidermoid carcinoma grade I) and high-grade (including mucoepidermoid carcinoma grade III and adenoid cystic carcinoma). Immunohistochemical staining for ALDH₁ and CD₅₆ markers was performed. Data were analyzed using SPSS (20) and the Chi-square test.

Results:

CD₅₆ expression was significantly higher in benign and high-grade malignant tumors (P=0.01). ALDH₁ overexpressed in all three salivary tumors, but not to statistically significant degree (P=0.54). There was no statistically significant correlation between ALDH₁ and CD₅₆ expression with demographic factors (age, gender, or location of tumor; P>0.05).

Conclusion:

It appears that the number of NK cells and their function change in different types of salivary gland tumors (benign/malignant) and stroma. NK cells are important components of the anti-tumor system; therefore immune dysfunction is associated with tumor progression in tumors of the salivary gland. ALDH₁ overexpression suggests its role in tumorigenesis, but ALDH₁ is not involved in the morphogenesis of salivary gland tumors.

Keywords:

Adenoid cystic carcinoma, ALDH₁, CD₅₆, Immunohistochemistry, Mucoepidermoid carcinoma, Pleomorphic adenoma.

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¹Department of Oral and Maxillofacial Pathology, School of Dentistry, Babol University of Medical Sciences, Babol, Iran.

²Dental Research Centre, School of Dentistry, Mashhad University of Medical Sciences, Mashhad, Iran.

³Health Research Institute, Babol University of Medical Science, Mazandaran, Iran.

*Corresponding Author:

Department of Oral and Maxillofacial Pathology, Faculty of Dentistry, Babol University of Medical Sciences, Babol, Iran. Tel: +98-11-3229-1408 E-mail: f_aliakbarpour@yahoo.com

Introduction

Salivary gland tumors are neoplasms with low prevalence and heterogeneous morphology in the jaw and oral region, and account for 3% of head and neck tumors. Pleomorphic adenoma is the most common salivary gland tumor, with 90% of cases occurring in the parotid. Mucoepidermoid carcinoma and adenoid cystic carcinoma are the most common malignant salivary gland tumors, and both show aggressive behavior and a tendency to metastasize. Due to differences in grades of mucoepidermoid carcinoma (low, intermediate, and high grade) and different histopathologic types of adenoid cystic carcinoma (including solid, cribriform, and tubular patterns), these tumors demonstrate varying biological behaviors (1,2).

Natural killer (NK) cells, part of the innate immune system, play important roles in the host defense against tumors. NK cells control different kinds of tumors by restricting their progress and inhibiting infiltration. This function is achieved by direct cell cytotoxicity without previous sensitivity and immune cytokine secretion such as IFN γ (3,4). NK cells demonstrate CD₅₆ and CD₄₅ adhesion markers, along with the absence of T-cell receptor (TCR) CD₃₃. These cells originate from the CD₃₄⁺ progenitor cells in the bone marrow, and migrate to the lymphoid tissues and peripheral blood for differentiation. Interleukin (IL)-15 is necessary for the development of these cells, while other factors such as TGF- β 1 and indoleamine 2,3-dioxygenase 1 (IDO₁) inhibit their function (5). CD₅₆⁺ NK cells, which constitute the majority of circulating NK cells, are the most potent cytotoxic NK cells against tumor cells (3).

The hypothesis that cancer arises from stem cells was suggested about 150 years ago, but it is only recently, thanks to advances in stem cell biology, that the necessary experimental framework to test the hypothesis has emerged. In the cancer stem cell model, tumors are constructed by tissue stem cells as well as progenitor cells, which have self-renewal features. The empirical evidence for the cancer stem cell hypothesis was provided by Dick in human myeloid leukemia, showing that tumor cells are derived from a small population of cells. This was then extended to solid tumors by Clark and colleagues (6,7). To identify

cancer stem cells, different markers such as CD₃₄, CD₁₃₃, CD₂₄, aldehyde dehydrogenase 1 (ALDH₁), and platelet endothelial cell adhesion molecule (PECAM) are used (8).

ALDH₁ is a detoxification polymorphic enzyme, which is responsible for the intracellular oxidation of aldehydes. ALDH₁ has an important role in the primary differentiation of normal and malignant stem cells through the conversion of retinol to retinoic acid. This enzyme is present in the liver and is metabolized by striated and cardiac muscle. High expression of ALDH₁ has been reported in normal and malignant stem cells (hematopoietic and central nervous system) and also lesions such as multiple myeloma, leukemia, breast carcinoma, and pancreatic adenocarcinoma. Cancer stem cells have features such as asymmetrical self-renewal, expression of active telomerase, increased permeability of the membrane, the ability to migrate and metastasize, and anti-apoptotic activity (9).

Because of the paradoxical and conflicting results of ALDH1 and CD56 expression in different neoplasms and the lack of an accurate assessment of them in salivary gland tumors, we aimed to assess the ALDH1 and CD56 expression in common benign and malignant salivary gland tumors, as well as their relation with each other and with a number of clinicopathologic factors.

Materials and Methods

In this cross-sectional descriptive-analytic study, 45 paraffin blocks (15 benign pleomorphic adenoma, 15 mucoepidermoid carcinoma, and 15 malignant adenoid cystic carcinoma) from Babol and Mashhad School of Dentistry archive files were selected. Clinical information, including age, gender, and the place of lesion were recorded. To confirm the histopathologic diagnosis and selection of appropriate block, as well as the grade of mucoepidermoid carcinoma differentiation and histopathologic patterns of adenoid cystic carcinoma, paraffin blocks were cut into 5-micron sections for hematoxylin-eosin staining.

The grade of differentiation of mucoepidermoid carcinoma samples was determined on the basis of Brandwein criteria (10). Then, malignant neoplasms were divided into two groups based on their biologic behavior: a low-grade malignant group consisting of mucoepidermoid carcinoma grade I, and a high-grade malignant

group consisting of mucoepidermoid carcinoma grade III and adenoid cystic carcinoma (11). Adenoid cystic carcinoma samples were classified into three groups: solid, tubular, and cribriform, in accordance with the system devised by Neville and colleagues (2).

For immunohistochemical staining of paraffin blocks, 4-micron slices were prepared, and then deparaffinized in xylene and dehydrated in different degrees of alcohol. We used hydrogen peroxide 0.03%, and phosphate-buffered saline (PBS) for washing. Because of antigen retrieval, slides were placed in the microwave (Panasonic 1280 Watt) for 30 min at 120°C and a pressure of 2 atm, then placed at room temperature for 20 min. After washing in PBS, we used superbloc solution for 30 min, and the slides were then washed with PBS.

Primary antibodies, anti-CD56 (Biocare-USA) and anti-ALDH1A1 (Biocare-USA) were applied at room temperature for 1 hour, and washed again with PBS. Next, we use polyvalent solution for 10 min, and then washed with PBS. Finally, horseradish peroxidase (HRP) (ScyTek-USA) was used and again washed with PBS, then 3, 3'-diaminobenzidine (DAB) chromogenic (ScyTek-USA) was added for 15 min.

The positive control for evaluation of ALDH1 and CD56 expression was breast ductal carcinoma and oral normal mucosa around the hyperkeratosis area, respectively. Furthermore, rat non-immunized serum was utilized as the primary antibody for the negative control. Brown staining with ALDH1 and CD56 in cytoplasm of tumor cells was considered positive.

To assess NK cell presence in tumors and CD56 expression, the percentage of cells that

stained in five microscopic fields with the greatest number of NK cells (hot spot) in $\times 40$ magnification were counted. If more than 10% of cells were stained with the CD56 marker, they were considered positive (12).

In terms of assessment of the ALDH1 expression, the percentage of stained cells as well as the intensity of cytoplasmic staining were considered. Intensity of the tumor cells stained with ALDH1 was recorded in four different degrees (I), consisting of: lack of staining, weak, moderate, and intense staining (0-3). Percentage of tumor cell staining (p) in five microscopic fields with $\times 40$ magnification or the highest number of tumor cells was also evaluated. Degree of staining percentage was based on: 0 (if there was no staining of tumor cells), 1 (staining in $\leq 1\%$ of cells), 2 (staining in 2–10% of cells), 3 (staining in 11–33% of cells), 4 (staining in 34–66% of cells), 5 (staining in $> 67\%$ of cells). The total score was calculated based on the sum of I and P. If the total score was 1 or 2, then ALDH1 was considered negative, while a total score of 3–8 was considered to show positive ALDH1 (13).

Data were analyzed using statistical software SPSS (20) and a Chi-square test (χ^2). $P < 0.05$ was considered statistically significant.

Results

The study included 45 blocks of paraffin, consisting of salivary pleomorphic adenoma (15 cases), mucoepidermoid carcinoma (15 cases) and adenoid cystic carcinoma (15 cases). The clinical characteristics of the patients (age, gender, and location of the lesion) is summarized in Table 1.

Table 1: Average age, gender and location of lesions in common benign and malignant salivary gland tumors

Salivary gland tumor	Number	gender		Age mean	Location of tumor	
		Female	Male		Minor salivary gland	Major salivary gland
Pleomorphic adenoma	15	10	5	44.2 \pm 9.51	8	7
Low-grade malignant tumors (Mucoepidermoid carcinoma grade I)	8	5	3	43.12 \pm 13.84	1	7
high-grade malignant tumors (Mucoepidermoid carcinoma grade III)	7	4	3	55.71 \pm 13.01	1	6
Adenoid cystic carcinoma	15	7	8	48.26 \pm 14.39	4	11

H&E staining:

From 15 cases of mucoepidermoid carcinoma, eight were of grade I and 7 were of grade III. From 15 cases of adenoid cystic

carcinoma, based on their histopathologic pattern, three were cribriform, seven were tubular, and five had a solid pattern.

Immunohistochemical:

Immunohistochemical expression of CD₅₆ and ALDH₁ markers in benign and malignant low-grade and high-grade salivary gland neoplasms are shown in Tables 2 and 3, respectively (Fig.1 to Fig.6).

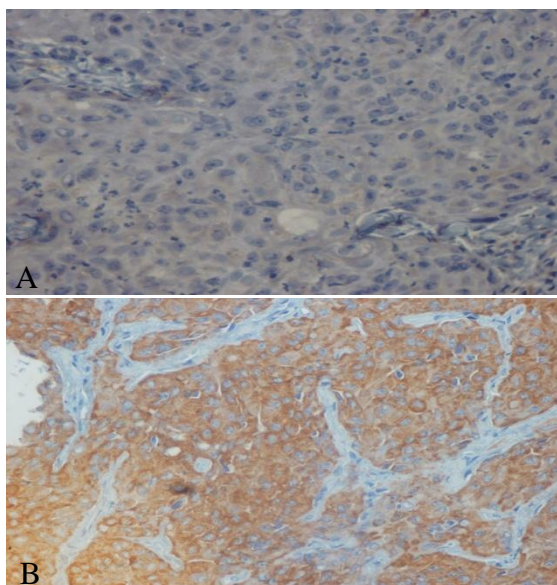


Fig1: Immunostaining with CD₅₆ in mucoepidermoid carcinoma (×40). A: negative staining of tumor cells in grade I. B: positive staining of tumor cells in grade III

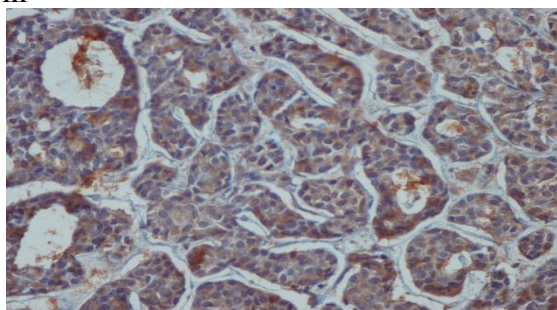


Fig 2: Positive immunohistochemical staining of tumor cells in solid adenoid cystic carcinoma with CD₅₆ (×40).

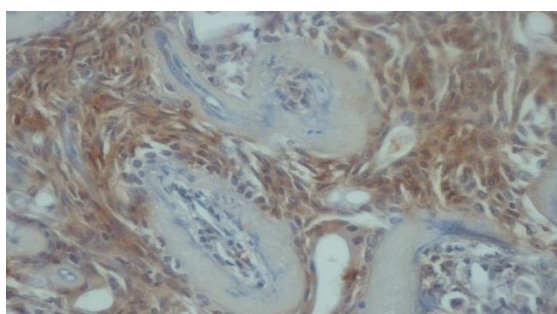


Fig 3: Positive immunohistochemical staining of tumor cells in pleomorphic adenoma with CD₅₆ (×40)

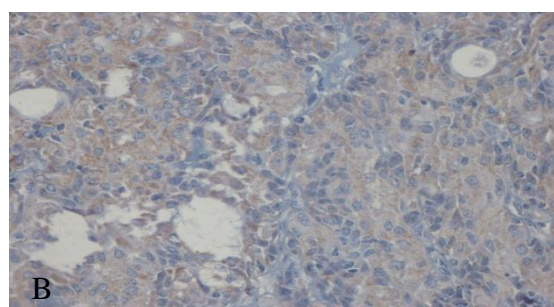
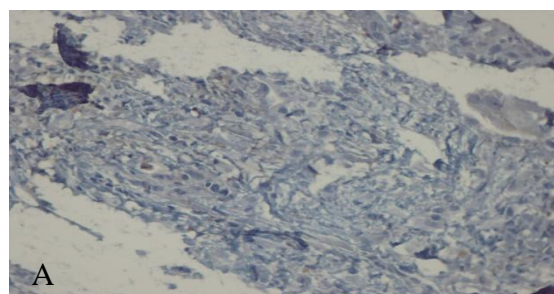


Fig4: Immunostaining with ALDH₁ in mucoepidermoid carcinoma (×40). A: negative staining of tumor cells in mucoepidermoid carcinoma grade III. B: positive staining of tumor cells in mucoepidermoid carcinoma grade I.

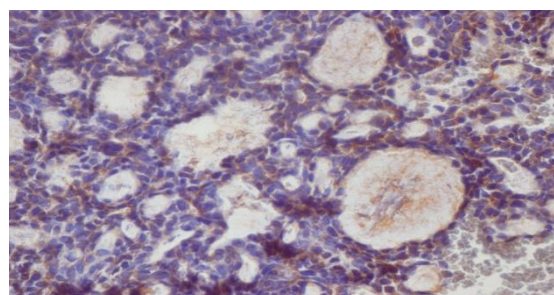


Fig 5: Positive immunohistochemical staining of tumor cells in Cribriform adenoid cystic carcinoma with ALDH₁ (×40)

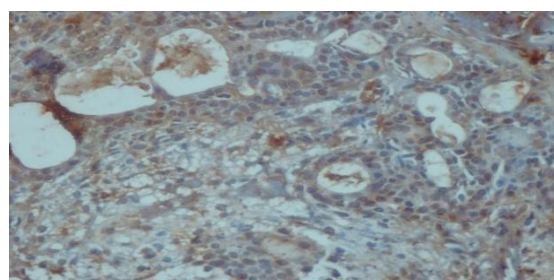


Fig 6: positive immunohistochemical staining of tumor cells in pleomorphic adenoma with ALDH₁ (×40)

According to Table 2, the differences in CD₅₆ expression between benign and low-grade and high-grade malignant tumors were statistically significant ($P=0.01$). CD₅₆ expression was higher in benign and high-grade malignant tumors. Also

according to Table 2, no statistically significant difference in CD₅₆ expression was observed between the mucoepidermoid carcinoma grade I and III (P=0.26). In addition, no statistically significant difference in terms of CD₅₆ expression was observed between different histologic patterns of adenoid cystic carcinoma (cribriform, tubular, or solid patterns) (P=0.58). Finally, CD₅₆ expression in salivary gland tumors showed no statistically significant relationship with location (major or minor salivary gland) (P=0.16), age (P=0.29), or gender (P=0.30).

According to Table 3, the difference between expression of ALDH₁ in benign and low-grade and high-grade malignant salivary gland tumor was not statistically significant (P=0.54). Also according to Table 3, no statistically significant differences in expression of ALDH1 between mucoepidermoid carcinoma grade I and grade III was observed (P=0.43). In addition, there was no statistically significant difference in expression of ALDH1 among the different histologic patterns of adenoid cystic carcinoma

(cribriform, tubular, or solid pattern) (P=0.54). Finally, ALDH₁ expression in salivary gland tumors showed no statistically significant relationship with location (major or minor salivary gland) (P=0.20), age (P=0.58), or gender (P=0.27).

Table 2: Immunohistochemical expression of CD₅₆ and ALDH₁ in benign and malignant Low-grade and high-grade salivary gland neoplasms

Tumor types	56 expression CD	
	Negative	Positive
Pleomorphic adenoma	6 cases (40%)	9cases (60%)
Low-grade malignant tumors (Mucoepidermoid carcinoma grade I)	8 cases (100%)	0 case (0%)
high-grade malignant tumors Mucoepidermoid carcinoma grade III	6 cases (27.3%)	1 case (4.5%)
Adenoid cystic carcinoma	3 cases (13.6%)	12 cases (54.5%)

Table 3: Immunohistochemical expression of ALDH₁ in benign and Low-grade and high-grade malignant salivary gland tumors

Tumor types	Frequency based on the percentage of stained cells (P)						frequency based on intensity of staining(I)				Total score(P+I)	
	p=0	p=1	p=2	p=3	p=4	p=5	No staining (I=0)	weak (I=1)	moderate (I=2)	intense (I=3)	Negative (0-2)	Positive (3-8)
Pleomorphic adenoma	1	3	1	2	6	2	1	6	8	0	4cases (26.7%)	11cases (73.3%)
Low-grade malignant tumors (Mucoepidermoid carcinoma grade I)	1	0	4	1	1	1	1	7	0	0	1 case (12.5%)	7 cases (87.5%)
high-grade malignant tumors Mucoepidermoid carcinoma grade III	2	0	3	0	2	0	2	4	1	0	2 cases (9%)	0 case (22.7%)
Adenoid cystic carcinoma	0	1	1	5	6	2	0	4	10	1	1 case (4.5%)	14 case (63.6%)

Also in this study, the relationship between positive expression of ALDH₁ and CD₅₆ with each other was investigated and it was found that there was no statistically significant association between these two markers in salivary gland tumors (P=0.13).

Discussion

The results of this study on salivary gland tumors indicate that expression of CD₅₆, as an

indicator of NK cells, is significantly higher in benign tumors and also high-grade malignant tumors. Based on these findings, it appears that a high density of NK cells in benign salivary pleomorphic adenoma represents the host immune system activity against cell damage. NK cells are the first line of the immune system response against harmful factors, but the number and function of NK cells and types (CD₅₆⁺ and CD₅₇⁺) varies

depending on the type of tumor and its stroma and grade in salivary gland tumors. Also according to the results of this study, it was observed that the number of CD₅₆⁺ NK cells declined in the early stages of malignant salivary gland tumors formation. In order to justify these findings, we can say that probably in the long-term vicinity of NK cells with tumor antigens, a kind of compatibility between immune cells and tumor stroma, and NK cell sensitivity to tumor cells has been lost. Also, secretion factors such as TGF- β 1, may cause a reduction in the number of CD₅₆⁺ NK cells and an increase in NK cell inactive receptors. On the other hand, a decrease in the number of CD₅₆⁺ NK cells may occur in parallel with an increase in other types of NK cell. However, with increasing tumor grade, malfunction of the normal NK cells occurs, meaning that the number of cytotoxic NK cells increases in the salivary gland tumors. Thus, an increase in cytotoxic NK cells is associated with tumor progression, high grade of malignancy, and metastasis in malignant salivary gland tumors.

Previous studies have reported controversial and conflicting results on the diagnostic and prognostic value of CD₅₆. So far, few studies have investigated the expression of CD₅₆ in salivary gland tumors. Nakatsuka and colleagues reported a case of invasive adenocarcinoma in the accessory parotid gland that showed positive expression of CD₅₆ in a scattered pattern, although they used CD₅₆ as a neuroendocrine marker (14). Weissferdt and Moran also reported five cases of rare salivary gland-type pulmonary tumors that showed characteristics of malignant mixed tumors, with neoplastic cells of these tumors showing positive staining for CD₅₆ (15). Also Dutsch and colleagues evaluated the immunohistochemical expression of CD₅₆ in parotid adenocarcinomas, and reported that 14% of the parotid adenocarcinomas were found to be CD₅₆ positive. These researchers assumed CD₅₆ as a marker of T lymphocytes that affect the performance of cytotoxic T cells (16). However, in the present study, approximately 43% of malignant tumors demonstrated positive expression for CD₅₆.

In a study on breast carcinoma, Wachter and colleagues reported that CD₅₆ expression was high in basal-like and luminal A-like breast

carcinomas (17). Also in the study by Kontogianni and colleagues on 20 samples of small cell lung carcinoma, they reported that CD₅₆ could be a valuable tool in the diagnosis of these cancers (18). Alegretti and colleagues reported that CD₅₆ expression in acute myeloid leukemia is related to poor prognosis and lower survival rate (19). In addition, Aloysius and colleagues demonstrated that in pancreatic cancer samples, CD₅₆ expression is associated with neural invasion and reduced survival (20), a finding that is consistent with the results of the present study. In a study on breast cancer, Mamessier and colleagues reported that aggressive types of disease have a reduced number of NK cell activating receptors such as CD₅₆. These researchers suggested that factors such as TGF- β 1 could play an important role in the reduced function of NK cells in tumors (21), contradicting the results of the present study.

In the present study, overexpression of ALDH₁, as one of the most commonly used markers to detect cancer stem cells, was observed in all groups of salivary gland tumors, showing its role in tumorogenesis of salivary gland neoplasms. Recent empirical evidence suggests the existence of a tumorigenic population of cancer cells that demonstrate stem cell-like properties such as self-renewal and multipotency. These cells are able to both initiate and maintain tumor formation and progression of human cancers, such as salivary gland tumors (22).

In the present study, ALDH₁ expression had no association with grade of mucoepidermoid carcinoma or histopathologic pattern of adenoid cystic carcinoma. This suggests that other factors and possibly other isoforms of ALDH, along with cancer stem cells, may play an important role in morphogenesis, cell differentiation, and development of salivary gland tumors.

In the study by Zhou and colleagues, ALDH₁ expression in salivary adenoid cystic carcinoma was investigated in three groups: stromal staining, both stromal and epithelial staining, and no stromal or epithelial staining. These researchers demonstrated that the pattern of ALDH₁ expression had no relation to histology, size, neural invasion, or survival of patients (23).

These findings are consistent with the results of the present study.

In a study by Fujita and Ikeda on salivary adenoid cystic carcinoma, it was suggested that the presence of small populations of cancer stem-like cells may play an important role in tumor morphogenesis through stimulating the extracellular matrix (24); in contrast with the results of the present study. A probable reason for this difference is that Fujita and Ikeda used CD₄₄ and CD₁₃₃ as the marker to identify cancer stem cells, while we used ALDH₁. Therefore, based on the present study, ALDH₁⁺ cancer stem cells appear to have no role in the morphogenesis of solid, tubular, and cribriform adenoid cystic carcinomas. However, perhaps the extracellular matrix, through CD₁₃₃⁺ and CD₄₄⁺ stem cells, plays an important role in regulating cell morphogenesis and various histologic patterns in adenoid cystic carcinoma.

Adams and colleagues demonstrated that salivary gland mucoepidermoid carcinomas contain a small population of cancer stem cells with enhanced tumorigenic potential and are characterized by high ALDH activity and CD₄₄ expression. These results suggest that patients with mucoepidermoid carcinoma might benefit from therapies that ablate these highly tumorigenic cells (25), a finding which is consistent with the results of the present study. In a study on an isolated side population from the salivary gland mucoepidermoid carcinoma cell line MC3, Zhang and colleagues observed that sphere-forming assays could enrich stem cell-like cells, exhibiting high cloning efficiency and possessing strong tumorigenic ability (26), supporting the results of the present study.

In our study, we anticipate that pleomorphic adenomas that show overexpression of ALDH₁ are more susceptible to malignant transformation. Indeed, Huang and colleagues demonstrated that ALDH₁ overexpression in tongue squamous cell carcinoma was associated with metastasis and aggressive behavior (27).

Liu and colleagues investigated the role of ALDH₁ in ovarian cancer and reported that overexpression of ALDH₁ was associated with poor prognosis (28). Qiu and colleagues discussed five isoforms of 19 family members of ALDH in breast cancer and reported that all five isoforms can be found in different amounts in the tumoral tissues, but only the ALDH₁ isoform is significantly associated with distant metastasis

and patient survival. Thus only this isoform can be used as a poor prognostic indicator (29). In the present study, benign and malignant salivary gland tumors showed no statistically significant difference between expressions of ALDH₁. In contrast, Schwartz and colleagues reported that benign breast tumors showed higher ALDH₁ expression compared with malignant breast tumors (30).

Based on various studies, it appears that the ALDH family has 19 isoforms that are expressed to a greater extent in the cancer of specific areas of the body; for example ALDH_{1A3} isoform in breast cancer, ALDH_{7A1} isoform in prostate cancer, and ALDH_{1A1} in pancreatic cancer (31). Therefore, further studies are needed to elucidate the role of other possible ALDH isoforms in salivary gland tumors to complete the results of the present study.

In conclusion, it seems that cancer stem cells, based on the type of tumor and its stroma, may express several markers, such as those seen in lung cancer, with adenocarcinomas showing CD₁₃₃ overexpression and squamous cell carcinomas showing ALDH₁ overexpression (32). No significant relationship between expression of CD₅₆ and ALDH₁ markers with gender, age of patient, or location of tumor were found. An increased number of specimens may allow us to offer more accurate results.

Conclusion

NK cells, which show high expression in benign and high-grade malignant salivary gland tumors, are important components of the anti-tumor immune response. Thus dysfunction of these cells may lead to tumor progression.

In the present study, overexpression of ALDH₁, one of the most commonly used markers for the detection of cancer stem cells, was observed in all salivary gland tumors, which indicates the role of these cells in tumorigenesis of salivary gland neoplasms. On the other hand, a lack of correlation between ALDH₁ expression and grade of differentiation or histopathologic pattern in malignant salivary gland tumors indicates that cancer stem cells might not have a role in the morphogenesis of salivary gland tumors, or that other isoforms of ALDH or other markers of cancer stem cells are effective in this regard. The results of the present study could be useful in the improvement of molecular-targeted therapies in salivary gland tumors.

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