Vasculogenic mimicry contributes to lymph node metastasis of laryngeal squamous cell carcinoma

Wei Wang1,3, Peng Lin3, Chunrong Han1, Wenjuan Cai1,4, Xiulan Zhao2 and Baocun Sun*1,2

Abstract

Background: Survival of laryngeal squamous cell carcinoma (LSCC) patients has remained unchanged over recent years due to its uncontrolled recurrence and local lymph node metastasis. Vasculogenic mimicry (VM) is an alternative type of blood supplement related to more aggressive tumor biology and increased tumor-related mortality. This study aimed to investigate the unique role of VM in the progression of LSCC.

Methods: We reviewed clinical pathological data of 203 cases of LSCC both prospectively and retrospectively. VM and endothelium-dependent vessel (EDV) were detected by immunohistochemistry and double staining to compare their different clinical pathological significance in LSCC. Survival analyses were performed to assess their prognostic significance as well.

Results: Both VM and EDV existed in LSCC type of blood supply. VM is related to pTNM stage, lymph node metastasis and pathology grade. In contrast, EDV related to location, pTNM stage, T stage and distant metastasis. Univariate analysis showed VM, pTNM stage, T classification, nodal status, histopathological grade, tumor size, and radiotherapy to be related to overall survival (OS). While, VM, location, tumor size and radiotherapy were found to relate to disease free survival (DFS). Multivariate analysis indicated that VM, but not EDV, was an adverse predictor for both OS and DFS.

Conclusions: VM existed in LSCC. It contributed to the progression of LSCC by promoting lymph node metastasis. It is an independent predictors of a poor prognosis of LSCC.

Background

Laryngeal squamous cell carcinoma (LSCC) is the second main upper respiratory tract tumor behind lung cancer in incidence and mortality rates. Despite many advances in the diagnosis and treatment of the disease, its overall survival rate has remained unchanged (at approximately 35-70%) over the past several decades. It is mainly due to uncontrolled recurrence and local lymph node metastasis[1]. Thus, it is necessary to develop new therapeutic targets for LSCC that can take advantage of the unique qualities of this disease.

It is traditionally known that tumor invasion and metastasis mainly depend on angiogenesis. Histological examination of human tumor specimens has confirmed that increased vascularity is a common feature of LSCC. However, the results of studies associating microvessel density and various clinical pathological parameters and/or outcome are still inconclusive in LSCC[2]. In addition, clinical uses of anti-angiogenic agents for head and neck squamous cell carcinoma(HNSCC), including bevacizumab, sorafenib, sunitinib, are currently limited to small clinical trials, and several ongoing large-scaled trials up to this point. Single-agent anti-angiogenic drugs so far have not shown activity in unselected HNSCC patients, with a response rate of less than 4%[3,4]. On the other hand, combinations of anti-angiogenic drugs with other treatments appear to be promising therapies, and biomarkers appear to have the potential to play an important role in anti-angiogenic treatment of LSCC in the future. Therefore, it is necessary to discover how blood supply contribute to LSCC biology, and to explore its characteristic biomarkers.

Vasculogenic mimicry (VM) is an alternative type of blood supplement formed by highly invasive and genetically dysregulated tumor cells with a pluripotent embryonic-like genotype[5]. Such tumor cells contribute to the plasticity and gain the ability to participate in the processes of neovascularization and ultimately constructing
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were plotted using the Kaplan-Meier method and different subgroups were compared using the log-rank test. Patients who dropped out during follow-up or died due to diseases other than laryngeal cancer were treated as censored cases. The Cox regression model was used to adjust for potential confounders. Comparison MVD expression between VM-positive and VM-negative group used t test. Significant level was set at 0.05. P values are two-tailed.

Results

Evidence of VM and EDV in LSCC

Both VM and EDV existed in LSCC. Forty-four (21.67%) of 203 cases were VM-positive by double-staining. VM appeared to be PAS-positive loops surrounding tumor cells (not endothelial cells), with or without red blood cells. In CD31-stained slides, there were no positive cells in VM (Fig. 1A). While endothelium dependent vessel showed a CD31-positive endothelial cell to form the vessel wall (Fig. 1B).

Characteristics and follow up of patients

Among the 203 patients, there were 154 men (75.86%) and 49 women (24.14%). The mean age at diagnosis was 66 years, ranging from 32 to 77 years. 166 (81.77%) cases reported history of tobacco use, and 37 (18.23%) cases without. 91 (44.83%) cases indicated history of alcohol consumption and 112 (55.17%) cases without. Patients with tumors located at super glottic were 93 (45.81%) cases, at glottic were 93 (45.81%) cases, and at subglottic were 17 (8.37%) cases. Patients in pTNM stage I, II, III and IV were 25 (12.32%), 60 (29.56%), 62 (30.54%) and 56 (27.59%), respectively. Patients in different T classification T1, T2, T3 and T4 were 27 (13.30%), 93(45.81%), 44(21.67%) and 39(19.21%), respectively.151 (74.38%) patients showed lymph node metastasis at diagnosis, and 19 (9.36%) patients appeared to show distant metastasis postoperative. In addition, histological grade 1 was in 30 (14.78%), grade 2 was in 149 (73.40%) and grade 3 was in 24 (11.82%) cases.

The mean follow-up time was 80 months (range 2-219 months). 121 patients (59.61%) were alive when the follow up ended. Eighty-two patients (40.39%) died as a result of their malignancy. The median DFS was 56 months. Local recurrence and local lymph node metastasis was observed in 157 patients (77.34%). The mean period from initial surgery to the first local recurrence or metastasis was 63.71 months (range 1-213 months). Nineteen (9.36%) patients developed distant metastasis. The metastatic sites included lung (n = 9), bone (n = 4), liver (n = 3), mediastinum (n = 2), and multiple concomitant metastasis (n = 1, including thoracic vertebrae, spinal cord and tibia).

Clinical significance of VM in LSCC patients compared with EDV

Clinical significance of VM and EDV are listed in Table 1. The positive rate of VM was significantly higher in progressive stage (III and IV) than primary stage (I and II) (27.97% vs. 12.94%) (p = 0.010) clinically, and it was significantly greater in patients with local lymph node metastases than those without local lymph node metastasis (36.53% vs. 16.56%) (p = 0.003). In addition, the positive rate of VM became higher with the raise of histopathological grade grade 1(6.67%), grade 2 (20.13%)
histopathological grade: grade 1 (66.7%), grade 2 (20.13%), grade 3 (50.0%) (p < 0.001). The incidence of VM did not differ with respect to the patients’ gender, age, tumor size, T stage, tumor location, recurrence or distant metastasis (all P > 0.05).

We performed immunohistochemical staining for CD31, a classic endothelial cell marker, to label endothelial dependent vessel, and analyzed whether it was associated with tumor clinicopathologic characteristic. The results showed MVD was correlated to location (ρ = 0.031), pTNM stage (ρ = 0.007), T stage (ρ = 0.019) and distant metastasis (ρ = 0.045). While, showed no association between MVD and gender, age, tobacco use, alcohol consumption, tumor size, lymph node metastasis, recurrence or histopathological grade (all P > 0.05).

Survival analysis
Univariate analysis showed that survival of VM-positive patients was significantly poorer than that of VM-negative patients in OS (p = 0.014) (Fig. 2A). Furthermore, pTNM stage (ρ = 0.009), T classification (ρ = 0.013), nodal status (ρ = 0.013), and histopathological grade (ρ = 0.038), tumor size (ρ = 0.028), radiotherapy (ρ < 0.0001) correlated with OS. However, there was no significant association between OS and gender, age at diagnosis, tobacco use, alcohol consumption, location, distant metastasis, recurrence and MVD (Fig. 2B) (all P > 0.05; Table 2). Multivariate analysis indicated that the presence of VM (risk ratio (RR) = -2.117, P = 0.003), recurrence (RR = -1.821, P = 0.020) and pTNM stage (RR = 1.367, P = 0.009) were adverse predictors for OS (Table 3), while radiotherapy were indicators of a good prognosis of OS (RR = 2.872, P < 0.0001).

In addition, univariate analysis of DFS showed that VM (P = 0.011) (Fig. 2C), location (P = 0.049), tumor size (P = 10.364) and radiotherapy (P < 0.0001) were proposed to correlate with DFS. While, gender, age at diagnosis, tobacco use, alcohol consumption, pTNM stage, T classification, nodal status, distant metastasis, recurrence, histopathological grade and MVD (Fig. 2D) (all P > 0.05; Table 2) showed no correlation with DFS. Multivariate analysis showed that VM (RR = -1.733, P = 0.003) and radiotherapy (RR = 2.756, P < 0.0001) were independent prognostic factors for DFS (Table 3).

Relationship between VM and EDV
To elucidate on the relationship between VM and EDV, the MVD between the VM-positive group and VM-negative group was compared. This determined patients of VM-negative group had a higher MVD (18.3403 ± 6.93218) than the VM-positive group (14.8643 ± 5.18685) (t = 3.096, P = 0.002) (Table 4). Correlation analysis revealed a negative correlation between VM and MVD (r = -0.198, P = 0.005).

Discussion
This study confirmed VM as a new type of blood supply in LSCC by double staining. Angiogenesis (the formation or sprouting of endothelium-lined vessels from pre-existing vessels) and vasculogenesis (the difference between precursor cells and endothelial cells which develop de novo vascular networks) are two kinds of traditional blood types [15]: Both have been reported in LSCC [16]. VM is a new pattern of matrix-rich networks surrounding tumors cells, being reported firstly in melanoma by Maniotis in 1999 [5]. It refers to the de novo generation of tumor microcirculation without participation by endothelial cells; it is independent of angiogenesis. Furthermore, it is not a vasculogenic event for the true vasculogenesis results in endothelial cell-lined vessels de novo formation. Majority of research on VM focuses on mesenchymal tumor [8,9,17], while only a few delve into epithelial tumor [6,10,11,18]. To date, there is dearth of research discussing squamous cell carcinoma. Thus, this study identifies VM existence in LSCC, in attempt to explain why anti-angi/vaculogenesis treatment remains to be clinically ineffective.

There is still no affirmative conclusion on the prognostic significance of the endothelium marker among CD31, CD34 and CD105. A long-term prognostic significance of angiogenesis in breast carcinomas compare with Tie-2/Teck, CD105, and CD31 immunocytochemical expression showed both CD31 and CD105 correlated with poorer survival [19]. Menio et al study on lung cancer reported that CD34-MVD and tumor vessel invasion not CD105, correlate with poor survival on multivariate analysis[20]. We selected CD31 to label endothelial-dependent vessel for the reasons: Because CD31/CD34 is a pan endothelial marker, and hence stains nearly all blood vessels, both stable vessels trapped inside the tumor and neoangiogenesis. However, CD105 (endoglin) is a proliferation-associated and hypoxia-inducible protein abundantly expressed in angiogenic endothelial cells. It is demonstrated that antibodies against CD105 reacted preferentially with active endothelial cells of angiogenic tissues. CD105 is a marker of neoangiogenesis and only stains a smaller proportion of blood vessels[21]. On the other hand, VM is an alternative type of blood supply different from endothelium-lined vasculature. It is becoming evident that VM, the intratumoral tumor-cell-lined, ECM-rich patterned network, can provide an extra vascular fluid pathway, now known as the fluid-conducting meshwork[22,23]. Here, we compared clinical significance of VM with CD31-MVD, to disclose their different contribution to tumor biology. Thus, we choose CD31 to label endothelial-dependent vessel rather than CD105 was in order to reflect the whole blood supply in a tumor, for both newly-forming vessels and stable vessels trapped inside the tumor acted in tumor invasion and metastasis.
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More work should be done in the future to enrich the theory of tumor blood supply pattern, which may provide reasonable theoretic evidence for tumor anti-angiogenesis.

In the current study, we identified that the positive rate of VM in LSCC is 21.67%, which is different from other tumors, such as inflammatory and ductal breast carcinoma (7.9%), ovarian carcinoma (36.4%), melanoma (5.3%), rhabdomyosarcoma (18.8%), and synovial sarcoma (13.6%). That is probably due to different tissue origin and judgment criteria variable across labs. More investigation of a larger sample is needed to illustrate the mechanism of VM formation in different tissue.

Previous research has demonstrated VM existed in most tumors, being a functional microcirculation [24,25], correlated with poor clinical outcomes among tumor patients [14,26]. The majority of studies in vitro have focused on the mechanism, until recently. However, rela-
tively few studies have interpreted VM’s influence on a tumor’s overall biological behavior using a large sample. In addition, there still no data which describes a significant difference between VM and other patterns of blood supply. In this study, we compared the significance of clinicopathology and prognosis between VM and EDV. This retrospective study of 203 LSCC patients showed that VM is associated with lymph node metastasis, pTNM stage and histopathology grade in LSCC. While EDV correlated with tumor location, pTNM stage, T stage and distant metastasis. This indicated that both VM and EDV played an important role in tumor progression.

Our study showed that VM is related to local lymph node metastasis intimately, which is an important feature and a key prognostic factor of LSCC[27]. It is different from a previous study[28], which reported that patients with breast carcinomas engaged in VM and had a higher rate of distant metastasis (liver, lung, and bone), but failed to find a significant correlation with lymph node metastasis status. In our study of 203 LSCC, only 9.36% appeared to have distant metastasis, while 74.38% developed local lymph node metastasis. We deduced from this that VM in LSCC may own the specific ability to facilitate metastasis by some modality. More studies are warranted to elucidate the effects of VM which use a larger sample on local lymph node metastasis in different types of tumors.

Table 2: Univariate analyses of factors associated with recurrence, metastasis and survival

<table>
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<tr>
<th>Variable</th>
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<th>Disease-Free Survival</th>
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<td>Sex, male vs female</td>
<td>χ² 1.809</td>
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<td>Age, y, ≥60 vs &lt;60</td>
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VM: vasculogenic mimicry; MVD: micro vessel density.

VM in tumors plays an important role in tumor aggression [5]. We also found VM is more common in the advanced stage of LSCC than in the primary stage. However, these results are different than the observations from a breast cancer study by Shirakawa et al[28], which showed that the VM group did not exhibit a more advanced pTNM stage than the non-VM group. However, there was no difference of VM exhibition among different T stage founded in Shirakawa’s and our studies. We suggested that the discrepancy result may due to different influence of VM on local lymph node metastasis or distant metastasis in diversity tumors. Therefore, the impact of VM on the survival of patients with LSCC needs to be confirmed further by some international collaboration of studies and systematic reviews by meta-analysis.

In addition, we found that positive rate of VM increased with the increase of histopathology grade, which is consistent with a previous study of hepatocellular carcinoma [13]. Nasu et al’s [29]in vitro study demonstrated that VM was linked to the aggressive tumor cell phenotype. Another in vitro study [6] also found that high invasive melanoma cell line MUM-2B, expressing both epithelial and mesenchymal phenotype was able to form VM, while MUM-2C, a low invasive melanoma cell line expressing only mesenchymal phenotype, failed to form VM. Taken together, these studies imply that the lower
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The lower histopathology grade of LSCC owning more cell heteromorphism, can change cancer plasticity by genetic reversion to a pluripotent embryonic-like genotype to ultimately form VM. However, in the study of EDV, it was both VM and EDV were related to pTNM, while no association was found between EDV and pTNM rather than distant metastasis. Therefore, we speculated that both VM and EDV contributed to LSCC progression, but through a diverse pathway. VM is a distinct pattern of blood supply from EDV. In general, VM may facilitate invasion and local metastasis in LSCC, indicating its role on aggressive behavior.

Previous study demonstrated that tumors with VM exhibited poor survival[9,13]. We found that VM was an unfavorable prognostic factor of LSCC patients both in OS and DFS, whereas EDV was not an independent predictor of outcome, consistent with Sun et al’s [14] investigation in hepatocellular carcinoma. Traditional microvessel density counts [30,31] within vascular hot spots of tumors using endothelial markers reflect only the vascular status of endothelial dependent vessel in a tumor, but ignore other patterns of the vascularity, including VM, leading to low microvessel density in the different tumor types. However, Eberhard et al[32] demonstrated that endothelial dependent vessel alone, there is wide variance in the endothelial proliferation index among the various tumor types. This indicated that there is marked heterogeneity of vasculature in human tumors. It is necessary for us to account for all types of blood supply and their contribution to tumor behavior when evaluating its clinical and prognostic value. Moreover, the phenomenon of VM existence can partly explain why we failed in anti-angiogenesis treatment of LSCC.

How do VM and EDV play their individual role in one neoplasm during tumor growth? In our retrospective of 203 cases LSCC, presentation of VM showed a negative correlation with EDV. Further investigation in vivo needs to be performed in order to detect the presence of VM and EDV to disclose the relationship between VM and EDV in the same tumor in a time-dependent way.

Conclusions
In conclusions, our results suggest that VM might be a new target of anti-vasculogenesis/angiogenesis therapy for LSCC. Those who rely on conventional markers of tumor “vascularity” as prognostic markers, and who are developing anti-cancer therapies by targeting angiogenesis should exercise caution concerning VM when interpreting their results. Vasculogenic mimicry is one example of the remarkable plasticity demonstrated by aggressive melanoma cells and suggests that these cells have acquired an embryonic-like phenotype. Several factors are involved in VM formation, including microenvironment, interaction between tumor cells and surrounding tissue, tumor cells changing to endothelial genotype by expressing embryo genotype. Further studies

| Table 3: Multivariate analyses of factors associated with recurrence, metastasis and survival |
|---|---|---|---|
| Variable | Hazard Ratio | 95% Confidence Intervals | p |
| | | lower | upper |
| Overall Survival | VM, Positive vs Negative | -2.117 | 1.286 | 3.425 | 0.003 |
| | Recurrence, Yes vs No | -1.821 | 1.363 | 3.639 | 0.020 |
| | TNM stage, Ivs IIvs IIIvs IV | 1.367 | 1.080 | 1.732 | 0.009 |
| | Radiotherapy, Yes vs No | 2.872 | 1.764 | 4.678 | <0.0001 |
| Disease-free Survival | VM, Positive vs Negative | -1.733 | 1.202 | 2.498 | 0.003 |
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VM: vasculogenic mimicry; MVD: micro vessel density.

| Table 4: Correlation between VM and MVD of 203 LSCC patients |
|---|---|---|---|
| n | MVD ($\overline{x}$ ± S) | t | P |
| VM+ | 44 | 14.8643 ± 5.18685 | 3.096 | 0.002 |
| VM- | 159 | 18.3403 ± 6.92318 | |

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Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
Before submission, all authors read and approved the final manuscript. Among the authors, WW designed the study, performed all experiments, and drafted the manuscript. While ZXL and LP collected the materials and conducted the statistical analysis. HCR participated in the instruction of the experiment, while CWJ revised the manuscript critically to ensure important intellectual content. The manuscript was read and reviewed by all authors.

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Before submission, all authors read and approved the final manuscript. Among the authors, WW designed the study, performed all experiments, and drafted the manuscript. While ZXL and LP collected the materials and conducted the statistical analysis. HCR participated in the instruction of the experiment, while CWJ revised the manuscript critically to ensure important intellectual content. LV and XP read and reviewed the sections, and performed follow-up observations on all patients. SBC provided the study concept and participated in its design and coordination.

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Key words: angiogenesis, immunohistochemistry, PAS reaction, vasculogenic mimicry.

INTRODUCTION

Vasculogenesis is a complex multistage process characterized by formation of new vessels from preexisting ones (1, 5, 7). Angiogenesis is essential for tumoral growing and metastasis, that’s why in more aggressive tumors the angiogenesis is more intense due to increased demands for newly formed structure. There are three main theories regarding intratumor angiogenesis, respectively (I) The theory of multistage angiogenesis, (II) The theory of cooption of preexisting vessels by the tumor, and (III) The theory of vasculogenic mimicry (1).

Angiogenesis is a complex process that leads to generation of new capillaries from preexisting vascular network (multistage angiogenesis), or by forming of blood flowing channels delimited directly by tumoral cells (vasculogenic mimicry). Endothelial cell proliferation is 30-40 folds higher in tumor structure comparing from normal tissues. Multistage angiogenesis begin with degrading of the basal membrane, followed by proliferation and migration of endothelial cells outside from the vessel structure. These cells are organizing into a tubular structure that forms
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some vascular buds originated in existing vessels. Finally, there it is formed a new blood vessel that supply a territory from tumor. Regarding the second theory of angiogenesis, the tumor entity subdue preexisting vessels, especially that ones from the periphery of tumor (1).

The vasculogenic mimicry pattern of angiogenesis shows the plasticity and increased adaptability of tumoral cells to some injurious conditions such as hypoxia. This model is characterized by the formation of some PAS positive channels lined directly by tumoral cells not by endothelial cells, contributing in this manner to intratumor blood flow. Vasculogenic mimicry was firstly described in uveal melanoma, malignant astrocytoma, breast cancer, osteosarcoma, etc (21).

There are many reports concerning intratumor angiogenesis and its importance in cancer progression and development. The vasculogenic mimicry pattern of tumor angiogenesis was and is an interesting idea that suggests the abilities of tumoral cells to avoid necrosis due to hypoxia. Vasculogenic mimicry was noticed also in cell cultures originated from aggressive melanomas; the cells had the abilities to form PAS positive channels without endothelium (4).

Furthermore, some studies proved that presence of vasculogenic mimicry is related with unfavorable prognosis. Initially was thought that blood flowing channels are generated by stromal cells originated in fibrovascular septa (3, 6, 18), but subsequent was noticed that the channels are bordered directly by tumoral cells, which generate PAS positive material to the channel’s lumen (19).

**MATERIAL AND METHODS**

Mammary tumor formations had been provided by corps or tumor biopsies reached to Pathology department from the University of Agricultural Science and Veterinary Medicine, Faculty of Veterinary Medicine Cluj-Napoca, Romania. There were utilized 7 malign and 1 benign tumors provided by different bitch breeds, such as: Cocker (3 subjects), Teckel (2 subjects), Amstaff (1 subject), German Sheppard (1 subject) and Mioritic Sheppard (1 subject). The mammary tumors are from 8 bitches, with the age of 2-13 years.

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Rb/Mo/Goat (DAB+) system (Dako); the counterstaining was performed by Mayer hematoxylin. To evaluate the antibody specificity were used negative control (replacing the primary antibody with antibody diluent) and internal positive tissue control (immunolabel of large vessels). Using PAS reaction may be highlighted mucopolysaccharides, which are present toward the inner part of the blood flowing channels. Double staining procedure (immunohistochemical and PAS reactions) had been realized at the end of immunohistochemical staining, before staining with Mayer hematoxylin. The slides were immersed in periodic acid (aqueous solution 0,5%) and Schiff reactive (30 minutes); Slides were rinsed with tap water and counterstained with Mayer hematoxylin.

To evaluate the microvessel number, perimeter and aria, we used a semiautomatic computerized analysis technique (Olympus Soft imaging solutions Cell B). There were analyzed 5 microscopic fields on every tumor, magnified of 200x. The microscopic images were obtained by Olympus BX51 microscope, connected to a photo digital camera (Olympus DP-25). Total vascular aria (total intratumor area expressed in µm²/image area, and its percentage; average vessel area for each tumor), total vascular perimeter (average perimeter expressed in µm/image area), and the microvessel number were related to microscopic image area (144352,00 µm²). Any isolated but immunohistochemically labeled endothelial cell (vessels without lumen) was quantified as distinct microvessel.

PAS positive blood flowing channels from different canine mammary tumor types were examined by monitoring all clear spaces bordered PAS positive material and/or directly by tumoral cells. The occurrence of vasculogenic mimicry pattern was evaluated as follow: relatively frequent encountered (++), rarely met but present (+), and absent (-).

RESULTS AND DISCUSSIONS

In 1948 in human pathology Willis et al. (1948) showed that some tumors with a fast growing rate presents some channels similarly in structure with blood vessels but without endothelium (20). The author mentions the bordering of the channel directly by tumoral cells. Later this feature was termed vasculogenic mimicry, being encountered in several aggressive tumors (1, 3, 4, 6, 14, 19, 21). Nasu et al. (1999) describe a similar type of intratumor angiogenesis, describing some non-endothelial channels where endothelial cells are scattered and without PAS positive material. The author considers these non-endothelial channels something different from vasculogenic mimicry (15). Elaborated work analyzed eight canine mammary tumors, respectively one benign and seven malignant tumors originated from different dog breeds of different age (2-13 years). Tumor size varied from 0,35 cm until to 20 cm. There were elected several histologic types of malignant tumors, such as: more differentiated mammary tumors and highly aggressive mammary tumors; it is known that vasculogenic mimicry is more frequent in poorly differentiated cancers.

Regarding intratumor angiogenesis, there were studied the main parameters which indicate angiogenic profile of a tumor, such as: microvessel number/microscopic field area, total vascular area and perimeter, average vascular area and perimeter, the structure of vascular walls, intensity of immunohistochemical reaction in vessel’s wall. All of these were monitored to detect blood flowing channels and to debate intratumor angiogenesis. Double staining procedure (immunohistochemical and PAS reaction) made possible evidence of aspects regarding vasculogenic mimicry almost in all poorly differentiated tumors (cases 1, 2, 4, 5, 6, 7). Blood flowing channels were more obvious using this method comparing with the other CD31-immunolabeling method. There should be mentioned that numerous blood
flowing channels without endothelium were noticed in vicinity of intratumor necrotic areas (case 5), being known that hypoxia is a stimulus for angiogenesis.

The location of blood flowing channels occur in both, connective tissue stroma (cases 2, 4, 7) and between neoplastic cells in the case of compact tumors with scattered sustaining stroma and numerous tumoral cells (cases 5, 7). In blood flowing channels without endothelium from sustentacular connective tissue, the misinterpretation of some empty spaces to be considered blood channels is minimal using double staining procedure. Also, there can be noticed blood flowing channels in which immunohistochemical reaction is discreet or more often restricted to a limited portion of the vessel wall not to all vessel circumference how is normal in blood vessels with continuous endothelium (cases 4, 5, 6, 7).

This aspect was also encountered by Nasu et al. (15). The PAS reaction highlights mucopolysaccharidic structure that line these channels, which don’t have endothelium (4, 14, 21). In many situations red blood cells can be seen into the channel’s lumen aiding with their notification.

Fig.1. Carcinoma in benign mixed tumor, grade II (case 8); double staining - IHC anti-CD31 and PAS reactions, counterstaining with Mayer’s hematoxylin x400.
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**Fig.1.** Carcinoma in benign mixed tumor, grade II (case 8); double staining - IHC anti-CD_{31} and PAS reactions, counterstaining with Mayer’s hematoxylin x400.
Table 1. General aspects regarding intratumor angiogenesis in different canine mammary tumors.

<table>
<thead>
<tr>
<th>Case nr.</th>
<th>Histologic diagnose</th>
<th>Histologic grade</th>
<th>Mitotic index</th>
<th>Intratumor angiogenesis</th>
<th>V M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IMD</td>
<td>TMA (%)</td>
</tr>
<tr>
<td>1</td>
<td>Solid carcinoma</td>
<td>II</td>
<td>19</td>
<td>27</td>
<td>5.81</td>
</tr>
<tr>
<td>2</td>
<td>Tubule-papillary carcinoma in benign mixed tumor</td>
<td>I</td>
<td>5</td>
<td>18.2</td>
<td>5.36</td>
</tr>
<tr>
<td>3</td>
<td>Adenoma</td>
<td>-</td>
<td>-</td>
<td>14.8</td>
<td>2.26</td>
</tr>
<tr>
<td>4</td>
<td>Cystic-papillary carcinoma</td>
<td>I</td>
<td>14</td>
<td>22.6</td>
<td>6.07</td>
</tr>
<tr>
<td>5</td>
<td>Solid carcinoma</td>
<td>III</td>
<td>24</td>
<td>24.4</td>
<td>2.34</td>
</tr>
<tr>
<td>6</td>
<td>Tubulopapillary carcinoma</td>
<td>II</td>
<td>33</td>
<td>49.2</td>
<td>5.40</td>
</tr>
<tr>
<td>7</td>
<td>Solid anaplastic carcinoma</td>
<td>III</td>
<td>13</td>
<td>22.3</td>
<td>3.28</td>
</tr>
<tr>
<td>8</td>
<td>Carcinoma in benign mixed tumor</td>
<td>II</td>
<td>12</td>
<td>18.8</td>
<td>4.10</td>
</tr>
</tbody>
</table>

IMD: Intratumor microvessel density (microvessel number)/area of microscopic image.
TMA: Total microvascular area (%)/area of microscopic image – average value obtained by monitoring five microscopic fields magnified of media 200x.
MA: Microvascular area (μm²) - average value obtained by monitoring five microscopic fields magnified of media 200x.
MP: Intratumor microvessel perimeter (μm) - average value obtained by monitoring five microscopic fields magnified of media 200x.

The new findings regarding angiogenesis deliver some important and useful dates not only about growing rate and prognosis, but also to improve antitumoral therapeutic protocols some of them involving the destruction of blood vessels which supply the neoformation. Anti-angiogenic therapies may be realized using natural or synthetic inhibitors of angiogenesis, such as angioatin, endostatin, tumtatina, etc. Endothelial cells were and are considered, genetically, more stable structure than cancerous cells. This genomic stability confers an advantage in elaboration of antitumoral therapies that have as target endothelial cells using anti-angiogenic agents. Because of that, endothelial cells may represent ideal targets for antitumor therapy. Nevertheless, antitumor therapeutic protocols using antiangiogenic agents (targeting vascular endothelium) may be useles for cancerous areas where the vascularisation occur using vasculogenic mimicry, which don’t have endothelium.
CONCLUSIONS

1. Utilizing either double staining procedure (immunohistochemical anti-CD31 and PAS reactions) or single immunohistochemical anti-CD31 technique made possible notification of vasculogenic mimicry in highly aggressive tumors, such as grade II and III canine mammary cancer. Nonendothelial blood channels were less extended to differentiated tumors, practically being absent in benign tumor.

2. The occurrence of blood flowing channels was higher in vicinity of intratumor necrotic areas knowing that hypoxia stimulate angiogenesis.

3. Vasculogenic mimicry was notified less frequent in sustaining connective tissue and more frequent in tumors with reduced stroma and numerous cancerous cells, such as compact carcinomas and simple carcinomas.

4. Some peculiarities of some blood flowing channels were represented by discreet immunohistochemical reaction that often was restricted only to a region of vascular wall not to all circumference of the vessel. This indicates that some vessels are incompletely lined by endothelial cells, the rest of the vessel’s lumen being bordered by tumoral cells. Furthermore, PAS reaction highlighted mucopolysaccharides which lined the channels without endothelium.

5. Occurrence of blood flowing channels was higher in tumors with increased microvessel density/microscopic field, and in tumors that had numerous microvessels with reduced caliber, both features indicating increased intratumor angiogenesis and alert tumor growth.

BIBLIOGRAPHY

Correlation between Intratumor Microvessel Density and Ki-67 Malignancy Marker in Bitch Mammary Cancer

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Abstract. The goal of the study is to evaluate and to correlate the significance of intratumor microvessel density with tumor malignancy degree. Tumor malignancy was appreciated using proliferating markers such as Ki-67, histology index, mitotic index, tumor size, and histology type. The study was realized using different histology types of bitch mammary tumors according to WHO classifications. Our results indicate a direct correlation between intratumor microvessel densities and mentioned proliferating marker, respectively Ki-67. The study of angiogenic profile of mammary tumors delivers enough dates about them, but intratumor microvessel density should be associated with reliable parameters that indicate correctly mammary tumor malignancy. Our dates obtained by Ki-67 marker quantification correlated with malignancy histological grade in studied canine mammary tumors confirming its utility in bitch mammary cancer.

Keywords: angiogenesis, tumor, proliferation, immunohistochemistry

INTRODUCTION

Mammary tumors in bitch like breast cancer in women have an increased incidence that determined scientific community to study them. Mammary tumors are the most frequent tumors in intact bitches representing about 52% from tumoral lesions in this specie. Histology diagnoses reveal that about 41 – 53% from mammary tumors in bitch is malign lesions (Baba et al., 2001; Baba et al., 2007, Pena et al., 1998). Histology features aren’t enough to prove mammary tumor malignancy because doesn’t involve obligatory a poor clinical evolution. That’s why is very important to evaluate prognostic factors to find out individual clinic evolution. Specialty literature describe some utile prognosis factors for canine malign mammary tumors, such as: tumor size, local lymph nodes status, metastasis presence, histology grade, nuclear differentiation grade, and others. On the other hand there are some useful proliferation markers like AgNORs, Ki-67, and PCNA, but markers such as intratumor angiogenesis could have an important role for their malignancy appreciation (Gal et al., 2004; Gal et al., 2005; Gal et al., 2007). In fact, intratumor microvessel density in breast cancer is one of the markers that deliver useful clues about tumor status and aggressiveness.

The aim of our study is to evaluate both expression of nuclear proliferation marker Ki-67 and angiogenesis. The intratumor angiogenesis was evaluated by some parameters such as: microvessel density, area and perimeter in varied neoplastic mammary tumors. The results are going to highlight the correlation (if there is one) between studied parameters (Ki-67 and angiogenesis markers) and some other classical and reliable parameters (tumor histology type and grade, mitotic index, tumor size and intratumor necrosis). Will be established their significance as malignancy marker in mammary tumors.
MATERIAL AND METHODS

Mammary tumor formations had been provided by corps reached to Pathology department from the University of Agricultural Science and Veterinary Medicine, Faculty of Veterinary Medicine Cluj-Napoca, Romania. There were utilized 7 malign and 1 benign tumors provided by different bitch breeds, such as: Cocker (3 subjects), Teckel (2 subjects), Amstaff (1 subject), German Sheppard (1 subject) and Mioritic Sheppard (1 subject). For histopathology exam the samples had been fixed in 10% buffered formalin and processed by paraffin technique, and stained by hematoxilin-eozin and trichrome Masson technique. The mammary tumors are from 8 bitches, with the age of 2-13 years. To make an appropriate histology grading of malignant tumors based on WHO classification, were evaluated nuclear degree, tubule formation and mitotic index.

Intratumor microvessel density and tumor proliferation was evaluated by LSAB immunohistochemistry technique using anti-CD\textsubscript{31} respectively anti-Ki-67 marker (Dako CD\textsubscript{31} monoclonal antibodies - clone JC70A, izotype IgG1 kappa; Dako Ki-67 monoclonal antibodies - clone KI-S5, izotype IgG1 kappa). Histological slides had about 5 \( \mu m \) thicknesses and were fixed on silanized slides (Dako) during 24 hours in 37°C, followed by deparaffination in xylene. Antigen retriever had been made using a pressurized cooker in citrate solution, pH=6.0 (Dako); endogenous peroxidase was inactivated by peroxidase blocking reagent (Dako - Peroxidase and PA blocking reagent 3%) during 5 minutes at the room temperature. Primary monoclonal antibodies (anti-CD\textsubscript{31} and anti-Ki-67) were maintained overnight, during 18 hours at 4°C, using a dilution of 1:30 respectively 1:75 in antibody diluent (Dako). The visualization of immunological reaction was performed using Universal LSAB+Kit/HRP, Rb/Mo/Goat (DAB+) system (Dako); the counterstaining was performed by Mayer hematoxylin. To evaluate the antibody specificity were used negative control (replacing the primary antibody with antibody diluent) and internal positive tissue control (large micro vessel immunolabel) and tonsil for Ki-67.

To evaluate the microvessel number, perimeter and aria, we used a semiautomatic computerized analysis technique (Olympus Soft imaging solutions Cell B). There were analyzed 5 microscopic fields on every tumor, totally of 40 microscopic images magnified of 200x. The microscopic images were obtained by Olympus BX51 microscope, connected to a photo digital camera (Olympus DP-25). Total vascular aria (total intratumor area expressed in \( \mu m^2 \)/image area, and its percentage; average vessel area for each tumor), total vascular perimeter (average perimeter expressed in \( \mu m \)/image area) and the microvessel number were related to microscopic image area (144352,00 \( \mu m^2 \)). Any isolated but immunohistochemically labeled endothelial cell (vessels without lumen) was quantified as distinct microvessel.

Ki-67 marker evaluation had been made by counting negative and positive cells (cells with varied brown intensities into nucleus). The counting was realized by 3 evaluators. Ki-67 evaluation was realized using 8-10 microscopic images magnified of 400x for every tumor, being counted a total of 1000 tumor cells. It was established the percentage of positive cells.

The dates had been statistically interpreted using SPSS program, considering significant statistic values of p<0,05. There were established if it is a correlation between histology grade and type, Ki-67 marker and studied intratumor microvessel parameters.

RESULTS AND DISCUSSIONS

In veterinary and human medicine is a sustained effort to add to classic prognosis factors (tumor size, ulcers, lymph nodes invading and so on) of new prognosis factors for mammary tumors. There are some markers which quantify genetic mutations or tumor
proliferation index, and some other studies highlight the possible prognosis significance of intratumor microvessel density in canine mammary tumors. Malignancy markers quantification importance resides from a quite different biological comportment for bitch mammary tumors, which could induce real problems for veterinary clinicians. Also, malignancy factors research is a priority field for this kind of tumors.

In the last period of time in comparative oncology had been introduced a series of tumor markers oncoproteins, such as: tumors suppressor genes products (p53), apoptosis inhibitors (bcl-2), cells adhesion molecules (CD44), stem cells markers (CD34) and tumor proliferation markers (Ki-67 and PCNA). Their clinic significance was established in several cancer types (Takes et al., 1997). From all presented factors it seems that only Ki-67 and PCNA markers could be considered real parameters, which could be utilized to make quick and useful evaluation regarding tumors proliferation degree. There are many studies indicating association of Ki-67 and PCNA proliferating markers and cancers’ prognosis and metastasis (Lindmark et al., 1996; Takes et al., 1997; Thompson et al., 1987).

Beneath mentioned proliferation parameters in some human and animal cancers, intratumor microvessel density represents another important malignancy and prognosis parameter. Also, in this study was evaluated the value of intratumor angiogenesis comparatively to some other classic parameters for bitch mammary cancer, such as histology tumor type and grade, mitotic index and Ki-67 proliferation index. Intratumor microvessel density is a measurement of angiogenesis. Angiogenesis is essential for tumor nutrition and have an important role in invasion and metastasis. Increased intratumor microvessel density is associated with quick tumor development and reduced survivor period (Fox et al., 1996; Misdorp et al., 1999).

There will be detailed the results obtained for evaluated cases following to notice some correlations between them. The dates will be compared with bibliographic dates.

<table>
<thead>
<tr>
<th>Case nr.</th>
<th>Histopathology diagnosis</th>
<th>HG</th>
<th>MI</th>
<th>IMD (Avg)</th>
<th>TMA (%</th>
<th>Average MA</th>
<th>Average MP</th>
<th>MP Sum</th>
<th>Ki-67 Average Value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Solid carcinoma</td>
<td>II</td>
<td>19</td>
<td>27</td>
<td>5,81</td>
<td>310,93</td>
<td>92,32</td>
<td>2492,59</td>
<td>22,6</td>
</tr>
<tr>
<td>2</td>
<td>Tubule-papillary carcinoma in BMT</td>
<td>I</td>
<td>5</td>
<td>18,2</td>
<td>5,36</td>
<td>425,89</td>
<td>110,63</td>
<td>2013,46</td>
<td>13,4</td>
</tr>
<tr>
<td>3</td>
<td>Simple adenoma</td>
<td>-</td>
<td>-</td>
<td>14,8</td>
<td>2,26</td>
<td>221,20</td>
<td>77,08</td>
<td>1140,75</td>
<td>12,76</td>
</tr>
<tr>
<td>4</td>
<td>Cystic papillary carcinoma</td>
<td>I</td>
<td>14</td>
<td>22,6</td>
<td>6,07</td>
<td>388,17</td>
<td>99,59</td>
<td>2250,75</td>
<td>22,13</td>
</tr>
<tr>
<td>5</td>
<td>Solid carcinoma</td>
<td>III</td>
<td>24</td>
<td>24,4</td>
<td>2,34</td>
<td>166,76</td>
<td>63,12</td>
<td>1540,16</td>
<td>26,7</td>
</tr>
<tr>
<td>6</td>
<td>Tubule-papillary carcinoma</td>
<td>II</td>
<td>33</td>
<td>49,2</td>
<td>5,40</td>
<td>158,72</td>
<td>62,72</td>
<td>3085,68</td>
<td>22,43</td>
</tr>
<tr>
<td>7</td>
<td>Anaplastic solid carcinoma</td>
<td>III</td>
<td>13</td>
<td>22,33</td>
<td>3,28</td>
<td>212,56</td>
<td>78,85</td>
<td>1760,94</td>
<td>55,5</td>
</tr>
<tr>
<td>8</td>
<td>Carcinoma in BMT</td>
<td>II</td>
<td>12</td>
<td>18,8</td>
<td>4,10</td>
<td>314,96</td>
<td>99,80</td>
<td>1876,28</td>
<td>45,05</td>
</tr>
</tbody>
</table>
Regarding intratumor angiogenesis, from all studied parameters (vessel density, area and perimeter) we observed a direct correlation with tumor malignancy (established using classic markers – histology grade and type, Ki-67 marker, mitotic index) only for IMD parameter. There are an increased number of microvessels in malign mammary lesions comparative to benign tumor. In simple adenoma IMD was about 14.8 vessels/microscopy field comparative to malign mammary lesions where IMD varied from 18.2 to 49.2 vessels/microscopy field. This feature indicates a direct correlation between tumors malignancy and angiogenesis. In malignant mammary tumors was a direct correlation between IMD and histological grade. There is one exception (case 4), this tumor being a differentiated tumor (grade I) despite of an increased IMD (22.6 vessels/microscopy field). Excepting case 4, there is an increased IMD in grade II and III (22.33 – 49.2 vessels/microscopy field) mammary tumors comparatively with grade I (18.2 vessels/microscopy field) and benign tumors (14.8 vessels/microscopy field). Despite of that IMD wasn’t correlated statistically with histological grade or Ki-67 proliferation marker. Also, IMD can’t be utilized like malignancy marker in canine mammary tumors (histological
grade/IMD – p = 0.420, r = 0.333; IMD/Ki-67 – p = 0.906, r = -0.050), only associated with some other reliable parameter.

Regarding bibliographic dates there are contradictory results. Restucci et al. (2000) utilized CD₃₁ marker to label intratumor microvascularization and concluded a direct correlation between IMD and canine mammary tumor malignancy. They highlighted an increased IMD in malignant tumors comparative with benign lesions, and indicate intratumor angiogenesis significance like malignancy marker in canine mammary tumors. Furthermore, there are some authors indicating IMD fidelity in prognosis and survivor period (Borsari et al., 1992; Kerns et al., 1994; Restucci et al., 2000, Spafford et al., 1996). Poorly vascularized tumors are going to spread more difficult comparatively with more vascularized tumors no matter of their origin and type, such as: prostate, breast, colon or lung cancer (Gal et al., 2003).

On the other hand there are some studies indicating some contradictions with presented results. Some reports indicate that IMD isn’t correlated with malignancy and prognosis (Gal et al., 2003, Gal et al., 2008, Gerdes et al., 1984, Restuci et al., 2000). Moreover, some studies noticed a more favorable prognosis in more vascularized tumors comparatively with poorly vascularized tumors (Luong et al., 2006). Thompson et al. (1987) investigated early angiogenesis stages in transplanted mammary adenocarcinoma in laboratory animals. During tumor development the vascularisation suffers a very fast growing in first stages, reaching to a plate in later stages comparatively with normal adjacent tissue. Intratumor microvessel development is based on host tissue vessels incorporation (Zuccari et al., 2008).

Evaluating average area and perimeter in studied tumors we noticed reduced values in aggressive tumors (grade II and III mammary tumors – cases 1, 5, 6, 7, and 8) comparatively with grade I mammary tumors (cases 2 and 4). Also, in poorly differentiated canine mammary
grade/IMD – \( p = 0.420, r = 0.333 \); IMD/Ki-67 – \( p = 0.906, r = -0.050 \), only associated with some other reliable parameter.

Fig. 5. Simple tubule-papillary carcinoma (case 6) – increased IMD and isolated labeled endothelial cells; CD\textsubscript{31} immunohistochemical reaction, Mayer hematoxylin counterstaining x100.

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Vasculogenic mimicry has no prognostic significance in pT3 and pT4 cutaneous melanoma

Daniela Massi, Alessandro Franchi, Milena Paglierani, Sheyda Ketabchi, Lorenzo Borgognoni, Umberto Maria Reali, Marco Santucci

Abstract

The concept of vasculogenic mimicry has been introduced to define periodic acid-Schiff (PAS)-positive channels and loops lined by tumor cells, instead of endothelium, able to contribute to microcirculation in uveal melanomas. Previous studies have shown that the PAS-positive patterns are associated with a poor prognosis in uveal melanoma. The aim of the current study was to investigate whether vasculogenic mimicry has a prognostic impact in pT3 and pT4 cutaneous melanoma. Fifteen patients with pT3 and pT4 cutaneous melanoma who did not experience progression after 10 years of follow-up and 30 matched controls who underwent progression were selected. Tumor sections were stained with PAS reaction, omitting the nuclear counterstaining. For immunohistochemistry, sections were stained with CD31, CD105 (endoglin), and laminin. Differences in the distribution of the PAS-positive patterns and a series of clinicopathological variables were evaluated by the Pearson $\chi^2$ and Mann-Whitney U tests. We observed PAS-positive linear sheets, arcs, elliptical loops, and networks encircling roundish to oval aggregates of melanoma cells. The overall distribution of the PAS-positive patterns did not match with the blood microvessels’ architecture as detected by immunohistochemical analysis. No statistically significant differences in the distribution of PAS-positive patterns were found between cases and controls. The presence of a parallel pattern correlated significantly with thickness ($P = 0.04$), whereas an inverse correlation was found with vessel area ($P = 0.05$). In conclusion, our results suggest that there is a mismatch between vasculogenic mimicry and tumor angiogenesis and do not support any prognostic role of vasculogenic mimicry in thick cutaneous melanoma.
Vasculogenic mimicry has no prognostic significance in pT3 and pT4 cutaneous melanoma

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Neoangiogenesis in cervical cancer: focus on CD34 assessment

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Abstract
Despite recent advances in understanding the immune mechanisms of cervical cancer (CC), relapse remains still an actual issue and recognition of new predictive biomarkers is essential. Aim: The purpose of this retrospective study was to investigate neo-angiogenesis in CC and its possible utility as prognostic biomarker. Material and Methods: Paraffin-embedded tissue samples from 61 consecutive women with CC were immunostained for CD34 and E-cadherin. Statistical analysis was performed in SPSS–12 software, \( p < 0.05 \). Results: Statistically significant differences between CD34 distribution among three interest tumor regions: micro-vessels density increase from central to peripheral area (\( \chi^2, p < 0.05 \)); statistically significant correlation between CD34 expression, particularly in stromal and peripheral sites, E-cadherin (Spearman \( r_1 = -0.321 \)) and lymphatic invasion (Spearman \( r_2 = 0.455 \)) (\( p < 0.05 \)) were reported. Overall five-year survival is clearly dependent on level and distribution of tumor angiogenesis among defined area of interest as suggested by Kaplan–Meier analysis. Conclusions: Angiogenesis is essential for guiding CC evolution and prognosis, particularly in squamous invasive types.

Keywords: cervical cancer, angiogenesis, CD34 antigen.

Introduction
Cervical cancer (CC) represents an important cause of morbidity and mortality in women worldwide. Despite complex therapeutic opportunities, ranging from classical surgery to newly approved anti-Human Papilloma Virus (HPV) vaccines, relapse still occurs in about 40% of women with CC [1].

At least three key factors are involved in tumor aggressiveness including uncontrolled proliferation activity, adhesion, migration and tumor cell invasiveness, and tumor neo-angiogenesis, resulting in a complex vicious circle [2–4].

Neo-angiogenesis or new micro-vessels formation advances through a multifaceted interaction between pro- and anti-angiogenic signals released by endothelial and stromal tumor cells and is critical for tumor growth, progression and metastases. The angiogenic activity, revealed by the development of novel micro-vessels in tumor tissue, can be quantified by intra-tumoral micro-vessel density, which, in turn, can be assessed by tissue expression of several representative molecules involved in angiogenesis such as VEGF (Vascular Endothelial Growth Factor), factor VIII-related antigen/von Willebrand’s factor, CD31 (Platelet Endothelial Cell Adhesion Molecule, PECAM-1), TSP-1 (Thrombospondin-1), UEA-1 (Ulex Europaeus Lectin 1) and CD34 [1–4].

The CD34 antigen, a member of a sialomucin family, is a single heavily chain transmembrane 67 kDa glycoprotein, expressed mainly on human hematopoietic stem and progenitor cells, vascular endothelial cells, but absent on fully differentiated hematopoietic cells; while the main function of CD34 is intercellular adhesion, anti-CD34 antibody is a highly sensitive biomarker for endothelial cell differentiation, that has been extensively studied in tumor angiogenesis [1–5].

Both classical (including tumor size, depth of stromal invasion, lymphatic metastasis, positive resection margins, histological type, tumor grading) and modern prognostic factors (tumor and immune biomarkers) have already been described, but not yet validated in cervical cancer [1, 2]. However, new predictive biomarkers are still necessary to identify and stratify patients according to their risk of relapse and to optimize disease management, especially in early cervical cancer.

Despite increased knowledge on tumor angiogenesis, research in cervical cancer have suggested rather conflicting data regarding the potential prognostic value of (neo)angiogenesis [6–9].

The aim of this work was to investigate cervical cancer angiogenesis by assessing tissue expression of CD34 and to evaluate the association between micro-vessel density and classical prognostic factors in cervical cancer.

Material and Methods
We performed a retrospective observational study on sixty-one consecutive women diagnosed with invasive cervical cancer undergoing radical hysterectomy with or...
without bilateral pelvic lymphadenectomy; all patients have attended Gynecology Department of “Cuza-Vodă” Hospital in Iassy between 2000 and 2003 and data regarding five-years overall free survival were retrieved from regional oncology files.

Paraffin-embedded cervical tissues were processed at the time of diagnosis at the Pathology Department of “Cuza-Vodă” Hospital and immunohistochemistry (IHC) was done at the Immunopathology and Genetics Laboratory of “Sf. Spiridon” Hospital in Iassy. The study was approved by the local Ethics Committee.

**Immunohistochemistry**

To identify neo-angiogenesis, tissue sections were immunostained for CD34 biomarker (CD34 class II mouse monoclonal antibody, Clone QBEnd, Code M7165, DAKO, in dilution of 1:25) and streptavidin–biotin method was used [10]. CD34 was assessed in tumor gradient on five representive microscopic fields, three area of interest being selected (central, median, stromal and peripheral tissue); distinction between low, mild and high micro-vessel density was based on micro-vessel count: 0–33, 33–66, and 66–99 vascular elements per examined field. Micro-vessels were counted in the area with the highest density (“hot spot”), after the identification with a smaller magnification; a brown-staining endothelial cell obviously separated from adjacent micro-vessels, tumor cells and other connective tissue elements was considered a single quantifiable micro-vessel.

Tissues have also been stained also for E-cadherin antibodies (DAKO); assessed as brown color of the cell membrane, E-cadherin was classified either negative (loss of expression), meaning loss of intercellular adhesion and increased tumor invasiveness, or positive, non-homogenous reaction.

**Statistical analysis**

Descriptive statistics, non-parametrical tests (Spearman’s correlation, Mann–Whitney and chi-squared tests) and Kaplan–Meier survival analysis were performed in SPSS–12 software, \( p<0.05 \).

**Results**

Baseline characteristics of selected cases with cervical cancer related to classical prognostic factors are shown in Table 1.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Percentage of cases [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FIGO classification</strong></td>
<td></td>
</tr>
<tr>
<td>IA</td>
<td>19.7</td>
</tr>
<tr>
<td>IB</td>
<td>31.1</td>
</tr>
<tr>
<td>IIA</td>
<td>4.9</td>
</tr>
<tr>
<td>IIB</td>
<td>39.3</td>
</tr>
<tr>
<td><strong>Lymph node invasion</strong></td>
<td></td>
</tr>
<tr>
<td>With invasion</td>
<td>31.1</td>
</tr>
<tr>
<td>Without invasion</td>
<td>68.9</td>
</tr>
<tr>
<td><strong>Five-years overall survival</strong></td>
<td></td>
</tr>
<tr>
<td>Death</td>
<td>42.6</td>
</tr>
<tr>
<td>Survival</td>
<td>57.4</td>
</tr>
<tr>
<td>Relapse</td>
<td></td>
</tr>
<tr>
<td>With</td>
<td>55.7</td>
</tr>
<tr>
<td>Without</td>
<td>44.3</td>
</tr>
</tbody>
</table>

Figure 1 presents different aspects of CD34 expression in cervical cancer, as defined by IHC.

Several critical differences regarding the distribution of CD34 expression between three-tumor area of interest and their relation with classical prognostic factors have been suggested in our study.

**CD34 expression in central tumor area – association between immunostaining and clinico-pathological variables**

CD34 expression in central tumor area – association between immunostaining and clinico-pathological variables: (i) no difference between levels of angiogenesis in CC in young, while patients aged more than 55-year-old mainly presented with low micro-vessel counts (72%); (ii) low CD34 expression in the up to 90% of micro-invasive CC, but also in more than half of squamous invasive type (55%); (iii) low micro-vessels count in both G1 (67%) and G2 (60%) grading; (iv) low angiogenesis in the majority (62%) of CC without lymph node metastasis (moreover, no lymphatic invasion has been reported in highly vascularized central tumor areas); (v) moderate neo-angiogenesis in 56% of recurrences, while (vi) increased global survival rate in CC with low CD34 expression (60%) have been noted in our study.

**CD34 expression in median tumor area – association between immunostaining and clinico-pathological variables**

CD34 expression in median tumor area – association between immunostaining and clinico-pathological variables: (i) no significant differences between young and elder CC based on angiogenesis in above mentioned area; moderate CD34 expression in the more than half of cases (67% under 35-year-old, 54% between 35–55-year-old, 61% above the age of 55 years); (ii) moderate neo-angiogenesis in about 50% MICC and 60% of SICC; (iii) moderate count of micro-vessels per field in G1 (73%) and G2 (47%) tumors; (iv) moderate angiogenesis in CC with lymphatic invasion (53%); (v) moderate angiogenesis in 65% of relapsed CC and (vi) worse five-years overall survival rate for women with moderate CD34 expression (65%) have been showed in studied CC.
Kaplan–Meier survival analysis

Low micro-vascular density assigned to central tumor sites is associated with high five-years overall survival rate in cervical cancer as suggested by Kaplan–Meier survival analysis (Figure 2a). Moreover, lower CD34 expression in median tumor area, higher disease free survival; as we have shown predominantly moderate CD34 expression in this particular area, we have reported an increased number of deaths during the monitoring interval. Survival rate was settled at 40% in both moderate and high angiogenesis CC and increases at 60% for those with low vascularized CC (Figure 2b).

High CD34 expression in stromal and peripheral area is associated with decreased free survival; about 30% of CC with high tumor angiogenesis in the above mentioned area and up to 90% of low vascularized CC are still alive five years after diagnosis (Figure 2c).

In the particular case of squamous invasive CC, Kaplan–Meier survival analysis based on micro-vessel density in central tumor area has suggested a dramatic course of the disease. Five-years global survival rate achieve only 25% for moderate neo-angiogenesis tumors, while low-vascular density up to 30%, suggesting that certain specific factors stimulate such an aggressive pattern (Figure 3a). Also, highly vascularized median tumor region denoted significant decrease in survival rate (about 20%), while mild micro-vessel density account for 30% survival rate; CC with low angiogenesis featured the best survival rate (40%) (Figure 3b).

According to CD34 expression in the last region of interest (stromal and peripheral area), low vascularized SICC displayed a good survival rate (up to 80%), while both moderate and high micro-vessel density resulted in decreased survival (mean value of 20%) (Figure 3c).

Discussion

Although performed on a limited number of patients (61 cases) and designed to evaluate vascular density in cervical cancer and the possible relation to clinical outcome, this study has pointed out on several particular aspects of angiogenesis in cervical cancer.

While other studies bring into attention several biomarkers for cervical neoplasia including anti-BNH9, anti-VEGF, anti-CD31 [3, 12, 13], we have applied only CD34 endothelial cell marker for the quantification of neo-angiogenesis. Commonly, the information acquired admit the data from literature with certain differences.

We have demonstrated a distinctive pattern of CD34 expression among three region of interest (low CD34 level in central tumor region, moderate angiogenesis in median area, while high micro-vessel formation was reported in stromal and peri-tumoral tissues) which is statistically significant ($\chi^2$, $p=0.001$). Micro-vessels
density significantly increases from central to peripheral area. In addition we have studied differences in CD34 distribution according to age, tumor grading, histological type, HPV-infection, pelvic lymph node metastases, relapse and 5-years overall survival rate.

Other studies have also reported major differences of CD34 expression among the above mentioned intra-tumoral regions [11]; moreover, intra-tumoral variation of the micro-vascular density has been demonstrated especially in poor differentiated carcinoma where the highest angiogenesis is reported in central and extra-tumoral area [11]. The same authors have also suggested significant discrepancy in CD34 expression based on tumor grading. Besides, the spatial variation of tumor angiogenesis is considered of predictive value for relapse and survival in women diagnosed with CC [11].

We have identified several relevant correlations between CD34 expression, mainly CD34 in stroma and peri-tumor tissues, and other factors of negative prognosis, such as E-cadherin expression (high peri-tumor neo-angiogenesis is associated with loss of E-cadherin expression) and lymphatic invasion (high neo-angiogenesis in peri-tumor tissues illustrated the direct spread of CC to lymph node). The same correlation between CD34 level and lymphatic metastases was suggested by Francu DL et al.; higher angiogenesis, higher potential of metastasis and poor prognosis [11].

As demonstrated by Vieira SC et al., higher micro-vessel density in frequently reported in squamous invasive CC type and undifferentiated carcinoma [12, 13].

Furthermore, research studies in literature reported statistically significant correlation between the intensity of angiogenesis and the presence of lymphatic invasion [12–14] as well as vascular involvement [14]. Also, anti-CD34 antibody reactivity is associated with pathological anatomical features indicative of poorer prognosis in cervical carcinoma [12, 13], particularly in SICC [14].

As reflected by Kaplan–Meier analysis, five-year overall median survival in our study is clearly dependent on micro-vascular density in selected tumor sites: lower CD34 expression among central and median tumor areas is associated with increased survival, while higher CD34 in stromal and peripheral tumor tissues advanced with decreased five-years overall survival. Moreover, the presence of a high angiogenesis in median tumor tissue associated with lymph node metastasis dramatically affects free survival; the same tendency was reported by Francu DL et al. [11].

Data from literature also support the idea that the five-year overall survival rate for patients with high micro-vessel density was significantly worse than for those with low angiogenesis [11, 14].

At the same time, Kaplan–Meier survival analysis for squamous invasive CC displayed a comparable pattern for CD34 expression in stroma and peri-tumor tissues, supporting the concept that high angiogenesis promote cancer invasion and death; the same is valuable for micro-vessel density in median tumor, while central tumor vascularization lead to a different design; specific squamous invasive CC factors result in aggressive disease pattern.

Conclusions

(Neo)angiogenesis (assessed by CD34 expression) and tumor invasiveness (defined by E-cadherin) represent additional factors that promote tumor aggressiveness. Moreover, angiogenesis is essential for guiding cervical cancer evolution and prognosis, particularly in squamous invasive types. However, larger cohort studies are necessary for the validation of CD34 as prognostic biomarker in cervical cancer.

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**References**


Abstract

Introduction: There have been few studies on lymphangiogenesis in the past due to the lack of specific lymphatic endothelial markers, and lymphatic-specific growth factors. Recently, these limitations have been relieved by the discovery of a small number of potential lymphatic-specific markers. The relationship between lymphangiogenesis and regional or distant metastasis has not previously been investigated in humans. Using these lymphatic markers, it is possible to explore the relationship between lymphangiogenesis and tumour metastasis. This study indirectly quantified lymphangiogenesis by measuring mRNA expression of all seven lymphatic markers described above in breast cancers and correlated these markers with lymphatic involvement and survival.

The cDNA from 153 frozen archived breast samples were analysed with Q-PCR for all seven lymphangiogenic markers. This was correlated with various prognostic factors as well as patient survival.

Results: There was significantly greater expression of all 7 markers in malignant compared to benign breast tissue. In addition, there was greater expression in lymph node positive/grade 3 tumours when compared to lymph node negative/grade 1 tumours. In 5 of the markers, there was a greater expression in poor NPI prognostic tumours when compared to favourable prognostic tumours which was not statistically significant. There was no association between recurrence risk and lymphangiogenic marker expression.

Conclusion: In summary, the findings from this study show that lymphangiogenesis, measured by specific lymphatic marker expression, is higher in breast cancers than in normal breast tissue. Secondly, breast cancers which have metastasised to the regional lymphatics show higher expression compared to those which have not, although the individual differences for all five markers were not statistically significant.
Introduction
Breast cancer is one of the leading causes of cancer death in the female population in the Western World affecting as many as one in ten women in the UK [1], and its incidence appears to be rising. Although earlier diagnosis and better treatment are now available, many of the mechanisms underlying its ability to metastasise are poorly understood. Breast cancer spreads primarily via the lymphatic system. Regional lymph nodes are usually the first metastatic sites to be involved, often followed by distant metastasis to the lungs, liver and bones. Although various prognostic factors are known, regional lymph node status is the single most important prognostic factor in breast cancer; patients with axillary metastasis at the time of diagnosis have a much worse prognosis than those without metastasis [2,3].

Clinical and pathological observations have long suggested that for many other tumours, the most common pathway of initial dissemination is also via lymphatics, with patterns of spread via afferent vessels following routes of natural lymphatic drainage. However, the lymphatic system has traditionally been overshadowed by the greater emphasis placed on angiogenesis, the formation of new blood vessels. Indeed, it is widely accepted that angiogenesis is necessary for the growth and metastatic spread of solid tumours [4,5]. There have been relatively few studies on lymphangiogenesis in the past due to the lack of suitable markers that distinguish lymphatic from blood vascular endothelium, and the lack of lymphatic-specific growth factors. Furthermore, it is not known whether pre-existing lymphatic vessels are sufficient to permit initial tumour metastasis, or whether tumour dissemination requires the development of new lymphatics [4].

In recent years, these limitations have been relieved by the discovery of a small number of potential lymphatic-specific markers [5]. These include: LYVE-1, a lymphatic endothelial receptor for hyaluronan [6], Prox1, a homeobox gene product involved in regulating early lymphatic development [7], podoplanin, a glomerular podocyte membrane mucoprotein which is also found on lymphatic endothelium, but not in blood vessels [8], 5'-nucleotidase, an enzyme whose activity is very high in the lymphatic endothelium, but not in blood vessels [9], and the vascular endothelial growth receptor-3 (VEGFR-3) which is a transmembrane tyrosine kinase receptor predominantly expressed on the lymphatic endothelium [10]. VEGFR-3 has been shown to control the development and growth of the lymphatic system [11] VEGF-C [12] and VEGF-D [13] are two polypeptide growth factors which are agonists of the VEGFR-3 receptor, and may therefore be considered to be lymphangiogenic [14] These factors have been shown to be associated with lymphatic and distant metastasis and shorter overall survival [15]. In contrast, the angiogenic growth factor, VEGF, does not bind to VEGFR-3.

Breast cancer angiogenesis has been clearly linked to tumour metastasis. Using histopathological staining methods for blood vessel endothelial markers, a significant direct correlation was found between the highest microvessel density in histological sections of human invasive breast cancer and the occurrence of metastases [16]. Notwithstanding these findings, no studies have shown that angiogenesis is correlated with regional lymph node metastasis in breast cancer. The relationship between lymphangiogenesis and regional or distant metastasis has not previously been investigated in humans. Real-time quantitative polymerase chain reaction (QPCR) has now become an established method of quantifying genetic sequences [17]. Using QPCR, a novel approach for quantifying lymphatic markers has been recently described in breast cancer, using LYVE-1 [18].

Using these new lymphatic markers, it is possible to explore the relationship between lymphangiogenesis and tumour metastasis. The aim of this study was to indirectly quantify lymphangiogenesis by measuring mRNA expression of all seven lymphatic markers described above in breast cancers and correlate these markers with lymphatic involvement and survival.

Methods
Specimens
153 frozen archived breast samples from 105 patients were kept at -80°C. The samples consisted of breast cancers and background benign breast tissue. Histopathological information and patient follow-up details of the specimens were collected but blinded until the end of the study. 7 µm sections were cut from each specimen for histopathology (see below) and ten adjacent 10 µm sections were stored for subsequent RNA extraction. Human umbilical vein endothelial cells (HUVECs), a fibroblast cell line (MRC-5), and two breast cancer cell lines (MB MDA 231 and MCF7), were also cultured.

Histopathology
All samples were stained with haematoxylin and eosin for routine histopathological assessment by a consultant pathologist to confirm whether or not tumour was present in the sample. The percentage of tumour, with respect to surrounding stroma, was also estimated. Each assessment was later compared with the patients’ original histopathological reports. In addition, sections were also stained with factor 8 monoclonal antibodies, using standard immunohistochemical techniques. Five microscope fields were counted for each case and summed to give a single figure. Ten sections were blindly counted a second time to exclude the presence of intra-observer error.