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Immunohistochemical study of the epithelial and stromal components of Warthin's tumor

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Abstract

Warthin's tumor is a benign monomorphic adenoma with unclear origin with the highest incidence in the sixth and seventh decades. The analysis of tumor markers involved in the pathogenesis of Warthin's tumor can improve the patients' prognosis. This study included 29 cases of Warthin's tumor, which were histopathologically and immunohistochemically examined for different compartments of tumors. For immunohistochemical study, we used as specific markers for epithelial compartment CD117, CEA and AMA, respectively S100 and D2-40 for the stromal compartment. The evaluation of immunoreactions was performed by semiquantitative analysis. The analysis of the CEA, CD117 and AAM immunoexpression allowed observing various patterns of immunostaining for tumor double-layered epithelia, which has the tendency of being similar to that in the normal ductal epithelia. S100 protein positivity similar to Langerhans cells suggests that delayed hypersensitivity can be involved in tumor development. The presence of D2-40 expression in majority of tumor subcapsular vessels, similar to lymph nodes structure, confirms the hypothesis that Warthin's tumor has its origin in regional lymph nodes.

***Keywords*:** Warthin's tumor, epithelial markers, stroma, immunoreaction.

Introduction

Benign salivary tumors are commonly met in practice [1], Warthin's tumor being the most frequent monomorphic adenoma of the major salivary glands [2].

This neoplasia, almost exclusively benign and asymptomatic [3], continues to be a studied theme in many researches. Throughout time had appeared varied theories about Warthin's tumor etiopathogenesis like that its origin develops from salivary duct inclusions in the lymph nodes [1, 4] or from the lymphocytic infiltration in a pre-existing adenoma [5].

Recently studies consider that autoimmune reactions can be responsible for Warthin's tumor development [6]. These researches concluded that epithelial proliferation is due to hypersensitive or allergic reaction, which can cause a increased reactivity of the germinal centers from the lymphoid stroma [6].

Histologically, Warthin's tumor is a well-encapsulated lesion with dual component, oncocytic epithelium and lymphoid stroma [7].

Use of immunohistochemisty to differentiate luminal cells and lymphoid stroma can help in understanding the complex architecture of Warthin's tumor. Our immunohistochemical study use a panel of specific antibodies for epithelial and stromal compartments represented by CD117, CEA (carcinoembryonic antigen), AMA (anti-mitochondrial antibody), S100 and D2-40 with aim to select the markers involve in tumor pathogenesis.

Materials and Methods

We reviewed medical records in the period 2011–2014 from the Laboratory of Pathology, Emergency County Hospital of Craiova, Romania, and identified 29 patients with Warthin's tumor diagnosis. The surgical pieces were processed by common histopathological technique using 10% formalin fixation, paraffin embedding and Hematoxylin–Eosin (HE) staining.

The immunohistochemical analysis was performed using LSAB+ System-HRP (Horseradish Peroxidase) technique (code K0690, Dako) and mouse antihuman monoclonal antibodies, addressed to epithelial and stromal compartments of the tumors (Table 1).

Table 1 – ***The antibodies used in the study***

Antibody	Clone	Dilution	Antigen retrieval	Positive controls
CEA	II-7	1:50	Citrate, pH 6	Breast carcinoma
CD117	Polyclonal	1:400	Tris-EDTA, pH 9	Tonsil
AMA	MUC213-UC	1:200	Citrate, pH 6	Liver
S100	Polyclonal	1:500	Citrate, pH 6	Schwannoma
D2-40	D2-40	1:100	Citrate, pH 6	Tonsil

The semiquantitative assessment of the immunohistochemical reactions took into account the percentage of labeled cells (<25%, 25–75% and >75%) and which have been considered as weak (+), moderate (++) or intense (+++). In this study, we used external positive and negative controls to validate the reactions.

The quantification of lymphangiogenic potential of tumors was performed using morphometric analysis. The immunostaining of lymphatic vessels (D2-40) was done in the context of assessing the lymphatic vessel density (LVD). For that, "hot spot" morphometric method was used, which consisted of manual quantification of vessels. Microscopic fields (MFs) with the highest vascular density at 20× objective were chosen, quantification being achieved at 40× objective, by choosing 10 intratumoral and advanced edge areas. The final result was the arithmetic mean of the vessels in the selected areas. All the results were statistically analyzed in SPSS 10 software using the *chi*-square test for dependence assessment.

Results

From 29 selected cases with Warthin's tumor, the majority developed in the seventh decade of life (13 cases), men being affected more frequently than women in a ratio of 2.6 to 1.

Histopathological analysis indicated that tumors had an oncocytic epithelial cell component arranged in double layers, which developed cysts and papillary projections and a variable stromal component with lymphoid tissue, which presented lymphoid follicles with germinative centers for 16 (55.1%) tumors. According to the two tumoral components, epithelial and stromal, we observed three distinct architectural patterns: typical form (with a balanced rapport between stroma and parenchyma) in 19 (65.5%) cases, with poor stroma in 8 (27.6%) cases and in two (6.9%) cases with abundant stroma.

The analysis of the CEA, CD117 and AMA immunoexpression allowed to observe various patterns of immunostaining for tumor double-layered epithelia, which has the tendency of being similar to that in the normal ductal epithelia.

Immunostaining analysis for CEA was positive in 26 (89.6%) cases, only into luminal columnar cells, in most (19) cases being positive in less than 25% of cells (Table 2). About immunostaining intensity of columnar cells, we found the presence of a weak reaction in most (14) cases. In only seven cases, the immunostaining was present between 25–75% of cells, with weak or moderate reaction intensity (Figure 1).

Table 2 – ***Immunostaining evaluation (No. of cases) for CEA and CD117***

Immunostaining		CEA			CD117		
		+	++	+++	+	++	+++
<25%	*luminal cells*	11	8	–	–	–	–
	basal cells	–	–	–	–	–	–
25–75%	*luminal cells*	3	4	–	–	3	8
	basal cells	–	–	–	–	–	–
>75%	*luminal cells*	–	–	–	–	3	6
	basal cells	–	–	–	–	–	–

The immunoreaction for CD117 was positive in 20 (68.9%) cases, limited to luminal columnar cells. We observed the absence of immunostaining corresponded to typical form of tumor in seven cases and form with poor stroma in two cases (Figure 1).

CD117 immunoexpresion analysis indicated positivity at the level of luminal epithelial cells with moderate and high intensity in six (20.6%) and 14 (48.2%) cases, respectively (Table 2).

We found the positivity between 25–75% of tumor cells in 11 cases compared to over 75% of tumor cells that were assigned the nine cases.

The immunoreaction for AMA indicated positivity in all analyzed cases, the staining being cytoplasmic at the level of the columnar cells with granular pattern and variable intensity. In addition, we observed diffuse positivity in rare cells of lymphoid stroma and more frequently in the peripheral lymphoid follicles (Figure 1).

At the level of the epithelial component of the AMA, the immunostaining indicated positivity in 11 (37.9%) cases in a percentage between 25–75% and in another 18 (62.1%) cases to more than 75% of columnar cells. The immunostaining of columnar cells was moderate in nine (31%) cases and strongly positive in the other 20 (68.9%) cases (Table 3).

Table 3 – ***Immunostaining evaluation (No. of cases) for AMA and S100***

Immunostaining		AMA			S100		
		+	++	+++	+	++	+++
<25%	*parenchyma*	–	–	–	17	8	–
	stroma	16	–	–	19	7	–
25–75%	*parenchyma*	–	4	7	1	3	–
	stroma	–	–	–	2	1	–
>75%	*parenchyma*	–	5	13	–	–	–
	stroma	–	–	–	–	–	–

For all studied tumors, we also got into consideration the immunoexpression of some markers that were addressed to stromal component, represented by S100 and D2-40.

The analysis of immunostaining of S100 indicated the presence of reaction in all investigated cases, both in the epithelial and frequently in the stromal component, (Figure 2).

For most cases (26 – 89.6%), the quantitative assessment of stromal S100 immunostaining indicated a positivity of less than 25% in tumor cells (Figure 2). In the epithelial component, positive immunoreactions were less than 25% of the cells in the majority of the investigated cases (25 – 86.2%), predominant of low intensity (Table 3).

Immunostaining analysis for D2-40 indicated positivity at the level of sinus blood vessels located subscapular or in follicular area and in some dendritic cells located in the lymphoid stroma, or in the lymphoid follicle structure (Figure 2). Also, the staining was observed in epithelial tumoral cells.

Comparative analysis of lymphatic vessel density (LVD) in the subcapsular lymphatics or lymphoid follicles showed different values for different variety of tumors (Table 4).

For cases of Warthin's tumor with stromal predominance, subcapsular LVD mean value was 12.7±1.2 vessels/MF, while the average value of 36.7±4.1 stromal vessels/MF. For cases with epithelial predominance, we found a subcapsular average of 6.5±1.3 vessels/MF and an average

of 22.8±3.4 stromal vessels/MF. For cases with epithelial–stromal balanced rapports, the subcapsular average value was 8.0±2.1 vessels/MF, and an average of 21.9±4.2 stromal vessels/MF. *Chi*-square test indicated a highly significant correlation ($p<0.01$) between the tumor type and subcapsular LVD ($p=0.014$). We found no other differences between the type of tumor and stromal LVD ($p>0.05$) or between stromal LVD and subcapsular LVD ($p>0.05$).

Table 4 – ***LVD values taking into account the tumoral type***

Histological type	*Stromal predominance*		*Epithelial predominance*		*Typical*	
Topography	***Subcapsular***	***Stromal***	***Subcapsular***	***Stromal***	***Subcapsular***	***Stromal***
LVD	12.7	36.7	6.5	22.8	8.0	21.9

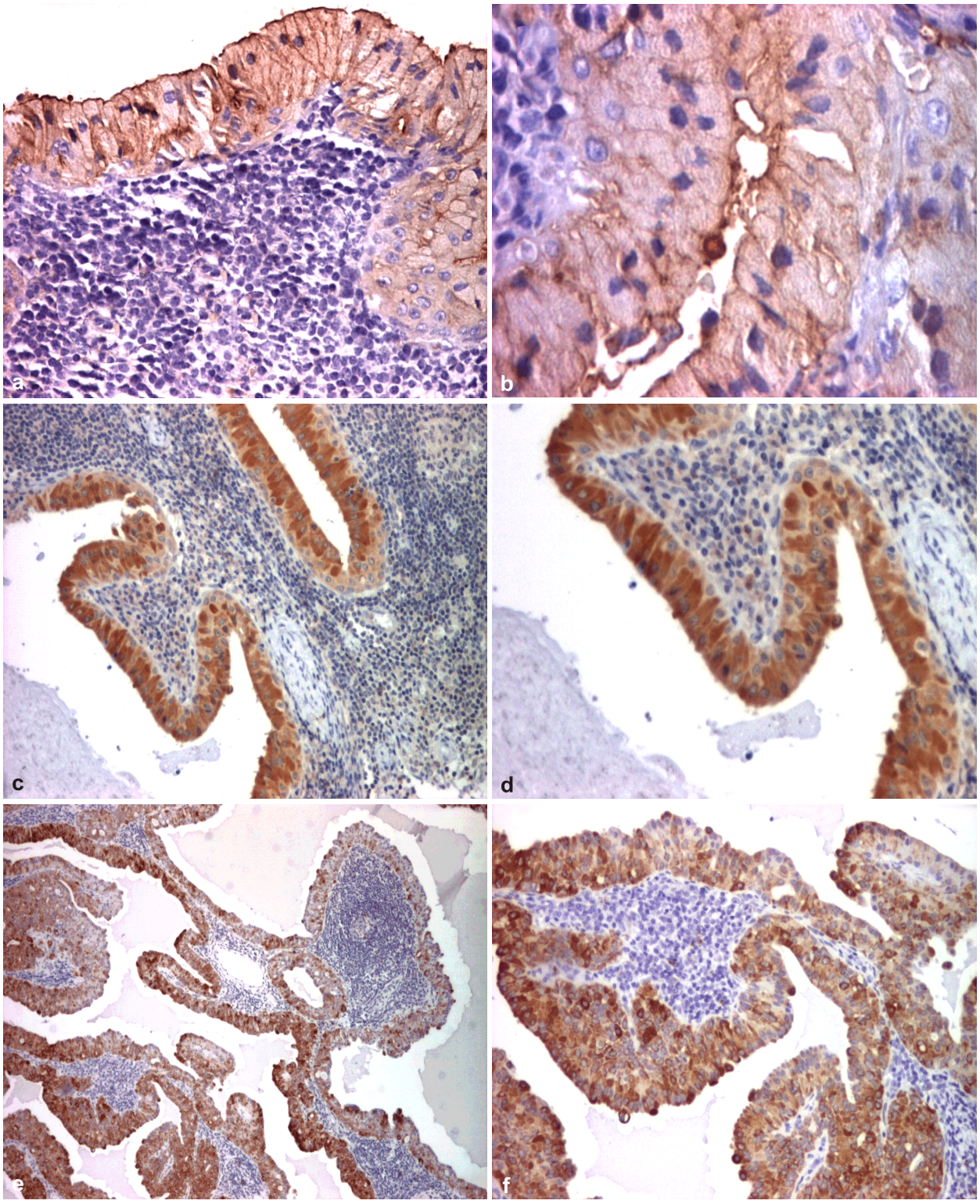

Figure 1 – ***Warthin's tumor. CEA immunostaining: (a) ×200, (b) ×400; CD117 immunostaining: (c) ×100, (d) ×200; AMA immunostaining: (e) ×40, (f) ×100.***

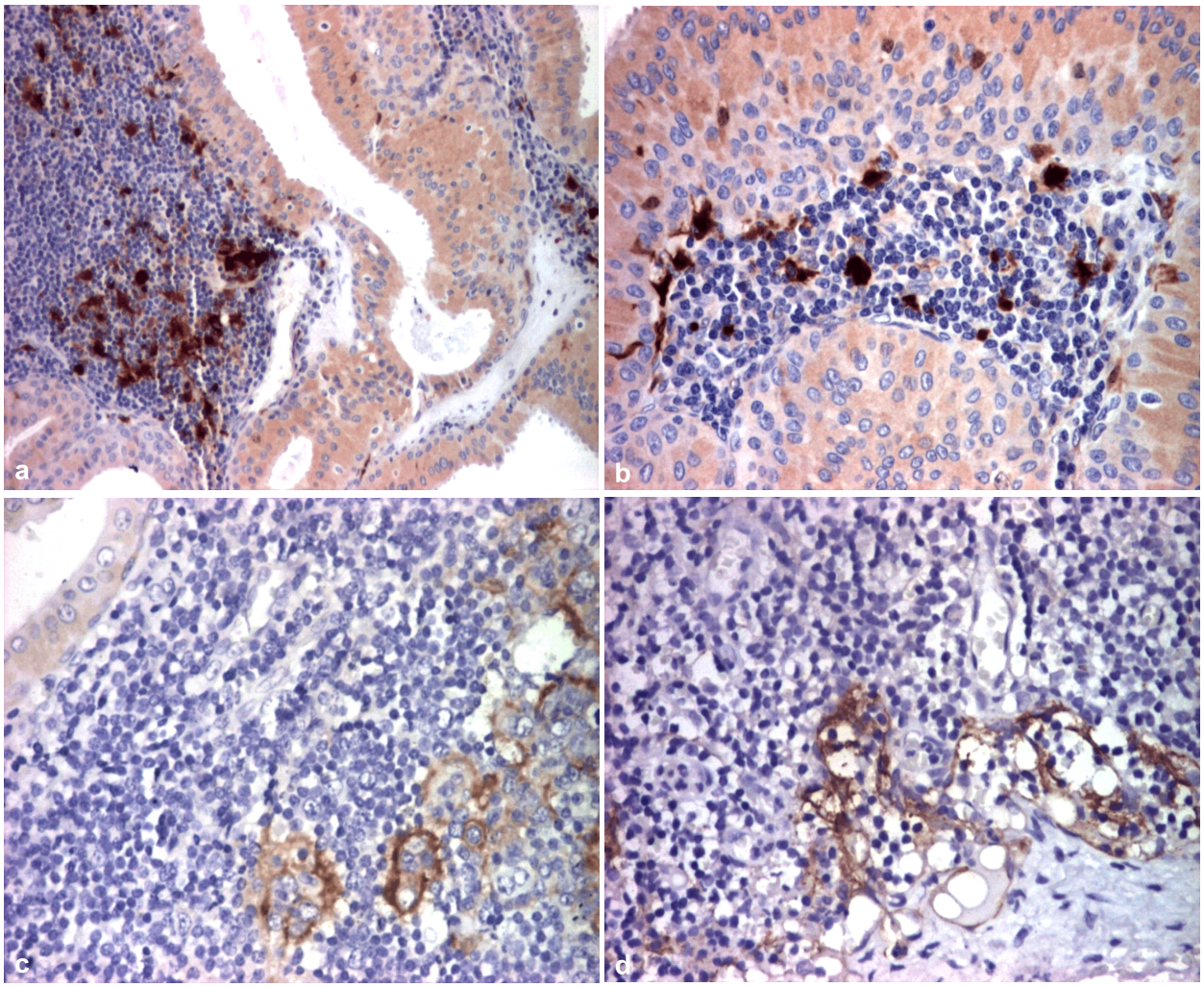

Figure 2 – ***Warthin's tumor. S100 immunostaining: (a) ×100, (b) ×200; D2-40 immunostaining: (c and d) ×200.***

Discussion

Warthin's tumor is the second most frequent benign neoplasm of salivary glands after pleomorphic salivary adenoma [8, 9], with a higher incidence at elderly men [10], although the recent study observed that the difference in male/female ratio is declining [11].

Histological sections showed papillary proliferation composed of bilayered oncocytic and basaloid epitheliums, which are accompanied by a dense lymphoid stroma usually consists of lymphocytes organized in follicles with germinal centers [12].

Many recent studies are focused on immunohistochemical examinations of that neoplasia [13].

In our study, immunostaining analysis for CEA was restricted to luminal columnar epithelium. Immunohistochemical studies regarding CEA immunostaining in Warthin's tumor reported that CEA was occasionally present in a narrow cytoplasmic luminal rim and its positivity was also communicated in papillary epithelial projections [14, 15]. In normal salivary gland, CEA immunoexpression is normally absent in striated ducts. This is the reason why some authors suggested that the positivity in the epithelial component of Warthin's tumor may be a useful marker for early diagnosis of neoplasia [16]. In contrast, recent immunohistochemical studies report that the oncocytes of the small Warthin's tumor and the ductular elements were negative for carcinoembryonic antigen and S100 protein [17].

C-kit expression in salivary glands was examined in a reduced number of studies, almost of these researches was made especially in the last year. These studies were directed mostly over the adenoid cystic carcinoma [18].

Immunohistochemical identification of CD117 can help highlighting the luminal cell component of various salivary gland tumors [19]. In our study, immunostaining for CD117 was positive in 20 (68.9%) cases, limited to luminal columnar epithelium. Andreadis *et al.* observed in his study that Warthin's tumor showed strong positivity in interspersed cells in tall columnar cell layer [20]. In contrast with these results, Mino *et al.* had proved that in all cases with Warthin's tumor is present only a weak positive staining in luminal epithelia of the intercalated and striated ducts of normal salivary glands. The results of Mino *et al.* study are similar with the other researches about staining in adenoid cystic carcinomas [21].

In our study, immunostaining analysis for AMA showed positivity in all analyzed cases. We found the presence of moderate intensity reactions for columnar cells in nine (31%) cases and in other 20 (68.9%) cases, the immunostaining was strongly positive. The intensity for AMA immunostaining at salivary glands was observed in a recent study. At normal salivary glands, the intensity for striated luminal oxyphilic cells was strongly positive

(+++) and at Warthin's tumor, luminal and non-luminal oncocytes was strongly expressed (+++) for AMA and also for CK7, CK14, but non-oncocytic cells of the basal layer were negative for AMA and myoepithelial markers [13].

Positivity for AMA is more useful in a panel of antibodies to distinguish oncocytary component if Warthin's tumor malignancies. Bell & Luna researched the malignant neoplasms developed from Warthin's tumor and calls them Warthin's adenocarcinomas [13]. That adenocarcinomas appear to be the results from malignant transformation of all Warthin's tumor components [13], but the pathogenesis is not fully elucidated [22, 23]. The immunohistochemical study of Warthin's adenocarcinomas showed marked reactivity (+++) for inner oxyphilic cells for AMA and CK14, CK18 and basal layer only focally positive (+) for AMA [13].

The analysis for protein S100 reaction indicated the presence of immunostaining in all investigated cases, both in the epithelial and stromal component, more frequently in the stromal component than the epithelial.

Because neoplastic modified myoepithelial cells have a key role in salivary gland pathology, some authors consider that the expression of S100 protein in these tumors have major histogenetic implications [24]. Immunohistochemical studies regarding S100 immunostaining in Warthin's tumor report cytoplasmic and nuclear positivity in the epithelial and stromal component, more frequently in the lymphoid tissue than in neoplastic epithelium [15]. The morphological aspect of these cells was similar to that of Langerhans cells, leading to the conclusion that the presence of such antigen-presenting cells in the immune responses indicates that delayed hypersensitivity may be the main factor in tumor development [25].

Many authors have tried to identify the true origin of the Warthin's tumor [26]. One of the most accepted hypotheses is that of heterotopia and other theory is that tumor is an adenoma with concomitant lymphocytic infiltration [27]. Honda *et al.* disproved these theories and showed that the tumor epithelial component is infiltrated with lymphocytes, which is a polyclonal population of cells, which makes this tumor not to be a true neoplasm [27]. Quantification of angiogenesis and tumor lymphangiogenesis is an important step in understanding the biology of these tumors [3, 28].

Immunostaining analysis for D2-40 showed positivity in all analyzed cases in the endothelial cells of the lymphatic sinus and some reticular cells. LVD values were higher in tumors with stromal predominance, indicating increased lymphangiogenesis for this type of tumor. On the other hand, the *chi*-square test indicated significant difference between the tumor type and the subcapsular LVD (p=0.014).

A relative recent study analyzed the intratumoral blood vessels density (BVD) as a measure of angiogenesis using CD34 staining and LVD as a measure of lymphangiogenesis using LYVE-1. They communicated significant difference between the values of BVD in Warthin's tumor (81; SD±19.3) and parotid gland (7; SD±4.2), but not to the parotid lymph nodes (69; SD±13.7) and similarly higher intratumoral LVD in Warthin's tumor (31; SD±4.6) as compared to parotid gland (7; SD±1.2) and parotid lymph nodes (6; SD±3.5) [3]. The LVD value significantly higher in Warthin's tumor compared to parotid lymph nodes indicate an increased intratumoral lymphangiogenesis and the fact that lymphangiogenesis seems to play an important role in the tumor pathogenesis [3]. These are in accordance with the hypothesis of Warthin's tumor heterotopia that is the result of proliferation of ductal cells from salivary glands that were caught in parotid lymph nodes during embryonal life [27].

In an immunohistochemical study of Warthin's tumor was analyzed podoplanin, a relatively new marker, using the monoclonal antibody D2-40. They reported numerous D2-40-positive sinus vessels, particularly in the inner layer of the tumor capsule [28]. Since the subcapsular sinuses are an essential morphological feature of the lymph nodes, the presence of podoplanin expression in most subcapsular vessels of Warthin's tumors confirms the theory that this tumor has its origin in regional lymph nodes [28].

Conclusions

Our immunohistochemical study is useful for markers selection involved in tumor pathogenesis. S100 protein positivity similar to Langerhans cells leads to the conclusion that delayed hypersensitivity is involved in tumor development. The presence of D2-40 expression in majority of tumor subcapsular vessels, similar to lymph nodes structure, confirms the hypothesis that Warthin's tumor has its origin in regional lymph nodes. Therefore, immunohistochemical assessment of Warthin's tumor may be useful to detect its origin.

Conflict of interests

The authors declare that they have no conflict of interests.

Author contribution

All authors contributed equally to the study and the publication.

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