Original paper

The number of CD163 positive macrophages is associated with more advanced skin melanomas, microvessels density and patient prognosis

Maciej Foks[1], Małgorzata Wągrowska-Danilewicz[2], Marian Danilewicz[1], Marta Bonczysta[1], Bogusław Olborski[3], Olga Stasikowska-Kanicka[2]

[1]Department of Pathomorphology, Medical University of Lodz, Poland
[2]Department of Nephropathology, Medical University of Lodz, Poland
[3]Health Care Institution Diagnostics Consilio, Lodz, Poland

The study was aimed to evaluate the number of TAMs and to investigate whether they have association with microvessels density and patients' survival times. 46 cases of melanomas, divided into four groups according to the Breslow scale, were tested immunohistochemically with antibodies anti-CD68, CD163, iNOS to vizualized macrophages and anti-CD34 antibody to stain microvessels. The number of macrophages and the microvessels density were counted by hotspot analysis using an image analysis system.
The study revealed increased numbers of CD68 and CD163 positive macrophages in successive stages of Breslow scale, but statistically significant differences were observed only between I and IV group for CD68 positive macrophages, and between I and III, IV group for CD163 positive macrophages. The mean number of the microvessels was significantly increased in group II, III, IV compared to group I. The correlative study showed significant positive correlations between the mean number of CD68 and CD163 positive macrophages and microvessels density. Moreover, the number of CD163 positive macrophages was associated inversely with patient's survival time.
The results of our study may indicate that higher infiltration of macrophages, especially CD163 positive cells, is associated with more advanced melanomas, microvessels density and worse patient's prognosis.

Key words: TAMs, M1 macrophages, M2 macrophages, microvessels density, survival time.

Introduction

Melanoma is the most aggressive and lethal malignant neoplasm of the skin. If diagnosed at late stages, the survival rates are extremely poor [1, 2]. Therefore, new indicators of melanoma progression and predictors of treatment response are urgently needed. Because melanoma has an immune-related pathogenesis, the composition of tumor microenvironment is considered as an important prognostic factor modifying the patient outcome [3].

Literature data documented that tumor-associated macrophages (TAMs) are one of the most prominent bone marrow derived cells recruited into tumor microenvironment. In the skin melanoma, TAMs play crucial role in promoting melanocytes neoplastic transformation, proliferation and invasion [4, 5, 6, 7]. CD68 is a scavenger receptor highly expressed on

macrophages and generally accepted as a pan-macrophage marker. Based on the expression of cytokines, receptors, and effector molecules, macrophages are classified into two main subsets – classically activated M1 macrophages with pro-inflammatory function and into alternatively activated M2 macrophages which are closely involved in tumor promoting processes [8, 9, 10, 11]. M1 macrophages are typically recognized by the expression of inducible nitric oxide synthase (iNOS) which is a crucial enzyme in arginine metabolism pathway [12, 13]. Nitric oxide (NO) has cytotoxic effect that contributes to the anti-tumor effect of M1 macrophages. M2 macrophages express the mannose receptors, scavenging receptors CD163 and CD204, high levels of the chemokines CCL17, CCL22 and CCL24, and low levels of IL-12 and have been suggested to contribute to tissue remodelling, angiogenesis, and tumor progression [8, 14]. The literature data suggest that melanoma's microenvironment tends to polarize macrophages toward M2 phenotype which is connected with higher expression of proangiogenic factors such as VEGF. Furthermore, the environment of melanoma was proven to be highly hypoxic which is also a very significant factor for angiogenesis [15, 16].

The aim of our study was to examine the number of TAMs and describe the macrophage subpopulations (M1 and M2) in 46 cases of skin melanoma divided according to Breslow scale from I to IV. Another purpose was to find possible associations between the number of M1 (iNOS positive), M2 macrophages (CD163 positive) cells, microvessels density (MVD) and patients' survival times.

Material and methods

The present retrospective study and experimental methods were approved by the university review board, and have been performed in accordance with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects. The study was conducted under the assumption that research findings would be kept anonymous.

We examined 46 formalin-fixed, paraffin-embedded tissue specimens of skin melanomas that were diagnosed in Health Care Institution Diagnostics Consilio in Lodz. Paraffin-embedded tissue sections taken from postoperative material were diagnosed using standard hematoxylin and eosin staining. The histological diagnoses were established according to the WHO classification of skin tumors [17]. All cases were divided into 4 groups according to the Breslow scale (group I: ≤ 1.0 mm, n = 12; group II: 1.01-2 mm, n = 10; group III: 2.01-4.0 mm, n = 10; group IV: > 4,0 mm, n = 14). None of the patients had metastatic disease at the time of diagnosis. The follow-up data including overall survival were received for all studied cases.

Immunohistochemistry

Immunohistochemical staining was carried out according to a standard method. 3-μm tissue sections were deparaffinized in xylene and rehydrated through a graded alcohol series. Heating in a microwave oven in a solution of target retrieval solution pH 9.0 (TRS, Dako), for 30 minutes was used for antigen retrieval. Endogenous peroxidase were quenched with 0,3% hydrogen peroxide in methanol for 30 minutes. The sections were washed with TBS and incubated from 30 to 60 minutes with monoclonal mouse primary antibodies against: CD68 (Dako, Denmark; clone: PG-M1, dilution 1:100), CD163 (Abcam, UK; clone 10D6, ready-to-use), iNOS (Thermo Scientific, USA; PA 1 - 21054, dilution 1:100), CD34 (Dako; clone: QBEnd 10, dilution 1:50). After washing, an adequate EnVision-HRP detection system (Dako, Carpinteria, CA, USA) was used. 3,3'diaminobenzidine was used as the chromogen. After counterstaining with Mayer's hematoxylin, the slides were washed, dehydrated, cleared in xylene and coverslipped. The negative controls involved the same procedure as for positive ones, with the primary antibodies replaced by antibody diluent.

Morphometry

CD68, CD163 and iNOS positive cells were evaluated using a computer image analysis system consisting of a PC equipped with a Pentagram graphic tablet, Indeo Fast card (frame grabber, true-color, real-time), produced by Indeo (Taiwan), and a color TV camera Panasonic (Japan) coupled with a Carl Zeiss microscope (Germany). This system was programmed (MultiScan 18.03 software, produced by Computer Scanning Systems, Poland) to calculate the number of objects (semiautomatic function).

Intratumoral macrophages were counted in the melanoma cell nests and the stroma by the hotspot analysis. The number of CD68, CD163 and iNOS positive cells was estimated by counting all positive cells in 7-10 high power monitor fields (HPF) (0.029 mm^2 each), marking immunopositive cells (semiautomatic function).

CD34 immunostains were evaluated in the vessels only (not in the individual cells), in the most vascular areas (hot spots) of the tumor. The results were presented as the mean number of CD34 positive vessels with visible lumina per HPF (0.029 mm^2).

Statistical methods

Differences between groups were tested using unpaired Student's t-test preceded by evaluation of normality and Levene's test. The Mann-Whitney U test was used where appropriate. Correlation coefficients were calculated using Spearman's method. For statistical purposes all cases were divided for those be-

low and above median of the particular parameters (mediana for CD68 was 20, for CD163 was 8, for iNOS was 5, and for CD34 was 7,5). The survival analysis between these groups was performed using Kaplan-Meier method. For comparison Kaplan-Meier curves the proportional hazard Cox regression test was used. For Kaplan-Meier estimator death was the point-of-interest and the observation period was 80 month. Results were considered statistically significant if $p < 0.05$.

Results

The quantitative data of the immunoexpression of CD68, CD168, iNOS positive macrophages and CD34 positive vessels (microvessel density – MVD) are presented in Table I.

To detect all TAMs, M1 and M2 type macrophages, tissue sections were stained with CD68, iNOS and CD163 antibodies, respectively. iNOS immunoreactivity was localized in the cytoplasm. Both CD163 and CD68 immunoreactivities were localized mainly in the cytoplasm, and in some cases also on the plasma membrane. Staining patterns were often granular.

In our study, the mean number of CD68 positive cells was increased in successive stages of Breslow scale, but statistically significant difference was observed only between I and IV group. The mean number of CD163 positive cells was significantly increased in group III and IV compared to group I (Fig. 1A, B). The mean numbers of iNOS positive cells were similar in all studied groups. There were no statistical differences between mean numbers of iNOS positive cells in studied groups. We also found a significantly lower number of microvessels in group I compared to all other studied groups (Fig. 1C, D)

In all cohort of the patients, there were positive significant correlations between the mean number of CD68 and CD163 positive cells and microvessels density ($R = 0.47$, $p < 0.001$; $R = 0.44$, $p < 0.002$ respectively), whereas the correlation with iNOS was not statistically significant.

Moreover, we observed positive significant correlation between iNOS positive cells and survival times ($R = 0.28$, $p < 0.02$), and negative significant correlations between CD163 positive cells and survival times ($R = –0.72$, $p < 0.001$), as well as between microvessels density and survival times ($R = –0.43$, $p < 0.003$). However, the Kaplan-Meier survival analysis showed that only accumulation of CD163 positive macrophages were associated inversely with patient's survival time ($p < 0.04$; Fig. 2).

Discussion

Tumor microenvironment is a field of constant struggle between malignant tumor cells and patient's immune system. Immune cells, fibroblasts, endothelial cells, pericytes and other cells that are included in microenvironment interact both with each other and with tumor cells [18, 19]. Literature data suggest that tumors may promote or alter different immune cells phenotypes and either suppress the immune system or raise the tolerance for tumor antigens which would lead to tumor progression and metastasis [4, 5, 6, 7].

Tumor-associated macrophages are the major immune component of the melanoma microenvironment. Literature data showed that TAMs presence is connected with tumor initiation and development, immunosuppression, angiogenesis, invasion and metastasis [20, 21, 22]. Our study showed that the number of CD68 positive TAMs were increased

Table I. Mean numbers of CD68+, CD163+, iNOS+ macrophages and CD34+ vessels (microvessels density – MVD) in melanoma in I-IV groups according to Breslow scale

Groups	CD68+ cells/1 mm^2	CD163+ cells/1 mm^2	iNOS+ cells/1 mm^2	CD34+ vessels/1 mm^2
I: ≤ 1.0 mm (n = 12)	15.3 ±7.1	7.9 ±4.4	6.1 ±3.9	2.1 ±2.4
II: 1.01-2.0 mm (n = 10)	21.0 ±9.1	17.4 ±16.0	6.6 ±5.4	9.5 ±4.2
III: 2.01-4.0 mm (n = 10)	28.1 ±21.1	24.4 ±19.8	6.8 ±4.8	10.2 ±5.4
IV: > 4.0 mm (n = 14)	29.8 ±14.6	23.4 ±20.7	8.1 ±6.7	10.8 ±5.8
Group I vs. group II	p = 0.09 (NS)	p = 0.06 (NS)	p = 0.8 (NS)	p < 0.01
Group I vs. group III	p = 0.06 (NS)	p < 0.02	p = 0.7 (NS)	p < 0.01
Group I vs. group IV	p < 0.01	p < 0.02	p = 0.4 (NS)	p < 0.01
Group II vs. group III	p = 0.3 (NS)	p = 0.39 (NS)	p = 0.9 (NS)	p = 0.7 (NS)
Group II vs. group IV	p = 0.8 (NS)	p = 0.45 (NS)	p = 0.6 (NS)	p = 0.6 (NS)
Group III vs. group IV	p = 0.8 (NS)	p = 0.9 (NS)	p = 0.6 (NS)	p = 0.8 (NS)

NS – not significant

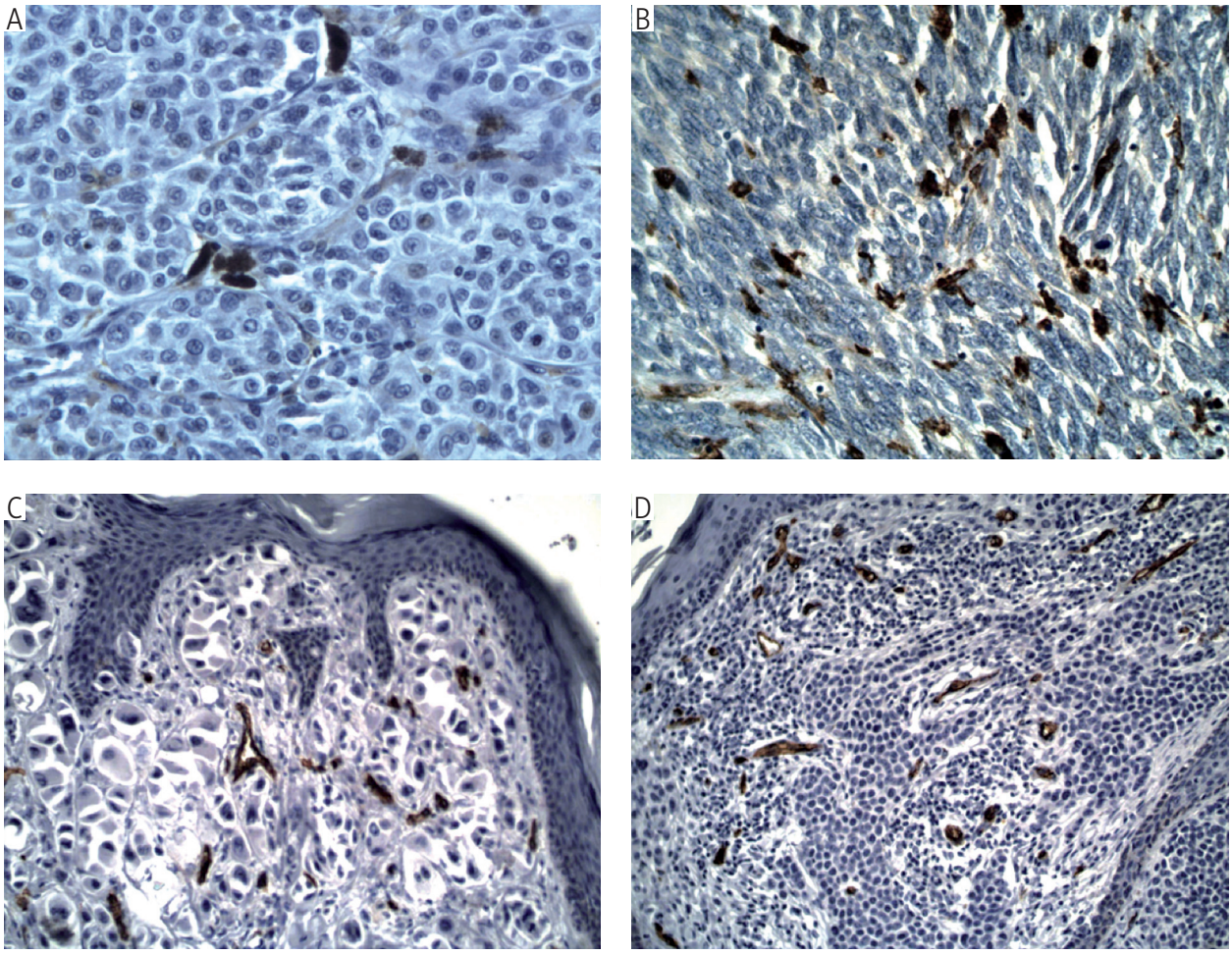

Fig. 1. A) Immunoexpression of CD163 positive macrophages in melanoma (group I of the Breslow scale). B) Immunoexpression of CD163 positive macrophages in melanoma (group IV of the Breslow scale). C) CD34 positive vessels in melanoma (group I of the Breslow scale). D) CD34 positive vessels in melanoma (group IV of the Breslow scale). Immunohistochemistry, light microscopy. Total magnification 200×

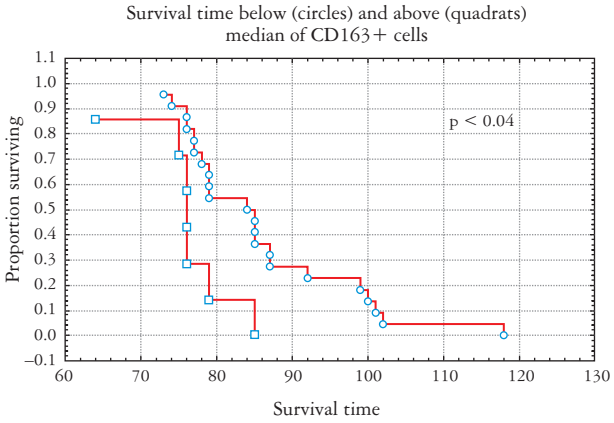

Fig. 2. CD163 positive macrophages and malignant melanoma patients' overall survival correlation

in subsequent stages of melanomas according to Breslow scale, even though statistically significant difference was observed only between I and IV group. In line with our findings, Hillen *et al.* [23] demonstrated that melanomas in the vertical growth phase contain more CD68 positive TAMs compared with melanomas in the radial growth phase. Recently, Falleni *et al.* [5] reported that CD68 accumulation was correlated with Breslow depth. All of this findings support the thesis that, TAMs can stimulate tumor invasion.

TAMs can display various functions depending on their polarization state [8, 10]. M1 macrophages are proinflammatory and antigen presenting subtype with cytotoxic NO as the product of their metabolism. They release IL-12, IL-23, TNF-α which, among other effects, stimuli T cells to induce cytotoxic response [9, 11, 14]. Literature data showed that the M1 infiltration is usually associated with immunostimulatory functions and extended patient survival in many human cancer [14, 24]. In our study, the mean number of intratumoral iNOS positive cells was similar in I, II and III group according to the Breslow scale. Only in group IV, the number of iNOS positive cells was slightly higher but with

no statistical significance. Our results may suggest that the intratumoral M1 macrophage infiltration is not associated with the skin melanoma depth. In contrary to our results, Falleni *et al.* [5] reported correlation between a high density of M1 macrophages in melanoma and the Breslow's thickness. The role of M1 macrophages in the tumor microenvironment is not completely clear what is reflected by many conflicting reports concerning the number and localization of M1 positive cells in the microenvironment of malignant melanoma [5, 25, 26, 27]. Differences concerning an association between the number of M1macrophages and Breslow depht in various studies may indicate that the precise role of M1 macrophages and immunological mechanisms taking part in malignant melanoma progression is still not fully understood. Furthermore, we should take into consideration that although iNOS was used in several studies to mark intratumoral M1 macrophages, it is not the best marker to evaluate M1 macrophages, because its expression was not restricted to infiltrating TAMs, but also involved the malignant melanoma cells.

M2 macrophages are known to produce various cytokines, chemokines and enzymes that are connected with tumor progression, angiogenesis and metastatic potential [4, 5, 7, 15]. In our study, the mean number of CD163 positive cells was increased in successive stages of the Breslow scale, and was significantly increased in group III and IV compared to group I. Previous reports also documented that a high density of M2 macrophages in melanoma correlates positively with Breslow's thickness [5]. Thus, our findings are in concordance with general knowledge in this field suggesting, that higher infiltration of M2 macrophages is associated with more advanced melanomas. Moreover, the results of our investigation show that the number of CD163 positive cells was associated inversely with patient survival. In line with our findings, Jensen *et al.* [28], and Falleni *et al.* [5] also reported that the density of M2 macrophages was correlated with poor overall patient survival, supporting the important role of CD163 positive macrophages in the melanoma development and prognosis.

Literature data revealed that TAMs play an important role in the process of angiogenesis also. TAMs tend to accumulate in hypoxic areas of tumors and produce multiple cytokines and chemokines that are known to accelerate angiogenesis such as VEGF, FGF, TNF-α, TNF-β, and CXCL [15, 16, 20, 29]. Increased number of TAMs were documented and positively associated with angiogenesis in several types of human cancers [30, 31, 32, 33]. In accordance with previous findings, we demonstrated that in cases of melanoma with higher TAMs infiltration, the microvessels density was also increased. The correlation between the presence of CD68 and CD163 positive macrophages and microvessels density described in our study is in concordance with current literature data [15, 16, 29, 30], and suggest that a high macrophages count may play an important role in angiogenesis.

By processing our data we managed to show some promising correlation between MVD and survival times of melanoma patients. Unfortunately, more thorough statistical analysis using the Cox proportional hazard model did not reveal expected results. Interestingly, literature data revealed many discrepancies concerning association between the microvessels density and patient's overall survival. Pastushenko *et al.* [34] revealed that the rate of intratumoral blood vessel proliferation was inversely associated with overall survival of patients with melanoma. On the other hand, Storr *et al.* [35] documented that angiogenesis estimated by microvessels density was not associated with overall survival. We should take into consideration that studies on human samples carries the intrinsic limitations, due to the biological variability of lesions, different number of studied cases and methodological issues (antibodies used, methods of evaluation, etc). We suggest that further, more homogenous and unified studies are necessary to make a final conclusion regarding the associations between microvessels density and survival of melanoma patients.

Tumor microenvironment immune cells are presently very promising subjects for the search of new prognostic factors and possible targeted therapies in melanoma. The present study documented that the higher density of CD68 and CD163 positive macrophages was positively associated with angiogenesis and Breslow depth. Moreover, the number of CD163 positive macrophages was associated inversely with patient's survival. Unfortunately, our study did not reveal correlation of MVD with survival times. Further studies are required to better understand relationships between individual cells of microenvironment, angiogenesis and tumor progression.

This work was supported by grant of Medical University of Lodz 502-03/6-038-01/502-64-085-17.

The authors declare no conflict of interest.

References

1. Liu Y, Sheikh MS. Melanoma: Molecular Pathogenesis and Therapeutic Management. Mol Cell Pharmacol 2014; 6: 228.
2. Rastrelli M, Tropea S, Rossi CR, et al. Melanoma: Epidemiology, Risk Factors, Pathogenesis, Diagnosis and Classification. In Vivo 2014; 28: 1005-1012.
3. Giavina-Bianchi MH, Giavina-Bianchi Junior PF, Festa Neto C. Melanoma: Tumor microenvironment and new treatments. An Bras Dermatol 2017; 92: 156-166.
4. Wang H, Yang L, Wang D, et al. Pro-tumor activities of macrophages in the progression of melanoma. Hum Vaccines Immunother 2017; 13: 1556-1562.

5. Falleni M, Savi F, Tosi D, et al. M1 and M2 macrophages' clinicopathological significance in cutaneous melanoma. Melanoma Res 2017; 27: 200-210.
6. Postovit LM, Seftor EA, Seftor REB, et al. Influence of the microenvironment on melanoma cell fate determination and phenotype. Cancer Res 2006; 66: 7833-7836.
7. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med 2013; 19: 1423-1437.
8. Mantovani A, Sica A, Sozzani S, et al. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 2004; 25: 677-686.
9. Mantovani A, Allavena P, Sica A, et al. Cancer-related inflammation. Nature 2008; 454: 436-444.
10. Gordon S. Alternative activation of macrophages. Nat Rev Immunol 2003; 3: 23-35.
11. Allavena P, Mantovani A. Immunology in the clinic review series; focus on cancer: Tumour-associated macrophages: Undisputed stars of the inflammatory tumour microenvironment. Clin Exp Immunol 2012; 167: 195-205.
12. Pekarova M, Lojek A. The crucial role of L-arginine in macrophage activation: What you need to know about it. Life Sci 2015; 137: 44-48.
13. Rath M, Müller I, Kropf P, et al. Metabolism via arginase or nitric oxide synthase: Two competing arginine pathways in macrophages. Front Immunol 2014; 5: 1-11.
14. Ma J, Liu L, Che G, et al. The M1 form of tumor-associated macrophages in non-small cell lung cancer is positively associated with survival time. BMC Cancer 2010; 10: 112. 15.
15. Huber R, Meier B, Otsuka A, et al. Tumour hypoxia promotes melanoma growth and metastasis via High Mobility Group Box-1 and M2-like macrophages. Sci Rep 2016; 6: 1-14.
16. Rofstad EK, Danielsen T. Hypoxia-induced angiogenesis and vascular endothelial growth factor secretion in human melanoma. Br J Cancer 1998; 77: 897-902.
17. Elder DE, Massi D, Scolyer R, et al. WHO Classification of Skin Tumours. 4th ed. Lyon, IARC 2018; 65-76.
18. Hanahan D, Coussens LM. Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment. Cancer Cell 2012; 21: 309-322.
19. Spaw M, Anant S, Thomas SM. Stromal contributions to the carcinogenic process. Mol Carcinog 2017; 56: 1199-1213.
20. Ribatti D. Mast cells and macrophages exert beneficial and detrimental effects on tumor progression and angiogenesis. Immunol Lett 2013; 152: 83-88.
21. Mantovani A, Sozzani S, Locati M, et al. Macrophage polarization: Tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 2002; 23: 549-555.
22. Kim S, Cho SW, Min HS, et al. The Expression of Tumor-Associated Macrophages in Papillary Thyroid Carcinoma. Endocrinol Metab 2013; 28: 192-198.
23. Hillen F, Baeten CI, van de Winkel A, et al. Leukocyte infiltration and tumor cell plasticity are parameters of aggressiveness in primary cutaneous melanoma. Cancer Immunol Immunother 2008; 57: 97-106.
24. Boström MM, Irjala H, Mirtti T, et al. Tumor-associated macrophages provide significant prognostic information in urothelial bladder cancer. PLoS One 2015; 10: 1-16.
25. Johansson CC, Egyházi S, Masucci G, et al. Prognostic significance of tumor iNOS and COX-2 in stage III malignant cutaneous melanoma. Cancer Immunol Immunother 2009; 58: 1085-1094.
26. Venza M, Visalli M, Beninati C, et al. Cellular mechanisms of oxidative stress and action in melanoma. Oxid Med Cell Longev 2015; 2015: 481782.
27. Spatz A, Batist G, Eggermont AM. The biology behind prognostic factors of cutaneous melanoma. Curr Opin Oncol 2010; 22: 163-168.
28. Jensen TO, Schmidt H, Møller HJ, et al. Macrophage markers in serum and tumor have prognostic impact in American Joint Committee on Cancer stage I/II melanoma. J Clin Oncol 2009; 27: 3330-3337.
29. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer 2004; 4: 71-78.
30. Valković T, Dobrila F, Melato M, et al. Correlation between vascular endothelial growth factor, angiogenesis, and tumor-associated macrophages in invasive ductal breast carcinoma. Virchows Arch 2002; 440: 583-588.
31. Straume O, Salvesen HB, Akslen LA. Angiogenesis is prognostically important in vertical growth phase melanomas. Int J Oncol 1999; 15: 595-599.
32. Straume O, Akslen LA. Expresson of vascular endothelial growth factor, its receptors (FLT-1, KDR) and TSP-1 related to microvessel density and patient outcome in vertical growth phase melanomas. Am J Pathol 2001; 159: 223-235.
33. Jonjic N, Zamolo G, Štifter S, et al. Cytomorphological variations, proliferation and angiogenesis in the prognosis of cutaneous melanoma. Clin Exp Dermatol 2003; 28: 310-314.
34. Pastushenko I, Van den Eynden GG, Vicente-Arregui S, et al. Increased Angiogenesis and Lymphangiogenesis in Metastatic Sentinel Lymph Nodes Is Associated With Nonsentinel Lymph Node Involvement and Distant Metastasis in Patients With Melanoma. Am J Dermatopathol 2016; 38: 338-346.
35. Storr SJ, Safuan S, Mitra A, et al. Objective assessment of blood and lymphatic vessel invasion and association with macrophage infiltration in cutaneous melanoma. Mod Pathol 2012; 25: 493-504.

Address for correspondence

Olga Stasikowska-Kanicka
Department of Nephropathology
Medical University of Lodz
Czechoslowacka 8/10
92-216 Lodz, Poland
e-mail: olga.stasikowska@umed.lodz.pl