Introduction

Malignant salivary gland tumors (MSGTs) constitute an extensive collection of highly heterogeneous tumors that exhibit a broad spectrum of histology. These tumors have variable biological behavior ranging from indolent and slow-growing to highly aggressive and rapidly fatal, reaching precise diagnosis and appropriate treatment strategy [1].

Recent research points out the critical role of cancer stem cells (CSCs) on tumor progression and virulence [2]. Therefore, targeting CSCs with specific CSC markers are a sensible way for optimal cancer therapy with less harmful effects on healthy cells [3].

One promising target of interest is the c-kit (CD117) receptor which causes specific expression of certain genes, regulates cell differentiation and proliferation, resists cell apoptosis, and plays a crucial role in tumor occurrence, development, migration, and recurrence through activating the downstream signaling molecules following interaction with its ligand stem cell factor (SCF) [4]. Although, c-kit dysregulation promotes malignant cells’ proliferative, invasive, and metastatic activities. The process of tumor invasion into the surrounding tissue is closely associated with the deregulation of the cell to cell and extracellular matrix adhesion apparatuses [5].

Tight junction protein (TJ) is a cell adhesion molecule that includes claudins, occludin, and junctional adhesion molecules. Claudin-1 is the most studied claudins member family in cancers, and either up or down-regulation plays a key role in tumor invasion and aggressiveness [6]. When tumor cells lose cell-to-cell contact, they can transverse vascular walls and colonize distant organs [7].

Lymph vessels provide the main avenue for nodal metastasis. The migration of cancer cells into the lymphatic circulation and entry into the regional lymph nodes is greatly facilitated by tumor lymphangiogenesis. This process generates new lymphatic vessels from pre-existing conduits [8]. Podoplanin is a specific marker for lymphatic endothelial cells (LECs) and lymphangiogenesis in tumors. It has a considerable
role in tumor metastasis and invasion through its ability to remodel the actin cytoskeleton of tumor cells [9].

Aim of the study

The objective of this work is to evaluate the immunohistochemical expression of c-kit, claudin-1, and measure the lymph vessels density (LVD) using D2-40 (podoplanin) antibody among common MSGTs.

Material and Methods

Tissue samples

A total of 50 specimens containing adenoid cystic carcinoma (AdCC) (20), mucoepidermoid carcinoma (MEC) (11), carcinoma ex pleomorphic adenoma (10), acinic cell carcinoma (six), and polymorphous adenocarcinoma (PAC) (three) were retrieved from the archival paraffin blocks from the files of the Oral and Maxillofacial Pathology Department, Faculty of Dentistry, Mansoura University, as well as, the files of General Pathology Department, Faculty of Medicine, Cairo University, and General Pathology Department, Faculty of Medicine, Ain-Shamas University, during the period from September 2015 to October 2020.

Thin (4 microns thick) paraffin sections of each tissue specimen were stained with hematoxylin and eosin stains to reconfirm the diagnosis according to the World Health Organization histological typing of MSGT (2017).

MEC cases were classified into five cases of low- and six cases of high-grade tumors depending on AFIP grading [10]. Cases of AdCC were classified into seven cases of Grade I – cribriform or tubular pattern, eight cases of Grade II – <30% solid pattern, and five cases of Grade III – more than 30% solid pattern according to Szanto et al., [11].

Paraffin sections were mounted on positively charged glass slides (Optiplus; Biogenex, Millmont Drive, CA, USA) for immunostaining. The immunohistochemical staining procedure was performed using P63, P-40, S100, pan-cytokeratin, DOG-1, AE1/AE3, and SMA for the appropriate diagnosis of obscure cases.

Immunohistochemical staining

Three sections were serially cut from each paraffin block and placed on positively charged (Optiplus) slides for the staining procedure. With a super sensitive biotin-streptavidin staining technique, these slides were prepared to receive antibodies (c-kit, claudin-1, and podoplanin antibodies). To block the endogenous peroxidase activity, tissue sections were deparaffinized, rehydrated, and treated with endogenous peroxidase in 0.3% H2O2 for 30 min. For antigen retrieval, the slides were boiled in 10 mL citrate buffer, pH 6.0 for 10–20 min, then cooled at room temperature for 20 m. The positive test slides were incubated with the primary antibodies of rabbit polyclonal anti-c-kit antibody (Cat. A0357; ABclonal Technology, U.S.A.), rabbit polyclonal anti-claudin-1 antibody (Cat. A2196; ABclonal Technology, U.S.A.), and mouse monoclonal anti-podoplanin antibody (Cat. MC0329; Medayesis, U.S.A.), with the appropriate dilution range 1:50 for 30 min at room temperature in a humidity chamber. On the other hand, the negative control slides were not exposed to the primary antibodies.

After washing with phosphate buffer solution, the slides were treated with the biotin-labeled link antibody, then the streptavidin conjugated to horseradish peroxidase was used. The diaminobenzidine chromogen was applied to visualize the antigen-antibody reaction. All these reagents belong to the universal Labeled Streptavidin-Biotin 2 System, Horseradish Peroxidase (code no. K0673 DakoCytomation, Glostrup, Denmark). All the slides were immersed in Mayer’s hematoxylin for counterstaining. Finally, the sections were covered by coverslips using an aqueous mounting medium.

Evaluation of c-kit and claudin-1 positive tumor cells

Image analysis Computer System is used to assess area percentage of positive cells and intensity of the immunostaining. Sections were examined with a magnification of 40x by light microscope (Olympus, Bx60, Japan) connected to a digital video camera (C5060, Olympus, Japan) and computer. The image analyzer computer system examined all stained sections using the Image J software (Image J, 1.41a, NIH, USA).

The degree of immunoreactivity was evaluated through summation of the score of area percentage of positive malignant epithelial cells and intensity of the immunostaining depending on Fan et al. [12] and Lu et al. [13] for c-kit and claudin-1, respectively. The percentage of positive tumor cells was scored as (0=0%; Score 1 =< 25%; Score 2 = 25–50%; and Score 3 = >51%), while, the intensity of immunostaining between cancerous cells was scored as (0 = 0; 1 = 1+; 2 = 2+; and 3 = 3+). The two scores were multiplied to get a total score which was ranged from 0 to 9. If the multiplication result was (1–2) that means a weak reaction, (3–6) moderate, and (9) strong immunoreactivity. Finally, weak expression was represented low immunoreactivity; meanwhile, moderate and strong expressions were considered as high immunoreactivity.
**Quantification of lymphatic microvessel density**

Evaluation of lymphatic microvessel density (LMVD) was carried out by counting positive podoplanin lymphatic vessels regardless of the position of packed vessels, peritumoral or intratumoral. Immunohistochemical D2-40-stained sections were scanned at low magnification to identify the most vascular areas (hot spot areas). The images were captured on the computer under \((x200)\) objectives to count the number of lymphatics using the Image J software. The average of 10 hot spot fields was taken to calculate LMVD [14].

**Statistical analysis**

Data were fed to the computer and analyzed using IBM SPSS Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. Monte Carlo test for comparison of two or more groups. The significance of the obtained results was judged at the \((0.05)\) level. Spearman’s rank-order correlation is used to determine the strength and direction of a linear relationship between two non-normally distributed continuous variables and ordinal variables. A one-way ANOVA test was used to compare more than two independent groups.

**Results**

**Clinical findings**

The present work was composed of 27 males (54%) and 23 females (46%) with a mean age of 49.96 ± 13.65 years (ranging from 19 to 76 years). The studied MSGTs were found between three different anatomical sites of salivary glands. About half of them were reported in the parotid gland (48%), followed by the palatal (36%) and the submandibular gland (16%).

On the other hand, the present study revealed four various tumor sizes, including T1, T2, T3, and T4. The highest frequent tumor size among current tumors was T2 (36%), followed by T3 (32%) and T1 (20%), whereas T4 (12%) was the lowest frequent tumor size. However, tumor involvement for regional lymph nodes was observed among 26% of studied MSGTs.

The evidence of perineural invasion was lacking in most current samples, while (14%) displayed positive invasion for neural tissue.

**Immunohistochemical findings with c-kit (CD117)**

Three histological patterns, cribriform, tubular, and solid of AdCC showed high reactivity for c-kit, which was strong in intensity between all stained sections. The expression of the c-kit was noticed as cytoplasmic/membranous and nuclear location (Figure 1a and b).

![Figure 1: Cases of adenoid cystic carcinoma showing high c-kit immunoreactivity throughout cancerous cells, (a, c-kit antibody ×400 and b, c-kit antibody ×200). A photomicrograph of high grade mucoepidermoid carcinoma demonstrating high c-kit immunoreactivity, (c, c-kit antibody ×200). A photomicrograph of carcinoma ex polymorphic adenoma displaying high c-kit immunoreactivity of neoplastic cells, (d, c-kit anti-body ×200). A high magnification of AcCC revealing membranous immunostaining outlining the neoplastic cells with faint cytoplasmic and negative nuclear reaction, (e, c-kit antibody ×400). A photomicrograph of polymorphous adenocarcinoma showing unequal distribution of high c-kit immunostaining between neoplastic cells, (f, c-kit anti-body ×200)](image)

Moreover, all six high-grade MEC cases interacted highly with the c-kit antibody. Meanwhile, 3/5 cases of low-grade malignancy showed low immunostaining reaction (Figure 1c).

Regarding to carcinoma ex polymorphic adenoma (CXPA), (80%) of cases expressed great variability of c-kit immunoreactivity. Three cases revealed high reactions for the c-kit biomarker. Meanwhile, five cases demonstrated low c-kit reaction (Figure 1d).

Four out of six studied cases (66.7%) of acinic cell carcinoma (AcCC) revealed low reactivity for the c-kit antibody. Furthermore, the reaction of c-kit was expressed as faint cytoplasmic/membranous immunopositivity throughout malignant cells (Figure 1e).

The entire samples of polymorphous adenocarcinoma (PAC) responded highly to the c-kit biomarker. The dissimilar pattern of c-kit immunostaining...
was prominent; some neoplastic cells had cytoplasmic/membranous and nuclear immunoreactivity, while the others were devoid of reaction (Figure 1f). A significant difference was observed between studied MSGTs and immunoreactivity of c-kit, Table 1.

**Immunohistochemical findings with claudin-1**

Claudin-1 immunostaining was observed in 14 (70%) of AdCC samples distributed between nine cases expressing low reactivity and five highly immunoreaction cases. Furthermore, the immunopositivity of Cld-1 was found in the membrane, cytoplasm, and nucleus of cancerous cells (Figure 2a and b). On the other side, 8/11 (72.7%) cases of MEC demonstrated Cld-1 immunostaining distributed equally between low- 4 (36.4%) and high-grade MEC, 4 (36.4%). The immunoreactivity of Cld-1 was decreased in high-grade MEC (grammar error) compared to the low grade of malignancy, (Figure 2c, and d). However, 60% of cases of CXPA exhibited low to high Cld-1 immunopositivity (Figure 2e and f).

Claudin-1 immunostaining was detected in 4 cases (66.7%) of AcCC as a high reaction. The positivity of Cld-1 was in the form of a diffuse cytoplasmic/membranous pattern. The membranous reaction was apparent surrounding tumor cells, whereas cytoplasmic staining was less tonality, (Figure 2g). In addition, all examined cases of PAC revealed low Cld-1 immunoreactivity, which was presented as cytoplasmic staining between malignant cells (Figure 2h). Statistical analysis showed no significant difference between the studied MSGTs and Cld-1 immunoreactivity (Table 2).

**Immunohistochemical findings with podoplanin (D2-40)**

The examined cases of MEC recorded the highest mean value of LMVD (17.53 ± 9.57) among the studied groups of MSGTs where the intratumoral LVs were prevalent in comparison to peritumoral LVs, particularly between the six cases of high-grade tumor (Figure 3a).

The second-highest density of LVs after MEC was shown between samples of CXPA (11.53 ± 10.29), which was detected only in the peritumoral region of the tumor (Figure 3b). Meanwhile, D2-40 expression was observed as peritumoral LVs among 9(45%) and intratumoral LVs between 11 (55%) of AdCC with a total mean value of LMVD (8.54 ± 5.214) (Figure 3c).

The positive lymphatic vessels between all current cases of PAC were concentrated in the peripheral area of the tumor with a mean value of LMVD (1.07 ± 0.16). However, the stained vessels in the bulk of malignancy were absent among all examined tumors (Figure 3d).

<table>
<thead>
<tr>
<th>Table 1: Distribution of c‑kit immunoreactivity between studied cases of malignant salivary gland tumors</th>
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</thead>
<tbody>
<tr>
<td>c‑kit Reactivity</td>
</tr>
<tr>
<td>Low</td>
</tr>
<tr>
<td>High</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

The lowest density of lymphatic vessels among the selected cases of MSGTs was identified in AcCC with a mean value of (0.92 ± 0.65) (Figure 3e and f).

Based on the previous results, the statistical analysis revealed a significant difference between studied MSGTs in terms of the mean lymphatic density values (Table 3).

Based on statistical analysis, there was a direct correlation between c-kit and lymphatic density. However, there was an inverse correlation between c-kit and cld-1 and cld-1 and lymphatic density (Table 4).

**Table 3**: Distribution of mean values of lymphatic density (D2-40) between studied malignant salivary gland tumors

<table>
<thead>
<tr>
<th>Lymphatic density</th>
<th>AdCC (n = 20), n (%)</th>
<th>MEC (n = 11), n (%)</th>
<th>CXPA (n = 10), n (%)</th>
<th>AcCC (n = 6), n (%)</th>
<th>PAC (n = 3), n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>8.54 ± 5.24</td>
<td>17.53 ± 9.57</td>
<td>11.53 ± 10.29</td>
<td>0.92 ± 0.65</td>
<td>1.07 ± 0.16</td>
</tr>
</tbody>
</table>

*ANOVA test. *Statistically significant if $p < 0.05$. AdCC: Adenoid cystic carcinoma, MEC: Mucoepidermoid carcinoma case, CXPA: Carcinoma ex polymorphic adenoma, AcCC: Acinic cell carcinoma, PAC: Polymorphous adenocarcinoma, SD: Standard deviation.

**Table 4**: Correlation between studied markers among all studied cases

<table>
<thead>
<tr>
<th>Marker</th>
<th>C-kit</th>
<th>Cld-1</th>
<th>Lymphatic density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rs</td>
<td>1.00</td>
<td>−0.125</td>
<td>0.565**</td>
</tr>
<tr>
<td>p</td>
<td>0.019</td>
<td>0.046</td>
<td>0.000</td>
</tr>
<tr>
<td>Rs</td>
<td>−0.349*</td>
<td>1.000</td>
<td>0.423</td>
</tr>
<tr>
<td>p</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

*p: p-value, Rs: Spearman correlation coefficient, Cld: Claudin-1.

**Discussion**

The role of CSCs and their specific markers are well documented in human malignancies. One of the compelling markers is c-kit/CD117, a tyrosine kinase receptor associated with cancer progression and normal stem cell maintenance. Further activation of CD117 by its ligand (SCF; kit ligand) in the progenitor cell niche stimulates several signaling pathways driving proliferation, survival, and migration [15].

Recent studies indicate that CSCs undergoing epithelial-mesenchymal transition (EMT) show higher migration and metastatic abilities and are resistant to chemoradiotherapy. CSCs undergoing EMT invade the blood or lymph vessels, circulate in the systemic network of vessels (circulating tumor cell/CTC), and metastasize in other organs [16].

The main observation of this study was that all cases of AdCC demonstrated intense positive staining for CD117 (c-kit). This finding was in agreement with the previous immunohistochemical investigation of Uraizee et al., [17], who considered that c-kit is a reliable marker for AdCC, so it supports the concept of using c-kit inhibitors as a suitable treatment strategy for AdCC.

The present work detected high c-kit immunoreactivity in the whole specimens of high-grade MEC. This was well supported by a previous study by Salehinejad et al. [18], who observed high c-kit immunoreactivity in the component of tumors that...
exhibited solid sheets of dedifferentiated anaplastic cells. An increase of c-kit positive staining indicates tumor cells’ raised stemness properties, which possess a high proliferative potential, self-renewal capacity, and aggressive behavior.

The analysis of c-kit expression between cases of CXPA displayed low to high immunoreactivity in (80%) of specimens. Discrepancies in c-kit patterns may reflect differences in histological subtypes of CXPA arising from the malignant transformation of ductal epithelial cells and myoepithelial cells of pleomorphic adenoma.

By screening the cases of AcCC, c-kit immunopositivity was demonstrated in (66.7%) of cases as a faint membranous reaction delineating the malignant cells. Membranous reaction might be reflected in the communication between tumor cells and their surrounding stroma through the c-kit receptor. Concerning PAC, all samples responded highly with a dissimilar pattern of staining. A similar result was compatible with the work of Salehinejad et al. [18]. Who clarified the role of mutated c-kit as a promotor for this tumor; on the contrary, it was disagreement with Tariq et al. [19], who found c-kit immunoreactivity may be used as a helpful marker to distinguish AdCC from PAC in cases where the diagnosis can be challenging. In this light, a distinct pattern of c-kit immunostaining can also be a proper ancillary method for further differentiation between both tumors, as noticed in our results.

Recent studies have also highlighted the fundamental role of EMT in acquiring and maintaining stem cell-like properties. The process is characterized by losing epithelial markers, including tight junction proteins. Claudin-1 is the most-studied TJ protein in cancers, and either up or down-regulation plays a key role in tumor invasion and aggressiveness [6]. Studies analyzing the expression of claudin-1 in SGTs are scarce and controversial. Thus, the current work was done to shed some light on the relation of claudin-1 with tumorigenic growth and aggressiveness. Studies of PAD depend on autophagy. Based on Soares et al. [23] demonstrated that the growth of PAD depends on autophagy. In addition, Kim et al. [24] reported that cytoplasmic immunostaining of Cld-1 is linked to autophagy, which acts as an autophagy regulator protein. All of this evidence confirms that Cld-1 can be promoted cancer progression through autophagy regulation.

Count down of stained lymph vessels among studied groups was MEC on the top, followed by CXPA, AdCC, and PAC; meanwhile, AcCC had the lowest number of stained LVs. The sequence reflects the ability of these malignancies to stimulate the formation of new lymphatic vessels in harmony with their biologic aggressiveness. The most significant number of LVD (17.53 ± 9.57) was recorded in the group of MEC. Moreover, the incidence of intratumoral LVD between MEC cases was higher than that of peritumoral LVs. Various studies have investigated the impact of intratumoral LVD on different types of cancer, including oral squamous cell carcinoma [25]. These studies have reported induction of intratumoral LVs, leading to a greater incidence of lymph vascular invasion and subsequently distant metastasis.

With respect to selected samples of CXPA, the mean value of LMVD was (11.53 ± 10.29). Furthermore, positive LVs were detected in the peritumoral side. The absence of intratumoral lymphatic vessels in CXPA may reflect collapsing the lymphatic vessels and pushing the preexisting vessels outside the tumor.
On the other hand, in examined cases of AdCC, the total mean value of LMVD was $(8.54 \pm 5.214)$. Moreover, D2-40 expression was observed in each of the peritumoral and intratumoral LVs. Therefore, the location of these vessels in AdCC is less effective in the traffic of tumor cells for this type of malignancy.

The samples of PAC demonstrated a low mean value of LMVD $(1.07 \pm 0.16)$. The vessels mainly were concentrated at the periphery of the tumor. A poor lymphatic vascularization may explain why metastasis is rarely found in PAC.

AcCC had the lowest mean value of density $(0.92 \pm 0.65)$ among all studied salivary neoplasms. This might probably be due to the metastatic dissemination of malignant cells through the lymphatic system being less effective in AcCC.

In the present study, the alterations in the immunohistochemical expression of CD117/c-kit (stem cell marker) were inversely correlated with claudin-1 immunoreactivity and parallel to lymphatic density. These findings prove the responsibility of CSCs in salivary malignancies progression and metastasis.

**Conclusion**

The stem cell marker c-kit (CD117) is a vital driver molecule for malignant salivary gland progression, survival, and aggressiveness. Moreover, up-regulation of that molecule promotes the capacity of cancerous cells to invade the surrounding stroma by reducing the tightness of adhesion between the cells involving claudin-1 and stimulating the formation of more lymph vessels to reach the distant organs. Therefore, further investigation is required to evaluate the potential therapeutic effects of small molecule inhibitors of c-kit on these tumors.

**Ethical Considerations**

The research followed the tenets of the Declaration of Helsinki. The Institutional Ethical Committee at Mansoura University (Dental Research Ethics Committee, approved all study protocols (IRB approval number: A07091019). The study was based on data collection and immunohistochemical analysis of positively charged slides prepared from paraffin blocks; therefore, informed consent did not apply to our research. This study was extracted from the philosophy Doctor Thesis of Marwa Mohamed Maghrabi registered at the Faculty of Dentistry Mansoura University. Besides, the authors have completely observed ethical issues (including plagiarism, data fabrication, and double publication).

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