

Original Article

CD44 expression in pleomorphic adenoma, carcinoma ex pleomorphic adenoma and their adjacent normal salivary glands

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ABSTRACT

Background: CD44 is a transmembranous proteoglycan, with a main role of cell adhesion to other cells and to extracellular matrix involved in the preservation of three-dimensional structure of organs. Pleomorphic adenoma (PA) is a common benign salivary gland tumor, composed of myoepithelial and ductal components. Carcinoma ex-PA (CXPA) is malignant transformation of the epithelial elements in PA. The aim of this study was to evaluate CD44 expression by immunohistochemistry in these two tumors and normal salivary gland near these tumors.

Materials and Methods: In this cross-sectional immunohistochemical study, 40 paraffin blocks (formalin fixed, paraffin embedded) with thirty belonging to PA and ten blocks of CXPA and 18 paraffinized blocks of normal salivary glands' tissue adjacent to tumoral tissue (13 in the vicinity of PA and 5 in the vicinity of CXPA) were selected. Immunohistochemical expression of CD44 was observed and compared with each other. For data analysis, we used Chi-square, Kruskal–Wallis, and Mann–Whitney tests and the quantity of *P* values was considered 0.05.

Results: There was a significant difference in CD44 expression frequency between PA, CXPA, and normal salivary glands with higher expression noted in CXPA. Furthermore, expression frequency of CD44 in adjacent normal salivary gland of CXPA was significantly higher than PA. There was no significant difference in the expression of CD44 with respect to lymph node metastasis.

Conclusion: Higher expression of CD44 in CXPA might illustrate CD44's role in malignant transformation of PA.

Key Words: CD44, pleomorphic adenoma, salivary glands

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INTRODUCTION

Pleomorphic adenoma (PA) or benign mixed tumor is a benign salivary gland neoplasm with notable morphologic variety. It is the most common primary neoplasm of both major (superficial lobe of parotid is a commonplace) and minor salivary glands (mostly at the region of hard palate). It is noted

within an age range of 30–50 years old. It presents with a minor preference in women.^[1]

PA consists of ductal epithelial components and myoepithelial cells in a mesenchyme-like stroma. Myoepithelial cells are the predominant component

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of the tumor and present with variable morphologies. Their products are responsible for the characteristic appearance of tumor stroma as myxomatous or chondroid or hyalinized.^[2] About 1.6%–7.5% of cases are at the risk of malignant transformation and developing carcinomatous foci, leading to the development of carcinoma ex-PA (CXPA).^[3-5]

CXPA thus is a malignant salivary gland neoplasm, which could arise from a malignant change in the epithelial components of preexisting benign PA.^[6] The average age of occurrence of CXPA is about 15 years after the onset of PA in older adults, and its frequency reported as a long-standing mass with rapid growth in recent times and with the presence of pain or ulceration.^[1,7] More than 80% of CXPA presents in parotid gland and 2/3 of cases in hard palate.^[1]

CD44 glycoproteins (homing cell adhesion molecules) are cell surface receptors and belong to cell surface adhesion molecules' family.^[8] CD44 mostly takes part in cell-to-cell and cell-to-external matrix adhesion to preserve tissue and organ organization by their bonding to specific ligands. The main CD44 ligand for extracellular adhesion is hyaluronic acid (extracellular matrix polysaccharide), but it interacts with other ligands including osteopontin, collagens, fibronectin, or laminin.^[9,10] The other roles of CD44 are similar to other cell adhesion molecules and are related to cell migration, signaling, proliferation, angiogenesis, lymphocyte function, and even metastasis.^[10] The diverse functions of these molecules are because of their varying extracellular protein structure which generated from multiple varying isoforms such as CD44 v6 or CD44 v3; alternative splicing and posttranslational modifications of single gene produce more than 1000 isoforms theoretically.^[11-13]

Endo and Terada and Bourguignon showed that expression of CD44 in its multiple isoforms and uncommon transcripts of CD44 could affect tumoral development^[14,15] in comparison with normal tissues. These studies have led us to investigate the role of CD44 as a diagnostic or prognostic factor of malignant transformation.^[16]

Expression and distribution of CD44 in salivary gland benign and malignant tumors have been investigated in some studies. It was shown in study of Ianez *et al.* that the expression of CD44 was higher in PA in comparison with normal tissue of salivary glands with reverse transcription-polymerase chain reaction method, but in a study of Fok *et al.*, the

expression of CD44 in mucoepidermoid carcinoma and PA was lower than normal salivary glands' tissue with immunohistochemical method.^[17,18] Comparing expression of CD44 in PA, CXPA and normal salivary glands adjacent to these tumors could help us to see if expression of CD44 may change in malignant transformation of PA to CXPA or not.

MATERIALS AND METHODS

In this cross-sectional immunohistochemical study, 40 paraffin blocks (formalin fixed, paraffin embedded) with thirty belonging to PA and ten blocks of CXPA and 18 paraffinized blocks of normal salivary glands' tissue adjacent to tumoral tissue (13 in the vicinity of PA and 5 in the vicinity of CXPA) were selected.

The tumor samples were collected from the archives of Amir Alam Hospital's Pathology Department (Tehran University of Medical Science, Tehran, Iran) with the certificate of Ethical Committee with the code of ir.sbmurids.rec. 1394.66. Information including the age, gender, neoplasm size, its location, and the lymph node metastasis was also collected from the corresponding patients' files.

The hematoxylin and eosin slides of the selected cases were reviewed by an oral and maxillofacial pathologist to confirm the previous diagnosis.

Immunohistochemistry

After verification of the samples, paraffin blocks were utilized for immunohistochemistry coloring through Envision method.

Four microns slices were prepared and put over silane-coated special glass slides.

After the deparaffinization and rehydration processes, the samples were placed in Tris-buffered saline (TBS) with pH = 6 to perform the antigen retrieval process and then heated inside a microwave device (Samsung Electronics, Co., Suwon, South Korea) with 750 W.

The H-CAM (CD44 stud/HCAM Ab-4 [156-3c11] Thermo scientific) antibody was diluted through diluting solution in the ratio of 1:2000, based on the manufacturer company instructions. After that, the slices were incubated with the primary antibody.

After washing with TBS, the slices were put inside the Envision tube (Envision Doublestain system, Dako, Carpinteria, CA, USA) for an hour and then rinsed in TBS buffer.

Diaminobenzidine was placed on the slides for 3–4 min as the chromogen and then washed with water.

In this technique, the primary antibody as well as the Novolink Polymer was omitted using peroxidase.

For counterstaining, the slides were placed in hematoxylin and lithium carbonate and then rinsed.

For microscopic assessment of the stained tissue sections, the membrane-stained areas were considered as positive CD44 expression.

For positive control of the study, squamous cell carcinoma tissue and for negative control, saline were utilized instead of primary antibody.

The staining scores for the CD44 immunostaining were assigned based on the percentage of stained cells in a section and 10 HPF was chosen to count. This way of grading was chosen among the previous studies^[19]

Score graded: (negative)

- 1%–10% (Score 1)
- 11%–50% (Score 2)
- 51%–75% (Score 3)
- 76%–100% (Score 4).

Layout of data investigation

The Kruskal–Wallis test analysis methods were used for investigating the relation between the CD44 staining frequency and age of presentation PA and CXPA cases. Furthermore, Chi-square test was used for investigating the amount of CD44 staining in PA and CXPA tissue sections and as well as that of normal salivary glands. The relation between the staining frequency of CD44 marker and patients' gender was investigated through Mann–Whitney test in samples. The first type error was 0.05 in this attempt. Therefore, the values lower than that were considered as statistically meaningful.

RESULTS

Slides were observed by two oral and maxillofacial pathologists by Leica optical microscope (Leica, Wetzlar, Germany) in Pathology Department of Shahid Beheshti Dental School. The stained cells could be seen in Figures 1-4.

Out of 30 PA tumors, 16 cases were observed in women and 14 cases in men. The mean age was 38.14 years old and most of the cases affected in the palate region.

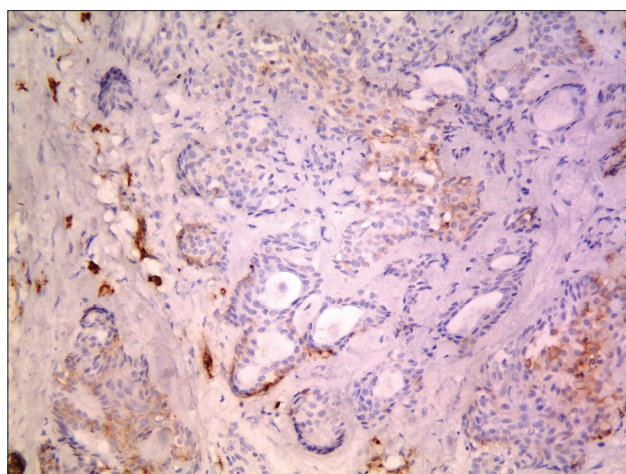


Figure 1: Pleomorphic adenoma with CD44 expression, ×200.

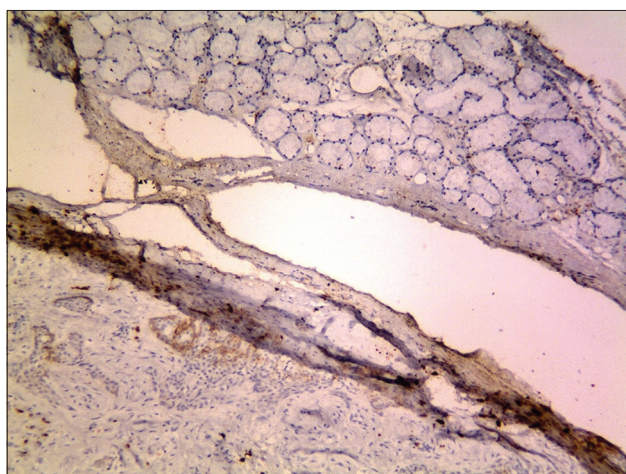


Figure 2: CD44 in pleomorphic adenoma and nearby normal salivary gland, ×100.

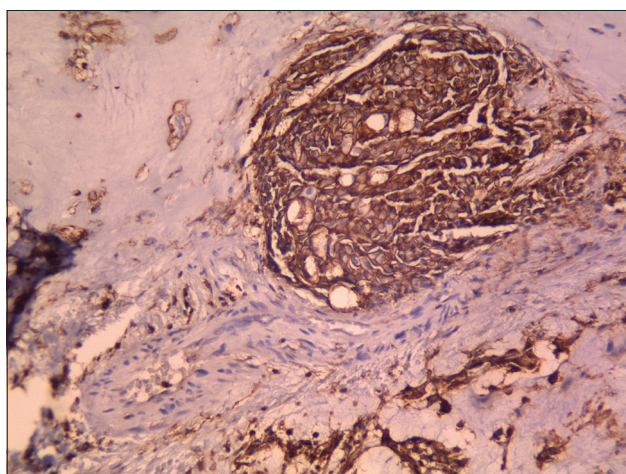


Figure 3: Carcinoma ex-pleomorphic adenoma with CD44 expression, ×200.

Analysis of the CXPA tumor showed that two cases out of ten were seen in women and eight cases were seen in

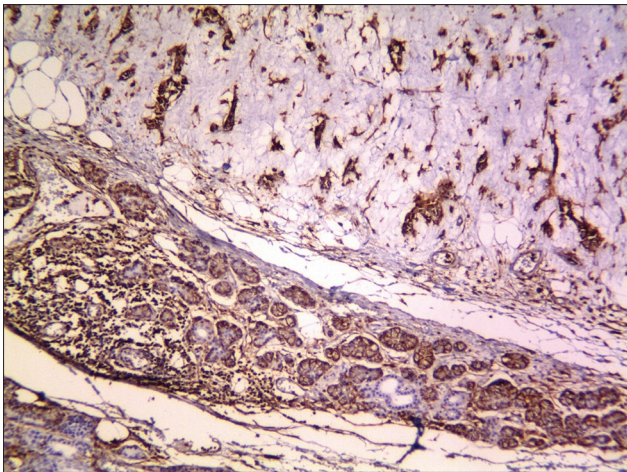


Figure 4: CD44 in carcinoma ex-pleomorphic adenoma and nearby normal salivary gland, ×100.

men. The mean age of occurrence of the neoplasm was 56.4 years and most of the cases affected the parotid.

In this study, the size of the PA tumors after surgery according to patient reports was ranged from 1 to 4.5 cm with a mean size of 2.02 cm; and the size of CXPA tumor ranged from 1.1 to 4.1 cm with a mean size of 2.87 cm.

Table 1 shows the staining frequency of staining score graded of CD44 marker in PA, CXPA, and the normal salivary glands. Glandular epithelium cells get stained, and there was a significant difference in CD44 staining scores between the tumors of salivary gland (PA and CXPA) and the normal salivary glands near both of them ($P = 0.001$); also, there was a significant difference between the staining scores of CD44 expression in PA and CXPA tumors ($P = 0.001$).

According to Table 2, the staining scores in the normal salivary glands adjacent to PA have been lower than that compared to normal glands adjacent to CXPA with a significant difference between them ($P = 0.003$).

The investigations showed that, in CXPA, of all the four samples exhibiting metastasis to lymph node, all had a score of 4. In the other six samples without metastasis, four samples had a score of 4 and two samples had a score of 3.

There was no significant difference in the staining frequency of CD44 marker with respect to metastasis in CXPA ($P = 0.467$).

DISCUSSION

The increase in CD44 upregulation in some studies has correlated with an invasive potential. In some animal

Table 1: Staining scores of CD44 marker in pleomorphic adenoma, carcinoma ex-pleomorphic adenoma, and the normal salivary glands

Tumor type	CXPA (%)	PA (%)	Normal salivary glands (%)
CD44 frequency			
0	0	10	42.1
Score 1	0	56.7	26.3
Score 2	0	23.3	5.3
Score 3	20	10	5.3
Score 4	80	0	21.1

PA: Pleomorphic adenoma; CXPA: Carcinoma ex-pleomorphic adenoma

Table 2: Staining scores of CD44 marker in normal salivary glands near pleomorphic adenoma and carcinoma ex-pleomorphic adenoma

Tissues	Normal salivary glands adjacent to PA	Normal salivary glands adjacent to CXPA
CD44 frequency		
0	61.5	0
Score 1	30.8	20.0
Score 3	0	20.0
Score 4	7.7	60.0

PA: Pleomorphic adenoma; CXPA: Carcinoma ex-pleomorphic adenoma

experiments, high intensity of CD44 immunoexpression has shown to have a capacity in transformation to malignant lesions as melanoma or human B-cell lymphoma.^[20,21] As a result, some studies have been conducted to investigate CD44 expression with respect to the aggressiveness and lymph node metastasis in benign and malignant tumors of salivary glands.^[17,22]

In the present study, the mean age of the PA patients was 38.13 years which is consistent with the study conducted by Ianez *et al.*^[17] and inconsistent with the studies by Fok *et al.*^[18] and Azúa-Romeo *et al.*^[23] in which the mean age was reported to be 44.1 and 48 years, respectively.

In the present study, women constituted 53.3% of the PA patients, which is in agreement with previous studies.^[1]

Moreover, the mean age of the CXPA patients was 56.4 years. It presented in women with a prevalence of 80% and the most affected gland was parotid, which is similar to the demographic information in a study conducted by Soave *et al.*,^[19] also as concluded in some studies, the occurrence age of CXPA is about 10 years more than that of PA.^[24]

In all of the samples of CXPA, CD44 marker was expressed immunohistochemically. The staining

frequency of CD44 in 80% of the CXPA cases showed a score of 4, which is consistent with the result of Soave *et al.*^[19] and is inconsistent with the observations of Franchi *et al.*,^[25] Mori *et al.*,^[26] and Yang *et al.*^[22] In the study by Franchi *et al.*,^[25] a reduced expression of both isoforms of CD44 v6 and CD44 v3 was seen in CXPA; and in the study by Yang *et al.*,^[22] there was a reduced expression of CD44 v6 isoform in CXPA, the same is true about the study by Isacke and Yarwood^[9] in which 47% of the CXPA samples implicated the expression of CD44. This incoherence might be due to the use of different isoforms of CD44 and the different scoring methods which was categorical use of + and – scoring, whereas, in the present study, a grading score comprising four grades was used to evaluate the staining frequency.

Moreover, in this study, in 90% of the PA samples and 58% of the salivary glands samples, next to PA and CXPA expression of CD44 was positive. In 21.1% of the normal salivary glands' samples next to PA and CXPA, the score of 4 was seen, whereas this score was not seen in any of the PA samples, and in 56.7% of them, the staining frequency score was 1; in the study by Franchi *et al.*,^[25] all the PAs showed the CD44 expression diffusely and in the study by Ianez *et al.*,^[17] they were shown focally, which is inconsistent with the present study. As well, in the study by Xing *et al.*,^[27] in all the PA samples, both the epithelial cells and mesenchymal components got stained using this marker.

In the present study, the staining frequency of the CD44 in CXPA was more than that in PA and normal salivary glands ($P < 0.001$), which is inconsistent with the study by Fok *et al.*^[28] and the study by Franchi *et al.*,^[25] in which the CD44 expression had reduced in malignant salivary glands and PA. This inconsistency might be due to the different methods of assessing and the different antibody manufacturing units.

In this study, there was a significant difference between the staining frequency of CD44 marker in the normal salivary glands near PA and CXPA ($P = 0.003$). It was actually higher in the normal salivary glands next to CXPA in which 60% were exhibited a score of 4. So far, this comparison has not been done in any study.

More studies are needed to study the expression of CD44 in normal salivary glands adjacent to lesional and tumor tissue and its comparison with normal salivary glands tissue, to consider it as a potential marker of malignancy.

In this study, the relation between the staining frequency of the CD44 with and metastasis in CXPA was studied with no significant difference between them ($P > 0.05$). However, in the study by Soave *et al.*,^[19] the CD44 expression had a significant relation with metastasis lymph node, but of course, in their study, several types of salivary glands carcinoma were studied, and the malignant samples were more in number.

Moreover, in the present study, there was a significant difference between the staining frequency of CD44 marker in tumoral tissue; the same result was reported in the study by Soave *et al.*^[19]

With the transformation of the CD44 gene by the use of a mouse model, it was seen that the expression of this marker changed with progression and tumor metastasis.^[29,30] It has, as well, been seen in other studies that this marker's isoforms expressions change with tumor growth and do not remain static.^[31,32] Moreover, the CD44 expression changes according to the cell type and the tumor types, and it is different and specific to each neoplasm.

CONCLUSION

The significant more CD44 expression in CXPA compared to PA could implicate the diagnostic role of this marker in the malignant transformation of PA components into CXPA. Considering the inconsistencies in its expression as reported in different studies, the existence of different isoforms of this molecule, and the changeability of its expression in the growth period of the tumor, there is a need for multiplex molecular assays to investigate its expression further.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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