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Original Article

Immunohistochemical Analysis of Endocan Expression in Salivary Pleomorphic Adenoma

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Abstract

Background: The tumors of salivary glands make up 3% of neoplasms in the head and neck region. Pleomorphic adenoma (PA) represents the most prevalent benign tumor of the salivary glands. Endocan is secreted by endothelial cells and is associated with tumorigenesis/tumor progression. This study aimed to analyze the expression and distribution pattern of endocan in salivary PA and the contribution of markers to tumor development.

Methods: Overall, 35 PA samples from parotid glands were collected from the Archive of the Pathology Department of Educational Hospital from 2016 to 2021. Hematoxylin and eosin staining confirmed the previous diagnosis. All samples were classified based on the differentiation of epithelial cells and the amount of the stroma according to the Seifert et al classification. Then, the specimens were processed for immunohistochemistry (IHC) analysis.

Results: Based on the Seifert et al classification, 37% of PAs were classified as classic type. The cellular or myxoid type was found in 31.4% of cases (each with 11 cases). There was a significant difference between the age of the patients and the histological subtype ($P<0.011$). Additionally, a statistically significant difference was found between the duration of the tumor and the histological subtypes ($P<0.018$). Endocan positivity was mainly observed in the ductal epithelium in the classic subtype. Moreover, plasmacytoid-like and spindle cells were stained for endocan primarily in the classic type.

Conclusion: The present study demonstrated a noticeable occurrence of endocan positivity in different cell types found in PA samples. The findings from our research offer evidence that supports the notion that endocan is crucial in the progression and potentially in the malignant transformation of PAs. The immunoreactivity shown by plasmacytoid-like and spindle cells provides a strong indication of the possible presence of the epithelial-mesenchymal transition phenomenon as a key factor in the development of these tumors. Thus, endocan can be considered a potential therapeutic target for PA.

Keywords: Endocan, Epithelial-mesenchymal transition, Pleomorphic adenoma, Salivary glands

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Background

Tumors of the salivary glands constitute 3% of neoplasms in the head and neck region. Pleomorphic adenoma (PA) represents the most prevalent benign tumor of the salivary glands, accounting for 40%–70% of all salivary gland tumors (1). PA primarily affects females, with a mean age of 43 years at the time of diagnosis. Most PAs occur in the parotid glands (85%), followed by minor salivary glands (10%) and submandibular glands (5%). PA may have a higher risk of local recurrence and distant metastasis, contrary to its benign histological appearance, along with malignant transformation (carcinoma ex PA) (2,3).

The histologic diversity, composed of epithelial and stromal/mesenchymal components, is the hallmark of PA (1,4); therefore, it is useful to investigate the role of different molecular markers in tumor growth, along with their relevance to clinical and pathological features.

Endocan, also called endothelial cell-specific molecule 1, is a dermatan sulfate proteoglycan secreted by endothelial cells and is associated with tumorigenesis and tumor progression (5,6). Endocan has been identified as a marker of endothelial cell activation (5,7). It is activated by inflammatory responses, cytokines, and proangiogenic factors, such as vascular endothelial growth factor (VEGF).

VEGF is involved in angiogenesis in both physiological processes and tumors and contributes to metastasis (8,9). A growing body of research has suggested that altered endocan expression levels in tumor vessels lead to angiogenesis and cancer progression through cancer cell proliferation, migration, invasion, and the stemness properties of cancer cells (10). This study aims to analyze the expression and distribution pattern of endocan in salivary PA and the contribution of markers to tumor development.

Materials and Methods

A total of 35 samples of PA from major salivary glands were collected from the Archive of the Pathology Department of Be'sat Educational Hospital, Hamadan, Iran, from 2016 to 2021. In addition, five normal parotid tissues were selected as the control group. Hematoxylin and eosin (H&E) staining confirmed the previous diagnosis. All PA samples were classified based on the differentiation of the epithelial cells and the amount of the stroma according to the Seifert et al classification (1). Then, the specimens were processed for immunohistochemistry (IHC) analysis. The monoclonal anti-mouse antibody used in the IHC assay was endothelial cell-specific molecule 1 (1:170; Abcam; 56914). IHC staining was performed according to previous studies (11, 12). Briefly, the paraffin blocks were cut into sections that were 4 µm thick. The sections were subsequently deparaffinized and dehydrated using graded alcohol. Antigen retrieval was conducted using citrate buffer (pH = 6). The Leica detection kit was utilized to inhibit endogenous peroxidase activity. Following three washes in Tris-buffered saline, the samples were treated with the primary antibody for one hour. The negative control was created by excluding the primary antibody. Following Tris-buffered saline washing, the slides were processed in a freshly made 3,3'-diaminobenzidine solution for six minutes and then counterstained with hematoxylin, dehydrated, and mounted.

The expression level of endocan was evaluated in the cytoplasm of tissue sample cells. The abundance of positive cells was graded as 1 (weak or moderate) for < 50% positive cells and 2 (strong) for > 50% positive cells.

Micro-vessel density (MVD) was detected through the light microscopic examination of stained sections; the most vascularized areas with the highest MVD within the tumor (hot spots) were identified. The micro-vessels were counted in the five hot spots at 400x magnification (Olympus BX51 optical microscope). The average vessel counts from the five fields were regarded as MVD. The MVD was classified as either high (≥ 5) or low (< 5); 5 was considered the median value for MVD. Large or thick-walled blood vessels were excluded from the investigation (11,12). The data were then analyzed using SPSS (Statistical Package for the Social Sciences), version 20.

The clinical and histopathological variables were statistically analyzed to compare the examined groups regarding the endocan expression levels and the Seifert classification. Statistical analyses were performed using both chi-square and Fisher's exact tests to establish associations. Variables that were significant on univariate analysis were subjected to multivariate analysis, and a *P* value of less than 0.05 was deemed statistically significant.

Results

In this study, there were 18 (51.4%) males and 17 (48.6%) females. In general, the ages of the patients ranged from 18 years to 68 years, with a mean age of 41.83 years. Based on the Seifert et al classification, 37% of PAs were classified as classic type. The cellular or myxoid type was found in 31.4% of cases (each with 11 cases). The extremely cellular variant (type IV) of the Seifert classification was not detected in our samples. There was a significant difference between the age of the patients and the histological subtype ($P < 0.011$). The classic subtype and the cellular type were more frequent in patients aged ≤ 42, while stroma-rich subtype was more common in patients aged > 42. Additionally, the results revealed a statistically significant difference between the duration of the tumor and histological subtypes ($P < 0.018$). The cellular and classic subtypes primarily developed in ≤ 61 months, whereas stroma-rich subtype mainly developed in > 61 months. Table 1 provides the demographic and histopathological characteristics of the samples. Table 2 presents a comparison of the average values of variables, including the count of endocan-positive cells categorized by histological subtype. Table 3 outlines the impact of age and tumor duration on various parameters according to the multivariate analysis.

Discussion

PA, a benign tumor of the salivary glands, predominantly manifests in females. The peak incidence is between the third and fifth decades (13,14). In the present study, although there was an agreement regarding the age of the patients in previous reports, the tumor was frequently found in males. Concerning tumor size, earlier reports showed that 7%–10% of the PA had a diameter of more than 40 mm (15,16). However, in the present series, 45.7% of samples had a diameter of more than 36 mm. In line with earlier research on endocan expression levels in pituitary adenomas, no correlation was identified between endocan expression and tumor size or tumor subtype (Table 1) (17).

In the current study, the expression level of endocan was investigated in 35 cases of PA. According to the histological findings, all types of cells represented endocan expression (Figure 1A-L). It is proposed that different types of cells in the salivary glands, such as acinar, ductal, and myoepithelial cells, are progenitor cells and have the potential to proliferate and differentiate (4, 18); therefore, they can overcome the limiting growth restriction and contribute to the development of a neoplasia. Interestingly, studies have shown that the overexpression

Table 1. A Summary of Demographic and Histopathological Findings of 35 Cases of Pleomorphic Adenoma and Normal Salivary Gland Tissues Stained With Endocan

Clinical and Histopathological Findings	Number of Cases (%)
Gender	
Male	18 (51.4)
Female	17 (48.6)
Age (year)	
Mean age (range)	41.8 (18-68)
≤42	22 (62.9)
>42	13 (37.1)
Size (mm)	
Mean (range)	35.6 (10-80)
≤36	19 (54.3)
>36	16 (45.7)
Duration (month)	
Mean	61 (4-360)
≤61	24 (68.6)
>61	11 (34.4)
Recurrence	
Yes	2 (5.7)
No	33 (94.3)
Subtype	
I	13 (37.1)
II	11 (31.4)
III	11 (31.4)
Invasion to capsule	
Yes	2 (5.7)
No	33 (94.3)
MVD	
≤5	16 (45.7)
>5	19 (54.3)
Tissue component	
Myoepithelial sheets and islands	35 (100)
Duct-like structures	35 (100)
Myoepithelial cords	32 (91.4)
Spindle-shaped cells	29 (82.9)
Myxoid area	25 (71.4)
Plasmacytoid-like cells	20 (57.1)
Chondroid area	20 (57.1)
Hyalinized area	10 (28.6)
Cystic structures	10 (28.6)
Squamous metaplasia	7 (20.0)
Clear cells	7 (20.0)

Note. MVD: Micro-vessel density.

Table 2. Comparison of Variables Based on the Seifert Classification

Variables	Classic	Stroma-rich	Cell-rich	*P* value
Gender				0.445
Male	5 (14.3%)	5 (14.3%)	7 (20.0%)	
Female	8 (22.9%)	6 (17.3%)	4 (11.4%)	
Age (year)				0.011*
≤42	11(31.4%)	3 (08.6%)	8 (22.9%)	
>42	2 (05.7%)	8 (22.9%)	3 (8.6%)	
Size (mm)				0.333
≤36	6 (17.1%)	5 (14.3%)	8 (22.9%)	
>36	7 (20.0%)	6 (17.1%)	3 (8.6%)	
Duration (month)				0.018*
≤61	8 (22.9%)	5 (14.3%)	11 (31.4%)	
>61	5 (14.3%)	6 (17.1%)	0 (0.0%)	
MVD				0.733
≤5	5 (14.3%)	6 (17.1%)	5 (14.3%)	
>5	8 (22.9%)	5 (14.3%)	6 (17.1%)	
Endocan positive ductal epithelium				0.598
≤50	3 (08.6%)	2 (5.7%)	4 (11.4%)	
>50	10 (26.6%)	9 (25.7%)	7 (20.0%	
Endocan positive myoepithelial cells				0.625
≤50	4 (11.4%)	4 (11.4%)	2 (5.7%)	
>50	9 (25.7%)	7 (20.0%)	9 (25.7%)	
Endocan positive epithelial lining of cystic structures				0.081
≤50	5 (14.3%)	8 (22.9%)	3 (08.6%)	
>50	8 (22.9%)	3 (08.6%)	8 (22.9%)	
Endocan positive plasmacytoid-like cells				0.172
≤50	1 (2.9%)	4 (11.4%)	4 (11.4%)	
>50	12 (34.3%)	7 (20.0%)	7 (20.0%)	
Endocan positive spindle-shaped cells				0.608
≤50	1 (2.9%)	1 (2.9%)	0 (00.0%)	
>50	12 (34.3%)	10 (26.6%)	11 (31.4%)	

Note. MVD: Micro-vessel density. *$P<0.05$.

of endocan in tumorigenic cells can also remarkably augment the proliferation rate of tumors and even induce the tumor formation of non-tumorigenic epithelial cells (19,20). A prior study demonstrated that myoepithelial cells are localized in the intercalated ducts and are absent in the acini (21). Various forms of myoepithelial cells are basaloid, plasmacytoid-like, spindle, epithelioid, and clear cells (22); however, some researchers have suggested that plasmacytoid-like cells originate from luminal cells rather than myoepithelial cells (23,24).

Furthermore, the presence of spindle-shaped cells raises the prospect of epithelial-mesenchymal transition (EMT) in PAs. The expression of E-cadherin, a cell adhesion molecule, decreases during the EMT phenomenon; consequently, cells acquire mesenchymal characteristics by elevating the expression of vimentin and N-cadherin (25). Notably, the transformation of plasmacytoid-like cells into a spindle-shaped morphology has been documented. Moreover, E-cadherin expression has been found to be subdued or absent in plasmacytoid-like cells and negative in spindle cells within PA samples (4). Additionally, vimentin has been indicated in plasmacytoid-like cells of PA (26). Another study on PA confirmed vimentin positivity in both plasmacytoid-like and spindle cells (27). These observations collectively

Table 3. Significant Results Derived From Multivariate Analysis

Variables	95% CI	*P* Value
Age		
Size	0.000-1	<0.001
Duration	0.000-1	<0.001
MVD	1-2	<0.001
Seifert classification	0.000-2.047	<0.001
Endocan positive ductal epithelium	1-2	<0.001
Endocan positive myoepithelial cells	2-2	<0.001
Endocan positive epithelial lining of cystic structures	0.001-1	<0.001
Duration		
Size	1-1	0.003
Seifert classification	1-2	<0.001
Endocan positive ductal epithelium	1-1	<0.001
Endocan positive myoepithelial cells	1-1	<0.001
Endocan positive spindle cells	1-2.078	<0.001

Note. MVD: Micro-vessel density; CI: Confidence interval.

suggest that both plasmacytoid-like and spindle cells are potentially involved in EMT. Given the robust endocan positivity detected in spindle-shaped and plasmacytoid-like cells within our study, it is plausible to infer that not only does the EMT phenomenon occur in PA, but also that endocan plays a role in this process. Therefore, it is conceivable that PA rich in plasmacytoid or spindle cells could be more susceptible to malignant transformation.

Endocan positivity was also detected in areas with squamous metaplasia (n = 7:20%, Figure 1I). Early studies have described squamous metaplasia as a result of trauma or infarction/ischemia and repair following infarction (28,29). Experimentally, squamous metaplasia was induced by arterial ligation in rat salivary glands (30). It is suggested that ischemia is the most probable etiology for this change (28). It is also believed that squamous metaplasia may result in squamous cell carcinoma (31). As endocan expression is associated with aggressive tumor progression, its expression in areas with squamous metaplasia may predict malignant transformation in PA samples.

Within our present study, endocan reactivity was detected throughout the entire tumor in both epithelial proliferative areas and stromal regions. Endocan positivity was indicated in both stromal chondroid-like and myxoid areas. It is noteworthy that neoplastic myoepithelial cells can arise through diverse mechanisms, including metaplasia and dedifferentiation (32). This observation could indicate that endocan is involved in aiding the transdifferentiation of myoepithelial cells into different cell types, possibly functioning in an autocrine fashion. Notably, a previous study on colorectal cancer revealed that endocan expression is positively associated with tissue differentiation, indicating the possible role of endocan in the development and differentiation mechanisms of colorectal cancer (33). Additionally, another study focusing on pancreatic neuroendocrine tumors showed a link between endocan expression and tumor recurrence. The researchers proposed that endocan expression levels might serve as a reliable biomarker for predicting tumor recurrence in patients with pancreatic neuroendocrine tumors. In addition, this study suggested that the elevated expression of endocan is associated with greater malignant potential (34). In lung cancer, increased endocan expression level is associated with poor clinical outcomes (35). It has been proposed that endocan targets malignant epithelial cells. At the same time, endocan boosts the impact of growth factors and prevents the migration of immune cells into the tumor (36). These relationships highlight the potential importance of endocan in these biological functions, which may also pertain to other neoplastic situations, such as PA.

Angiogenesis in tumors is a critical process, regulated by multiple factors. Despite VEGF being the most powerful angiogenic factor, tumor cells can find alternative growth signaling pathways (11). Endocan has additionally been identified as an indicator of angiogenesis (5,6). The prognostic importance of angiogenic biomarkers, such as endocan, has been assessed in various cancers (34, 35). VEGF promotes the secretion of endocan, which subsequently regulates angiogenic genes, such as *VEGF-A* (35). In the current study, micro-vessel formation was assessed by endocan positivity (Table 1). In agreement with other studies, the present study suggested that endocan might serve as a useful biomarker to monitor angiogenesis and, hence, disease progression. Based on these findings, endocan may be involved in mediating the growth of PA.

In this study, endocan positivity was also found in ductal epithelial cells of normal salivary glands (Figure 1L). Interestingly, acinar cells did not stain with endocan, confirming the presence of myoepithelial cells only in the ductal cells; therefore, the acinar cells lack myoepithelial cells (21). A prior study on the expression profile of endocan in adrenal cortical tumors detected the expression of endocan in 30.7% of subjects in the control group (37). Interestingly, endocan expression was found in normal endocrine tissues but not in the normal islets of Langerhans (34). Early studies showed that the basal cells of excretory and intercalated ducts are reserve cells and can replace all types of cells in normal salivary glands. Furthermore, it is believed that these cells are the source of neoplastic transformation (38,39). However, another study indicated that differentiated cells, including luminal, basal, and acinar cells, are capable of mitosis (40). Our findings may confirm the role of ductal cells as the reserve cells and a source of neoplastic transformation.

Conclusion

Our findings confirmed a noticeable occurrence of endocan positivity in different cell types found in PA samples. The findings suggest evidence supporting the notion that endocan is vital in the progression and potentially in the malignant transformation of PAs. The

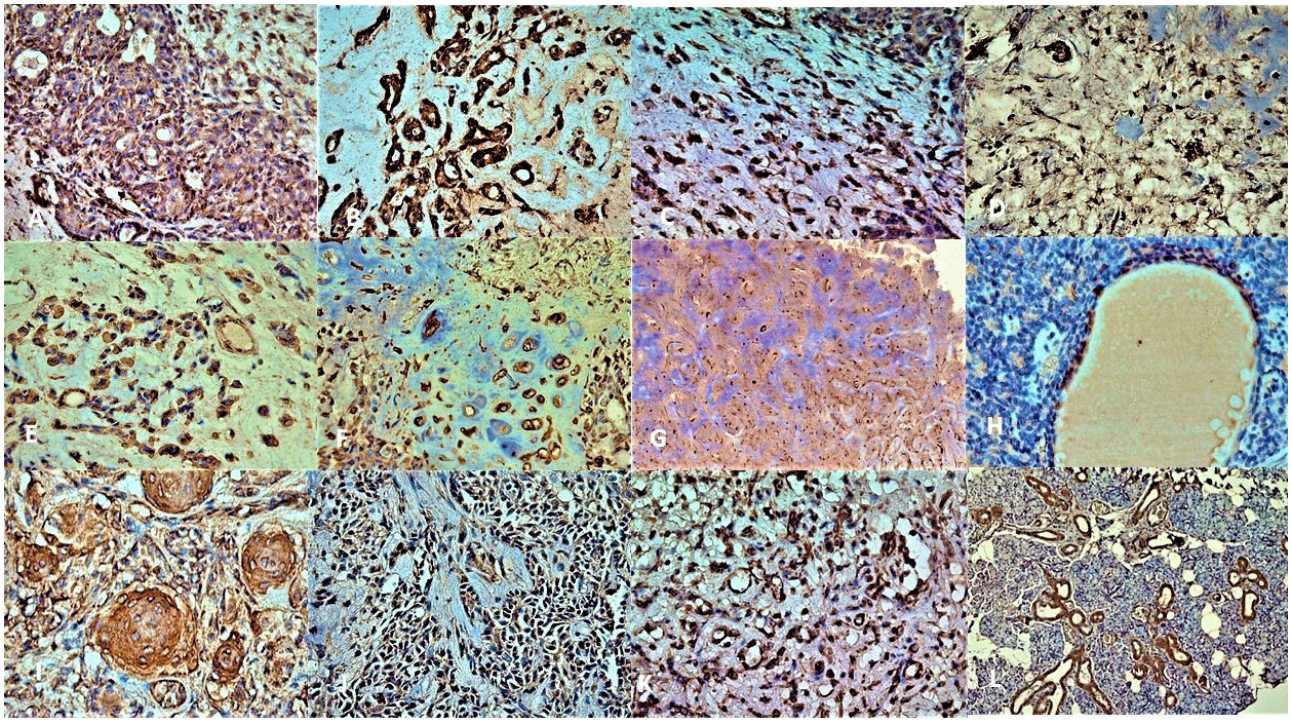

Figure 1. Immunohistochemical Staining of PA Samples Showing the Endocan Positivity of **(A)** Sheets of Myoepithelial Cells, **(B)** Myoepithelial Cells Lining Duct-Like Structures, **(C)** Spindle-Shaped Myoepithelial Cells in a Stroma Matrix of Mucoid (×250), **(D)** Spindle-Shaped Myoepithelial Cells in a Variable Stroma Matrix of Myxoid and Hyalinized Types (×250), **(E)** Plasmacytoid-Like Myoepithelial Cells (×250), **(F)** Myoepithelial Cells in a Cartilaginous Stromal Component (×250), **(G)** Myoepithelial Cells in a Hyalinized Stroma (×100), **(H)** Myoepithelial Cells Lining a Cystic Structure (×250), **(I)** Metaplastic Squamous Structures (×250), **(J)** Myoepithelial Cells Arranged in Sheets and Cords, **(K)** The Capillary Endothelium, and **(L)** Normal Parotid Tissues. *Note*. PA: Pleomorphic adenoma. **(A)** A few ducts can be observed as well (×250). **(B)** Unstained surrounding stroma is evident (×100). **(J)** A clear cell variant of myoepithelial cells does not stain with endocan (×250). **(K)** Note the high number of the vessels (×250). **(L)** Only the ductal cells are stained with endocan, while the acinar cells are not stained (×100)

immunoreactivity represented by plasmacytoid-like and spindle cells provides a strong indication of the possible presence of the EMT phenomenon as a key factor in the development of these tumors. These results indicated that PA samples marked by the significant presence of plasmacytoid-like and spindle cells may be especially susceptible to malignant transformation. Additionally, endocan is a biomarker for angiogenesis, a crucial process in tumor growth. Accordingly, it is important to highlight the potential function of endocan in influencing the development and specialization of normal salivary tissue. Thus, endocan can be considered a potential therapeutic target for PA. Nevertheless, additional studies with more extensive sample sizes are essential to thoroughly clarify endocan functions and significance.

Authors' Contribution

Conceptualization: Soussan Irani.
Data curation: Soussan Irani, Arash Dehghan, Erfane Amini.
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Methodology: Soussan Irani.
Project adminstaration: Arash Dehghan.
Resources: Soussan Irani.
Software: Soussan Irani.
Supervision: Soussan Irani.
Validation: Soussan Irani, Erfaneh Amini.
Visualization: Soussan Irani.
Writing-original draft: Soussan Irani.
Writing-review & editing: Soussan Irani, Arash Dehghan, Erfaneh Amini.

Competing Interests

None declared.

Ethical Approval

This study was evaluated and approved by the Ethics Committee of Hamadan University of Medical Sciences (Institutional Review Board approval No. Res.Proj.1399.316).

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