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Original article

CORRELATION BETWEEN MICROVESSEL DENSITY AND MORPHOLOGICAL FEATURES IN SKIN SQUAMOUS CELL CARCINOMA

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Abstract

Introduction: Abnormal angiogenesis is described in tumor growth and it facilitates its metastatic spread. Tumors with high angiogenic activity belong to the category of aggressive tumors with a poor prognosis for patients.

The **aim** of this study was to determine neovascularization at the invasive front of the tumor stroma in skin squamous cell carcinoma (SCC) in relation to the healthy skin and the ratio of blood vessel density in the skin SCC with a different depth of invasion and different degree of histological differentiation.

Materials and methods: The material consisted of surgical specimens obtained from 30 patients with skin SCC, who underwent surgery at the University Clinic for Plastic and Reconstructive Surgery and University Clinic for Maxillofacial Surgery. Samples were analyzed by standard paraffin technique stained by hematoxylin-eosin and immunohistochemically with antibodies against smooth muscle actin (SMA) and CD34.

Results: The difference found in the neovascularization density in neoplasms with different degree of differentiation (G1, G2, G3) was statistically significant. The differences in the depth of stromal invasion in skin SCC registered in neoplasms with different degree of histological differentiation (G) showed a statistical significance for G1, G2, G3. The density of neovascularization in skin SCC was in a positive correlation with the depth of invasion.

Conclusion: The increased vascularization at the invasive front of a neoplasm in SCC with deeper invasion and higher grade has pointed out to its possible role in neoplasm progression.

Keywords: squamous cell carcinoma, CD34, neovascularization, histological differentiation

Introduction

Angiogenesis or neovascularization is a process of forming new blood vessels from preexisting capillaries. The blood vessels in the human skin are usually passive, but neovascularization is noticed in follicular angiogenesis that takes place in the separate phases of the follicle cycle[1].

The connective tissue located under the epidermis, in the dermal papillae, in healthy human skin lies under the basement membrane and inhibits the angiogenesis that keeps the blood vessels passive. New data of vascular biology put in place key factors that are in control of the vascular growth and are incorporated in the hypothesis encouraging the fact that there is vascular inactivity in normal tissues due to the potent influence of endogenous angiogenic inhibitors in despite of the angiogenic stimulators.

Vascularization is managed by the potent influence of the principal physiologic inhibitors of the skin angiogenesis, the endogens thrombospondin-1 (THSP-1) and thrombospondin-2 (TSP -2). They are part of the matrix glycoproteins family and are kept in the basement membrane by which they represent the angiogenic barrier segregating avascular epidermis from vascular dermis. These inhibitors along with angiostatin, vasostatin, endostatin and interleukin 12 (IL-12) are known as inhibitors of tumor angiogenesis and tumor growth *in vivo*[2,3].

Dominant pro-angiogenic factors in the skin angiogenesis are: vascular endothelial growth factor (VEGF-A), basic fibroblast growth factor (bFGF-2) and interleukin-8 (IL-8). VEGF-A is the leading angiogenic growth factor. VEGF-A is tethered to two types of tyrosine receptors VEGFR-1 and VEGFR-2 that are present in vascular endothelial cells. VEGF-A is produced by tumor cells and impact the releasing of matrix metalloproteinases (MMPs) from endothelial cells. The released MMP-2 and MMP-9 deteriorate the extracellular matrix (ECM) charting invasive passage of endothelial cells in the neighboring tissue. MMPs also dissolve ECM which leads to releasing new concentrations of bFGF-2 and VEGF-A[11,14,22]. In case of disturbed equilibrium, when the production of angiogenic stimulators is increased and the regulation of endogenous angiogenic inhibitors is lowered, the risk for tumor angiogenesis is developed[8,9].

In the 70s, Judah Folkman suggested the hypothesis that tumors need vascularization for growing - a process regulated by diffusible molecules. This process of angiogenesis is balanced by pro- and anti-angiogenic factors. Folkman believed that by deranging and preventing angiogenesis in tumors, the process can be slowed down, the tumor mass would decrease, which would lead to tumor regression.

The increased production of angiogenic inhibitors or inhibition of receptors' activity and signaling of blood vessels and inhibition of angiogenic growth factor production take part in the angiogenesis inhibition[3-6].

Proliferation and migration of the endothelial cells from existing blood vessel and organizing themselves in tubular vascular structure was the beginner phase of the mechanism of tumor vascularization named vascularization by sprouting[7,8]. In the next years, other mechanisms of tumor vascularization were discovered and named as: angioblast recruiting, co-opting vessels, vasculogenic mimicry (vascular imitation) and mosaic vessels[9,10].

Not all tumors are angiogenic in the beginning. The formation of tumors can be influenced by chemical cancerogenic substances, confirming that tumor progression happens by a switch from the prevascular to the vascular phase.

In the epithelial organs, premalignant lesions develop and they are characterized by disarranged proliferation, lack of cell uniformity and architecturally different organization. These appearances can be part of a reversible process, or can develop further towards carcinoma *in situ* and then progress to invasive carcinoma[4,5].

The activation of vascularization in premalignant lesions starts with proliferation and migration of endothelial cells. New blood vessels form from the preexisting ones, in higher density, and aside with the increased expression of VEGF-A/VEGFR-2 and decline of TSP-1 indicate an early angiogenic switch in skin squamous cell carcinoma (SCC). Growth factors from the VEGF family play an essential role in the growth and invasion of SCC. The blockade of VEGFR-2 receptors leads to inhibition of angiogenesis and invasion of SCC. The levels of TSP-1 and TSP-2 physiologic inhibitors of skin angiogenesis are decreased in SCC, which leads to a phase that precedes the invasion

Skin squamous cell carcinoma (SCC) is a malignant tumor of the epidermal tissue (keratinocytes), characterized by destructive growth and metastasizing potential. Normal cells undergo through multiple phases and genetic changes turning into malignant cells. Following

actinic keratosis, skin SCC develops in 60% of patients. This premalignant condition becomes malignant in 20% of patients due to a genetic mutation of p53 tumor suppressor gene[1].

Treatment of vascular and solid skin tumors such as SCC includes an oncologic therapeutic approach that prevents tumor growth by interfering tumor angiogenesis[12].

Angiogenesis, density of the vascular net in the neoplasm and its progression are closely associated with tumor aggressiveness and poor prognosis[13].

Winter J. *et al.* analyzed the density of blood vessels in basal cell carcinoma and benign trichogen tumors. The consecutive data showed a statistically significant difference in the blood vessel density; basal cell carcinoma had the highest vascular density[14].

Staibano S. *et al.* examined tumor angiogenesis in basal skin cell carcinoma, a tumor with good prognosis in the largest number of patients. Evidence showed that this neoplasm had developed more aggressive course and metastasis in certain number of cases[15].

Abulafia O. *et al.* studied angiogenesis in SCC *in situ* and microinvasive SCC of the uterine cervix in order to connect angiogenesis and depth of invasion. The microvessel counts in both types of the neoplasm showed a significant polarity[16].

The study of the tumor angiogenesis in SCC in the head and neck region revealed a correlation between the degree of tumor angiogenesis and clinical outcome in patients with SCC[17].

Data in another study showed that angiogenesis in oral SCC had a different microvasculature in comparison to other neoplasms according to the degree of histological differentiation of the carcinoma[18].

Literature-focused tumor angiogenesis has pointed to an increased microvascular density during tumor progression[19], increased peritumoral microvascular density during neoplasm progression[20] or a significantly increased microvasculature in invasive SCC compared to superficially invasive SCC and solar keratosis[19].

The aim of this study was to present the neovascularity and to determine the density of blood vessels at the invasive front of tumor stroma in skin SCC in relation to the healthy surrounding skin. Whether the change in the density of neovascularization depends on the degree of histological differentiation of a neoplasm, or whether there is an influence of tumor stage and correlation between the blood vessels density and the depth of carcinoma invasion are questions to which we have tried to give an answer.

Materials and Methods

This retrospective study included surgical specimens obtained from 30 patients with skin SCC, treated at the University Clinic for Plastic and Reconstructive Surgery and University Clinic for Maxillofacial Surgery in Skopje. The histological analysis of the specimens was done at the Institute of Pathology, Faculty of Medicine in Skopje.

Archival materials used in this study included: paraffin blocks, histopathological specimens and histopathological reports. Patients were categorized by sex, age and location of the neoplasm. Paraffin blocks were additionally used for making new histopathological sections from the carcinoma segment as well as from the resection margins of the surgical material, which were used as control groups for each patient separately. Sections taken for histopathological analysis were examined and stained with hematoxylin-eosin. Special, immunohistochemical staining by using specific primary monoclonal antibodies against smooth muscle actin (SMA) and CD 34 were used.

Visualization of tumor neoangiogenesis was achieved by staining with CD34 antibody, and hot spots in the invasive front of the neoplasm were identified at a magnification of x40 (Figure 1); well-formed vascular channels were counted at a magnification of x400 (Figure

2). The vascular channels had a clearly differentiated lumen along the invasive front, in a set of 10 visual fields.

Smooth muscle actin (SMA) staining was used to identify the presence of non-capillary blood vessels that had smooth muscle cells in their structure and furthermore to visualize blood vessels in the normal dermis representing as a control for staining with CD34. The procedure for density determination was identical with that applied in staining with CD34 (Figure 3).

Immunohistochemical staining was performed in line with the standard procedure used at the Institute of Pathology, Faculty of Medicine in Skopje, that is, visualization system (EnVision Flex from Dako manufacturer) using specific primary monoclonal antibodies.
Pretreatment was done with PT link apparatus with an adequate 3 in 1 buffer. Incubation of the primary antibodies diluted in an antibody diluent (Antibody Diluent Dako-Cytomation) at 1:100 for smooth muscle actin (SMA) and 1:50 for CD34, for 20 minutes in a wet chamber at room temperature was done. DAB (diaminobenzidine) as a chromogen and hematoxylin (ENVision Flex Hematoxylin) as a counterstain were used in the procedure. The negative control was obtained by omitting the primary antibody, and positive control when carcinoma tissue was found.

Histopathological analysis included determination of neoplasm type, tumor stage (pT), degree of histological differentiation (G) and depth of neoplasm invasion (Figures 4, 5, 6 and 7). A sum of all stained vascular areas found in the 10 visual fields was determined as density of neovascularization in each separate case. The minimum and maximum count of blood vessels was set on from all visual fields in each and every case and their mean value.
An identical procedure was conducted on the sections from the surrounding healthy skin, involving determination of vessel density in the dermis (Figures 8, 9 and 10).

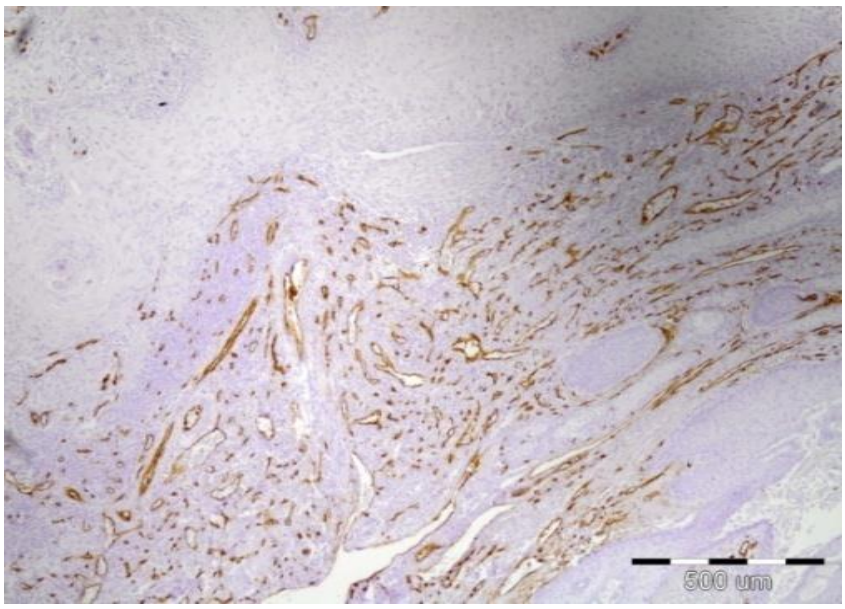

Fig. 1. Skin squamous cell carcinoma (CD34, x40)

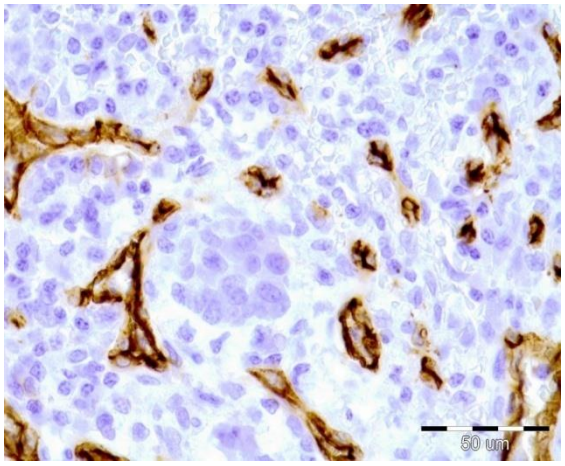

Fig. 2. Skin squamous cell carcinoma (CD34, x400)

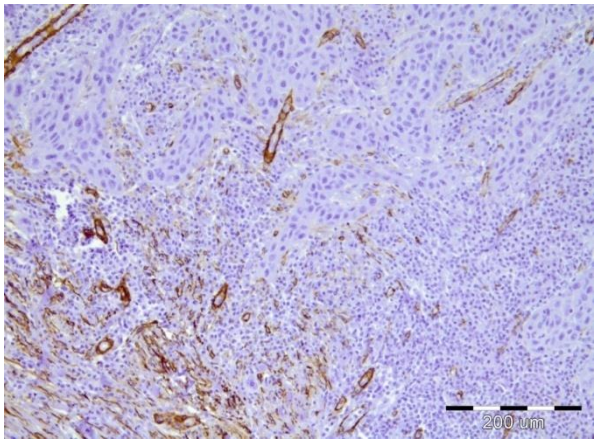

Fig. 3. Skin squamous cell carcinoma (Actin, x100)

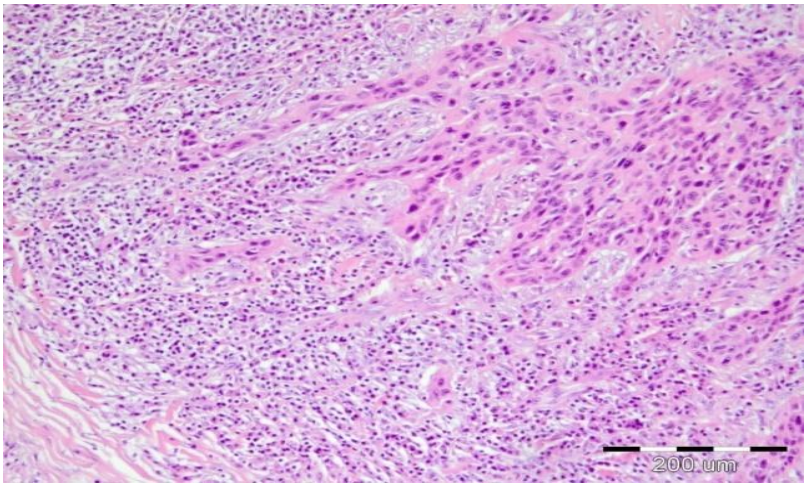

Fig. 4. Skin squamous cell carcinoma, invasive front (hematoxylin-eosin, x100)

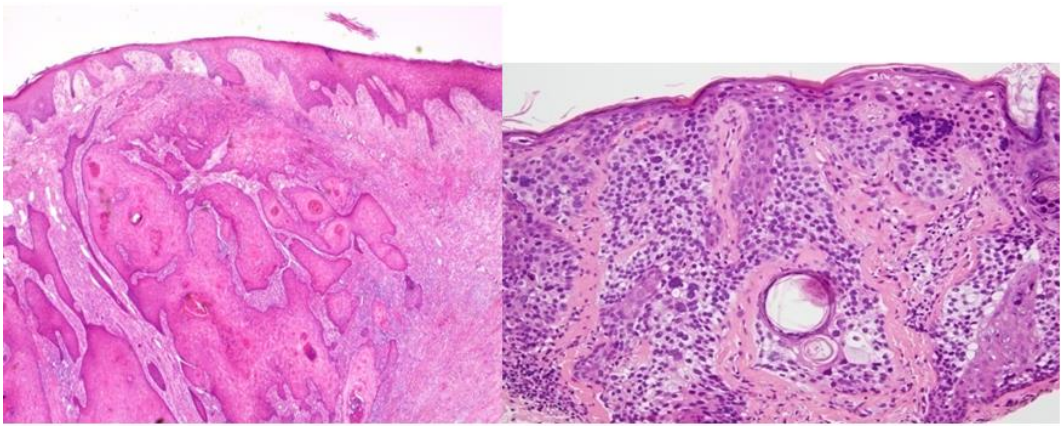

Fig. 5. Skin squamous cell carcinoma, well-differentiated (G1) (hematoxylin-eosin, x40)

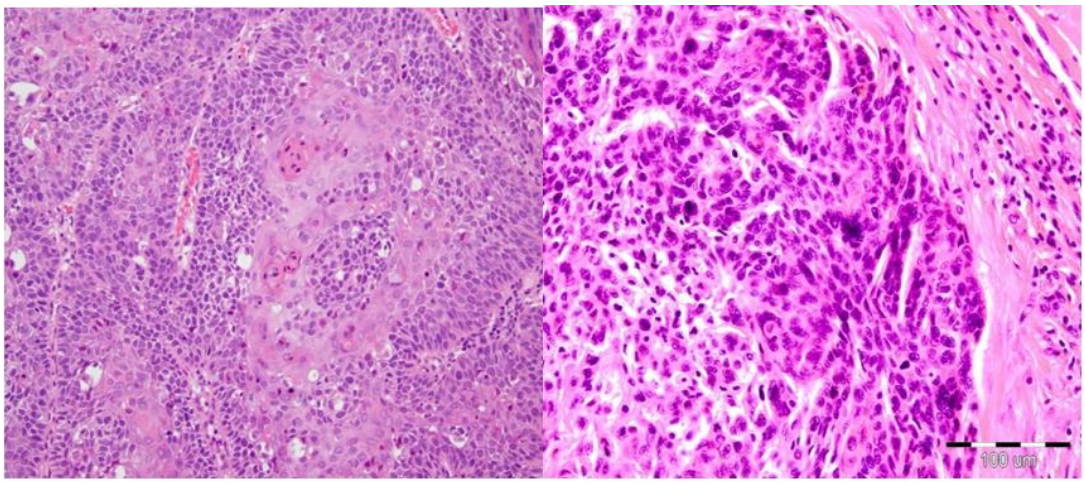

Fig. 6. Skin squamous cell carcinoma, moderately differentiated (G2) (hematoxylin-eosin, x100)

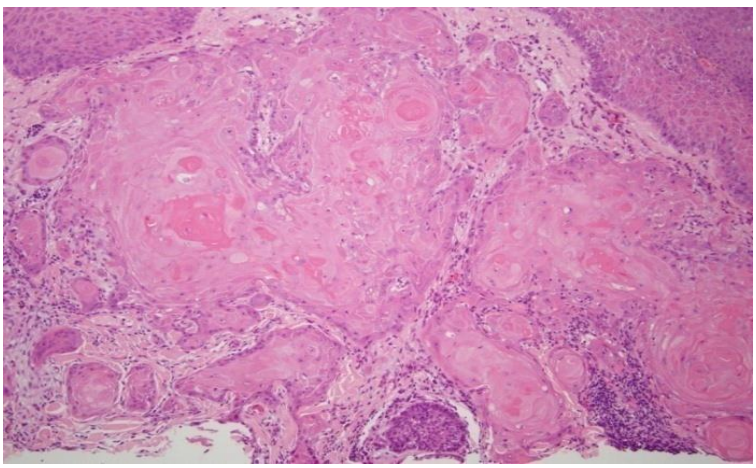

Fig. 7. Skin squamous cell carcinoma, poorly differentiated (G3) (hematoxylin-eosin, x100)

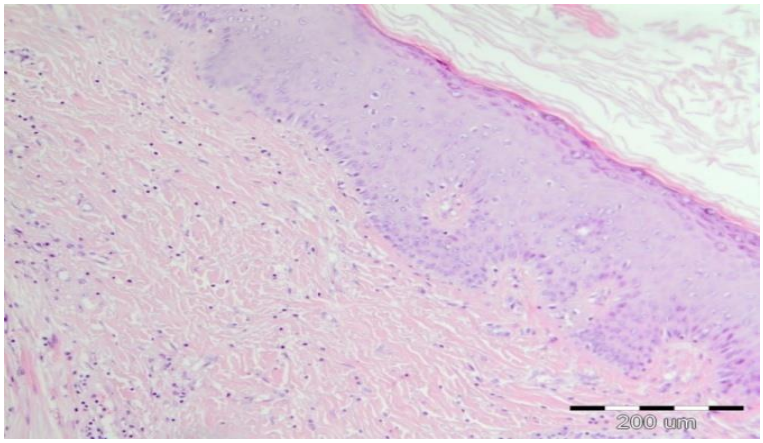

Fig. 8. Normal skin (hematoxylin-eosin, x100)

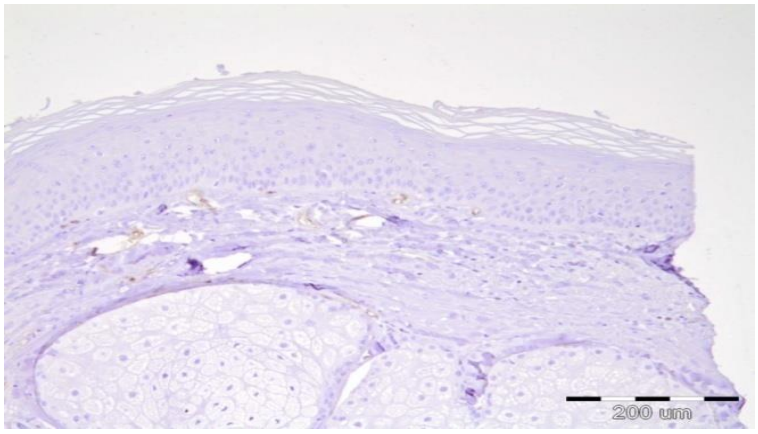

Fig. 9. Normal skin (CD34, X100)

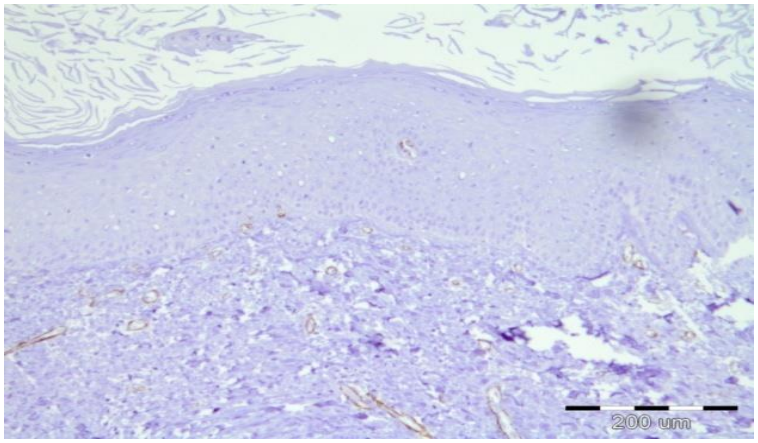

Fig. 10. Normal skin (Actin, x100)

Statistical analysis

The results were descriptively analyzed and presented in attributive and numerical statistical series. The applied descriptive methods were: mean value, standard deviation; presentation of data in figures and tables.

For the evaluation of the results modern statistical methods of analyses were used by employing a computer software. The statistical package SPSS 11.0 was used for creation of databases.

Results

The total number of patients was 30, 10(33.3%) were females, aged 70-98 years (average 85.7±8.4), whereas 20(66.7%) were males, aged 57-89 years (average 74.2±10.4) (Table 1).

The skin of the face was the most common region in the examined group - 13 cases (43.3%), and the least present were regions of the neck, forehead, eyelid, breast, arm, and leg with one case each (3.3%) (Table1).

In accordance with the degree of histological differentiation of the tumor, well-differentiated (G1) skin squamous cell carcinomas were present in 12 patients (40.0%), moderately differentiated (G2) skin squamous cell carcinomas in 13 patients (43.3%) and poorly differentiated (G3) skin squamous cell carcinomas in 5 (16.7%) patients (Table 1).
Values obtained by measuring the depth of invasion ranged from the lowest 1561.2 μm to the highest 13000.1 μm, average 4991.71 ± 1741.9 (Table 1).

Table 1. Distribution of cases according to location, tumor stage of neoplasm (pT), degree of histological differentiation (G) and depth of invasion

Location	**Number and % presence**
Skin of face	13(43.33%)
Skin of nose	3(10.0%)
Skin of ear auricle	3(10.0%)
Skin of abdomen	3(10.0%)
Skin of scalp	2(6.66%)
Skin of forehead	1(3.33%
Skin of eyelid	1(3.33%)
Skin of neck	1(3.33%)
Skin of breast	1(3.33%)
Skin of hand	1(3.33%)
Skin of leg	1(3.33%)
Degree of differentiation	***Number and % presence***
G1	12(40.0%)
G2	13(43.33%)
G3	5(16.66%)
Depth of invasion	***Mean ± sd.***
	4991.71±2741.9μm
Depth of invasion	***min. / max.***
	1561.2 μm/13000.1 μm

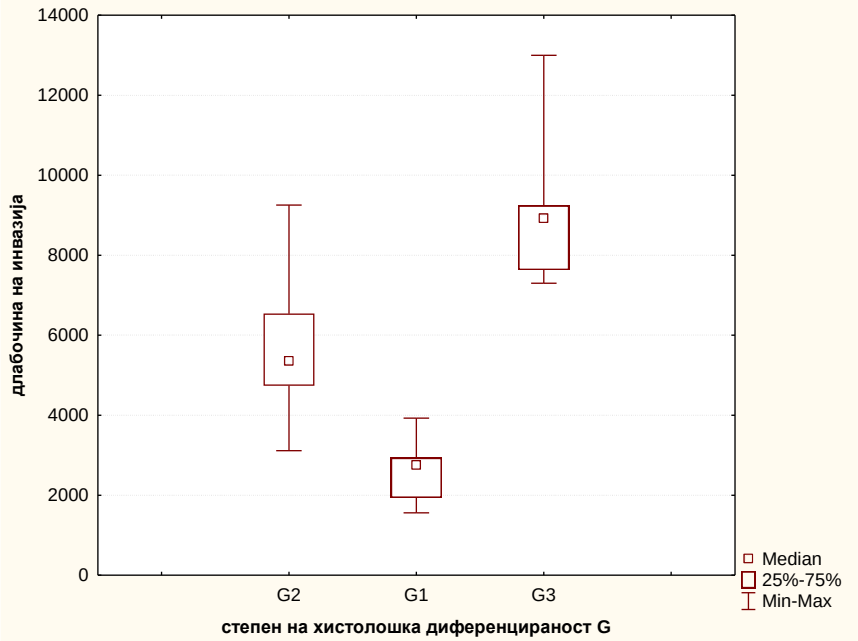

Fig. 11. Statistically significant difference between depth of invasion in skin SCC with different degree of histological differentiation
Legend: (G1)-well-differentiated carcinoma, (G2)-moderately differentiated carcinoma, (G3)-poorly differentiated carcinoma

The difference in depth of stromal invasion in skin SCC, which was registered in neoplasms with different degree of histological differentiation, was statistically significant (Kruskal-Wallis test: H (2, N= 30) =23.47711, p =0.00008) (Figure 11).

If the depth of stromal invasion is linked to the degree of histological differentiation, then it can be said that the smallest depth of stromal invasion was seen in well-differentiated (G10) squamous cell carcinomas (2579.28 ± 697.26), and the largest in the poorly differentiated (G3) squamous cell carcinomas (9219.896 ± 2268.882) (Table 2), (Figure 11).

Table 2. Average depth of stromal invasion in skin SCC expressed in micrometers according to the degree of histological differentiation (G) of neoplasms

Degree of histological differentiation	Depth of stromal invasion (μm)
G1(N=12)	2579.28±697.26
G2(N=13)	5592.33±1532.48
G3(N=5)	9219.896±2268.882

The smallest mean density value of neovasularization in well-differentiated tumors (G1) was 15.08±3.44, and the highest value was 24.75±5.59. In moderately differentiated carcinomas (G2), the smallest mean value was 13.07±2.53, while the highest mean value reached 33.0±9.88. In poorly differentiated carcinomas (G3), the smallest mean value was 17.40±4.16, and the highest 39.0±4.63. In normal skin, the values ranged from 3.80±1.78 for the lowest mean value to 13.69±3.22 for the highest mean value (Table 3).

Table 3. Ratio between density of vascular areas at the invasive front of skin SCC and of normal surrounding skin and degree of histological differentiation of neoplasms (G)

	G1 (N = 12)	G2 (N = 13)	G3 (N = 5)
Total number of blood vessels in visual field	187.92±37.99	256.61±83.34	364.80±30.13
Minimum number of blood vessels in visual field	15.08±3.44	13.07±2.53	17.40±4.16
Maximum number of blood vessels in visual field	24.75±5.59	33.0±9.88	39.0±4.63
Average number of blood vessels in visual field ± SD	18.79±3.79	25.44±8.43	36.48±3.01
Density of vascular areas in normal surrounding skin			
Total number of blood vessels in visual field	66.33±15.95	68.61±9.13	63.20±8.70
Minimum number of blood vessels in visual field	4.16±1.19	4.92±2.10	3.80±1.78
Maximum number of blood vessels in visual field	12.33±3.20	13.69±3.22	11.60±3.28
Minimum number of blood vessels in visual field ± SD	6.63±1.59	6.86±0.91	6.32±0.87

Table 3 illustrates that the lowest density of neovascularization was found in well-differentiated neoplasms (G1) - 18.79±3.79, and the highest in poorly differentiated neoplasms (G3) - 36.48±3.01. The statistical analysis of the results showed a statistically significant difference in the density of neovascularization between the groups of carcinomas with different degree of differentiation and between the groups with normal surrounding skin (H (2, N=30) =16.02890, p =0.0003) (Figure 12).

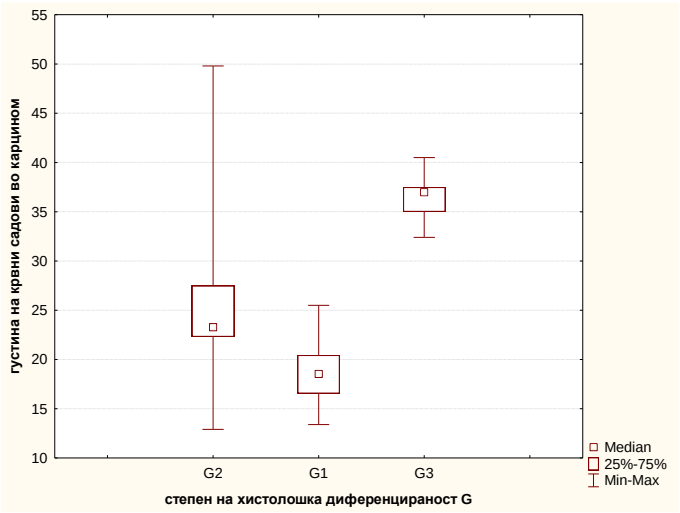

Fig. 12. Statistically significant difference in the density of neovascularization in skin SCC according to the degree of histological differentiation (G1, G2, G3)
Legend: (G1)-well-differentiated carcinoma, (G2)-moderately differentiated carcinoma, (G3)-poorly differentiated carcinoma.

The statistical analysis of the results have shown a statistically significant difference in the density of neovascularization between the groups of carcinomas with different degree of differentiation and between the groups with normal surrounding skin (Kruskal-Wallis test: H (2, N=30) =16.02890, p =0.0003) (Figure 12).

The difference registered in the mean density values of neovascularization in tumors compared to that in normal skin was statistically significant (p=0.0000001) (Figure 13).

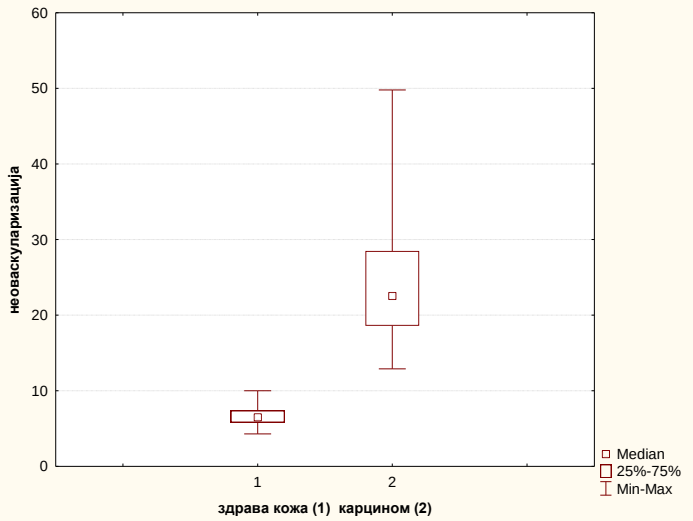

Fig. 13. Statistically significant difference in density of neovascularization in skin SCC compared to density of vascularization in normal surrounding skin
Legend: 1-healthy skin, 2-carcinoma

The difference registered in mean density values of neovasularization in tumors compared to that in normal skin was statistically significant (Mann-Whitney U тест-z=6.652991, p=0.0000001) (Figure 13).

The density of neovascularization in skin SCC was in a quite positive correlation with the depth of invasion of carcinoma (p=0.00018) (Figure 4).

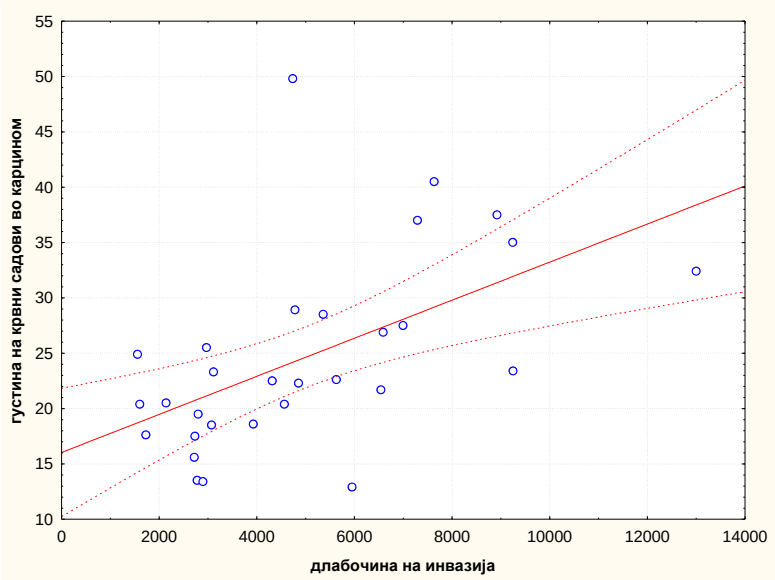

Fig. 14. Correlation between density of neovascularization at the invasive front of skin SCC and depth of invasion

The statistical analysis of the examined parameters: tumor status (pT1/pT2), degree of histological differentiation (G1, G2, G3), depth of invasion and density of blood vessels at the invasive front of skin SCC according to sex and age of patients as well as according to location of neoplasms showed no statistical significance for $p<0.05$ (Table 4).

Table 4. Values of $p<0.05$ of the parameters sex, age and location in correlation with tumor stage (pT), histological differentiation (G), depth of invasion and density of blood vessels in skin SCC and in healthy skin

Histological differentiation	Blood vessel density in neoplasms		Sex	Age	Location
	Mean value, SD	p<0.05			
G1	18.79±3.79				
G2	25.44±8.43	p=0.0003			
G3	36.48±3.01		p=0.187	p=0.729	p=0.561
Depth of invasion	p=0.00018		p=0.929	p=0.311	p=0.651
Blood vessel density in healthy skin	6.86±0.91	p=0.0000001	p=0.428	p=0.302	p=0.747

Discussion

Based on the capacity of the tumor to secrete angiogenic factors, tumor cells stimulate proliferation and migration of endothelial cells from the host to create irregular vascular network[11].

In accordance with the hypothesis proposed by Folkman, the manifestation of angiogenesis in tumor samples is determined by the number and structure of tumor-associated blood vessels, by the existence of endogenic pro-angiogenic and anti-angiogenic molecules and development of their specific receptors[3-6].

There are data that indicate increased microvascular density during tumor progression[19], heighten peritumor microvascular density during neoplasm progression, or a notably increased microvasculature in invasive SCC compared to superficially invasive SCC and solar keratosis[20].

Analysis of angiogenesis in solar keratosis, in superficially invasive and invasive skin SCC, showed a significant increase in microvasculature compared to the surrounding normal skin, indicating that angiogenesis appears early in the development of cutaneous SCC and confirming that neovascularization is parallel with tumor progression[26].

The results obtained in this study showed a statistically significant increase in the microvascular areas of the invasive front in skin SCC versus normal skin. Density of neovascularization had considerably been diverted with the increased invasive front depth, which was statistically confirmed.

Histological analysis of our specimens taken from 30 patients with skin SCC showed a highly statistical difference in the density of blood vessels at the invasive front of skin SCC in comparison to healthy skin ($p=0.0000001$).

The difference in the neovascularization density of neoplasms with different degree of differentiation (G1, G2, G3) was statistically significant ($p=0.0003$).

Similar findings are presented in the study by Winter J. *et al.*, who studied the density of blood vessels in basal cell carcinoma and benign trichogenic tumors. There were statistically higher counts of blood vessels in basal cell carcinoma than in benign trichogenic tumors[19].

This study found differences in the depth of the stromal invasion in skin SCC registered in the neoplasms with different degree of histological differentiation (G) and showed a statistical significance (p=0.00008) for G1, G2, G3. The density of neovascularization in skin SCC was in a positive correlation with the depth of carcinoma invasion (p=0.00018).

The study designed by Staibani S. *et al.* emphasized the importance of the density of blood vessels in determination of neoplasm aggressiveness and outcome of the disease. Tumor angiogenesis in skin basal cell carcinoma was analyzed. This tumor has a favorable outcome in the largest number of cases. Still, it was noticed that neoplasms in certain cases had demonstrated an aggressive course and metastasized. Studying the density of blood vessels in non-aggressive type and aggressive type of skin basal cell carcinoma, they discovered a statistically significant difference in the neovascularization between the two types of carcinomas. It was pointed out that the increased neovascularization at the invasive front of the basal cell carcinoma can serve as a useful indicator in assortment of patients at an increased risk of recurrence and metastasizing during the course of the disease, which would help in determining a more aggressive therapeutic treatment[20].

The investigation of tumor angiogenesis in SCC in the head and neck region revealed a correlation between the degree of tumor angiogenesis and the clinical outcome in patients with SCC[21].

Immunohistochemical staining with CD34, which is a good visual indicator for blood vessel detection, supplemented by determination of VEGF level have proven to be useful tools in discriminating the neoplasm aggressiveness. Thus, in the study comparing skin squamous cell carcinoma and basal cell carcinoma high levels of VEGF were found and high microvascular density in patients with skin SCC[22,23].

Angiogenesis in SCC was studied in tumors that occur in several different anatomic sites in order to discover its role in progression of tumors or their aggressiveness[25].

The poorer histological differentiation, the higher vessel density. This results in a greater expansion of malignant cells and metastasizing. In our examined cases, at the time of establishing the diagnosis no metastases in the neighboring lymph nodes were found, and hence these data were not included in the analysis.

A study on angiogenesis in oral SCC found a relation in neoplasm microvasculature with the degree of histological grading of the carcinoma, and presence or absence of nodal metastases. There were nodal metastases in patients with high vessel density with poorly differentiated SCC[22].

Studying angiogenesis in solar keratosis, in superficially invasive and invasive SCC of the skin, showed a higher microvessel density in comparison to the neighboring normal skin, stating the fact that angiogenesis appears early in the developmental stage of cutaneous SCC and that neovascularization is parallel with tumor progression[21].

In oral SCC, the finding of higher microvessel density or neovascularization along with the presence of positive lymph node metastases were the evidence of progression and spread of the malignant process as one of the key events in oral carcinomas[25-27].

In our study, differences found in the vessel density in neoplasms of different degree of histological grading, tumor stage and depth of invasion correlated to sex, age of patients and tumor location were not statistically significant and were a result of the randomly selected specimens.

Having in mind that in skin SCC determination of pT parameter from the pTNM classification means measuring the largest diameter of the neoplasm, which is not always clearly defined, many authors have examined the depth of invasion of SCC as a prognostic factor in expansion of the neoplasm.

Our findings are consistent with observations from a number of other studies that we have consulted.

We found an association of the depth of invasion with neovascularization density as well as with the degree of histological differentiation of neoplasms, factors that are predictors for tumor prognosis according to many authors.

Conclusion

Numerous molecular events, crucial for tumor progression and expansion, include: increase or loss of adhesive molecules, secretion of proteolytic enzymes, increased cell proliferation and initiation of angiogenesis. These processes develop in the front between the tumor and the surrounding tissue of the host, i.e., the invasive front. The hypothesis postulated by Bryne M. *et al.* has confirmed that molecular or morphological characteristics at the invasive front of different carcinomas might reflect tumor prognosis much better than the other tumor segments.

By analyzing the invasive front in skin SCC and its depth we have shown the changing of the number of newly formed vascular channels compared to the healthy neighboring tissue and the differences found were not only accidental but statistically significant. A different degree of differentiation in this type of neoplasm has shown different vessel density, which was statistically confirmed along with the positive correlation between them.

Angiogenesis as an important process in malignant neoplasms and by increased ingrowth and formation of new vascular channels plays a key role in sustenance, expansion and metastasizing of neoplasms.

Conflict of interest statement. None declared.

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