Abstract. Background/Aim: Tumor angiogenesis has been the subject of intensive research in recent years in many tumor types. Studies involving epithelial skin tumors are few to date. We evaluated tumor angiogenesis and lymphangiogenesis in cancerous and pre-cancerous lesions of the eyelids using immunohistochemical techniques.

Materials and Methods: The study included 147 formalin-fixed, paraffin-embedded samples. We studied cancerous lesions of the eyelid skin such as basal cell carcinoma, squamous and basosquamous cell carcinoma and pre-cancerous lesions such as actinic keratosis and Bowen’s disease. We applied immunohistochemical staining using antibodies to investigate angiogenic and lymphangiogenic molecular factors, such as vascular endothelial growth factor (VEGF) and its receptors. We recorded the microvascular density of these tumors by using the marker CD-105, a specific antibody against endoglin protein. Results: Data analysis showed that the molecular factors that control angiogenesis are expressed in high proportions in the tumors studied and that this expression is positively-correlated with tumor microvascular density. Furthermore, correlations emerged with the mean diameter of these tumors. We also found differences in microvascular density between pre-cancerous and cancerous eyelid lesions. Conclusion: Activation of the angiogenic molecular factors results in intratumoral and peritumoral microvascularity formation at initial tumor growth. As the tumor attains a certain size and microvascular network, some VEGF receptors appear to decrease. Tumor angiogenesis appears to be active in cutaneous malignancies of the eyelids; therefore our hypothesis of a potential anti-angiogenic therapy for the studied tumors needs investigation in future studies.

Basal cell carcinoma (BCC) is the most common cutaneous malignancy in humans (1). It is the most frequent malignant tumor in the eyelids and represents 90% of malignancies identified in this area (2). Squamous cell carcinoma (SCC) is also a frequent type of skin cancer in humans. Treatment includes a range of options (surgical removal, cryotherapy, radiotherapy, photodynamic therapy, chemotherapy etc.) (2, 3) and the prognosis is usually excellent and rarely do complications occur, such as relapse (4) or metastasis (5).

The most frequently identified pre-cancerous lesions in the eyelid are actinic or solar keratosis and Bowen’s disease (2). These lesions usually have a less aggressive behavior and a benign course, but malignant transformation can occur. Treatment options are the same as previously mentioned, with preference for non invasive techniques.

Among many factors that contribute to tumor growth and spread, neoplastic angiogenesis plays a pivotal role. Angiogenesis in solid tumors is a multistep process widely studied in various tumor types, particularly in the last 20 years. It is controlled by cancer cells and helps create sufficient vascularity to enhance further tumor growth (6, 7). The loss of regulatory mechanisms in malignant neoplasms results in disruption of the balance between stimulators and inhibitors of angiogenesis, with dominance of pre-angiogenic factors and activation of the angiogenesis cascade (8).

Vascular endothelial growth factor (VEGF) or VEGFA, a member of the VEGF protein family, is one of the many factors involved in this process and has been the focus of many investigations (9-11). VEGF acts mainly through the interaction with its receptors: FMS-like tyrosine kinase-1 (FLT1) and fetal liver kinase-1 (FLK1). FLT4 is a VEGFC and-D receptor and has been used as a marker for lymphangiogenesis in solid tumors (12). Lymphangiogenesis

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is a process similar to angiogenesis occurring in the lymphatic system and is an event that has not been adequately studied in cutaneous malignancies (13-15).

The importance of tumor angiogenesis in the development and outcome of malignant neoplasms has created the need to quantify this process. The contribution of immunohistochemistry, with the use of specific antibodies against endothelial cells in histological sections of tumors, to fulfilling this need was decisive. Such an antibody is CD-105, a specific endothelial marker of endoglin protein. The angiogenic potential of various tumors through assessment of VEGF and CD-105 as a measure of microvascular density (MVD) has been studied intensively. In the vast majority of studies on malignant neoplasms, overexpression of VEGF by tumor cells was noted. Recognition of MVD, as determined by CD-105-positive staining, as a strong and independent prognostic marker associated with survival of patients, was supported by a series of studies (16-19). Thereafter, numerous publications showed a strong correlation between the expression of VEGF by tumor cells and MVD in solid tumors (20-23).

Regarding cutaneous malignancies, most studies involve malignant melanomas (24-26) and fewer basal and squamous cell carcinomas (27, 28). Investigation of angiogenesis in cancerous and pre-cancerous lesions of the eyelid does not boast, as we know so far, studies in the international literature.

The purpose of this study was to study the angiogenic status of cancerous and precancerous eyelid lesions, using immunohistochemical techniques. Specifically, particular emphasis was placed on quantitative assessment of expression of the main factors controlling these processes, to investigate the correlation between them and the macroscopic tumor features, and to report, if any, the differences of the studied eyelid skin lesions, according to their angiogenic status.

Materials and Methods

The study design was retrospective and covered a 5-year period. The present study included formalin-fixed, paraffin-embedded samples of BCC, basosquamous cell carcinomas (BSC), SCC and pre-neoplastic lesions from the archives of the Pathology Laboratory of the University Hospital of Ioannina. Simple tumor surgical removal was performed with a surrounding healthy tissue rim of 3-4 mm diameter. After careful examination of the samples in hematoxylin–eosin stain, a suitable block was chosen from the available paraffin blocks. Selection criteria were the presence of a representative section of the tumor and the surrounding healthy tissue, and the absence of areas with intense inflammation and necrosis. From each paraffin block, 2-μm-thick sections were cut and placed on slides for immunohistochemistry.

The sequence of recorded sections was continuous. The study recorded patients’ sex, age and diagnosis. Subsequently, we evaluated certain descriptive characteristics of tumors such as the mean diameter (MD) and the existence or not of ulceration and inflammation. Finally stains were evaluated for MVD using the CD-105 marker, as well as angiogenic factors VEGF, FLT1, FLK1, and FLT4. The information was recorded in the data file with only arithmetic coding, making the data anonymous.

Immunohistochemical staining was performed with an automated instrument BenchMark XT IHC/ISH Staining Module (Ventana Medical Systems, Inc., Tucson, AZ, USA) following the protocol process of automated steps (XT iVIEW DAB V.1). For each specific antibody, we used the standardized protocol consisting of about 100 automated steps, with the only manual step being the addition of the antibody.

Evaluation of expression of VEGF, FLT1, FLK1, FLT4 and CD-105. The immunohistochemical expression of VEGF, its receptors and CD-105 was evaluated by two independent and experienced observers who were unaware of the clinicopathological data of patients, using an Olympus BX-52 microscope.

Evaluation involved the expression of VEGF and its three receptors (FLT1, FLK1 and FLT4). In particular, in each section, we evaluated the positive cytoplasmic staining in 10³ measured tumor cells and quantified the result by the use of a staining index. The index was calculated by multiplying the staining intensity (0=no staining, 1=mild staining, 2=moderate staining, 3=strong staining) and a number established in accordance with the percentage of positive staining of tumor cells (≤10%=1, 10-50%= 2, >50%=3). The staining index therefore ranges from 0 to 9, with the samples with an index of 4 or more being identified as having high expression and those with less than 4 as having low expression. Inflammatory cells with positive staining and areas of necrosis were excluded from the evaluation.

Vascular endothelial marker CD-105 was used to study MVD in consecutive histological sections. We performed the Weidner method. Specifically we identified in each section by using a low magnification (×40), the three areas with the greatest vessel concentration, ‘hot spots’, followed by counting of vessels per area at high magnification (×200) and the number was then divided by 3. Counts were made of blood vessels in the stroma surrounding the tumor (peritumoral, PTMVD) and also for vessels in the body of the tumor (intratumoral, ITMVD). The result corresponded to the MVD per field (with the size of the field being equal to 0.7386 mm²). For the counting of vessels, immunohistochemical expression of the endothelial marker was sufficient, without necessarily identifying the vessel lumen.

For the statistical analysis of the data, a sequential procedure was applied. Firstly, we explored the most robust sample of patients with BCC. The remaining clinical cases (BSC, SCC and pre-neoplastic) were all sufficiently represented. Only factors with significant predictive power were kept in the final model.

All analyses were performed using the statistical program SPSS 19.0 (SPSS Inc., Chicago, IL, USA).

Results

BCC. By far the most cases analyzed in the present study refer to BCC (113 cases out of 147 cases in total). In this group, the molecular factors of angiogenesis (VEGF and its receptors) were all significantly correlated with each other (Spearman Rho 0.26-0.34, p<0.01). All variables exhibited all degrees of expression (i.e. absence, low and high) except FLT4, which was expressed in all cases. In general, high
expression was more frequent (Chi-square, \( p=0.000-0.002 \)) except for FLT1, which in contrast, was mostly absent or had low expression (Chi-square, \( p<0.001 \)).

Expression of some angiogenesis factors, such as VEGF and FLT4, was not significantly related to sex or age of patients, nor to macroscopic tumor-describing variables, such as MD, ulceration or inflammation (multinomial logistic regression). Nevertheless, both FLK1 and FLT1 were significantly related to the MD of tumors (\( p=0.002 \) and 0.001 respectively, Figure 1). As Figure 1 shows, increase in the MD of tumor was negatively associated with expression of FLK1 but positively associated with low expression of FLT1.

The MVD expressed as number of CD-105-positive cells per field showed a clear correlation (\( R^2=0.353; \ p<0.001 \)) between intratumoral and peritumoral areas. The abundance of CD-105-positive cells was significantly higher in peritumoral areas (median values of 23 and 26 cells per field respectively, Wilcoxon related samples rank test, \( p<0.001 \)). Some degree of angiogenesis was usually present in BCC tumors, as there were only three cases of non-stained samples.
Dependence of MVD on the age and sex of patients, macroscopic parameters and angiogenic factors (VEGF and its receptors) was studied by linear modeling on the log-transformed values. Results showed that the same independent variables slightly but significantly affected both ITMVD and PTMVD (Table I). Vascularization was more developed in large or inflamed tumors expressing high levels of VEGF. The effect of FLK1 was less pronounced; for ITMVD it considerably improved the Akaike information criterion (AIC) value but was itself not significant. Partly, this was due to the non-linear component of the relationship between FLK1 and MVD. In fact, within the group of low FLK1 expression, higher average MVD was detected (median: 31 CD-105-positive cells per field) in comparison with the groups of no or high FLK1 expression, where the median MVD was 25 and 24 cells per field, respectively and this difference was statistically significant (Kruskal-Wallis test, \( p<0.05 \)).

**Other types of eyelid carcinomas.** The rare categories of eyelid lesions in our study (represented by six BSC, 10 SCC and 18 precancerous lesions) were not statistically different from the reference group (BCC) with regard to age, sex and any macroscopic or molecular angiogenic factors (multinomial logistic regression, forward entry using Wald statistic). However, it was the MVD, both intra-and peritumoral, that had a statistically significant difference between those lesions and BCC. Both BSC and SCC had higher values of ITMVD (median values 41.5 and 42, respectively, vs. 23 for BCC). The probability for more aggressive malignancy increased with increasing ITMVD \[ \text{Exp}(B)=1.117, 95\% \text{ confidence interval (CI)}=1.039-1.201, \ p=0.003 \text{ and } \text{Exp}(B)=1.204, 95\% \text{ CI}=1.102-1.315, \ p<0.001 \text{ for BSC and SCC, respectively}. \] Moreover, SCC had slightly but statistically significantly lower PTMVD values (23.5 vs. 26 in BCC) \((\text{Exp}(B)=0.857, 95\% \text{ CI}=0.765-0.961, \ p=0.008)\). An example of different ITMVD between these cancer types can be seen in Figure 2. Finally, the precancerous lesions exhibited weaker peritumoral CD-105 staining in comparison with the BCC reference group \([\text{median}=14.5 \text{ CD-105-positive cells per field}]; \ \text{Exp}(B)=0.917, 95\% \text{ CI}=0.865-0.971, \ p=0.003\]. Differences are illustrated in Figure 3.

**Discussion**

Most studies about cutaneous tumor angiogenesis involve malignant melanoma correlated with increased VEGF expression and MVD, which in turn appears to play an important role in the vertical growth phase of the tumor. Increased MVD was also associated with a greater MD of
the tumor, the presence of ulceration and finally with worse patient prognosis (29-33). Angiogenesis in epithelial skin tumors (BCC, BSC and SCC and its precancerous lesions) is addressed in few studies (34-37). To our knowledge, this is the first study on angiogenesis for epithelial skin tumors in such a specific anatomical location as the eyelids.

Mainly in patients with BCC being the reference group, we observed higher MVD in inflamed or larger tumors. In addition, the present study showed a positive correlation between CD-105-measured MVD, both intratumoral and peritumoral, and the expression of VEGF, meaning that high expression of angiogenic molecular factors results in higher tumor microvascularization. Furthermore, PTMVD differed in preneoplastic lesions and the BCC group. Pre cancerous lesions exhibited some neovascularization, but less compared to the neovascularization of BCC. The aggressive cancer types (BSC and SCC) had mainly increased ITMVD in relation to BCC. In addition, our study confirmed the positive correlation that exists between ITMVD and PTMVD, showing their common mechanisms of development.

Oh and colleagues separated BCC into two categories, classic-type BCC with a relatively benign course and aggressive BCC. They observed that MVD was significantly increased in the second ‘aggressive’ category and was correlated with high VEGF expression (38). A recent study published similar results and correlated MVD and VEGF expression with invasiveness of BCC (39). Loggini et al. observed statistically significant correlation between VEGF expression and MVD in 26 BCC and 11 SCC, with the SCC group exhibiting increased MVD in relation to the BCC (40). In 41 cases of sinonasal SCC, Tang et al. reported that high VEGF expression resulted in greater neovascularization of the tumors (41). Chin and colleagues found PTMVD to be higher in SCC compared to BCC (42). Finally, in a relatively recent study, the researchers compared the MVD between BCC and benign adnexal skin tumors histologically resemble BCC. A distinctive feature between those tumors was PTMVD, which was significantly higher in BCC than in purely benign neoplasms. This group proposed high PTMVD as useful information in the differential diagnosis of BCC from their benign histological mimics (43).

Furthermore, we evaluated the expression of the three VEGF receptors (FLT1, FLK1 and FLT4) in epithelial skin tumors. Expression and function of the VEGF receptors in vascular endothelial cells is generally known, the significance of the expression by the tumor cells is not yet fully demonstrated (44). VEGF receptors have been observed in breast cancer (45), melanoma tumor cells (46), lung cancer (47), prostate cancer (48) and head and neck SCC (49). For some of these tumors, autocrine action of VEGF has been documented, therefore we studied this hypothesis in our samples.

The expression of VEGF has been shown to correlate positively with the expression of its receptors, mainly in BCC. Furthermore, the expression of some VEGF receptors related to the diameter of the tumor, and FLK1 presented the strongest negative correlation, while low FLT1 expression was positively correlated with high MD. In our study, tumors which can attain a certain size appear to have a relatively low expression of FLT1 and low or lack of FLK1 expression, while low FLK1 expression was also associated with high expression of CD-105, meaning that when the tumor attains a sufficient microvascular network there is no need for high FLK1 expression and usage. The tumor cells probably produce VEGF not only to stimulate tumor angiogenesis but also for self usage, as an autocrine growth factor activity for tumor spread until a satisfactory microvascular network is achieved. Our observations on the expression of VEGF and its receptors in epithelial skin tumors should be confirmed in larger series of tumors with more samples of the types of precancerous lesions, BSC and SCC to draw safer conclusions about their role in dermal angiogenesis.

The general conclusion from all the studies so far is that MVD and VEGF expression in epithelial skin tumors can provide useful information for separating patients into groups with different prognosis and therefore requiring different therapeutic strategies and monitoring protocols. Furthermore, the presence of increased PTMVD at the excision margin is a common finding in our study. This fact may imply microscopic invasion of the remaining neoplastic microvessels and a possible increased risk of local recurrence, especially in more aggressive squamous carcinomas. This hypothesis certainly requires further investigation with larger series of patients under close and long-term postoperative follow-up. The high expression of angiogenic markers in a sizable proportion of the eyelid tumors we studied generates justifiable promise for a potential antiangiogenic therapy, as already applied in other malignancies (50) and could be investigated by future trials. Blocking angiogenic pathways in the early stages may prevent or stabilize further tumor growth. In addition, a topical anti-VEGF therapy when increased PTMVD at the excision margin is present could be used as a prevention measure for recurrence, or in order to avoid additional surgery or plastic eyelid reconstruction, which can be a challenge. This is particularly important if one considers the anatomical peculiarity of the eyelid region in relation to the need of surgical resection of the tumor with healthy boundaries and the achievement of a functionally and esthetically acceptable outcome.

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References


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