Original paper

**Vascular morphogenesis in Warthin’s tumor and insights into its origin**

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**Abstract**

Although the morphological aspect of Warthin’s tumor (WT) is known since 1895, its pathogenesis is still unclear. Because neovascularization in a tumor is considered the main mechanism for its development, in this study we analyzed the immunoexpression of CD31, D2-40 and vascular endothelial growth factor (VEGF) antibodies in 10 cases of WT. We found a significantly elevated vascular density (p=0.000) in all the 10 WT as compared to the surrounding normal parotid gland and VEGF expression in the oncocytic epithelial component of WTs (9/10 cases). Our results highlight the importance of studying the vascularization in WTs, the role of the epithelial component in the tumorigenesis of WTs and support the hypothesis that these tumors develop in lymph nodes, on heterotopic salivary gland tissue. To the best of our knowledge, our study is the first study to analyze and correlate the immunoexpression of the three specific vascular and angiogenic markers on a series of WT cases and with results that underline the similar morphological aspects between the histopathological characteristics of WTs and the changes that appear in the context of a neoplastic process in lymph nodes.

**Keywords**

Warthin’s tumor, angiogenesis, lymphangiogenesis, tumorigenesis, parotid gland.


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Introduction

Although the first descriptions of Warthin’s tumor morphology were made by Hildebrand in 1895, the first 2 cases of Warthin’s tumor (WT) were reported in American literature by Alfred Warthin in 1929 (TEYMOORTASH & al [1]; SIMPSON & al [2]). Immediately after the histological characterization of WT, controversy about its origin also appears. While Nicholson considers WT an adenoma developed on ectopic salivary tissue in parotid lymph nodes, Warthin classifies it as a teratoma and Allegre claims that WT’s origin is based on an immune reaction of delayed hypersensitivity type and that oncocytic cells represent the tumor component, the lymphoid stroma being a cell-mediated immune reaction to tumor proliferation. Albrecht and his resident Artz are the first to publish images with the histology of WT, explaining its genesis on ectopic salivary tissue in parotid lymph nodes and they name the tumor papillary cystadenoma in typical lymphoid tissue (TEYMOORTASH & al [1]; TEYMOORTASH & al [3]).

Ectopic salivary tissue is clinically unapparent. Most of the time, salivary gland inclusions in parotid lymph nodes are discovered incidentally on parotidectomy specimens or tissue excised for various pathological conditions of the head and neck region. The presence of glandular inclusions in parotid lymph nodes was linked to embryogenesis of the parotid gland. It is considered that the parotid gland develops synchronously with the lymphoid tissue, allowing salivary ducts and acini to remain trapped in developing lymph nodes. These salivary tissue remains can represent the origin site for various pathologic processes, like the one represented by WT (TEYMOORTASH & al [1]; TEYMOORTASH & al [3]; MADRIGAL-MARTINEZ & al [4]).

Currently, tumor angiogenesis is the main subject of oncolgic research. So far, the results of the studies on neovascularization proved useful in distinguishing some histological features of normal and pathologic tissues, as well as for further broadening the knowledge about cell mechanisms involved in tumor pathogenesis (HOEBEN & al [5]).

CD31, a transmembrane glycoprotein, is considered one of the most effective markers in identifying vascular tumors, but it is also expressed in other cells like plasma cells, monocytes and megakaryocytes (PUSZTASZERI & al [6]). During the last decade, the study of tumor vascularization was enriched with a new marker, D2-40, which highlights only the endothelium of lymphatic vessels. D2-40 is an antibody against human podoplanin, a protein expressed in lymphatic endothelial cells. This antibody is used in many studies that aim to show the lymphatic origin of a tumor or to demonstrate the presence of tumor emboli in lymphatic vessels. Recently, D2-40 immunopositivity was observed in myoepithelial cells, this aspect being used at present in the diagnosis of breast tumors (REN & al [7]).

VEGF, another key element of tumor angiogenesis is involved in vascularization development, tumor and endothelial cell survival. Numerous data showed its proangiogenic function and its role in the potentiating of microvascular hyperpermeability, which can precede or accompany angiogenesis, but also the chemotactic effect that VEGF has on endothelial cells and macrophages (BERENDSEN & al [8]).

In current literature there are only few studies that analyzed the histopathology and immunohistochemistry of both the lymphoid and the epithelial components of WT in order to obtain a complete morphological profile of the tumor (SONG & al [9]; OHMORI & al [10]). Given this data, our study aims: 1) To evaluate the microvascular density and analyze the distribution and morphology of vessels in WT using two endothelial markers, D2-40 and CD31; 2) To quantify the immunohistochemically (IHC) expression of VEGF in WT, and 3) To analyze the IHC expression of the studied markers, in the tumor and the surrounding normal salivary gland and to correlate the results obtained with the morphological aspects of WT.

Materials and Methods

In this study, we analyzed the archived data from the Pathology Department of the Emergency City Hospital of Timisoara from the last 7 years. We identified 32 cases diagnosed with Warthin tumor (25 men; 7 women), with ages between 41-80 years. All tissue samples stained with hematoxylin and eosin (HE) and all the paraffin blocks were reevaluated. Tissue samples with hemorrhage, inflammation, infarction and insufficient tissue, as well as patients with incomplete clinical data were excluded from the study. We decided to examine IHC a final number of 10 cases with Warthin tumors (7 males and 3 females, median age 69.5 years, range 48-80 years, the median size of WT at diagnosis was 3.1 cm (range 1-6.5 cm). All the patients were previously informed and they gave their consent on the protocol and interventions included in this study. 9 of the WT's analyzed were located in the parotid gland (6 in the right parotid and 3 in the left one), one being discovered in a lymph node.

The paraffin blocks extracted from the laboratory’s archives were used to make additional sections that were stained with HE and studied with IHC techniques.

a) Immunohistochemistry

One representative paraffin block was selected for each case and further IHC studies were conducted, using the following antibodies: monoclonal-mouse anti-human D2-40 (clones D2-40, code M3619), monoclonal mouse
anti-human CD31 (endothelial cell, clone JC70A, code M0823) and monoclonal mouse anti-human VEGF (clone VG1, code 7273) from Dako (Glostrup, Denmark).

For the IHC detection of blood vessels we used the mouse anti-CD31 monoclonal antibody and the EnVision + technique (code K4004, Dako). Sections were pretreated by boiling for 20 minutes in a steamer immersed in Target Retrieval solution (Code S1700, Dako) at 95-99°C, further treated with the 1:25 diluted primary antibody for 30 minutes and afterwards with the EnVision polymer HRP for another 30 minutes. The visualization system included diaminobenzidine+ (DAB+) as chromogen, and hematoxylin was used for counterstaining.

For the mouse anti-human VEGF monoclonal antibody we used the EnVision + technique (code K4004, Dako). Pre-treatment of slides with heat-induced epitope retrieval was required and the sections were treated by boiling for 20 minutes immersed in Target Retrieval solution, pH 9 (Dako, S2368). The incubation time with the 1:25 diluted primary antibody was 30 minutes. The visualization system included DAB+. Counterstaining was performed with hematoxylin.

For the IHC detection of lymph vessels we used the D2-40 monoclonal mouse antibody and the EnVision + technique (code K4004, Dako). Sections were pretreated by boiling for 20 minutes in a steamer immersed in Target Retrieval solution (Code S1700, Dako) at 95-99°C; then, the sections were treated with the primary antibody (dilution 1:150) for 30 minutes at room temperature and afterwards with the EnVision polymer HRP for another 30 minutes. The visualization system included DAB+ as chromogen, and hematoxylin was used for counterstaining.

Negative control was made by processing the sections with the same method but the primary antibody was omitted. For the positive control we used sections from a squamous cell carcinoma and a normal salivary gland.

b) Evaluation of staining

Lymphatic and blood microvessel counting:

Lymphangiogenesis and angiogenesis were analyzed by measuring the microvessel density. Vessel count was made by examining tissue sections at low magnification (objective x 40), with evaluating vessel distribution, the highly vascularized areas and the adjacent parotid salivary tissue. In each case, we selected the most vascularized areas. Blood vessel density (BVD) and lymphatic vessel density (LVD) were determined by selecting 3 intensely vascularized areas in the microscopic high-power fields (HPF; objective x 400) within the tumor mass and another 3 HPF from the salivary gland adjacent to the tumor. All CD31- and D2-40-positive vascular structures from each microscopic field were counted and the final value for tumor areas and salivary tissue were recorded as a mean value.

VEGF expression: VEGF expression was evaluated according to staining intensity, as follows: 0=no positive cells; +1=mild staining; +2=moderate staining; +3=intense staining.

The IHC reactions were applied on all 10 cases of WT; the evaluation of results obtained followed previously described methods (TEYMOORTASH & al [11]; COSTA & al [12]; ZHANG & al [13]), with slight changes and adapted on the cases we analyzed.

Statistical analysis

For the statistical analysis we used the Minitab software for Windows (Minitab 16; Minitab Inc., UK). The statistical data analysis consisted of calculation of arithmetic means and standard deviations (SD) for quantitative variables, statistical comparison of sample averages using the ANOVA test (n average) and student t-tests. The results with p<0.05 were considered significant.

Results and Discussion

Warthin’s tumor is still one of the most controversial benign tumor entities of the salivary glands because of its curious histopathological aspect and uncertain origin. Morphologically, WT shows clear-cut margins, a thin capsule, solid and cystic areas made up of epithelial and lymphoid elements.

On the common HE stain, WTs analyzed showed a solid architecture, with cystic areas (that varied in shape and size) and papillary structures (Figure 1A). The papillae had fibrovascular cores and lymphoid stroma. Cystic spaces contained eosinophilic secretion material and infrequently cholesterol crystals. The epithelial component of the tumor is formed by oncocytes-cells with slightly granular eosinophilic cytoplasm, which delineate the cystic spaces and intra-cystic papillary projections, or have a gland-like pattern. In most areas, the epithelial component is made up of two layers of oncocytes-cells – the luminal cells, which are tall, columnar, with palisading oval nuclei and a basal layer (discontinuous here and there) of flat or cuboidal cells, with cytoplasm similar to luminal cells, but quantitatively less and small, flattened nuclei and indistinct nucleoli. Nuclear atypia or mitotic activity was not observed. Occasionally, on the examined sections, there were small foci of squamous metaplasia. The stromal component is made up of lymphoid tissue, lymphoid follicles with germinative centers being frequently observed. We also identified multiple mast cells, macrophages/histiocytes (intra-tumor or at its periphery) and plasma cells. In one case we identified the WT by making serial sections in an intra-parotid lymph node (Figure 1B); in this case we also observed the presence of histiocytes, predominantly at the periphery of the tumor, subcapsular.
The origin of lymphoid stroma is the most debated subject related to the pathogenesis of Warthin’s tumor. Regarding the debates on Warthin’s tumor etiopathology, some authors consider the presence of lymphoid stroma to be a reactive response of the human organism to tumor proliferation, while others affirm that the presence of WT in a lymph node represents a source for prolonged antigenic stimulation of the lymphoid tissue, hence being the cause of lymphoma development that is sometimes associated with WT (PARK & al [14]; SAXENA & al [15]). As to the epithelial component of WT, the oncocytes show morphological and structural similarities with striated ducts of normal salivary glands, like the presence of numerous mitochondria. Moreover, in the normal salivary gland, with age, numerous oncocytes may be observed – a process called oncocyteosis or oncocytyc hyperplasia. These oncocytes can go through metaplastic transformation, becoming squamous, mucinous or ciliated, aspects found also in WT (MADRIGAL-MARTINEZ & al [4]; THOMPSON & al [16]; CHAPNIK & al [17]).

LVD and BVD quantification, vascular distribution and morphology in the WT studied and the adjacent parotid gland

In all 10 cases of WTs, as well as in the salivary tissue surrounding the tumor, we identified vascular structures immunopositive for CD31 and D2-40. After LVD and BVD counting, we obtained 4 groups of values, 2 from the adjacent salivary gland and 2 from the WTs studied. One-way ANOVA showed statistically significant differences (in both blood and lymphatic vessels) between the vascularization in WTs and the adjacent normal salivary gland investigated (p=0.000). The results are presented in Figures 2 and 3.
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Figure 2. One-way ANOVA results showing statistically significant differences in BVD and LVD among the four study groups from normal parotid gland (NP) and Warthin tumors (TW).

Figure 3. The Box plot-type graphical representation of ANOVA One-way statistical test results. The BVD and LVD in Warthin tumors (TW) is significantly higher than in the normal adjacent salivary gland (NP).

The sample t-test also demonstrated significant differences between LVD and BVD in WTs with a mean value for LVD of 14.8 (SD±3.88) and a mean value for BVD of 27.8 (SD±10.4). The adjacent parotid tissue had a mean BVD of 3.6 (SD±3.86) and LVD of 1.267 (SD±0.450). In WTs we noted more pronounced LVD and BVD as compared to the adjacent salivary tissue and the values obtained showed statistically highly significant differences (p<0.000).

In all WT cases that we examined, we identified D2-40-positive vascular structures, irregularly shaped, with thin vascular walls, delineated by flattened endothelial cells, some dilated and others with a compressed lumen. Regarding distribution in WT, most D2-40-positive vascular structures were located especially at the inner layer of the capsule (Figure 4A). A high number of lymphatic vessels were also identified between the epithelium and the stroma, in the areas adjacent to the oncocytic epithelium, following the basal layer of the epithelial proliferation, in vicinity and/or parallel to oncocyttes (Figure 4B). Alongside D2-40 positive vascular structures, we also found some D2-40 negative blood vessels, filled with erythrocytes. In the epithelium and lymphoid stroma of WTs, we observed some isolated cells (probably myoepithelial cells) and lymphoid aggregates immunopositive for D2-40.

Figure 4. D2-40 expression in the WTs: highlights dilated and compressed subcapsular vessels (A) and D2-40 positive cells lining the oncocytic epithelium (B).

CD31-immunopositive vascular structures had variable shapes and sizes, most with conspicuous vascular lumen, erythrocyte-filled and thicker vascular wall; in a small number of vessels, we observed thin vascular walls, like those of lymphatic vessels. Numerous CD31-positive blood vessels can be seen in all tumor mass, but are concentrated mostly in areas adjacent to oncocyttes or subcapsular, with a pattern similar to those D2-40 positive (Figures 5 A, B).

Figure 5. CD 31-positive subcapsular blood vessels filled with erythrocytes (A) and also lining the oncocytic epithelium (B).
In the adjacent salivary gland, the number of CD31- and D2-40 positive vessels is significantly low; the vessels are usually small, with an even spatial distribution and are mainly found in the interlobular connective tissue.

In agreement with the results of the above cited publications, in our study, we noticed CD31 and D2-40 immunoreactive blood and lymphatic vessels, dilated or thin-walled and with flattened endothelium, and some erythrocyte-filled or lymphocyte-filled vessels. Using CD31 and D2-40 markers, we highlighted vascular structures adjacent and parallel to the epithelial component, in a lower number than those found in the inner lining of the tumor capsule. Our results show a significant number of CD31-immunopositive vessels (most with a large calibre lumen and endoluminal erythrocytes), as compared to the D2-40 positive ones. On sections with D2-40 positive vascular structures, we also identified vascular structures that were immunonegative for this marker, filled with erythrocytes. Our results support the use of D2-40 marker in identifying lymphatic vascularization and for the characterization of their density, both in WTs investigated and in salivary glands.

The histological features of lymph nodes are made up of lymphoid lobules with a reticular meshwork, arterioles, venules and capillaries. Each lobe is surrounded by a complex network of lymphatic sinuses (subcapsular, transverse, medullary sinuses). The number of capillaries is reduced in lymphoid follicles and in the center of lymph nodes (WILLARD-MACK & al [26]). The presence of a tumor may induce changes in the microenvironment and biology of the lymph nodes. So, aspects like the increase of the proliferation rate of endothelial cells and blood and lymphatic vessel reorganization were observed, the lymph node becoming a functional organ, with increased vascularization. Moreover, it was argued that vessel function can shift from lymphocyte recruitment to becoming a large blood vessel (LEE & al [25]).

Other authors report vascular transformations of lymph node sinuses as secondary to the incentive effect of some mediators secreted by neoplastic cells or due to the obstructive effect from near-by tumors (FUKUNAGA & al [27]).

In this context, these transformations were interpreted as benign vascular lesions identified occasionally in lymph nodes excised for various pathologies, mostly tumoral. Morphologically, the authors describe vascular transformations of lymph nodes sinuses as proliferations of vessels lined by a uniform layer of endothelial cells, with a minimal quantity of inter-vessel connective tissue (MIRANDA & al [28]; SAMET & al [29]). The studies of Chang JK et al [30] on the morphological aspects that can be identified in vascular transformations of lymph nodes sinuses prove their histopathological versatility. The authors describe an increased number of vessels that form thin vascular channels, round vascular spaces of variable sizes, plexiform ducts (with lymphocytes/erythrocytes inside the lumen or emptied of content), solid or spindle cell aggregates, with or without fibrosis (CHANG & al [30]).

There are few studies on angiogenesis and lymphangiogenesis and the impact on tumoral cell behavior in in Warthin’s tumor (TEYMOORTASH & al [11]; RABIA & al [18]; NAKAMURA & al [31]; KUZENKO & al [32]; FAUR & al [33]). Using immunostaines for the vascular structures in WTs, with CD34 and LYVE-1 markers, TEYMOORTASH & al [11] reported marked lymphangiogenesis in WTs, as compared to pleomorphic adenomas, parotid lymph nodes and normal parotid gland, the authors suggesting that tumor lymphangiogenesis seems to have a role in WT pathogenesis and that epithelial tumor cells might stimulate lymphangiogenesis. KUZENKO & al [32] focused on the stroma of WT and considered the tumor as being of inflammatory etiologi. RABIA & al [18] results showed similar levels of blood over lymphatic vascular density between WT and lymph nodes with inclusions. HANSEN & al [19] observed that D2-40 positive vascular structures are found mainly subcapsular in WT. The authors assess that, as subcapsular sinuses represent structures characteristic for lymph nodes, it can be concluded that WTs originate from regional lymph nodes. In our study, in the 10 WT cases, we identified numerous D2-40 positive vascular structures, of variable shapes and sizes, with thin walls, evident or collapsed lumens, located subcapsular in a manner similar to lymph node sinuses.

Evaluating our results from the perspective of the above-mentioned data, we observed a series of common elements. Most studies show marked vascular density in tumors as compared to normal tissues. Also, in our study, LVD and BVD in WTs that we investigated were significantly higher than the surrounding salivary gland, the values that we obtained showing statistically highly significant differences (p=0.000). Furthermore, the large number of CD31-positive vessels proved a marked angiogenesis in these tumors. When comparing the quantitative values of BVD and LVD in WTs, we also found statistically significant differences in the microvessel density, with a higher number of blood vessels in the tumors analyzed. Morphologically, blood vessels are found in high numbers both subcapsular and near the oncocyti component of WT, but less in its lymphoid stroma.

The role of VEGF in tumor progression is demonstrated by a series of studies over the years; recent data support the stimulative role that VEGF has for endothelial cell differentiation from mesenchymal stem cells (BERENDSEN & al [8], ROSKOSKI JR & al [23]). To the best of our knowledge, up to the completion of this study, there is only one study, with results published in English that analyzed VEGF expression on a group of Warthin tumors. In their study, NAKAMURA & al [31] analyzed the IHC expression of VEGF, some growth factors and their receptors, cell adhesion molecules and
extracellular matrix components in WTs, but tumor vascularization was not assessed. These authors affirm that the results obtained support the theory that WT represents an epithelial tumor that induces a lymphocytic response (NAKAMURA & al [31]).

In our study, we identified a mild and moderate VEGF expression in 9 out of the 10 cases that we studied. Our results confirm data presented in literature, in all 9 immunopositive cases, VEGF expression being found mainly in the epithelial component of WTs.

The immunohistochemically VEGF expression in Warthin tumors

Regarding VEGF expression, 9/10 tumors were VEGF-positive, all 9 cases of WT showing tumor areas with mild and moderate VEGF expression (Figures 6 A, B), in the cytoplasm of oncocytes and in some occasional stromal cells (especially lymphocytes from lymphoid follicles and some macrophages).

Our results confirm data presented in literature, in all 9 immunopositive cases, VEGF expression being found mainly in the epithelial component of WTs. In the adjacent parotid gland, we observed moderate VEGF expression (mainly cytoplasmic and less in the membrane) in striated and intercalated ducts, in endothelial cells of vascular walls, in serous acini and in some cells from their periphery, probably representing VEGF expression in myoepithelial cells.

In WTs studied, we consider that morphological aspects, density and distribution of vessels, correlated with VEGF expression of oncocytes offer important clues on the pathogenesis of these curious tumors. VEGF immunopositivity of the epithelial component of WT, as well as the high number of vessels found near these structures support the contribution of this component to the tumor process; we consider that these elements can represent more evidence of the epithelial origin of WT, on ectopic salivary tissue in a lymph node.

Analyzing vascular density, by immunostaining with CD31 and D2-40 in the 10 cases of WT, we observed similar histological aspects in the pattern of distribution and vessel morphology and the vascular changes that appear in lymph nodes during vascular transformations of lymph node sinuses: numerous pericapsular vascular structures, some of them D2-40 positive, with thin endothelium and lymphocytes in the lumen, others CD31 positive, dilated, with thicker walls, with/without the presence of erythrocytes in the lumen. Subcapsular arrangement and vessel morphology in the investigated WTs (highlighted both on HE and IHC stainings) are similar to the changes that appear in the context of a neoplastic process in lymph nodes. We consider that these aspects can be proof of vascular transformations of lymph node sinuses in which a WT developed.

We hypothesized that these changes can also be present in the case of a tumor that develops in a lymph node, like Warthin’s tumor. Subcapsular vascular changes that we described in WT can be the result of the compressive effect of WT (developed on salivary ectopic tissue) in a lymph node where the tumor evolved. As venous or lymphatic obstruction can be the consequence of a near-by tumor (FUKUNAGA & al [27]), the mechanism of vascular transformation in WT is most probably an obstructive one. Plus, the behavior of lymphoid stroma in studied WTs emphasized by the presence of lymphoid follicles, some with germinative centers, is also an element that is similar to reactive changes that can be observed in lymph nodes in various pathologies. In our study, we also used a WT discovered (incidentally) in a lymph node. On the serial sections from this tumor, we observed the presence of histiocytes, with a distribution similar to the one from a reactive lymph node.

In the 10 cases of WT we also identified isolated cells, dendritic follicular cells and lymphoid follicles with germinative centers positive for D2-40. Considering the data that confirmed the presence of podoplanin expression in myoepithelial and myofibroblastic cells, we appreciate that D2-40 expression in these individual cells identified in the stroma and at the periphery of the epithelial component probably are of myoepithelial origin (HANSEN & al [19]; RAICA & al [35]).

Conclusions

To the best of our knowledge, our study is the first study to analyze and correlate the immunoexpression of the three specific vascular and angiogenic markers (CD31, D2-40 and VEGF) on a series of WT cases. Also up to the finalization of this study we did not find in the English literature any specific data to underline the similar morphological aspects between the histopathological

References

7. S. REN, M. ABUEL-HAIJA, J.S. KHURANA, X. ZHANG. D2-40: an additional marker for myoepithelial cells of breast and the precaution in characteristics of WTs and the changes that appear in the context of a neoplastic process in lymph nodes, as we had shown above. However, there are few published studies on WTs and our study was a preliminary investigation, with a small sample size therefore, a larger study and more data are needed to confirm the results.

We conclude that our results: (i) the distribution and morphology of the vascular structures in WTs examined emphasizes by CD31 and D2-40 immunostaining, and the (ii) VEGF-positive expression in the epithelial component support the contribution of oncocytes to the process of proliferation, hence the epithelial origin of WT, while the stromal component behaves more like a lymph node whith aspects of vascular transformations of the sinuses in the presence of a tumour.

Declaration of conflicting interest

The author(s) declare no potential conflict of interest with respect to the research, authorship and publication of this article.


