IMMUNOHISTOCHEMICAL ANALYSIS OF CD34 EXPRESSION IN BENIGN PLEOMORPHIC ADENOMA ACCORDING TO HISTOPATHOLOGICAL SUBTYPE
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ABSTRACT
Pleomorphic adenoma (PA) is a common tumor which involves both the major and minor salivary glands. Its growth depends on angiogenesis which is assessed by measuring the tumor microvessel density (MVD) through CD34 immunostaining. The present study was performed to evaluate the situation of angiogenic activity in PA according to the histopathological subtype. Tissue specimens of (42) PA were sectioned and stained for hematoxylin and eosin, and immune-stained for CD34 protein. The most vascularized areas at low power magnification (hotspots) were selected for vessel counting at (×400) magnification, and the mean number of microvessels in three fields within the tumor mass was calculated. The stroma-rich was the most common subtype, followed by cell-rich and classic. The mean number of the CD34 positive micro vessels in all cases of PAs studied was 7.88±4.381. The mean number of the CD34 positive micro vessels in stroma-rich, cell-rich, and classic types was 11.90±0.867, 2.8±0.941, and 5.4±0.894 respectively. Statistical analysis showed a highly significant difference present between them regarding the MVD (P=0.0001). Salivary gland PA of stroma-rich type showed a higher vascular density than other subtypes, the reason for this higher angiogenic activity could be related to metabolic characteristics, this may add an insight about the type of treatment needed for PA especially the stroma-rich subtype.

Keywords: Angiogenesis, CD34, Pleomorphic adenoma, Salivary gland tumors

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INTRODUCTION
The salivary gland neoplasms make up 1–4% of all human tumors and the majority of these lesions are PA1. PA although classified as benign, but can cause problems in the clinical management since it has tendency for recurrence and malignant transformation 2, 3. It mostly occurs in the superficial lobe of the parotid gland, and in the region of the hard palate and is more frequent in adults and among females 4.

Histologically, the tumor is characterized by histomorphologic heterogeneity and a wide variety of structures may be seen arise mainly from duct epithelium or myoepithelial cells, these varied tissues are completely disordered in arrangement and the proportions of the different components are also vary widely. The fibrous, myxoid or cartilaginous mesenchymal elements are due to the properties of myoepithelial cells 5, 6. Recurrence of PA is observed in 10-12% of cases and malignant transformation is not rare 7. However, the mechanisms of oncogenesis and tumor progression remain unclear 8.

The tumor growth depends on angiogenesis which is assessed by measuring the tumor MVD through the CD34 immunostaining. Counting tumor blood vessels by immunohistochemistry is the most common
method used for evaluating the angiogenic activity of PA. Several studies have shown that CD34 antibodies are more specific to tumor vessels and therefore it is suitable for determining the MVD.

There were no previous study correlated immunohistochemical expression of CD34 of PA of salivary glands according to the histopathological subtype in Karbala city/ Iraq, so the present study was designed to evaluate the angiogenic activity in benign pleomorphic adenoma according to the histopathological subtype.

MATERIALS AND METHODS

The materials used in this study consist of (42) formalin fixed, paraffin-embedded salivary gland biopsy specimens of benign PAs. They were retrieved from the archives of Al-Kafeel HospitalLab, Karbala/ Iraq and the specialized labs in the periods 2016, 2017, and 2018. Sample collection was authorized by Ministry of Health in Karbala/ Iraq. Sections were made and stained with hematoxylin and eosin, and the slides were examined to find the subtype classification of PAs (stroma-rich, cell-rich or classic which contains balanced amount of epithelial and stromal components). Five randomly selected fields in each slide were examined to find the mean percentage of stroma-rich or cell-rich area, using microcomputer imaging device (MCID) image analysis software. Additional sections were made for immunohistochemical study.

The EnVision labeled peroxide systems (Dako, Carpentaria, CA, USA) was used for immunohistochemical staining. The antigen retrieval was performed by DakoCytomation target retrieval solution with pH = 9, for 20 min. Tissue sections were incubated for 30 min with the anti-CD34 monoclonal antibody (mouse, Dako Corporation, Denmark) at 1/10 dilution. Pyogenic granulomas were employed as positive control. Brown cytoplasmic staining for CD34 was considered positive. The negative tissue controls indicates a tissue specimen processed using a non-immune serum and applying the antibody diluents alone, this was done under the same test conditions throughout the work time and run with each batch of the stain. The research project was approved by the Research Ethics Committee at Department of Dentistry, Al-Hussein University College under protocol.

MVD is defined as the number of positive CD34 micro vessels (hot spots) stained per individual lesion. First, the tissue was screened at low power to identify areas of the highest vascularization. Then, the vessels were counted in three such high density areas at high power magnification (x400). The average of the MVD which was carried out by two independent observers was considered as the MVD of each individual lesion.

Any brown staining of endothelial cells or cluster of endothelial cells with or without a lumen that is clearly separate from adjacent micro vessels and other tissue elements is considered as a single vessel. The vessels with muscular walls were excluded. Intensity of staining was not considered for evaluation. The sections were observed with Nikon light microscope.

Statistical analysis

To compare MVD between the three groups, the results were analyzed by analysis of variance (ANOVA). Data were presented as mean ± standard deviation (SD) and the result with P < 0.05 was considered significant. SPSS (Statistical Package for Social Science) software was used to analyze the data.

RESULTS

Hematoxylin and eosin results
The histopathological evaluation showed that the chief component of most PAs was Stroma-rich (22 case, 52.38%), fifteen cases (35.71%) were cell-rich,and only five cases (11.91%) were classic (Table-1). Figure (1) and Figure (2) shows different histopathological subtypes.

**Table 1:** The histopathological subtypes of salivary glands pleomorphic adenoma

<table>
<thead>
<tr>
<th>Histopathological subtype</th>
<th>No.</th>
<th>%</th>
</tr>
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<tbody>
<tr>
<td>Stroma-rich</td>
<td>22</td>
<td>52.38</td>
</tr>
<tr>
<td>Cell-rich</td>
<td>15</td>
<td>35.71</td>
</tr>
<tr>
<td>Classic</td>
<td>5</td>
<td>11.91</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>42</td>
<td>100.00</td>
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</tbody>
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Figure1: (A) Mesenchymal-like components of pleomorphic adenomas showing areas of calcification, arrow.(B) Cell-rich pleomorphic adenoma shows the epithelial component with ductal structures, arrow (H&E, x400).

Figure2: Classic pleomorphic adenoma showing both (A) squamous differentiation, arrow and (B) mesenchymalmyxoid component, arrow (H&E, x100).

**Immunohistochemical results**

CD34 was expressed in all studied cases (n = 42). Figure-3 illustrates the CD34 expression in mesenchymal and epithelial components. In the present study, the MVD was more numerous in stroma-rich areas than cells rich regions. In addition, the myxoid areas showed a higher number of vessels.

The mean number of the CD34 positive micro vessels in all cases of PAs studied was 7.88±4.381. The mean number of the CD34 positive micro vessels in stroma-rich, cell-rich, and classic types was 11.90±0.867, 2.8±0.941, and 5.4±0.894 respectively. Statistical analysis showed a highly significant difference present between them regarding the MVD (P=0.0001).

Figure 3: Immunohistochemical expression of CD34 in pleomorphic adenoma. Note the lower microvessel density in cell-rich type (A) than the stroma-rich type (B) (A1,B1, Immunohistochemistry x100; A2,B2, Immunohistochemistry x400).

DISCUSSION

In the present study, the histopathological classification showed that the chief component of most PAs was Stroma-rich, followed by cell rich and classic. This result agrees with that of Ito et al12, they found that the stroma rich type constitutes 52.4% followed by cell-rich (36.5%), and the classic (11.1%). Stennert et al13 also found nearly the same result, in which 51% of pleomorphic adenomas studied were classified as stroma-rich type, 35% specimens as cellular type, and 14% as classic subtype. Khandker et al14 study showed stroma rich sub-type in 38.9% of cases, cell-rich subtypes in 36.1% of cases and classic sub-type in 25% of cases. Bhat et al15 and Paris et al16 were having a similar distribution of the subtypes with stromal subtype being the most common. But these results disagree with that of Nezhad et al17 study, in which they found that the cellular rich constitutes the higher percentages (38%), followed by the classic (32%) and stroma rich (30%). The dissimilarity to the present study probably due to different sample size and period of time.
Pleomorphic adenoma is the most common neoplasm of salivary glands and was shown sometimes to undergo malignant transformation in its course. Carcinoma ex pleomorphic adenoma is considered to be malignant transformation product of pre-existing PA. The pathogenic mechanisms involved in progression of normal salivary gland to PA and to a carcinoma remain unclear, requiring evaluation of molecular events.

Several studies investigated the role of neoplastic microenvironment in proliferation, invasion and metastasis of tumor cells. The increased MVD plays an important role in many physiologic and pathologic conditions and play an important part in cell nutrition in both malignant and benign tumors. The major factor contributing to vessel density is the metabolic demand, which is frequently increases during tumor progression.

The present study showed that, the MVD was more numerous in stroma-rich areas than cells-rich regions. This may be because of a higher metabolism and more oxygen demand. Moghadam et al and Soares et al found that the CD34 positive vessels were more numerous in cell-rich areas than cell poor regions of PA, and a lower number of vessels were detected in myxoid and chondroid areas. Xu et al found that the CD34 staining demonstrated many microvascular vessels in both the chondroid and osseous tissues. The difference in the expression of this marker in different studies could be related to the type of antibody used (monoclonal or polyclonal), and the differences in how the cells are counted.

The angiogenesis is refers to the development of new blood vessels. The multiplication of a tumor cell population is always preceded by an increased number of new capillaries. Tumors with a high vascular density are always associated with an increased metastatic potential and decreased survival. Czader et al and Rodriguez-Fernandez et al studies found that metastasis of PA of the salivary glands is a very infrequent but can occur and suggested that its metastases may be the first stage in the transformation of a tumor into a carcinoma. Thus, antiangiogenic drugs are considered as a potential target for cancer therapy.

The present study showed that, the MVD was more numerous in stroma-rich areas and a higher number of vessels were detected in myxoid areas. Trandafirescu et al found that the proliferation marker Ki67 presented a positive, intensely reaction, especially in the nuclei of the myxoid areas, so the target therapies which utilize the antiangiogenic agents aim at reducing the blood flow to the proliferative cancer cells and can slow down tumor growth especially in stroma-rich type and reduces formation of metastasis.

CONCLUSION

The stroma-rich subtype of the studied PAs being the most common than the cell-rich and classic subtype, and the MVD was more numerous in stroma-rich subtype, this may add an insight about the type of treatment needed for PA especially the stroma-rich subtype.

ETHICAL CLEARANCE

The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.
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REFERENCES


