Immunohistochemical Assessment of the Role of Tumor Microenvironment Associated with Odontogenic Neoplasms

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Submitted: 24-05-2024
Accepted: 22-06-2024

Abstract

Background: The diverse clinical behavior of the odontogenic neoplasms remain indistinct and may be related to the surrounding microenvironment of these neoplasms. The objective is to evaluate the immunohistochemical expression of CD163 and CD34 as elements of tumor microenvironment in different odontogenic neoplasms of variable nature and correlate them with the known clinical behavior. Methods: 15 cases of odontogenic hamartomas, 15 benign odontogenic tumors, and 15 cases of malignant odontogenic neoplasms were investigated by IHC. The count of the number of macrophages and blood capillaries in the stroma surrounding the tumor cells was detected in each section of the study groups; then, the data analysis was done for each study group by SPSS version 25. Results: Statistical analysis by SPSS version 25 revealed a significant difference regarding the count of macrophages (expressing CD163) and blood capillaries (expressing CD34) between hamartomas, benign, and malignant neoplasms groups. A statistically significant positive correlation was detected by the Pearson Correlation test between macrophages expressing CD163 immunostain and a number of microvessels expressing CD34 immunostain in odontogenic neoplasms of variable nature and behavior. Conclusion: A positive link is observed between the increase in the number of M2 macrophages (expressing CD163) and microvessel density (expressing CD34) surrounding tumor cells; both elements are strongly related to the clinical aggressiveness of the odontogenic neoplasms of variable nature.

Keywords: Benign odontogenic tumors / odontogenic hamartomas / malignant odontogenic tumors/ CD34 / CD163/ odontogenic tumor microenvironment

I. INTRODUCTION

Odontogenic neoplasms are usually considered an important sector of oral and maxillofacial tumors. These types of neoplasms arise mainly from any part of the odontogenic apparatus, which gives rise to the dental organ [1]. Aggressive-type odontogenic neoplasms are well known for their detrimental effects, including accelerated growth, severe bone expansion, and elevated rate of recurrence [1].

Odontogenic neoplasms include a variety of heterogeneous lesions extending from tumor-like malformations as odontoma (OD), benign neoplasms as ameloblastoma (AB), and their malignant counterparts as ameloblastic carcinoma (ABC) (known for their aggressive behavior and metastatic potential). Some benign odontogenic neoplasms are indolent lesions; others exhibit locally aggressive behavior. Benign odontogenic neoplasms are known for their indolent and self-limiting growth rate. However, the locally aggressive odontogenic neoplasms are usually described as probable local destruction [2].

According to the literature, the explanation of the diversity of the clinical behavior of these neoplasms is still indistinct and may be related to the surrounding Tumor microenvironment (TME)
of these neoplasms. Regarding the current literature, there is a great attention to the TME elements surrounding neoplasms, as the development of these types of oral neoplasms is probably determined by the components of the TME. Therefore, these factors will finally impact the biological behavior of these neoplasms and their mode of treatment [1&3]. Odontogenic neoplasms usually comprise mesenchymal stromal components such as blood vessels, fibroblasts, and immunological cells (principally macrophages and lymphocytes). Usually, the elements of the mesenchymal stroma of the odontogenic neoplasms support tumor cells with nutrients and oxygen supply [5]. Earlier clinical studies have specified that macrophages are one of the main regulators affecting progression of various medical conditions, dissemination, and clinical course [6]. Moreover, it was previously mentioned that macrophages are vital element in TME associated with the progression of some malignancies and influence tumor angiogenesis [7].

Tumor-associated macrophages (TAM) promote tumor growth and angiogenesis through its paracrine effect. This is mediated by the signaling pathway of endothelial nitric oxide synthase, which later stimulates macrophages polarization into immune-suppressive M2 phenotype. Indeed, the polarization process of TAM into M2 phenotype and abnormal hypoperfused vessels have been known as hallmarks of aggressive malignant neoplasms. Furthermore, the increase in M2 macrophage density is mainly correlated with rapid tumor growth and metastasis [1]. Myofibroblasts and blood vessels are crucial elements in connective tissue stroma for their participation in process of angiogenesis and tumor growth, as investigated previously by Kumar et al., 2016. Tumor immune histochemical (IHC) markers can be categorized into diagnostic and prognostic markers. Usually, prognostic markers such as CD163 and CD34 can predict the potential biological behavior of oral neoplasms [8&9].

The rational of this study was to assess the role of some elements of the TME surrounding odontogenic neoplasms. Therefore, we investigated vital element of TME surrounding different odontogenic neoplasms as density of M2 macrophage (using valuable CD163 IHC marker) and also the microvessel density using CD34 IHC marker (as an ideal marker for tumor-associated endothelial cells). Investigating the relation between both elements of TME and the known clinical behavior of these rare neoplasms could be considered as a novelty of this study, which would be helpful in altering patient’s life by developing targeted therapeutic approaches and reduction of the rate of extensive oral & maxillofacial surgeries [11].

II. SUBJECTS AND METHODS

The protocol for this study was revised and approved previously by the Research and Ethics Committee of the Faculty of Dentistry, Cairo University. The patients' files, which are registered in Oral and Maxillofacial Pathology Department (in the last seven years) were reviewed for case selection. Therefore, all the cases in this study have robust clinical data and were previously diagnosed in the Oral and Maxillofacial Pathology Department. The excised tissues of odontogenic neoplasms are stored as paraffin blocks and kept under certain storage conditions approved by the ethical committee. Therefore, we have the ethical committee's approval to use these neoplastic tissues without the need of the patient’s consent.

Forty-five total excised cases are included in this study from the Oral and Maxillofacial Pathology Department for immunohistochemical staining with CD163&CD34, divided into three main groups as 15 cases in odontogenic hamartomatos group (10 cases of OD & 5 cases of ameloblastic fibroodontome (AFO)), 15 cases in benign neoplasms group (10 cases of AB & 5 cases of Pindborg tumor (PT)) and 15 cases in malignant odontogenic neoplasms group (5 cases ABC, 5 cases clear cell odontogenic carcinoma (CCOC) & 5 cases primary intraosseous carcinoma (PIOC).

Three micrometers histological sections were cut from the paraffin-embedded tissue blocks for IHC protocol. All slides underwent a deparaffinization process, and then the rehydration step was done by submersing the slides in descending concentrations of ethanol. The antigen retrieval step was mainly performed by inserting slides to be heated in a microwave device for twenty-five minutes. At that point, all slides underwent blocking step (to stop the endogenous peroxidase activity), followed by immersing the glass slides in 3% hydrogen peroxide, considered an obligatory step. Phosphate-buffered saline (PBS) washing for all slides was done, followed by the antibody application step. The primary mouse, monoclonal antibodies of both immune markers, were diluted by phosphate buffer saline (PBS)1:100 to be used in this study (Santa Cruz Biotechnology Inc., Dallas, TX, USA). After that, the sections were submerged with the primary antibodies in a moist chamber, followed by two successive cycles.
of PBS washing. The necessary treatment was done at room temperature with a polymer-based complex anti-CD163 and anti-CD34 antibodies. Subsequent dipping the tissue sections in diaminobenzidine (liquid DAB+ substrate; Dako), which finally led to brown staining production. Ultimately, all the prepared tissue sections were counterstained with Harris’s hematoxylin and were protected coverslips [12].

IHC analysis was performed using SOPTOP EX20 biological microscope (China), HD camera (model No. XCAM1080PHB), and Image view software at X200 magnification power to assess all the immune stained histological sections by two blinded experienced pathologists as external examiners and using the image analyzer computer system applying QuPath-0.3.2 software, UK for image analysis. Scanning of the prepared slides were done at low magnification to select the fields with the highest number of microvessels and macrophages (hot spot fields). Then, five fields were measured from every histological section of each case using a high magnification (x400); this method was illustrated before by Pinheiro et al., 2020. In addition, the count of M2 macrophages immunostained with CD163 and the microvessel density expressing CD34 immunostain was measured.

Statistical analysis was performed using data collected from the image analyzer program, followed by checking the normality of the distributed data using (SPSS version 25; IBM ® Company). The parametric data were then presented as mean and standard deviation (±SD) values for each group of cases. Next, ANOVA test was statistically introduced for comparison between the 3 groups, this may be followed by Tukey’s Post-Hoc Test for pairwise comparison. Finally, the Pearson Correlation test was utilized to check the relation between CD163 and CD 34 immune markers. Data of 5% significance level will be considered significant.

III. RESULTS

As for the diagnosis of the H&E-stained sections was done according to the WHO classification (2017). Regarding cases of OD in the odontogenic hamartomas group, areas of dentine and pulp tissues linking with enamel spaces were observed in prepared sections. The arrangement of these structures was found to be organized in all cases of compound odontoma and disorganized in all cases of complex odontoma (Figure 1a). Concerning all cases of AFO, the neoplastic odontogenic epithelium is composed of peripheral ameloblast-like cells and central stellate reticulum-like cells. These cells are arranged in the form of small follicles and small branching cords and surrounded by primitive mesenchymal stroma, enclosing areas of retinoids and enamel spaces (Figure 1b).

As for the benign neoplasms group, cases of AB showed the neoplastic odontogenic epithelium composed of stellate reticulum-like cells surrounded by ameloblast-like cells. The neoplastic epithelial cells were arranged in follicles of variable appearance (follicular pattern) or anastomosing stands (plexiform pattern) and surrounded by mature connective tissue stroma (Figure 2a). Cases of PT showed neoplastic polyhedral epithelial cells with central hyperchromatic and pleomorphic nuclei. Prominent intercellular bridges were observed between the epithelial neoplastic cells. The surrounding fibrous tissue stroma showed amyloid-like production in many cases, with Liesegang calcifications in some cases (Figure 2b).

Regarding the H&E stained sections of the malignant odontogenic neoplasms, cases of ABC showed malignant odontogenic epithelium showing signs of dysplasia, arranged in a follicular or plexiform pattern and surrounded by fibrous connective tissue stroma (Figure 3a). All cases of PIOC revealed dysplastic epithelial tumor cells arranged in the form of nests or neoplastic islands. Some invaded dysplastic epithelial cells were masked by inflammatory cells within the surrounding fibrous stroma (Figure 3b). Regarding cases of CCOCs, some neoplastic epithelial cells showed clear cytoplasm, and others appeared as small polyhedral cells, all surrounded by hyalinized fibrous stroma (Figure 3c).

Upon examination of the immunohistochemically stained sections of the odontogenic hamartomas group, very few numbers of macrophages immunostained by CD163 were detected in many areas in the prepared sections. In contrast, other areas showed a complete absence of macrophages in cases of OD (Figure 1c) and AFO (Figure 1d). At the same time, few blood vessels expressed by CD34 immunostaining were observed in all cases of the odontogenic hamartomas group (Figure 1e & f). As for the benign odontogenic neoplasm group, many macrophages immunostained by CD163 were detected in the connective tissue stroma surrounding the tumor.
cells in most of the AB cases (Figure 2c) and some PT cases (Figure 2d). On the other hand, an obvious elevation of microvessel density was found in the cases of AB (Figure 2e) and PT (Figure 2f) compared with odontogenic hamartomas group cases. Regarding sections of all malignant odontogenic neoplasms group cases, numerous macrophages expressing CD163 were observed in the fibrous stroma surrounding the malignant neoplastic cells (Figure 3d, e &f). In addition, a marked increase in the microvessel density expressed by CD34 immunostaining was noted (Figure 3g, h &i) compared to the odontogenic hamartomas group.

Regarding the count of M2 macrophages (+ve CD163), the most outstanding mean value was noted in the malignant odontogenic neoplasms group. In contrast, the lowest mean value was documented in the odontogenic hamartomas group. ANOVA test revealed that there is significant difference between the 3 groups (P=or<0.05). Tukey’s post hoc for pairwise comparison test exposed a significant difference between the groups. All the recorded statistical values are represented in Table (1).

Regarding the microvessel density examination by counting blood vessels (+ve CD34), the greatest mean value was recorded in the malignant group, whereas the lowest value was documented in the odontogenic hamartomas group. ANOVA test revealed that the difference between the 3 groups was significant (P<0.05). However, Tukey’s post hoc revealed no statistically significant difference between the odontogenic hamartomas and benign neoplasms groups as seen in Table (2).

Statistical linking of the number of M2 macrophages expressing CD163 immunostain and microvessel density expressing CD34 immunostain, Pearson correlation test was done. It was found to be a moderate positive and statistically significant relation (r=0.6) between TAMs & microvessel density, independently of the type of the group. This test showed that the increase in the number of TAMs (defined by CD163 immune expression) is related to the increase in microvessel density (defined by CD34 immunostaining) in the tumor microenvironment (Table.3 & Fig.4).
Figure (1): Photomicrographs of odontogenic hamartomas group-stained sections showing (a) areas of dentine, pulp tissue and enamel spaces found in the OD, (H&E x200). (b) Odontogenic epithelium arranged in small follicles, anastomosing cords and surrounded by primitive mesenchymal stroma with areas of dentinoid found in AFO, (H&E x200). Very few numbers of macrophages expressing CD163 immunostain were found in (c) OD and (d) ABF (CD163 x200). (e) Absence of blood capillaries expressing CD34 immunostain in OD, (CD34 x200). (f) Small number of blood capillaries found in the surrounding stroma of AFO, (CD34 x200)
Figure (2): Photomicrographs of benign odontogenic neoplasms group stained sections showing (a) network and branching cords of neoplastic odontogenic epithelium composed of ameloblast like cells and central stellate reticulum like cells in plexiform AB , (H&E x200). (b) Sheets of odontogenic polyhedral epithelial cells with central pleomorphic nuclei and prominent intercellular bridges observed in PT(H&E x200). (c) Large number of macrophages expressing CD163 immunostain was found in AB (CD163 x200). (d) Some macrophages were detected in connective tissue surrounding the tumor cells in PT, (CD163 x200). (e) Obvious number of small blood capillaries expressing CD34 immunostain was noted in AB, (CD34x200). (f) Some large blood capillaries expressing CD34 immunostain are found to surrounding the tumor cells in PT (CD34x200)
Figure (3): Photomicrographs of malignant odontogenic neoplasms group stained sections showing (a) malignant odontogenic epithelium, arranged in both follicular and plexiform patterns and surrounded by fibrous connective tissue stroma in ABC, (H&E x200). (b) Islands of dysplastic epithelial cells masked by inflammatory cells found within the surrounding fibrous stroma in PIOC (H&Ex200). (c) Neoplastic epithelial cells showed clear cytoplasm or small polyhedral cells, surrounded by hyalinized fibrous stroma in clear cell odontogenic carcinoma (H&Ex200). (d) numerous macrophages expressing CD163 immunostain were found in the surrounding the malignant neoplastic cells in ABC, (e) PIOC and (f) CCOC (CD163 x200). (g) Obvious number of small blood capillaries expressing CD34 immunostain was noted in ABC, (h) PIOC and (i) CCOC (CD34x200).

Figure (4): Simple scatter and fit line diagram showed the positive and statistically significant correlation between CD34 &CD163
**Table (1): Count of macrophages expressed CD163 immunohistochemical stain in the examined groups**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean</th>
<th>SD</th>
<th>SE</th>
<th>Min.</th>
<th>Max</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign neoplasms</td>
<td>12.3a</td>
<td>10.7</td>
<td>3.4</td>
<td>0</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Locally aggressive neoplasms</td>
<td>119.7b</td>
<td>110.4</td>
<td>34.9</td>
<td>22</td>
<td>287</td>
<td>17.840</td>
<td>0.000*</td>
</tr>
<tr>
<td>Malignant neoplasms</td>
<td>238c</td>
<td>109.6</td>
<td>28.3</td>
<td>83</td>
<td>392</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Significant at p<0.05

Tukey’s post hoc test means sharing the same superscript letter are not significantly different.

**Table (2): Microvessel density expressed by CD34 immunohistochemical stain in the examined groups**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean</th>
<th>SD</th>
<th>SE</th>
<th>Min.</th>
<th>Max</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign neoplasms</td>
<td>7.2a</td>
<td>5.1</td>
<td>1.6</td>
<td>0</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Locally aggressive neoplasms</td>
<td>18.4a</td>
<td>11.6</td>
<td>3.7</td>
<td>6</td>
<td>39</td>
<td>28.5</td>
<td>0.000*</td>
</tr>
<tr>
<td>Malignant neoplasms</td>
<td>48b</td>
<td>18.7</td>
<td>4.8</td>
<td>17</td>
<td>83</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Significant at p<0.05

Tukey’s post hoc test means sharing the same superscript letter are not significantly different.

**Table (3): Pearson correlation test between density of macrophages expressing CD163 and microvessel density expressing CD34**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Mean</th>
<th>SD</th>
<th>r</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Counting the number CD163 immune expression</td>
<td>139.7</td>
<td>131.8</td>
<td>0.6</td>
<td>0.00*</td>
</tr>
<tr>
<td>CD34 immune expression</td>
<td>27.9</td>
<td>22.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Correlation is significant at the 0.01 level

**IV. DISCUSSION**

Tumor progression is known to be a complex route involving numerous contributors. An earlier study confirmed that angiogenesis is probably encouraged by the role of M2 macrophages. And both elements could contribute to the pathogenesis of different tumors with heterogeneous clinical behavior and prognosis [1].

TAMs (M2 macrophages) are noticeably significant in tumor development and progression. Several documented studies also recorded a high density of M2 macrophages in the TME, indicating a strong relationship between tumor progression and poor prognosis [1&13]. Nevertheless, the impact of the increase of M2 macrophages density and their relation with the microvessel density in the tumorigenesis of variable odontogenic neoplasms has not illuminated previously.

CD163 is documented to be specifically used to distinguish between polarized types of macrophages (M2 and M1). Although angiogenesis is not a straightforward process, with many vital factors controlling it, the selection of CD34 immunostain was made as it is considered the gold standard immune marker for angiogenesis [1].

In this study, the count of M2 macrophages (+ve) CD163 immunostain in the stroma surrounding tumor cells was significantly higher in the malignant odontogenic neoplasms than in any other study group according to statistical analysis. Even though there is a significant difference between the numbers of M2 macrophages in the benign odontogenic neoplasms group (as AB and PT) and the odontogenic hamartomas group (as AFO), such results are in accordance with previous study findings showing a high number of M2 macrophages in the stroma surrounding the malignant tumor cells. Moreover, this number is closely related and supports a clue regarding the progression and prognosis of oral cases of squamous cell carcinoma [13&14]. Also another study reported that there is a lack of significant difference in the number of TAMs between the benign and aggressive odontogenic lesions as AB & odontogenic keratocyst and other indolent odontogenic lesions as dentigerous cysts. Such results indicated that M2 macrophages might not be linked to the clinical behavior of the tumors [15].

In tumorigenesis, the most common technique utilized for semiquantitative assessment of angiogenesis is the microvessel density (MVD) using endothelial markers [16], commonly CD34.
and CD31. In this study, CD34 immunostain was used to assess microvessel density in the odontogenic hamartomas group, benign odontogenic neoplasms group, and malignant odontogenic neoplasms group. We observed that the mean value of microvessel density recorded in the malignant odontogenic neoplasms group was significantly greater than other groups of benign nature.

Our results are on the same track as the findings of a previous study inspecting microvessel density in variable odontogenic neoplasms and confirmed that an increase in microvessel density is associated with an increase in tumor aggressiveness and worsens its prognosis [17]. Furthermore, another earlier study proposed that angiogenesis is a main factor influencing the clinical aggressiveness of AB (benign and aggressive neoplasm) when compared to benign odontogenic (indolent) neoplasm as adenomatoid odontogenic tumor [18]. Also, comparison between the microvessel density was earlier done and showed a statistically significant difference in the expression of CD34 between odontogenic keratocyst (bening and aggressive odontogenic cyst) and dentigerous cyst (known for less aggressive behavior than odontogenic keratocyst) [19].

These points are consistent with the study's results, which showed a prominent increase in the neoangiogenesis found in malignant odontogenic neoplasms as ABCs compared to a locally aggressive benign neoplasm as AB. All of those discussed findings confirmed that the increase in angiogenesis is related to aggressive clinical behavior and impact the prognosis [20]. Another study mentioned in literature showed that lack of statistically significant difference in CD34 and CD163 immune expression between odontogenic keratocysts, radicular cysts and pericoronal follicle, although the highest numerical expression of these proteins were found in odontogenic keratocyst (most aggressive group of lesions). They concluded that their different findings compared to earlier studies may be related to the variation in the methodology used in application of the immune staining [21].

Finally, we tested the correlation between M2 macrophage count and microvessel density through the Pearson correlation test, which indicated a statistically significant positive relationship between both elements. Our study results are inconsistent with previous studies on esophageal squamous cell carcinoma, demonstrating that microvessel density strongly correlates with the number (density) of M2 macrophages [13 & 22].

Recently a study was done on cases of adenoid cystic carcinoma (malignant salivary gland neoplasm) compared to normal salivary gland tissue; it reported that the elevation of many M2 macrophages (expressing CD163) is correlated with an increase in the microvessel density (expressing CD31). This finding confirms that M2 macrophages have a leading and stimulating role in the process of angiogenesis. Furthermore, this correlation signifies their role progression and prognosis of such tumors. Nowadays, targeting therapy for elements of tumor microenvironment has positive impact on improving the line of treatment of these tumors instead of the extensive surgical removal approach. [23].

At the end we must clearly mention the limitations for this work was the small number of cases included in our study, which is attributed to the rarity of these neoplasms and excluding cases of incomplete clinical data. In addition, we couldn't investigate our study elements by other methods due to the restricted amount of tissue sections available for research study and the rarity of these types of neoplasms.

V. CONCLUSION

We noted that the density of M2 macrophages and microvessels are critical elements in the TME of odontogenic neoplasms. The elevation in the microvessel density and the number of M2 macrophages strongly correlated with the aggressiveness of most odontogenic neoplasms. A statistically significant and positive relationship is observed between the number of M2 macrophages (expressing CD163) and microvessel density (expressing CD34) surrounding odontogenic tumor cells. Through this conclusion, clinicians can hinder the progress or recurrence of such neoplasms by targeting treatment to such elements (TAMs) and angiogenesis. Finally we recommend investigating other elements of TME surrounding these types of odontogenic neoplasms.

Conflict of Interest:
The authors declare no conflict of interest.

Funding:
This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors

Ethics:
This study protocol was approved by the research ethics committee of the faculty of dentistry- Cairo university on approval June 2022 with ID number: 27622.
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