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Original Article

CD44 and EPCAM expression in pleomorphic adenoma and mucoepidermoid carcinoma: An immunohistochemical method

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ABSTRACT

Background: Recent studies have indicated that assessing the expression levels of cancer stem cell markers is critical in predicting the behavior of these neoplasms. This study aimed to evaluate and compare the expression levels of CD44 and epithelial cell adhesion molecule (EpCAM) markers in pleomorphic adenoma (PA) and mucoepidermoid carcinoma (MEC) using immunohistochemistry.
Materials and Methods: In this cross-sectional descriptive-analytical study, 20 samples each of PA and MEC were selected from Kashani Hospital, Isfahan, Iran, based on inclusion and exclusion criteria. Specimens were prepared using immunohistochemical methods and analyzed under an optical microscope. Pathologists evaluated microscopic grade, staining intensity and percentage, and the staining intensity distribution (SID) index. Statistical analysis was conducted with SPSS (version 26), employing the Kolmogorov–Smirnov test, *t*-test, Chi-square, and Fisher's exact test.
Results: The mean frequency of stained cells for both CD44 (P = 0.39) and EpCAM (P = 0.40) markers showed no statistically significant differences between the PA and MEC groups. Similarly, the mean intensity of staining did not differ significantly for either CD44 (P = 0.40) or EpCAM (P = 0.18). The average SID index for the EpCAM marker in the MEC group was significantly higher than the PA group (P = 0.03) and for the EpCAM marker, there was a significant difference between the average SID index and all three variables of microscopic grade (P = 0.01), clinical stage (P = 0.00), and 3-year prognosis (P = 0.02).
Conclusion: The use of EpCAM immunohistochemical marker may help to predict the behavior of salivary gland tumors and obtain better treatment measures for patients.

Key Words: CD44, epithelial cell adhesion molecule, immunohistochemical, mucoepidermoid carcinoma, pleomorphic adenoma

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INTRODUCTION

Salivary gland tumors represent a relatively common occurrence within oral and maxillofacial pathology, comprising a diverse group of neoplasms that affect the major and minor salivary glands. The significant morphological and clinical variability associated with these tumors presents considerable diagnostic

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and therapeutic challenges for surgeons and pathologists.[1]

Pleomorphic adenoma (PA), also known as a benign mixed tumor, is the most prevalent benign neoplasm of the salivary glands, accounting for approximately 60% of benign salivary gland tumors.[2] These tumors frequently present as asymptomatic masses in the parotid gland, hard palate, or upper lip[3] and are most commonly diagnosed in middle-aged individuals, with a higher incidence in women.[4] The tumor is composed of ductal and myoepithelial cells, as well as underlying connective tissue with a myxoid and chondroid structure and is frequently surrounded by an incomplete capsule.[3] On the malignant spectrum, mucoepidermoid carcinoma (MEC) is the most frequently occurring salivary gland malignancy.[5] It primarily affects the parotid gland but may also arise in the submandibular, sublingual, and minor salivary glands.[5] Patients often experience painless swelling; however, in high-grade cases, symptoms may include pain and facial nerve paralysis.[5] Microscopically, MEC consists of mucous, epidermoid, and intermediate cells, with some cases exhibiting clear cell differentiation.[6]

Cancer stem cells (CSCs) are a subset of cancer cells with stem cell-like properties, such as self-renewal and differentiation into various cell types.[7,8] These cells contribute to tumor initiation, growth, progression, treatment resistance, and metastasis.[7,8] CD44 is a CSC marker that serves as a receptor for hyaluronic acid and plays a crucial role in cell motility and cell-to-cell adhesion.[7]

The epithelial cell adhesion molecule (EpCAM) is another CSC marker involved in critical processes such as cell proliferation, metabolism, angiogenesis, and epithelial–mesenchymal transition.[9] While CSCs marker has been studied extensively in various cancers, including those of the breast, prostate, lung, pancreas, colon, as well as in melanoma and leukemia, their role in salivary gland tumors remains less explored.[10] The diversity and complex histology of salivary gland tumors often complicate diagnosis. Identifying CSC markers such as CD44 and EpCAM in these tumors could enhance diagnostic accuracy, improve prognostic evaluations, and support innovative treatment strategies. Considering the markedly different biological behavior and prognosis of benign and malignant tumors, a comparison was made between PA, the most common salivary gland neoplasm, and MEC, the most common malignant salivary gland tumor. Given the tendency of both tumors to recur, this comparison aimed to investigate the role of CSCs in the pathogenesis of these lesions. Therefore, this study aimed to evaluate and compare the expression levels of CD44 and EpCAM markers in PA and MEC using immunohistochemistry.

MATERIALS AND METHODS

In this cross-sectional descriptive-analytical study, 20 samples of PA and 20 samples of MEC were selected from the cases recorded in the archive of the pathology department, Kashani Hospital, Isfahan, Iran, which had been prepared by the excisional biopsy method prior to 2020. The total sample size was calculated assuming $\alpha = 0.05$ and study power of 90%.

Sample selection

Specimens were selected by nonrandom sampling based on specific inclusion criteria as follows: Paraffin blocks were prepared under excisional biopsy, containing high-quality and sufficient tissue samples, making them suitable for immunohistochemical staining. Samples with an inconclusive diagnosis, lacking the necessary information for research, exhibiting necrosis, or having insufficient tissue for specific staining were excluded from the study.

Immunohistochemical method

Tissue sections (3–4 μm thick) were prepared from paraffin blocks, placed on slides, and processed for immunohistochemical staining. After deparaffinization and rehydration, samples were treated with 3% hydrogen peroxide for 3 min and then subjected to antigen retrieval using 0.01M citrate buffer (pH 6) at boiling temperature for 10 min. Tissue sections were washed with Tris Buffer Saline for 1–5 min and were protein blocked for 10 min. Sections were incubated with primary antibodies for CD44 and EpCAM at 4°C. Specimens were then treated with a secondary antibody (mouse EnVision Horseradish Peroxidase) for 60 min at the room temperature, stained with diaminobenzidine, counterstained with hematoxylin, dehydrated, and mounted. A squamous cell carcinoma sample served as a positive control for CD44, whereas colon adenocarcinoma was the positive control for EpCAM, with normal saline as the negative control.[11,12]

Microscopic evaluation

Two independent oral pathologists and a final-year oral pathologist resident examined the specimens and were

calibrated for the detection of them by evaluating the prepared slides under an optical microscope (Olympus BX41TF, Tokyo, Japan) with the ×400 magnification with H and E staining. The immunohistochemical staining was performed using the HCAM Ab4 monoclonal antibody (Proteogenix company on behalf of the Samatashkhis company**)** to evaluate CD44 expression at a concentration of 1.2000, and the EpCAM monoclonal antibody (MOC31, Proteogenix company on behalf of the Samatashkhis company) at a concentration of 1.200 to evaluate EpCAM expression.[13,14] Specimens that exhibited brown color with the cytoplasmic, membranous, or both types of staining in tumor cells of the salivary gland were considered positive. In case of disagreement and inability to reach a consensus, more experienced oral pathologists (S.E) would make the final decision.

The microscopic grade of MEC samples was determined based on the Armed Forces Institute of Pathology system and their clinical stage was determined based on the Tumor, Nodes and Metastases system.

The intensity and percentage of staining for CD44 and EpCAM were used to determine their expression levels in the samples. To determine the percentage of staining, the score 0–4 indicated 1%–25%, 26%–50%, 51%–75%, and more than 75% of the cells were stained, respectively. In addition, the intensity of cell staining was scored on the scale from 0 to 4, with 0 indicating no stained cells, and higher scores representing mild, moderate, moderate to severe, and severe staining. This scoring system was selected based on the methodology described by Meyerholz and Beck.[15]

The staining intensity distribution (SID) index was calculated by multiplication of the percentage of staining score by the intensities of cell staining score. In addition, staining was evaluated based on the patient's age, sex, tumor location, microscopic grade, and clinical stage. The patient's prognosis after 3 years in terms of recurrence or death was assessed by calling and asking for those with MEC.

Statistical analysis

To assess inter- and intra-examiner agreement, three examiners re-evaluated a random selection of 25% of the images 3 weeks after the initial assessment.

Data were analyzed using IBM SPSS Statistics for Windows, version 26 (IBM corp, Armonk, N.Y, USA). The Kolmogorov–Smirnov statistical test was used to assess the normal data distribution. In addition, the *t*-test and Chi-square, and Fisher's exact test have been used. Statistical significance was set at $P < 0.05$.

The protocol of this study was approved by the ethics committee of the Isfahan University with the ID number of IR.MUI.DHMT.REC.1402.011.

RESULTS

The mean age of individuals with MEC (54.5 ± 14.62 years) was significantly higher than that of those with PA (45.3 ± 12.65 years). No significant differences were observed regarding gender ($P = 0.52$) or the location of the lesions ($P = 0.89$).

The mean frequency of stained cells for both CD44 ($P = 0.39$) and EpCAM ($P = 0.40$) markers showed no statistically significant differences between the PA and MEC groups. Similarly, the mean intensity of staining did not differ significantly for either CD44 ($P = 0.40$) or EpCAM ($P = 0.18$). Figures 1 and 2 illustrate the mean staining index distribution (SID) for EpCAM and CD44 based on lesion type, gender, age ranges, and lesion locations.

Although the mean SID for CD44 ($P = 0.7$) marker was not significantly different between the two groups, it was higher in the MEC group compared to the PA group. This discrepancy was significantly higher for the EpCAM ($P = 0.03$).

Moreover, the mean SID did not have significantly different between genders ($P_{CD44} = 0.41$, $P_{EpCAM} = 0.66$) various age groups ($P_{CD44} = 0.34$, $P_{EpCAM} = 0.19$), and location of the lesions ($P_{CD44} = 0.06$, $P_{EpCAM} = 0.62$) for both markers.

The results of this study illustrated that most of the MEC tumors were in the category of moderate microscopic grade (40%) and clinical stage I (40%). In addition, the majority of patients' tumors did not recur after a 3-year follow-up.

The mean SID for the EpCAM marker was significantly associated with the microscopic grade ($P = 0.01$), clinical stage ($P = 0$), and 3-year prognosis of patients ($P = 0.02$) with MEC; however, no significant differences were found for any of these three criteria for CD44 ($P > 0.05$).

While the frequency of stained cells for the EpCAM marker did not show significant differences related to the microscopic grade of MEC ($P > 0.05$), it was significantly associated with clinical stage ($P = 0.007$) and the patient's 3-year prognosis ($P = 0.04$).

Although for the EpCAM marker, there was a significant difference between the staining intensity of stained cells and all three variables of microscopic grade ($P = 0.02$), clinical stage ($P = 0$), and 3-year prognosis ($P = 0.02$); for the CD44 marker, there was no significant difference neither for the intensity of staining nor for the frequency of stained cells ($P > 0.05$).

Figures 3 and 4 demonstrated the staining level of MEC samples for CD44 and EpCAM markers, respectively.

The Cohen's kappa coefficient ranged from 0.891 to 0.915 for intraexaminer agreement and 0.931 to 0.947 for interexaminer agreement.

DISCUSSION

The average age of patients with PA was 45.3 ± 12.65 years in this study, which was lower than previous studies.[12,16] In addition, the average age of patients with MEC was 54.5 ± 14.62 years, which was significantly higher than the PA group. On the other hand, the average age of these two lesions was almost equal in the study of Phattarataratip *et al*.[17]

In this study, the average age of patients with PA was lower than that reported in previous studies.[14,18] Conversely, the average age of patients with MEC was significantly higher than that of the PA group. However, in the study by Phattarataratip *et al*.,[17] the average ages of these two lesions were reported to be nearly equal.

The results of the present study indicated that the frequency of stained cells for the CD44 marker was at least 25% in both PA and MEC groups. These findings align with those reported in the literature.[16,19] In addition, Moura *et al*. and other studies have shown no significant differences in the percentage or intensity of CD44 staining between the two groups.[20]

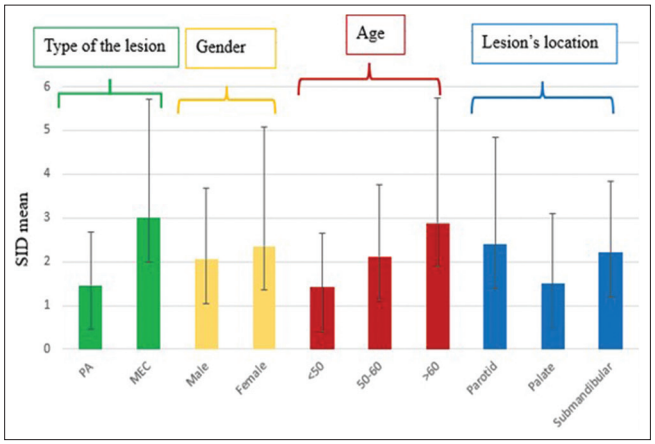

Figure 1: The staining intensity distribution mean for the epithelial cell adhesion molecule based on the type of the lesions, gender, age ranges, and lesion locations. SID: Staining intensity distribution.

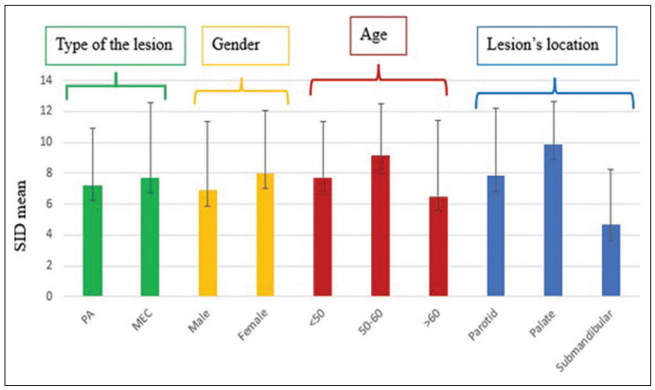

Figure 2: The staining intensity distribution mean for the CD44 based on the type of the lesions, gender, age ranges, and lesion locations. SID: Staining intensity distribution.

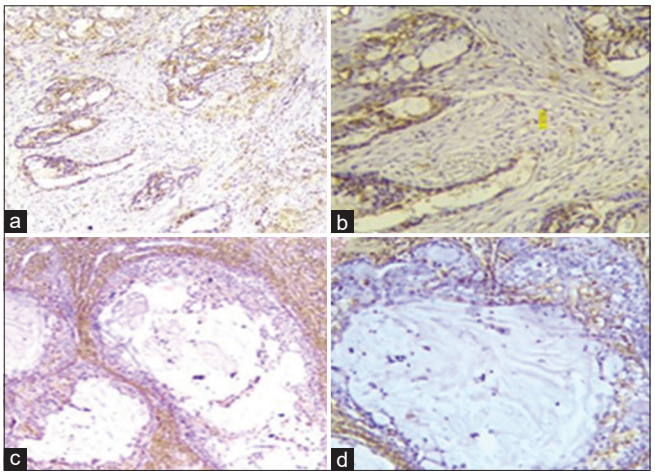

Figure 3: Staining level of mucoepidermoid carcinoma samples for EPCAM marker: (a): ×100 mild, (b): ×200 mild, (c): ×100 moderate, (d): ×200 moderate.

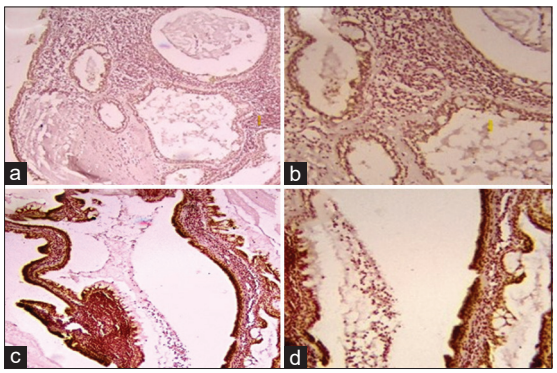

Figure 4: Staining level of mucoepidermoid carcinoma samples for CD44 marker: (a): ×100 mild, (b): ×200 mild, (c): ×100 moderate to severe, (d): ×200 moderate to severe.

Although the mean SID for the CD44 marker was not statistically significantly different between the PA and MEC groups, it was higher in the MEC group, consistent with findings from Moura *et al*.[20] The samples from both groups in this study exhibited moderate-to-severe staining; however, Alsheddi *et al*.[16] reported that most PA and MEC tumors demonstrated intense severe staining for this marker.

Similar to our findings, the study conducted by Phattarataratip *et al*.[17] revealed a significantly higher mean SID for the EpCAM marker in the MEC group. In addition, in our study, most PA tumors exhibited either no staining or minimal staining, which aligns with the results reported by Phattarataratip *et al*.[17]

According to our results, the percentage and intensity of CD44 staining did not show significant differences across age groups and genders. However, the percentage of EpCAM marker staining was significantly higher in men compared to women. This finding has not been reported in previous studies. Zhu *et al*.[21] conducted a study evaluating the expression of EpCAM and β-catenin markers and found a significant difference in EpCAM expression related to patient age (P = 0.004). However, this variable was not associated with patient gender or tumor location.

In the current study, no significant relationship was found between the SID of these two biomarkers and the location of the neoplasms. However, Kalaitsidou *et al*.[11] indicated that the intensity of EpCAM marker expression in parotid gland neoplasms was significantly higher compared to that in submandibular and sublingual glands.

Previous studies reported similar results to our research indicating that there were no significant differences between the SID mean of the CD44 marker and the microscopic grade, clinical stage, and prognosis of MEC samples.[13,18]

The results of Kalaitsidou *et al*.[11] study confirmed that the frequency of EpCAM expression had a significant association with the clinical stage and prognosis, but there was no significant association with the microscopic grade of this neoplasm.

The results of Phattarataratip *et al*.[17] research were inconsistent with the findings of the present study so that the lower expression of the EpCAM marker was associated with the higher microscopic grades of MEC. The reason for the difference in the results of the studies can be related to the difference in the sample size of the studies, methodology, and types of tumors investigated.

Due to time and cost limitations, collecting more samples was one of the limitations of this study. Considering the importance of this issue, it is suggested that other studies be conducted in a multicenter manner with a larger number of samples.

CONCLUSION

The present study showed a higher expression of EpCAM marker in the malignant tumor of MEC and its association with microscopic grade, higher clinical stage, and poorer prognosis. Therefore, the use of this immunohistochemical marker can help to predict the behavior of salivary gland tumors and lead to better treatment strategies for patients.

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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 non-financial in this article.

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