

ORIGINAL PAPER

CANCER STEM CELL MARKERS IN INTRAORAL MINOR SALIVARY GLAND TUMORS

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Emerging evidence suggests the existence of a tumorigenic population of cancer cells that demonstrate stem cell-like properties. Cancer stem cells have been associated with tumor initiation and progression. The purpose of this study is to evaluate whether cancer stem cells play a functional role in the tumorigenesis of salivary gland tumors.

24 malignant, 24 benign salivary gland tumors, and seven normal salivary gland tissues were immunohistochemically stained for cancer stem cell markers ALDH1, CD44, CD24, and CD166. We scored the expressions of these proteins based on staining intensity and the ratio of positive cells.

ALDH1 expression was down-regulated in malignant tumors ($p = 0.034$), while CD166 expression was upregulated ($p = 0.002$). CD44 and CD24 showed decreased expression in malignant tumors. Downregulation of ALDH expression by age also showed statistical significance ($p = 0.007$).

ALDH1, CD166, CD44, and CD24 are potential stem cell markers for salivary gland tumors. Particularly CD166 and ALDH1 have a role in the pathogenesis and prognosis of these tumors. Loss of ALDH1 by aging may play an essential biological role in malignancy. The potential diagnostic role of ALDH1 and CD44 should be investigated.

Key words: minor salivary gland tumors, cancer stem cell, ALDH1, CD44, CD24, CD166.

Introduction

Recent 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, termed cancer stem cells (CSC), can initiate and maintain tumor formation and progression. They are also reported to be resistant to treatment in many tumor types [1, 2].

Different markers are used to identify cancer stem cells. ALDH1, CD44, CD24, and CD166 are the most studied CSC markers used in adenoid tis-

sues such as SG and breast [2, 3]. CD44 is an essential receptor for hyaluronic acid. The functions of this transmembrane receptor include coordination of cell motility, cell-cell adhesion, lymphocyte activation, cell migration, and cellular-extracellular matrix interaction [4, 5]. CD24 is a mucin-like adhesion molecule expressed by neutrophils, pre-lymphocytes, and many solid tumors. Functionally, it is identified as an alternate ligand for P-selectin, an adhesion receptor on platelets and endothelial cells. Their interaction facilitates the passage of tumor cells in the bloodstream during metastasis. This metastatic

association of CD24 suggests its prognostic value as a CSC marker [6]. Besides the interaction with hyaluronic acid, CD44 protein has also been shown to interact with other proteins in the extracellular matrix, including fibronectin, collagen types I and IV, serglycin, and osteopontin.

ALDHs are a family of enzymes involved in maintaining cellular homeostasis by metabolizing both endogenous and exogenous reactive compounds. ALDH plays a vital role in protecting against various environmental stressors such as dehydration and ultraviolet radiation. Mutations in multiple ALDHs are associated with numerous pathological conditions in humans, highlighting the fundamental importance of these enzymes in physiological and pathological processes, including cancer [7, 8].

CD166 is a transmembrane glycoprotein of the immunoglobulin superfamily of adhesion molecules and transduces signals to the intracellular signaling pathway. It mediates heterophilic and hemophilic cell-cell interactions. It also regulates N-cadherin [9, 10]. All these biological properties are essential to the physiological activities of normal cells, but they are also associated with the pathologic activities of cancer cells [11].

The concept of CSC has been studied in several human cancers, including breast, prostate, lung, pancreas, colon cancers, melanoma, and leukemia [2]. However, there is limited research on salivary gland tumors. Salivary glands are composed of various cell types, which may give origin to different tumor types. The diversity of SGTs, rarity, and varied morphologic aspects often make the diagnosis challenging [12]. Knowledge of CSC in SGT may contribute to the understanding of the pathobiology of these tumors. It can provide molecular markers to designate prognosis as well as an alternative therapeutic approach.

This study aimed to investigate the presence of CSCs in minor SGTs. We also aimed to examine the correlation of CSC with the clinicopathologic features of these tumors.

Material and methods

Tissue samples

We conducted a retrospective study on 55 cases. Twenty-four malignant tumors (8 adenoid cystic carcinomas [ACCs], 6 mucoepidermoid carcinomas [MECs], 2 acinic cell carcinomas [AcCCs], 2 carcinoma ex pleomorphic adenomas [ExPAs], and 6 polymorphous adenocarcinomas [PACs]), 24 benign tumors (21 pleomorphic adenomas [PAs], three basal cell adenomas [BAs]) and seven normal salivary gland tissues (NSGTs) obtained from the archive of the oral pathology department were included in the study. The NSGTs were isolated from intact salivary gland tissues of mucocele biopsies. We analyzed

medical files. We recorded age, gender, tumor evolution, histological classification, tumor recurrences, and metastasis. The age of 60 years was determined as the reference age [13]. We revised HE stained slides from 55 cases to confirm the histopathological diagnosis and tumor grade.

Immunohistochemical staining

All 55 samples were fixed in 4% neutral formalin and embedded in paraffin. Deparaffinized and dehydrated 4 μ m thick paraffin sections were processed in a microwave at low, medium, and high degrees for a total of 15 min in 1X Tris EDTA solution to reveal the masked antigenic structures in the tissue. After cooling and washing with PBS (phosphate buffer solution, pH: 7.60), the sections were immersed in 4% hydrogen peroxide (H_2O_2) for ten min to block endogenous peroxidase activity. We performed immunohistochemical staining using Ultra Vision Large Volume Detection System Anti-Polyvalent, HRP (Lab Vision Corporation, USA). Non-immune blocking serum was applied for 5-10 min. The slides were incubated in primer antibodies ALDH1, CD44, CD24, and CD166 for 1 hour, then set in secondary antibody for 15 min and washed with PBS again. The reaction was developed with DAB (3,3-diaminobenzidine) chromogen. Sections were counterstained with Mayer's haematoxylin, and dehydrated in a graded series of alcohols.

Immunohistochemical assessment

Protein immunoexpression was analyzed semi-quantitatively and quantitatively for positive cell ratio, staining intensity, and cellular compartment. Stained cell percent was estimated using the percentage of the cells stained in 4 high magnification fields to total cell count for CD44, CD24, CD166, and we used the scoring system described previously in the literature [9, 14, 15]. Immunohistochemical staining of ALDH1 was classified as positive when more than 1% of tumor cells showed evident cytoplasmic positivity [16]. Assessment criteria are given in Table I.

Statistical analysis

We analyzed data using the Mann-Whitney U test, Kruskal-Wallis test, and Spearman's rho correlation analysis using the IBM SPSS Statistics 23 package program. A p of ≤ 0.05 was considered to indicate statistical significance.

Results

All specimens were from minor SGs. Four recurrent cases (2 PAC, 1 MEC, 1 ACC) and two loco-regional metastases (1 MEC, 1 ACC) were recorded. Table II shows the clinical features of the cases.

Table I. Properties of primer antibodies and immunohistochemical assessment criteria

Parameter	ALDH1	CD44	CD24	CD166
	Mouse monoclonal antibody, Novocastra Lyophilized	Mouse monoclonal antibody, Novocastra Lyophilized	Mouse monoclonal antibody, Novocastra Lyophilized	Mouse monoclonal antibody, Novocastra Lyophilized
Dilution	1: 100	1: 100	1: 150	1: 100
Control	Lung ca	Tonsil	Tonsil	Skin
Localization	Cytoplasm	Membrane	Membrane, cytoplasm, nucleus	Membrane, cytoplasm
Scoring	$\leq 1\%$ (+); positive (94)	0-10%(+) = 0; negative 10-25%(+) = 1; positive 25-50%(+) = 2; positive $\leq 50\%$ (+) = 3; positive	0-10%(+) = 0; negative 10-25%(+) = 1; negative 25-50%(+) = 2; positive $\leq 50\%$ (+) = 3; positive	0-10%(+) = 0; negative 10-25%(+) = 1; positive 25-50%(+) = 2; positive $\leq 50\%$ (+) = 3; positive

Table II. Clinical features

VARIABLE	N
Normal	7
Tumor	
Benign	Σ 24
PA	21
BCC	3
Malignant	Σ 24
MEC	6
PAC	6
ADCC	8
ACCC	2
Ca Ex-mix	2
Metastasis/Recurrence	
Yes	6
No	18
Grade	
High	8
Low	16
Age	
< 60	40
≥ 60	14

Expression of CSC markers

Normal salivary gland tissue expressed all four markers, but their staining ratio and intensity showed variations. Generally, ductal cells expressed CSC markers more widely. Serous acinar cells expressed

CD44 more extensively, while mucous acinar cells showed more extensive ALDH1 and CD166 expression. CD24 expression did not show any difference based on acinar cell type.

Table III gives the expression of CSC proteins in different tumor types.

In PAs, ductal and myoepithelial tumor cells showed similar expression for CSC markers (Fig. 1A-D). In BCAs, there was no ALDH or CD166 expression. Tumor cells showed similar expression levels of CD44 and CD24.

In ACCs, tumors that have cribriform patterns showed higher CSC protein expression than solid tumors. There was no ALDH1 expression in ACCs (Fig. 2A). Tumor cells showed similar expression for CD166, CD24 and CD44 (Fig. 2B-D).

In MECs, ALDH1 expression was higher in mucous cells than epidermoid and intermediate cells (Fig. 3A). There was no difference regarding CD166 and CD24 expression (Fig. 3B, 3C). CD44 showed higher expression in epidermoid cells (Fig. 3D).

In PACs, tumor cells showed similar expression for all the CSC markers (Fig. 4A, 4B). In AcCCs, tumor cells showed identical expression for ALDH, CD166, and CD24 (Fig. 4C). There was no CD44 expression in AcCC (Fig. 4D). In EX-MIX tumors, ductal and myoepithelial cells showed similar expression for CSC markers.

The lack of ALDH1 expression in adenoid cystic carcinomas ($p = 0.000$) and basal cell adenomas ($p = 0.026$) was statistically significant. The lack of CD166 expression in basal cell adenomas also showed statistical significance ($p = 0.039$).

Table IV presents an analysis of CSC marker expression based on tumor biology and age.

Table III. Expression of CSC proteins in different tumor types

TUMOR TYPE	N	ALDH		CD 166		CD44		CD24	
		N (+) %	SCORE	N (+) %	SCORE	N (+) %	SCORE	N (+) %	SCORE
MEC	6	100	2.16	100	1.33	50	1.33	50	1.33
PLGA	6	67	0.83	83	1.33	83	2.0	50	1.0
AdCC	8	0	0	75	1.0	50	1.25	50	1.25
AcCC	2	100	2.5	50	2.5	0	3.0	50	2.0
EXMIX	2	100	3.0	100	2.5	100	3.0	100	2.5
PA	21	90	1.7	52	1.7	90	2.35	81	1.95
BCA	3	0	0	0	0	33	1.33	100	2.66
NSG	7	100	2.57	100	1.42	100	2.71	57	1.57

Table IV. Analysis of CSC markers expression on the basis of tumor biology and age

	Σ N	ALDH		CD166		CD44		CD24		P
		N +	%	N +	%	N +	%	N +	%	
Tumor										
Normal	7	7	100	2	29	7	100	4	57	ALDH* p= 0.034
Benign	24	20	83	12	50	21	88	20	83	CD166* p= 0.002
Malignant	24	14	58*	21	88*	16	67	14	63	
Met/rec										
Yes	6	4	67	4	67	5	83	3	50	
No	18	10	56	17	94	11	61	7	39	
Grade										
High	8	3	38	6	75	4	50	5	62	
Low	16	11	69	15	94	12	75	9	56	
Age										
≤ 60	40	22	53	15	38	30	75	23	58	ALDH* p = 0.007
≥ 60	14	3	21*	5	36	7	50	8	57	

ALDH1 expression was highest in NSGs, followed in descending order by benign and malignant tumors. Malignant (MSGTs) had statistically significant down-regulated ALDH1 expression when compared with NSG tissues (p = 0.034). For CD166, the highest expression was by MSGTs, followed in descending order by benign SGTs (BSGTs) and NSGs. MSGTs had statistically significantly up-regulated CD166 expression when compared with NSGTs (p = 0.002). MSGT showed prominently decreased CD44 expression, and there was no significant difference regarding CD24 expression.

There was a prominent decrease in CD166 and a slight increase in CD44, CD24, and ALDH1 expression in metastasizing/recurrent tumors. We noted decreased ALDH1, CD166, and CD44 expression in high-grade tumors. The patients over 60 years old had lower ALDH1 expression (p = 0.007).

CD44/CD24 immunophenotypes did not show any significant difference regarding tumor biology.

Discussion

Researchers have studied various cell surface markers, including CD44, CD24, ALDH1, and CD166, to identify CSCs in adenoid tissues [4, 17]. CD44 is one of the most frequently studied CSC markers; however, the results show different correlations in each tissue analyzed [4, 5]. Certain studies have shown that CD44 plays a significant role in initiation, metastasis, and promoting tumorigenesis [5, 17], while other studies reported opposite results as its high expression was not related to carcinogenesis [3]. CD44 is expressed in both normal and cancer cells and has various functions [6]. It could promote apoptosis through the activation of caspase-3. It also

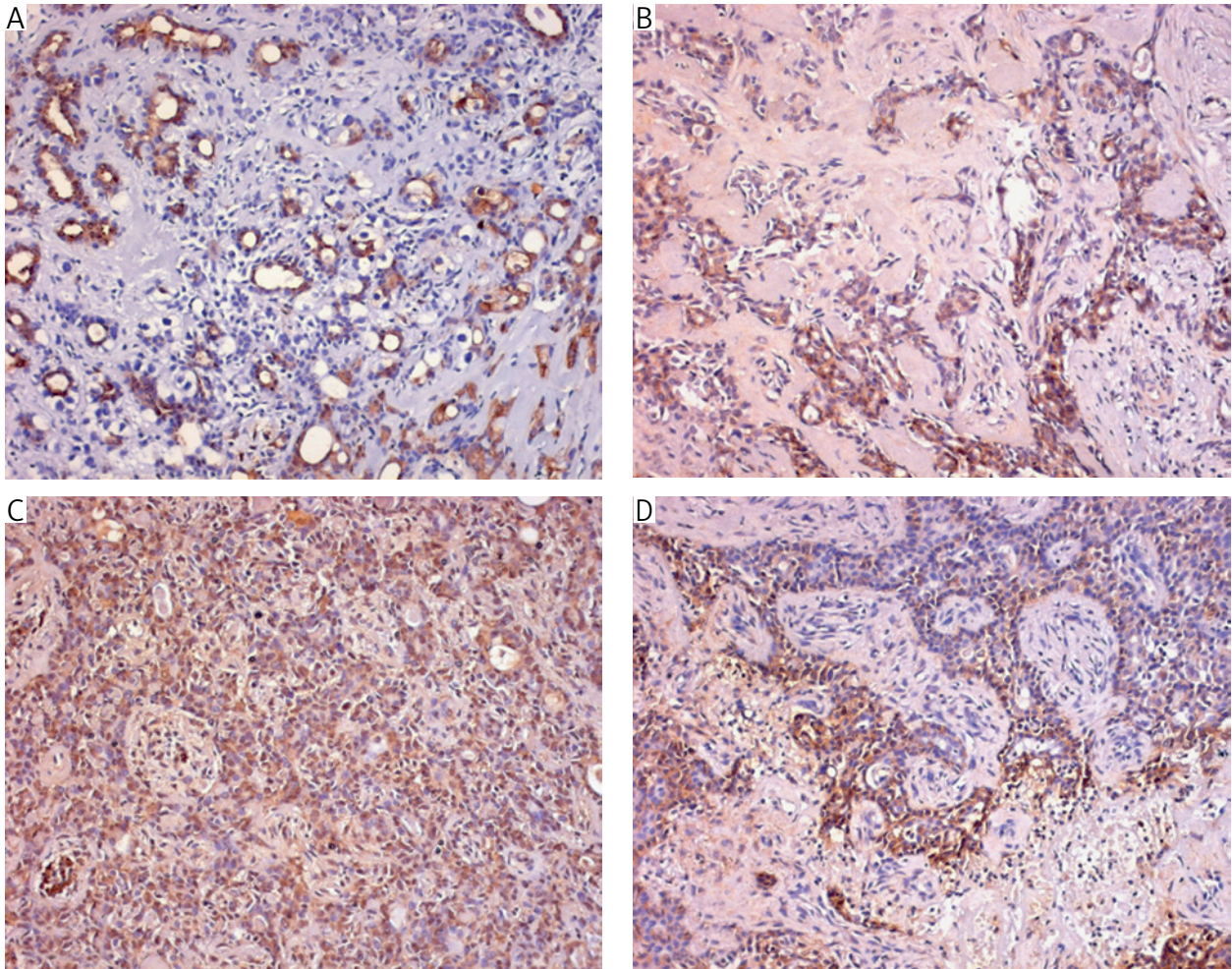


Fig. 1. A) ALDH positive ductal cells in PA (ABC $\times 20$). B) CD166 positive tubular structures in PA (ABC $\times 20$). C) CD24 positive solid areas in PA (ABC $\times 20$). D) CD44 positive solid areas in PA (ABC $\times 20$)

inhibits PI3K activation and AKT, thus inhibiting tumor initiation [3, 5, 6].

Furthermore, CD44 has been implicated in inhibiting angiogenesis, particularly by high molecular weight (HMW) hyaluronan engagement, which can inhibit the induction of the immediate-early genes *c-fos* and *c-jun* from suppressing migration of endothelial cells [3, 6]. As all these properties of CD44 are crucial in preventing carcinogenesis, the loss of CD44 may facilitate tumor initiation and progression. Consistent with this information, we detected a prominent decrease in CD44 expression in MSGTs compared to BSGTs and NSGs.

CD44 also initiates a string of events that facilitate adhesiveness, matrix degradation, proliferation, cell survival and motility, features that together may lead a tumor cell through a metastatic cascade [18]. In the present study, metastasizing tumors displayed higher CD44 expression in comparison to non-metastasizing tumors. So it is suggested that loss of CD44 may be a crucial step toward early SG malignancy. In

contrast, the increase of this molecule in the course of tumor progression may facilitate metastasis.

In the current study, all types of malignant tumors displayed variable CD44 expression except AcCC. Both acinic cell carcinoma cases were negative for CD44. AcCC shows prominent serous acinar differentiation. In normal SG tissues, serous acinar cells had expressed extensive CD44 protein. Lack of CD44 in these tumors could be associated with loss of differentiation of these cells; hence it may play a role in carcinogenesis of AcCC, and it might be a candidate for a diagnostic marker. Advanced research on large series should be carried out to elucidate this suggestion.

Different CD24 expression levels and functions have been observed in various cancer types. In a recent study conducted on breast, pancreas, and ovarian cancers, high expression of CD24 was associated with tumor progression and metastasis [6]. Similarly, Soave *et al.* [19] observed positive CD24 expression in 9 malignant salivary gland tumors correlated with

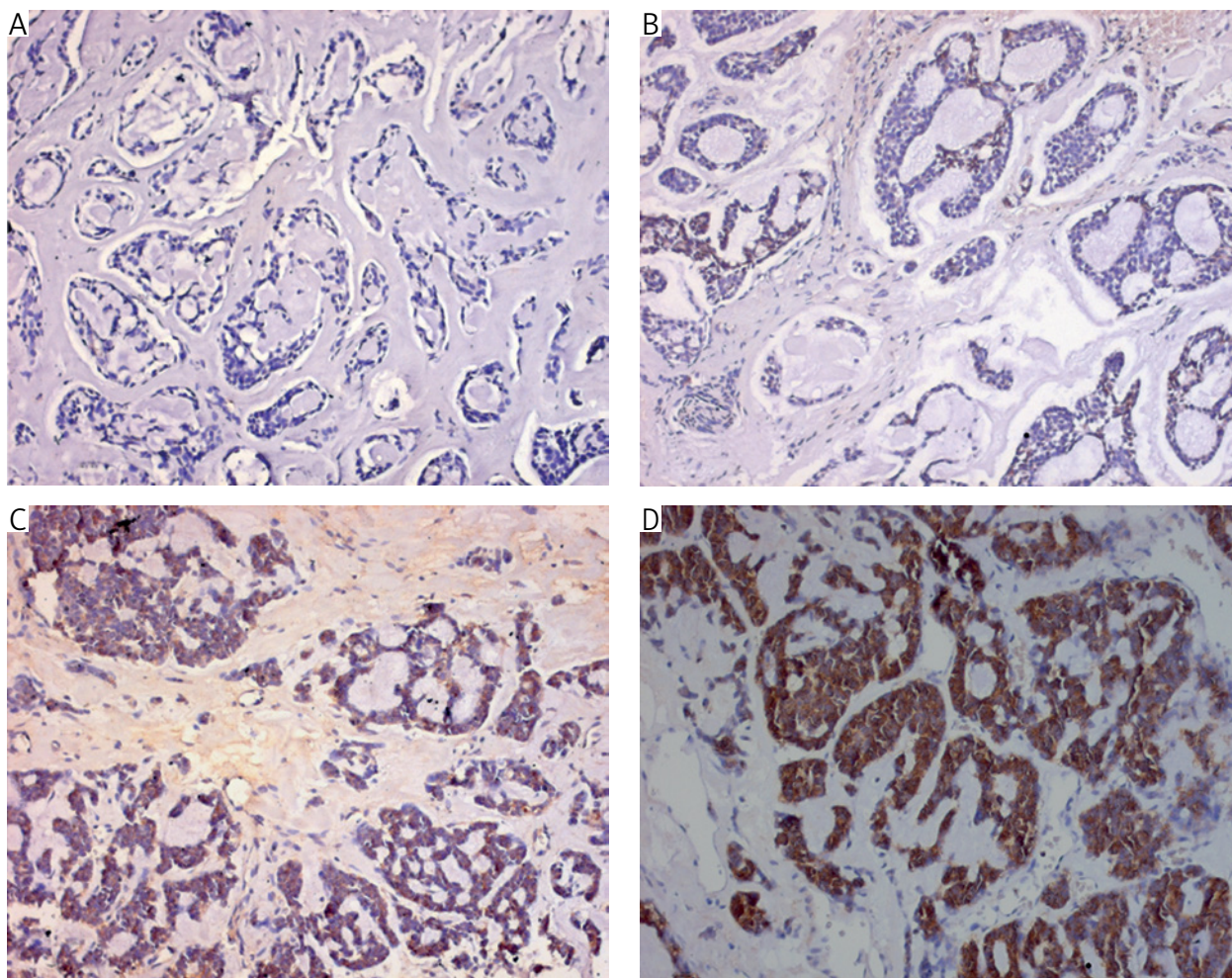


Fig. 2. A) AdCC cells were negative for ALDH (ABC $\times 20$). B) Scattered CD166 positive cells in AdCC (ABC $\times 20$). C) CD24 positive cells in AdCC (ABC $\times 20$). D) Strong CD44 expression in AdCC (ABC $\times 20$)

the clinical stage. On the other hand, Ma and colleagues [20] reported that metastatic ACC cell lines lack CD24 expression. The levels of CD24 expression may show significant variation between cell lines even in cells of the same cancer subtype. They affect different functions at different periods during tumor progression and metastasis. In the present study, there was no significant difference regarding CD24 expression between NSGs, BSGTs, or MSGTs, whereas metastasizing/recurrent tumors showed higher CD24 expression. So it is suggested that CD24 may have a role in tumor progression rather than initiation.

With the progress of studies in CSCs, researchers have been questioning the co-expression of CSCs in tumor initiation, invasion, and metastatic properties. However, co-expression of surface markers in CSCs is generally contentious in several cancer types. Every marker shows an independent expression level but they seem to coordinate with each other in developing tumors at different stages [19, 20] Saove *et al.* [19] presented evidence that the CD44/CD24

immunophenotypes could give prognostic information about MSGT. They reported that MSGTs with the CD44+/CD24+ profile might represent the most aggressive tumors and worst prognosis. In the present study, the CD44+/CD24+ immunophenotype was the most prevalent in MSGTs (55%) and BSGTs (70%). However, we did not find a correlation between any CSC immunophenotype and tumor grade or metastasis. Also, we did not detect any significant difference between benign and malignant SGTs. In the same study, Soave *et al.* [19] stated that all the immunophenotypes were more prevalent in major SGs except for CD44-/CD24- and suggested lower stem cell activity in minor SGTs. Our results oppose this controversial suggestion as all SGTs were from minor SGs.

Data about ALDH in salivary gland tumors are limited. Sreerama [21] documented an elevated level of ALDH in the Warthin tumor and mucoepidermoid carcinoma of the parotid gland. In the same study, they reported a lower level of ALDH in pleo-

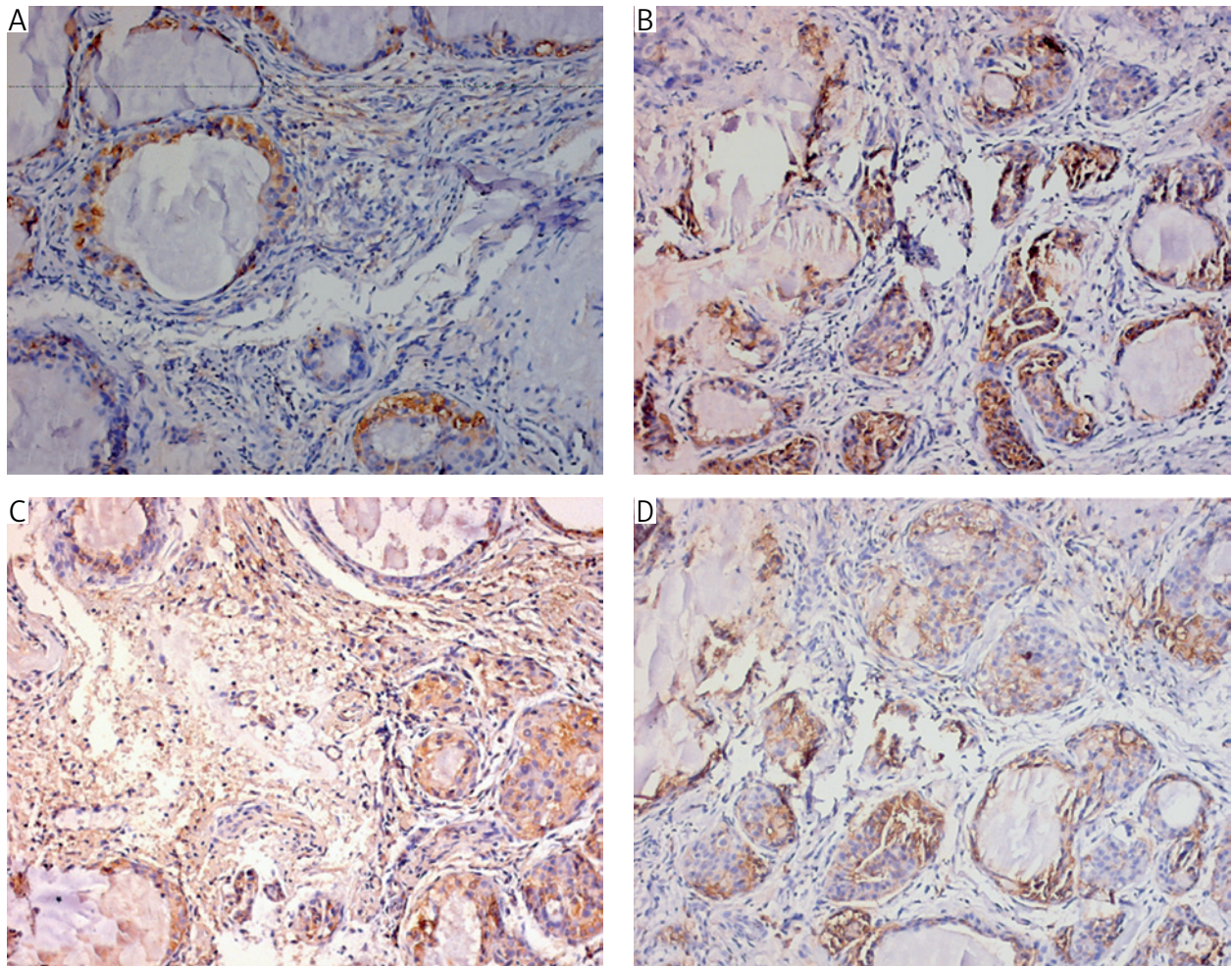


Fig. 3. A) ALDH1 positive cells in MEC (ABC $\times 20$). B) Strong CD166 positivity both in cystic and solid areas in MEC (ABC $\times 20$). C) CD24 positive cells in MEC (ABC $\times 20$). D) CD44 positive cells in cystic and solid areas in MEC (ABC $\times 20$)

morphic adenoma, undifferentiated carcinoma, and an adenoid cystic carcinoma of the parotid compared to normal salivary gland tissue.

In the present study, the highest expression of ALDH1 was in normal salivary gland tissues, followed by benign and malignant salivary tumors. MSGTs showed statistically significantly lower ALDH1 expression in comparison to NSGs ($p = 0.034$). Also, decreased ALDH1 expression was noted in high-grade tumors. These data suggested that loss of ALDH1 might be a step toward SG malignancy and may lead to more aggressive and higher-grade tumors. ALDH1 enzyme has a role in protecting against various environmental stressors such as dehydration and ultraviolet radiation. Furthermore, it is responsible for oxidation of the carcinogenic reactive form of aldehydes to their corresponding non-reactive state carboxylic acids [7, 22]. So the decreased protective and detoxifying effect of ALDH1 may induce carcinogenesis and hence tumor progression.

When we analyzed ALDH1 expression based on tumor types, we found a statistically significant differ-

ence in the expression of ALDH1 in ACC ($p = 0.000$). The same results were also noted for BCA ($p = 0.026$). Both tumors lack ALDH1. These data were interesting as both tumors have basal cell components. The lack of ALDH1 in ACC and BCA could be significant in the differential diagnosis of these tumors. Thus further studies should be carried out to elucidate the value of ALDH1 as a diagnostic marker. We also detected a significant loss of ALDH1 with age. Patients over 60 years old had lower ALDH1 expression ($p = 0.007$). The loss of ALDH1 with the normal process of aging may decrease its protective capacity against carcinogenic agents. Expectedly, together with the reduced ability of DNA repair genes, this will facilitate carcinogenesis.

Since its discovery, CD166 expression has been regarded as a cause of tumor progression and metastasis in a subset of tumors, such as breast cancer [11], head and neck cancer [22], cutaneous melanoma [23], and prostate carcinoma [24]. However, previous studies addressing the role of CD166 in cancer have yielded conflicting results [11]. Depending on the tumor

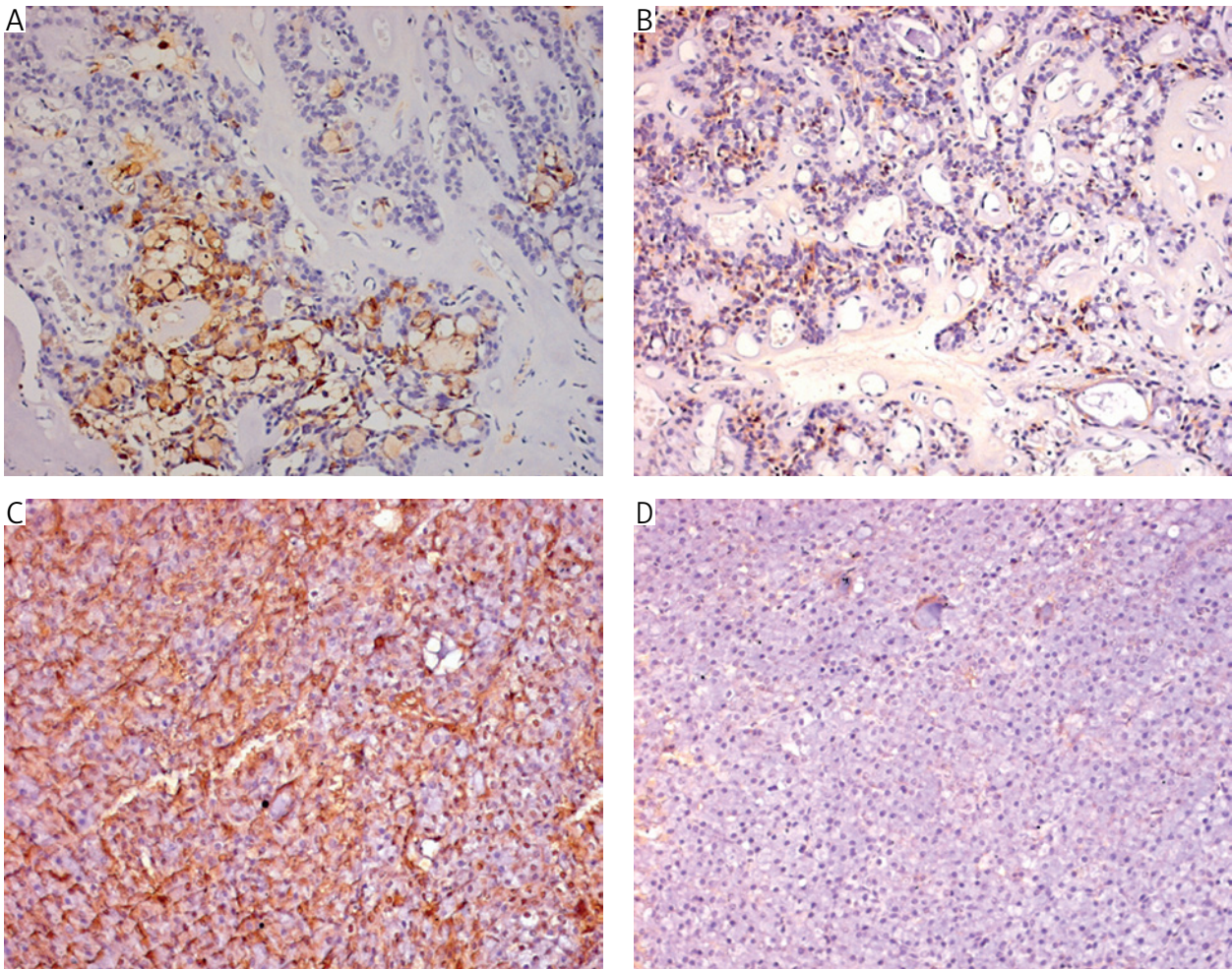


Fig. 4. A) ALDH positive cells in PLGA (ABC $\times 20$). B) CD166 positive cells in tubular areas of PLGA (ABC $\times 20$). C) diffuse CD24 positivity in AcCC (ABC $\times 20$); D) AcCC were negative for CD44 (ABC $\times 20$)

cell type, ALCAM expression has been positively and negatively correlated with cancer progression and metastasis [25]. There is only one published article concerning CD166 expression in salivary gland tumors [9]. That study found significantly higher CD166 expression in malignant salivary gland tumors (MEC and ACC) than in benign salivary gland tumors (PA) and higher in PA than normal salivary glands. They also found that CD166 expression was significantly higher in high-grade tumors compared to low-grade ones.

In our study, the highest CD166 expression was in malignant tumors, followed in descending order by benign tumors and normal salivary gland tissue. MSGTs showed statistically significant higher CD166 expression in comparison to NSGs ($p = 0.002$). Contrary to Tadbir *et al.*'s [9] study, we noted decreased CD166 expression in the metastasizing/recurrent and high-grade tumors compared to non-metastasizing/non-recurrent and low-grade ones.

There are conflicting results regarding CD166 expression and the tumor's biological behavior. Yet

studies pointed out mainly up-regulated CD166 in the early malignancy and down-regulated CD166 in advanced malignancy and metastasis [25], which correlated with our data. In SG malignancy, the increase of CD166 might be a critical step in the early stages of tumors. However, in aggressive malignancies, the decreased level of the CD166 adhesion molecule could be a crucial step as its loss facilitates detachment of the invading cells from their contacts and extracellular matrix. The present CD166 negativity in BCCs was statistically significant, indicating it as a possible candidate for a diagnostic marker, which should be elucidated.

Conclusions

The loss of ALDH1 by aging is a crucial step toward SG malignancy, and it might be a CSC marker for malignant salivary gland tumors. ACC and BCC are devoid of ALDH1 protein. Further studies should be carried out to determine the value of ALDH1 as a diagnostic marker for ACC and BCC. CD166

is mostly up-regulated in early SG malignancy and down-regulated in advanced malignancy and metastasis. Loss of CD44 may be an essential step toward early SG malignancy, whereas the increase of this molecule in the course of tumor progression may facilitate metastasis. AcCC lacks CD44 expression. Advanced research on large series should be carried out to elucidate the significance of CD44 as a potential diagnostic marker in AcCC. CD24 expression could be associated with tumor progression and metastasis rather than initiation.

This work was supported by Gazi University Scientific Research Projects Coordination Department (03/2012-28).

The authors declare no conflicts of interest.

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