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Association of vascular endothelial growth factor expression with patohistological parameters of cutaneous melanoma

Udruženost ekspresije vaskularnog endotelnog faktora rasta sa patohistološkim parametrima kožnih melanoma

Milomir Gačević[*†], Milena Jović[‡], Lidija Zolotarevski[†‡], Ivan Stanojević[†§], Marijan Novaković[*†], Karolina Miller[‖], Vesna Šuljagić[†¶], Željko Mijušković[†], Danilo Vojvodić[†§]**

***Clinic for Plastic Surgery and Burns, [‡]Institute of Pathology and Forensic Medicine, [§]Institute for Medical Research, [¶]Department of Hospital Infection Control, [**]Clinic for Dermatovenerology, Military Medical Academy, Belgrade, Serbia; [†]Faculty of Medicine of the Military Medical Academy, University of Defence, Belgrade, Serbia; [‖]Dorset County Hospital Foundation Trust, Dorchester, UK**

Abstract

Background/Aim. Melanoma is the most aggresive malignant tumor of the skin. Contradictory data was published on vascular endothelial growth factor (VGEF) in tumor samples and its role in skin melanoma progression and prognosis. The aim of this study was to investigate the significance of VEGF expression as a prognostic parameter in melanoma. **Methods.** The experimental group included 81 patients with primary skin melanomas treated from 2009 to 2013 at the Military Medical Academy, Belgrade. The control group included 20 patients with dysplastic and 20 with benign naevi. Stratification was done according to gender, age, clinical and patological stage, localization, histologic type, Clark's, Breslow, mitotic count, regression and ulceration, tumor infiltrating lymphocytes and metastatic spread. Immunohistochemical staining was performed on skin biopsies using DAKO anti-VEGF antibodies (Ab), LSAB™ +HRP, DAB and microvawe antigen (Ag) retrieval in DAKO pH 9.0 solution. For statistical data analysis was done with ANOVA, Bonferroni, Mann Whitney and Wilcoxon test. **Results.** The mean intensity of VEGF staining was statistically significantly higher in melanomas than in benign or dysplastic naevi. Furthermore, the highest recorded values were in Ia and IV clinical stages. The majority of melanomas with high intensity of VEGF staining were in pT1a pathological stage. Melanomas with the highest mitotic count (> 6) had a significantly higher intensity of VEGF staining than those with < 2 mitoses. The higest intensity of staining was in melanomas without significant lymphocytic infiltrate and the lowest was in those with brisk lymphocytic infiltrate, thus a statistical difference was siginifant. The mean intensity of VEGF staining was highest in melanomas with lymphovascular invasion. There was no statistically significant difference between VEGF and any other parameter. **Conclusion.** VEGF in primary skin melanomas plays an important role in tumor progression and is linked to the absence if tumor infiltrating lymphocytes and the presence of lymphovascular invasion. More detailed studies have to be done on VEGF prognostic value in melanoma on a larger number of patients.

Key words:
melanoma; skin; vascular endothelial growth factors; histology; immunohistochemistry; sensitivity and specificity.

Apstrakt

Uvod/Cilj. Melanom je najagresivniji maligni tumor kože. Do sada su objavljeni kontradiktorni podaci o vaskularnom endotelnom faktoru rasta (VGEF) prisutnom u uzorcima tumora, kao i njegovoj ulozi u progresiji i prognozi melanoma kože. Cilj ove studije bio je da se ispita značaj ekspresije VEGF kao prognostičkog parametra kod melanoma. **Metode.** Eksperimentalna grupa sastojala se od 81 bolesnika sa primarnim melanomom kože lečenih na Vojnomedicinskoj akademiji, Beograd, od 2009. do 2013. godine. Kontrolna grupa sastojala se od 20 bolesnika sa displastičnim i 20 sa benignim nevusima. Stratifikacija je izvršena prema polu, starosti, kliničkom i histološkom stadijumu, lokalizaciji, histološkom tipu, Clark-u, Breslovu, broju mitoza, regresiji i ulceraciji, prisustvu tumorinfiltrišucih limfocita i načinu širenja. Imunohistohemijsko bojenje izvedeno je na biopsijama kože uz upotrebu DAKO anti-VEGF antitela, LSAB™ + HRP, Liquid DAB i mikrotalasnog demaskiranja u DAKO pH 9.0 rastvoru. Za statističku obradu korišćeni

Correspondence to: Milomir Gačević, Clinic for Plastic Surgery and Burns, Military Medical Academy, Crnotravska 17, 11 000 Belgrade, Serbia. E-mail: milomir.gacevic@gmail.com

su testovi ANOVA, Bonferroni post-test, Mann-Whitney i Wilcoxon test. **Rezultati.** Prosečan intenzitet VEGF bojenja bio je značajno veći u melanomima nego u benignim ili displastičnin nevusima, a u melanomima bio je najveći u Ia i IV kliničkom stadijumu. Većina melanoma u pT1a patološkom stadijumu imala je najveće prosečne vrednosti VEGF. Melanomi sa najvećim brojem mitoza (> 6) imali su značajno veći intenzitet VEGF bojenja nego oni sa < 2 mitoze. Melanomi bez limfocitne infiltracije imali su najveće vrednosti VEGF, dok su oni sa najintenzivnijom infiltracijom imali najniže vrednosti. Ova razlika je bila statistički visokoznačajna. Melanomi sa limfnim i hematogenim načinom širenja imali su najveće prosečne vrednosti VEGF. Nije bilo statistički značajne korelacije između intenziteta VEGF bojenja i bilo kog drugog parametra. **Zaključak.** U primarnim melanomima kože povećana ekspresija VEGF značajna je za tumorsku progresiju, a povezana je kako sa odsustvom limfocitne infiltracije, tako i sa prisustvom limfovaskularne invazije. Potrebna su detaljnija prospektivna istraživanja ekspresije VEGF kao prognostičkog parametra kod melanoma na većem broju bolesnika.

Ključne reči:
melanom; koža; vaskularni endotelni faktori rasta; histologija; immunohistohemija; osetljivost i specifičnost.

Introduction

Melanoma is the most aggressive malignant tumor of the skin. In the period from 1999 to 2008 the Cancer Registry of Central Serbia reported the incidence of melanoma to be 1.4% for men and 1.5% for women. According to the same source, melanoma was recoded as the main cause of death in 1.1% of the population [1]. Patients with stage III and IV disease had a 5-year survival rate of only 14% due to exceptionally high chemo- and radiotherapy resistance of melanomas [2]. Surgical resection of primary tumor at an early stage (*in situ* melanomas) is usually curable, but treatment for metastatic melanoma is still a challenge. Numerous genetic and biological studies of factors relevant for pathogenesis and progression of melanoma may be split into two groups. The first group relies on the identification of new reliable biomarkers of early melanoma progression, predictive and prognostic factors, while the second is dependent on the identification of resources and/or procedures to reduce chemoresistance, to induce apoptosis and decrease proliferation and metastatic potential of melanoma cells [3–5]. Angiogenesis plays a significant role in the progression and metastasis of melanoma [6–8]. Tumor angiogenesis is a net outcome of an interplay between pro-angiogenic and inhibiting factors of angiogenesis [8]. A key event in the activation of angiogenesis is an increase in angiogenic factors and/or decrease/loss of inhibitors of angiogenesis. Hypoxia is one of the physiological stimuli responsible for the increase in production of various pro-angiogenic cytokines such as vascular endothelial growth factor (VEGF). Expression of VEGF is triggered by hypoxia-induced factor-1α (HIF1α), the oxygen-sensitive transcription factor also known as angiogenic trigger (angiogenic switch). HIF1α is activated by low pO2, low pH, hyper/hypoglycemia, hyperthermia, mechanical stress, immune/inflammatory response or by genetic mutations [9, 10]. The activity of pro- and anti-angiogenic factors is regulated by multiple genes, many of these often mutated in cancers. For example, in normal cells, p53 can stimulate the expression of anti-angiogenic molecules, such as thrombospondin-1, and inhibit the expression of pro-angiogenic molecules such as VEGF. Thus, the loss of p53 in tumor cells not only affects the cell cycle, but also angiogenesis. Transcription of VEGF is also regulated by the RAS-MAP kinases signals and mutations in RAS and MYC genes.

The role of VEGF in metastatic melanoma has been the main focus of research in recent years [11–15]. It has been shown that VEGF stimulates tumor angiogenesis in autocrine and paracrine fashion. VEGF is secreted by melanoma cells, stromal cells, dendritic cells, macrophages, and fibroblasts. A number of members of the VEGF family are identified: VEGF-A, B, C, D, E, and placental growth factor (placenta growth factor – PlGF). Different forms of VEGF bind to the VEGF-receptor with tyrosine kinase activity [6]. VEGF stimulates endothelial cell proliferation, migration, vasodilatation and vasculogenesis and recruitment of hematopoietic progenitor cells in the bone marrow [16, 17].

Contradictory data was published on VEGF in tumor tissue samples and its role in melanoma progression and prognosis [18, 19]. In previous publications VEGF was analyzed in melanoma tissue or in blood serum of melanoma patients, but a very small number of studies was done simultaneously on both tumor tissue and serum obtained from the same patient. The therapy based on inhibition of the VEGF signaling pathway so far appears promising, although further research is needed. Main prognostic factors are melanoma thickness (Breslow score), ulceration, sentinel lymph node status and anatomical site, the latter considered to be an independent prognostic factor in primary melanoma [15]. The aim of this study was to introduce VEGF-A as a reliable new prognostic factor in melanoma, to compare this new factor with those already in use and to examine the link between initial expression of VEGF with local tumour progression and metastatic spread.

Penetration of blood or lymph vessels depends on the profile of generated chemotactic and angiogenic factors and physical tissue barriers [20]. Blood and lymphatic vessels share a common embryologic origin, and they respond to the same growth factors: VEGF-A, VEGF-C, VEGF-D, fibroblast growth factor (FGF2), platelet-derived growth factor (PDGF-A), hepatocyte growth factor (HGF), etc. [21]. It is, therefore, to be expected that tumors simultaneously induce lymphangiogenesis and angiogenesis [22] but, for an unknown reason, this is often not the case. Proliferation of lymphatic vessels was detected in melanoma [22], as well as in squamous cell carcinomas of the head and neck [23] while in other cancers it is poorly documented. A possible reason for the lack of proliferation of lymphatics in tumours may be caused by

anti-lymphangiogenic factors [24]. Vascular endothelial growth factor C (VEGF-C) released by macrophages and melanoma cells adjacent to the main tumor mass is the key lymphangiogenic factor. Skin metastases, subcutaneous and lymph node metastases were associated with longer survival than visceral metastases. In recent years it has been established that chemokines and their receptors play a major role in metastasis [25, 26] and that certain chemokines and their receptors are directly involved in molecular control mechanisms in the spread of melanoma [27].

Methods

Tissue samples of cutaneous melanoma were obtained from 81 patients aged between 23 and 87, diagnosed between 2009 and 2013 at the Institute of Pathology and Forensic Medicine of Military Medical Academy in Belgrade, Serbia. The distribution of patients according to clinical stage of melanoma was 22 in stage I, 18 in stage II, 27 in stage III and 13 in stage IV. The control group comprised of 20 patients with dysplastic naevi and 20 patients with benign melanocytic naevi.

Out of 81 patients, 43 were males and 38 females. All the patients were stratified by age, sex, clinical stage, histological TNM stage, Clark's level, Breslow score, histologic type of melanoma, mitotic count, the presence of tumor infiltrating lymphocytes (TIL), anatomical localization and route of spread (lymphatic, hematogenous or combined).

Immunohistochemical staining with mouse monoclonal anti-human VEGF antibody (Ab) (DAKO clone VG1, dilution 1 : 25) was performed on surgical skin biopsies that were fixed in 10% neutral buffered formalin and embedded in paraffin. Antigen (Ag) retrieval was performed using a pH 9.0, DAKO Code S2367 Ag retrieval solution and microwave oven. We used a LSAB$^{TM\ +}$ labelling system HRP and DAKO Liquid DAB + substrate chromogen system. For negative controls primary Ab was replaced by mouse IgG1 DAKO Ab, diluted to the same concentration as the primary antibody. Keratinocytes in the epidermis adjacent to melanomas served as VEGF internal positive control. The intensity of immunohistochemical staining was scored semiquantitatively from 0–3 according to the publication of Rajabi et al. [19]: 0 – no difference in staining between malignant melanocytes and keratinocytes; 1 – staining slightly more intense than in keratinocytes; 2 – staining moderately more intense than in keratinocytes, and 3 – staining more intense than in keratinocytes (Figure 1).

Statistical analysis of our data was performed with GraphPad Prism software using ANOVA test (with Bonferroni post testing), Mann Whitney (MW) test and Wilcoxon test.

Results

VEGF expression in correlation with clinical stage showed that 89% of melanomas in the earliest clinical stage had the strongest intensity of staining for VEGF (Table 1), even stronger than melanomas in stage IV (84%) (Table 1). There was no VEGF expression in 15% of primary melanomas in stage Ib and 40% of melanomas in stage IIa (Table 1). As expected, we found that all primary melanomas with metastatic disease (clinical stages III and IV) were positive for VEGF, although the intensity of this staining was variable.

VEGF values in tumor stage IA were significantly higher

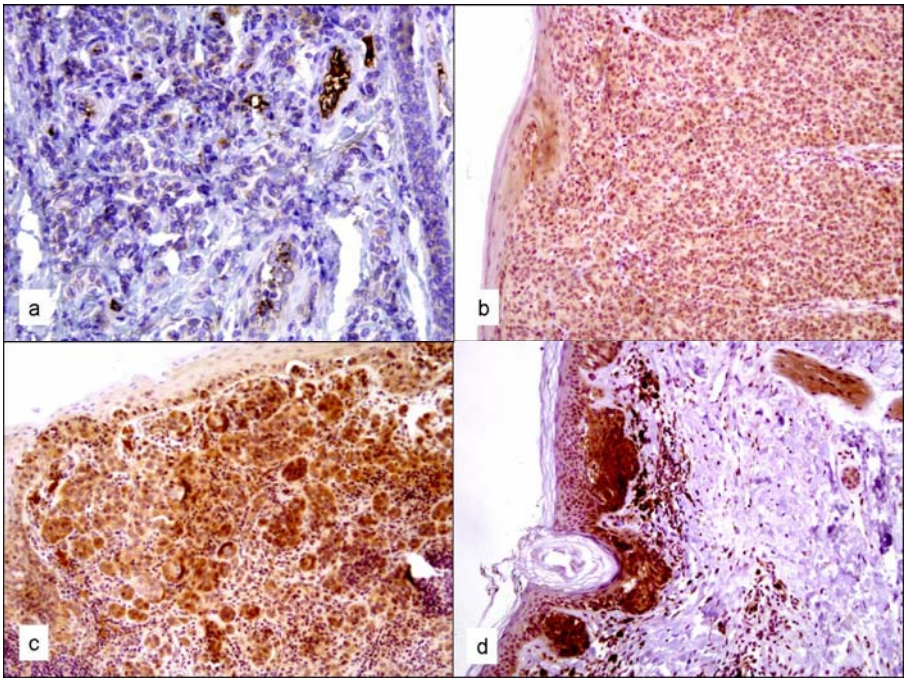

Fig. 1 – Vascular endothelial growth factor (VEGF) in primary melanomas: a) score 0, magnification 200 ×; b) score 1, magnification 100 ×; c) score 2, magnification 100 ×; d) score 3, magnification 100×.

than in the tumor samples in stages IIA, IIB, IIC and IIIA (p = 0.0175, MW test) (Figure 2a). In addition, VEGF in tissue samples of patients with stage IV was significantly higher than in samples of patients in stages IB, IIA, IIB, IIC, IIIA and IIIB.

Analysis of VEGF expression according to the histological staging showed similar results, whereby the majority of patients with strongest intensity of VEGF staining belonged to the group with the earliest pT1a histological stage (Table 1). The average levels of VEGF expression were high in the patients with pT1a, pT2a, pT3b and pT4b stages, with the highest absolute value in pT3b group (Table 2). The average degree of VEGF expression in pT2a stage was significantly higher than in pT2b (p = 0.0346,

Table 1

Frequency of vascular endothelial growth factor (VEGF) expression in melanoma samples

Variables	VEGF immunostaining level, n (%)							
	0		1		2		3	
Clinical stage								
Ia	0	(0)	1	(11)	0	(0)	8	(89)
Ib	2	(15)	1	(8)	5	(38)	5	(38)
IIa	2	(40)	1	(20)	1	(20)	1	(20)
IIb	0	(0)	2	(40)	2	(40)	1	(20)
IIc	1	(12)	3	(38)	2	(25)	2	(25)
IIIa	0	(0)	9	(39)	6	(26)	8	(35)
IIIb	0	(0)	1	(25)	2	(50)	1	(25)
IV	0	(0)	1	(8)	1	(8)	11	(84)
Histological stage								
pT1a	0	(0)	1	(13)	0	(0)	7	(87)
pT1b	2	(66)	0	(0)	1	(33)	0	(0)
pT2a	0	(0)	0	(0)	4	(36)	7	(64)
pT2b	0	(0)	1	(33)	1	(33)	1	(33)
pT3a	1	(25)	1	(25)	1	(25)	1	(25)
pT3b	0	(0)	2	(66)	1	(33)	0	(0)
pT4a	1	(13)	2	(29)	2	(29)	2	(29)
pT4b	0	(0)	8	(29)	6	(21)	14	(50)
Mitoses								
0	1	(3)	8	(22)	9	(24)	19	(51)
1	4	(24)	5	(28)	4	(24)	4	(24)
2	0	(0)	1	(11)	3	(33)	5	(56)
3	0	(0)	1	(25)	0	(0)	3	(75)
4	0	(0)	2	(50)	0	(0)	2	(50)
6	0	(0)	1	(20)	2	(40)	2	(40)
> 6	0	(0)	0	(0)	1	(25)	3	(75)
Lymphocyte infiltration								
0	0	(0)	6	(23)	6	(23)	14	(54)
1	3	(14)	4	(19)	6	(29)	8	(38)
2	2	(7)	8	(30)	5	(19)	12	(44)
3	2	(33)	2	(33)	1	(17)	1	(17)
Clark								
I	/	/	/	/	/	/	/	/
II	2	(20)	1	(10)	1	(10)	6	(60)
III	0	(0)	4	(17)	6	(26)	13	(57)
IV	2	(8)	4	(15)	9	(35)	11	(42)
V	1	(11)	4	(44)	2	(22)	2	(22)
Spreading								
no	5	(11)	9	(21)	8	(19)	21	(49)
lymphatic	0	(0)	7	(29)	9	(38)	8	(33)
lympho-hematogenous	0	(0)	0	(0)	2	(22)	7	(78)
Anatomical localisation								
head	0	(0)	0	(0)	3	(25)	9	(75)
trunk	0	(0)	11	(30)	10	(28)	15	(42)
arm	4	(100)	0	(0)	0	(0)	0	(0)
leg	1	(6)	5	(28)	4	(22)	8	(44)
foot	0	(0)	0	(0)	2	(50)	2	(50)

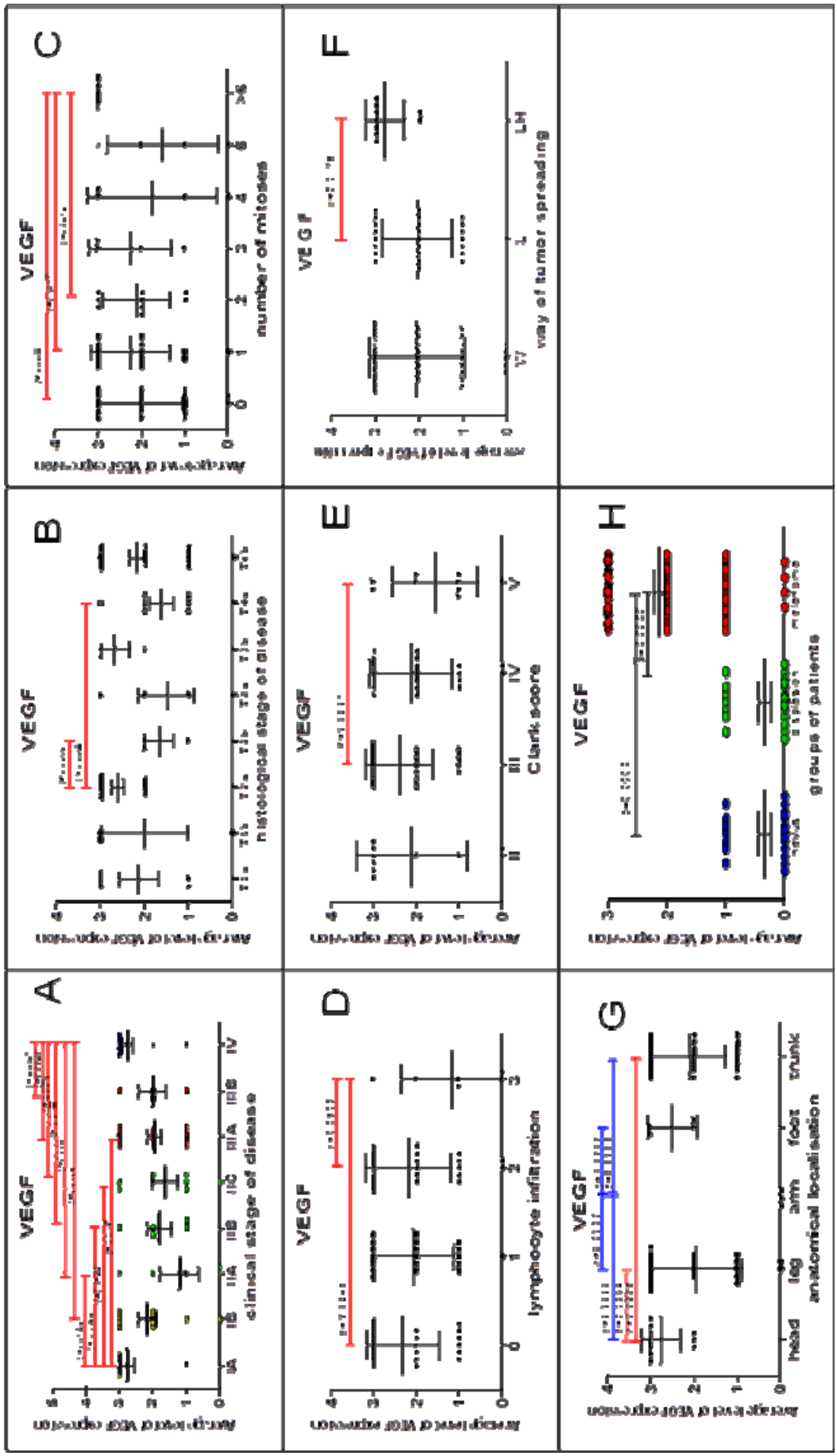

Fig. 2 – Average level of tumor vascular endothelial growth factor (VEGF) expression in the investigated groups: A) Average level of VEGF expression *vs* clinical stage of disease; B) Average level of VEGF expression *vs* histological stage of disease; C) Average level of VEGF expression *vs* number of mitoses; D) Average level of VEGF expression *vs* lymphocyte infiltration; E) Average level of VEGF expression *vs* Clark score; F) Average level of VEGF expression *vs* way of tumor spreading; G) Average level of VEGF expression *vs* anatomical localisation; H) Average level of VEGF expression *vs* groups of patients.

W – without spreading; L – lymphatic way of spreading; L/H – lymphatic and hematogenous way of spreading.

Table 2

Average level of vascular endothelial growth factor (VEGF) tissue expression in primary melanomas and control groups according to the investigated parameters

Clin stage,	IA	IB	IIA	IIB	IIC	IIIA	IIIB
x̄ ± SD	2.78 ± 0.67	2.17 ± 0.94	1.20 ± 1.30	1.80 ± 0.84	1.63 ± 1.06	1.96 ± 0.88	2.00 ± 0.82
significance	IA / IIA $p = 0.0159$ IA / IIB $p = 0.0286$ IA / IIC $p = 0.0169$ IA / IIIA $p = 0.0167$	IB / IV $p = 0.409$	IIA / IV $p = 0.0081$	IIB / IV $p = 0.0155$	IIC / IV $p = 0.0076$	IIIA / IV $p = 0.0062$	IIIB / IV $p = 0.0407$
Hist stage,	pT1a	pT1b	pT2a	pT2b	pT3a	pT3b	pT4a
x̄ ± SD	2.13 ± 1.25	2.00 ± 1.73	2.63 ± 0.51	1.67 ± 0.58	1.50 ± 1.29	2.67 ± 0.58	1.63 ± 0.74
significance			pT2a / pT4a $p = 0.0065$ pT2a / pT2b $p = 0.0346$				
Mitoses,	0	1	2	3	4	6	> 6
x̄ ± SD	2.24 ± 0.96	2.38 ± 0.96	2.44 ± 0.73	2.00 ± 1.15	2.00 ± 1.41	2.00 ± 1.42	2.75 ± 0.50
significance	0 / > 6 $p = 0.0200$	1 / > 6 $p = 0.0417$	2 / > 6 $p = 0.0319$				
Ly Infiltr,	0	1	2	3			
x̄ ± SD	2.31 ± 0.84	2.05 ± 0.95	2.19 ± 1.00	1.17 ± 1.17			
significance	0 / 3 $p = 0.0245$		2 / 3 $p = 0.0500$				
Clark,	I	II	III	IV	V		
x̄ ± SD	none	2.10 ± 1.29	2.39 ± 0.78	2.12 ± 0.95	1.56 ± 1.01		
significance			III / V $p = 0.0307$				
Spreading,	W	L	LH				
x̄ ± SD	2.05 ± 1.09	2.04 ± 0.81	2.78 ± 0.44				
significance		L / LH $p = 0.0175$					
Anat Loc,	Head	Trunk	Arm	Leg	Foot		
x̄ ± SD	2.92 ± 0.29	2.33 ± 0.83	0.00 ± 0.00	2.16 ± 1.01	2.75 ± 0.50		
significance	H / L $p = 0.0339$ H / A $p = 0.0000$ H / T $p = 0.0235$		A / L $p = 0.0000$ A / F $p = 0.0000$ A / T $p = 0.0000$				
Patients,	Naevus	Dysplasia	Melanoma				
x̄ ± SD	0.333 ± 0.483	0.381 ± 0.498	2.127 ± 0.952				
significance	N / Mel $p = 0.0000$	D / Mel $p = 0.0000$					

Mitoses – number of mitoses; Ly Infiltr – lymphocyte infiltration; Clark – Clark score; Spreading-W – without spreading; L – lymphatic way of spreading; LH – lymphatic and hematogenous way of spreading; Anat Loc – Anatomical localisation.

MW test) and pT4a ($p = 0.0065$, MW test) stages (Figure 2b). Melanomas with the highest number of mitoses (> 6) had a significantly higher intensity of VEGF expression in comparison with melanomas with no mitosis ($p = 0.0200$), with 1 mitosis ($p = 0.0147$) or with 2 mitoses ($p = 0.0319$: MW test) (Figure 2c).

The highest average value of VEGF expression was detected in the melanomas without lymphocyte infiltration, and the minimum value was detected in the group with brisk lymphocytic infiltration. The difference between these two groups was statistically significant ($p = 0.0245$, MW test) (Figure 2d).

There was a significant difference in the intensity of VEGF staining in relation to the Clark level (Figure 2e). Melanomas Clark's level III had a significantly higher intensity of VEGF staining than melanomas Clark's level V ($p = 0.0307$; MW test).

Average values of intensity of VEGF staining were highest in melanomas with lymphatic and hematogenous invasion. In addition, we found a significant difference in the intensity of VEGF staining between melanomas with only lymphatic invasion and those with both lymphatic and vascular invasion ($p = 0.0175$; MW test) (Figure 2f).

Melanomas were divided into groups according to the anatomical localization of the primary tumor (Table 1). The highest average intensity of VEGF staining was detected in melanomas in the skin of the head (2.92 ± 0.29) (Table 2). This intensity of VEGF staining in the head region was significantly higher than in the melanomas of the trunk or extremities (Table 2). Interestingly, after further substratification of melanomas arising in the skin of extremities on the arm, foot and leg, there was complete absence of VEGF staining in the melanomas of the arm (Figure 2g).

As we expected the average value of the tissue expression of VEGF was significantly higher in the samples of the melanoma patients in comparison to the average value of VEGF in the patients with nevi or dysplastic nevi ($p < 0.0000$; MW test) (Figure 2h).

There was no statistically significant difference between the intensity of VEGF staining and the following parameters: growth phase, histological type of tumor, Breslow index, the presence of vascular invasion, regression and ulceration, and the presence of metastatic disease.

Discussion

Our results show that the average value of the intensity of VEGF staining was significantly higher in melanoma (2.127 ± 0.952) compared to benign naevi (0.333 ± 0.483) and dysplastic naevi (0.381 ± 0.498). We found that 5 out of 81 patients with melanoma (2 in stage I, 2 in stage II and 1 in stage III) were completely negative to VEGF (5.7%). In the study of Konstatina et al. [28] from 2011, 13.69% patients with melanoma were negative for VEGF, but, in contrast with our control group results, 75% of patients with dysplastic nevi

had weak VEGF staining. The opinions on VEGF positivity in benign melanocytic lesions are divided. Some authors consider that the intensity of positivity depends on the nature of melanocytic lesions and that benign lesions have a low level of VEGF expression in comparison to the changes in malignant melanocytic lesions [29]. Pisacane and Risio [30] reported positive VEGF staining in 91% of their analyzed naevi and this was in contradiction with fidnings of Simonetti et al. [31] who did not find any VEGF positivity in benign melanocytic naevi, atypical melanocytic naevi or Spitz naevi. Some authors consider that the intensity of positive staining depended on the nature of the melanocytic lesions and that benign lesions have a low level of VEGF expression in comparison to the changes in malignant melanocytic lesions [29]. Such variations in immunohistochemical staining for VEGF may be explained by different clones of primary antibodies, antigen retrival techniques, labelling systems and chromogens used in various studies. The most important of all is probably the difference in tissue fixation and processing [32].

Our most interesting finding is the high intensity of VEGF staining in 90% of melanomas in clinical stage I, a finding that was expected in more advanced stages. Some researchers associated increased expression of VEGF in melanoma cells with tumor transition from horizontal to vertical growth phase [33, 34]. As melanomas in only 6 out of 22 patients in clinical stage I (27%) and in 1 patient out of 18 in clinical stage II (5%) were in the horizontal phase of growth, we can conclude that angiogenic switch and locally enhanced synthesis of VEGF has already occured. These results would indicate that in the early stage, small melanomas secrete and/or induce local production of VEGF, priming the tumor's microenvironment for subsequent growth, invasion and metastasis.

The highest percentage of patients with histological stage pT1a demonstrated greatest intensity of VEGF staining, whereas the maximum value of the average level of VEGF staining was detected, in decreasing order, in patients with stage pT2a, pT3b and pT4b. In the study of Gajanin et al. [11] the most intensive staining for VEGF was detected in patients with pT3b / pT4b histological stage. They found a significant difference in the intensity of expression of VEGF staining in patients with melanomas in different pT stages ($p < 0.05$). They stated that the higher the pT stage, the higher the levels of VEGF expression (scores 2 and 3). Discrepancies between Gajanin's and our results could be explained by the greater average thickness of the tumor in the Gajanin et al. [11] series (7.09 mm *vs* 4.34 mm), by a smaller number of melanomas in the vertical growth phase, and by a difference in percentages of specific histological types of melanoma. In their study, nodular melanomas were present in 80% of cases, while in our study 23% of melanomas were nodular melanomas. Furthermore, they found that 15% of all melanomas were of the superficial spreading type and 5% of the lentigo malignat type, while our study showed 73% and 4%, respectively.

The presence of large numbers of mitotic figures is a sign of high metabolic activity, potential for tumor growth and metastastatic spread. We found that the average intensity of VEGF staining in primary melanomas was in melanomas with the highest mitotic activity of > 6 mitoses. The difference in intensity of VEGF staining in melanomas with the highest number of mitoses (> 6) and those with no mitotic activity was statistically highly significant ($p = 0.0200$), in melanomas with 1 mitosis ($p = 0.0147$), and in melanomas with 2 mitoses ($p = 0.0319$). Our results correlate well with the results of Gajanin et al. [11].

Tumor-infiltrating lymphocytes (TIL) are a host immune response to melanoma cells, and use of this parameter is the accepted and widely used prognostic parameter for patients in clinical stage I melanoma [35]. There is evidence that melanomas induce specific humoral and cellular immune responses. Our results show that VEGF values were highest in melanomas without tumor infiltrating lymphocytes, and lowest in melanomas with the highest degree of tumor infiltrating lymphocytes ($p = 0.0245$; MW test).

We found that the highest average intensity of VEGF staining was in melanomas Clark level III, and the lowest in melanomas Clark level V ($p = 0.0307$). Rajabi et al. [19] found that melanomas with higher intensity of VEGF staining exhibited deep dermal invasion. In the same study, the comparison between the intensity of VEGF staining in melanomas with Clark's level showed that all patients with invasion of the reticular dermis and subcutaneous adipose tissue (Clark IV and V) had intensity of VEGF +2 and +3. The average Breslow thickness in their study was 1.84 mm. Gajanin et al. [11] found no statistically significant difference between the intensity of VEGF staining in the tumor and Clark level of invasion.

Our results show that the average value of intensity of VEGF staining was highest in melanomas with lymphatic and hematogenous invasion and comparison with the intensity of VEGF staining in melanomas without lymphatic and hematogenous invasion we found a statistically significant difference ($p = 0.0175$, MW test). We also found a positive correlation between the increase in the intensity of VEGF staining and disease progression.

A correlation of the intensity of VEGF staining in melanomas with its anatomical localisation show that the highest average value of the intensity of VEGF staining was detected in patients with melanoma localized in decreasing order in the head, foot, trunk and leg. In melanomas located on the skin of the hand there was no VEGF staining. Previous publications reported a good correlation between anatomical localization and different paths for tumor progression, and it was suggested that this was influenced by various etiologic factors such as sex, history of multiple serious sunburns, number of nevi, hairs bearing skin or not, the number of normal melanocytes in the particular skin localization and the presence of mast cells [36–40]. For example, the highest density of melanocyte was found to be in the skin of the head that contains hair follicles and is constantly exposed to UV radiation. The next localization with a slightly lower number of melanocytes was in the skin of the extremities and trunk [36]. It is generally accepted that melanoma in men is more frequent in the head and neck region than on the trunk. In women the most commonly affected region with melanoma is the skin of the lower extremities. This difference in melanoma localization beween males and females could be explained by differences in the maximal sun exposure of dif-

ferent body parts in these two groups [15]. VEGF has been shown to play the important role in the biology of hair [41]. It was also found that the peritumoral accumulation of mast cells and, consequently, their release of potent angiogenic factors (including VEGF) leads to stimulation of angiogenesis and tumor progression [37]. According to the study of Callender et al. [15] patients with melanoma of the head and neck have increased synthesis of VEGF and worse prognosis than patients with skin melanomas in other localizations. This is related to the higher number of hair follicles, continuous exposure to UV radiation and a larger number of melanocytes and mast cells in the skin of the head. It is believed that, as the epidermal layer of the skin of the foot is thick, the melanomas in this location more frequently exhibit early vertical growth pattern and invasion into the deeper skin layers. This allows the tumor to grow to a considerably bigger size prior to any visible manifestations on the skin. Relatively high levels of VEGF together with relatively poor prognosis in trunk melanoma may be associated with multiple lymphatic drainage routes [42]. It is believed that patients with melanoma of the upper extremities have the best survival rate as they are more visible in this location than in others. Therefore, upper extremity melanomomas draw appropriate medical attention while still in earlier stages [37]. In our study, only 4 patients had melanomas localized on the arm, and all of them were in the earliest stage, a possible explanation for the absence of VEGF staining (score 0). Gajanin et al. [11] found the highest average intensity of VEGF staining in patients with melanoma of the extremities (2.36), followed by melanoma of the trunk (1.81) and melanoma of the head or neck (1.58). The above discrepancy between our study and that of Gajanin et al. [11] could be explained by a smaller experimental group (39 *vs* 81 patients in our study) of the latter, and a greater number of non-invasive, thin melanomas with horizontal growth phase of the same.

Conclusion

We did not find a statistically significant difference between the intensity of VEGF staining and the following parameters: growth phase, histological type of tumor, Breslow index, the presence of vascular invasion, regression and ulceration, and the presence of metastatic disease. Our results indicate that VEGF plays the important role in the earliest stage of melanoma invasion, in further local tumor progression and in lymphovascular invasion and subsequent metastatic spread. The absence of VEGF expression in melanomas with brisk lymphocytic infiltrate was statistically significant. We conclude that the expression of VEGF in primary skin melanomas plays the important role in tumor progression and that more detailed studies must be done on VEGF prognostic value in melanoma on a larger number of patients.

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