

The Significance of the Lymphocytes Infiltration and Endothelial-dependent Angiogenesis in Choroidal Melanomas

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Abstract

Objective: Posterior uveal melanoma is the most common primary intraocular malignant tumor in adults. Despite the clinical significant treatment, still many patients die because of unpredictable metastases. It is difficult to predict clinical outcome in individual cases of ocular melanoma basing only on the intraocular size, because of the spectrum of clinical, morphologic, and cytologic changes and lack of discrete stages.

Design: The aim of the study was to evaluate CD3, CD20 and CD34 expression within choroidal melanoma in correlation with clinic and pathological features.

Participants: 35 cases of choroidal melanoma were evaluated.

Methods: Immunohistochemical reaction was used to evaluate CD3 and CD20 (T and B cells markers) and CD34 (endothelial cells marker) expression, with positive and negative controls. Obtained results were estimated under the light microscope.

Results: T lymphocytes were present in 94.3% choroidal melanoma specimens, the most significant CD3 expression was noticed in choroidal melanoma in pT3 stage. Presence of B lymphocytes was observed only in 22.8% cases, but only in advanced tumors (pT3). CD34 expression was observed in 77.1% choroidal melanomas. A statistically significant correlation was observed between CD34 expression and staging- 84.2% choroidal melanomas in pT3 stage were positive for CD34 expression.

Conclusions: T cell infiltration is present in both low and high advanced ocular melanomas. B cells and thin wall vessels are present mainly in advanced choroidal melanoma and due to may be associated with tumor progression.

Keywords: Choroidal melanoma, CD3, CD20, CD34 expression

Introduction

Posterior uveal melanoma, together with metastatic carcinoma, is the most common primary intraocular malignant tumor in adults [1]. Standardized incidence rates of uveal melanoma in Europe (from 1983 to 1994) increased from south to north, from minimum of <2 per million in registries of Spain and southern Italy, up to >8 per million in Norway and Denmark [2]. It is seen much commonly in white than in non-white races. It usually presents from middle age onwards (40-70 years). Uveal melanoma may also be seen in the ciliary body and iris, but so far the greatest numbers are found in choroid [3]. Approximately 40% of patients with posterior uveal melanoma develop metastatic melanoma to the liver within 10 years after initial diagnosis [4,5]. Despite high accuracy of diagnosis and availability of various methods of treatment; the mortality due to choroidal melanoma has remained unchanged. The overall mortality has been reported as 35% in 5 years and 50% in 10 years. Up to 50% of patients with intraocular melanoma develop metastatic disease [6-8]. Most patients with choroidal melanoma patients have no symptoms and the melanoma is discovered on routine eye examination. If patients have symptoms they are usually: floating objects in the vision, "flashes of light", distortion or loss of vision. The diagnosis of ocular melanoma is made by indirect ophthalmoscopy, transillumination, fundus photography, fluorescein angiography, ultrasonography, computed tomography or magnetic resonance imaging [9,10]. However the final diagnosis is always made by histopathologist, with the staging of the disease. It is difficult to predict clinical outcome in individual cases of ocular melanoma on the basis of intraocular tumor size because of the spectrum of clinical, morphologic, and cytological changes and a lack of discrete stages [11]. Best known clinical factors that relate to prognosis include location,

size and configuration of the tumor. Iris melanomas have the best and ciliary body melanomas have the worst prognosis. Tumor size and optic nerve infiltration are the best parameters used to predict the survival and metastatic potential of the tumor [1,3,8,10]. Histopathologically choroidal melanoma has a spectrum of cell types, ranging from thin and plump spindle cells to epithelioid cells (spindle-cell, mixed-cell, and epithelioid-cell types) [5]. The most common cytogenetic changes in ocular melanoma include loss of DNA on chromosome 3, gain on 6p, loss on 6q, and gain on 8q, loss of an entire chromosome 3 (or monosomy 3), which is an early event in tumorigenesis, and is detected in approximately 50% of tumors [6]. The local treatment of ocular melanoma is now well established depending on the size, the extrascleral extension or the localization of the tumor. It consists either of radiation therapy or/and enucleation [1,5,10].

The aim of the study was to evaluate the presence of T and B lymphocytes within the choroidal melanomas, as well as the measure the endothelial-dependent blood vessels proliferation within the tumors.

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The obtained results were than correlated with histopathological and clinical features.

Methods

The specimens were collected in the period of 12 years. Choroidal melanomas are quite rare tumors that are so we have only 35 cases. We took the tumors which were diagnosed by the histopathologist as choroidal melanoma and were staged as pT1, pT2, pT3. For the histopathological evaluation the specimens, taken during surgery enucleation from Department of Ophthalmology Medical University of Bialystok, were fixed in 10% buffered formalin. Evaluation of lymphocytes and endothelial cells markers expression was performed using immunohistochemical method. Following the deparaffinisation and rehydration, epitope retrieval was carried out in the EnVision Flex Target Retrieval Solution (DAKO) in high pH (pH=9.0). Endogenous peroxidases were blocked by incubating the sections in methanol and 3% hydrogen peroxidase for 20 minutes. Next slides were incubated with special types of antibodies, CD3: which is a common antigen for all of the T cells types, also for B cells (CD20 receptor is a common B-cells receptor). CD34 antibody: common endothelial cells marker- was used to count a number of blood vessels in tumor. Monoclonal mouse antibody against CD20 receptor was used (DAKO, M0755) in 1:200 dilution overnight in 4°C and polyclonal rabbit antibody against CD3 receptor (DAKO, A0452) in 1:100 dilution overnight in 4°C. To evaluate the number of the blood vessels mouse antibody against CD34 receptor was used (DAKO, IR632) in 1:100 dilution incubated overnight in 4°C. Visualization reagent (LSAB plus DAKO) was applied for 30 minutes followed by DAB solution for 5 minutes. The slides were than counterstained with hematoxylin and examined under the light microscope Olympus BX45 by histopathologist. Following data the staining for T and B lymphocytes was evaluated as the percentage of positive cells as follows: ≤10% positive cells - negative (-), between 11-49% (+), and 50-100% positive cells (++) in random 10 fields under 20X magnification [12]. To estimate of blood vessels number we counted all of the CD34 positive areas in random 10 fields

under 10X magnification. The scale was: negative (-), if there were no CD34 positive cells in all of the examined areas, (+) if less than 50% of the examined fields were CD34 positive; (++) if more than 50% of the examined fields were CD34 positive [13]. Appropriate positive and negative controls were performed. Negative controls were performed using a nonimmunized IgG replacing the primary antibody. Knowing CD3 and CD20 expression in normal lymph node and CD34 in angiosarcoma, those specimens were used as positive controls.

Statistical analysis

Chi squared and Pearson's correlation tests and Statistica 10.0 StatSoft software were used for statistical analysis. Values of p<0.05 were considered as statistically significant.

Results

All of the examined cases were estimated as choroidal melanoma, 2 out of 35 cases were epithelioid type of melanoma (graded as G3), 3 spindle cell (graded as G1) and the rest were mixed type (graded as G2). Only 2 specimens were in pT1 stage, 14 in pT2 and 19 in pT3 stage. In our group there were 19 female and 16 male patients, the mean age was between 31 and 88 years (Table 1). The staging was correlated with the sex (p=0.003) - more advanced tumors were observed in male group (in 10 out of 16 patients tumors were in T3 stage) less commonly in female group (pT1 and pT2 stages were observed in 10 out of 19 patients).

CD3 expression – T lymphocytes

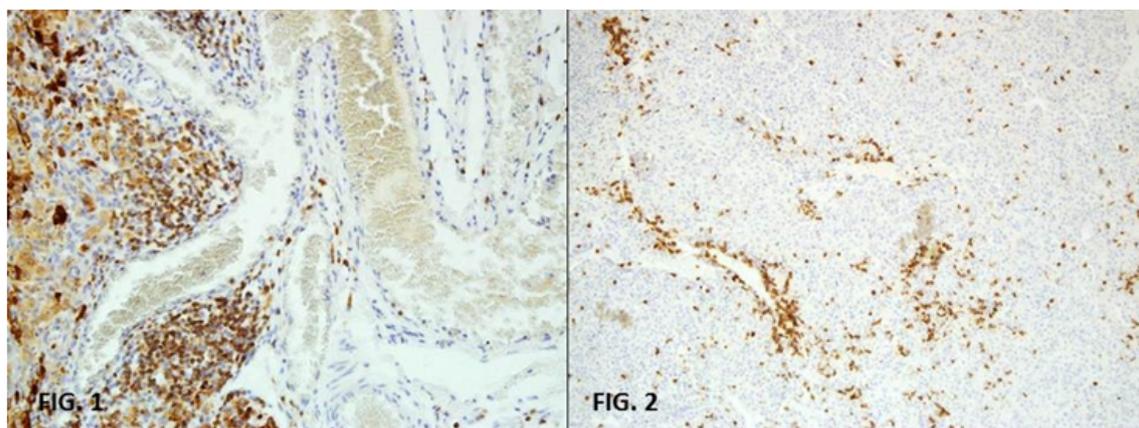
CD3 positive cells were observed in 33 out of 35 melanoma specimens (94.3%). The intensity of the T cells infiltration was there estimated as (+).

T lymphocytes were present in 14 out of 16 melanomas in pT1 and pT2 stages (87.5%) and in all (100%) pT3 cases (p=0.112) (Figures 1-4).

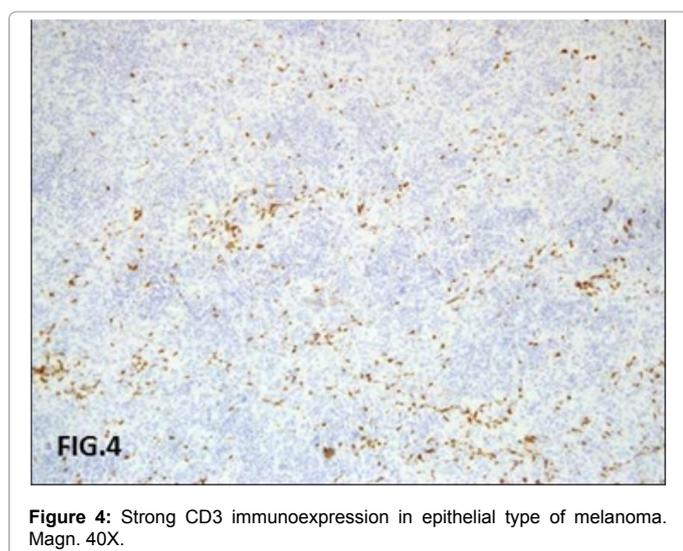
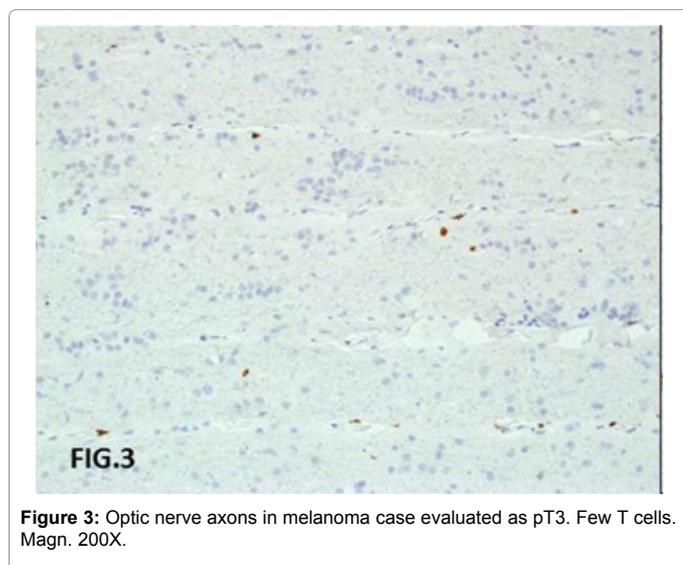
Although there was no statistically significant correlation between CD3 expression and staging (p=0.204) it is worth to notice, that T cells were observed in most of the examined melanomas (Table 2).

Type	pT stage		Gender		CD3	CD20	pT1	pT2	pT3	F	M	Age
	No	CD34	CD34	CD34								
Mixed	30	23	29	5	1	13	16	16	14	54-88		
Epithelioid	3	3	3	3	0	0	3	2	1	71-80		
spindle-cell	2	1	1	0	1	1	0	1	1	13-44		

Table 1: Histopathological and demographic features of examined uveal melanomas.



Figures 1 and 2: CD3 expression observed next to blood vessels within the melanoma. Magn. 200X, 100X.



CD34 expression – endothelial dependent angiogenesis

CD34 expression was observed in 27 out of 35 cases (77.1%), all were estimated as (+), presenting number of small thin-wall vessels within the tumor.

There was a statistically significant correlation between CD34 expression and the staging ($p=0.026$) - in 16 out of 19 choroidal melanomas in pT3 stage (84.2%), we observed numerous of thin-wall blood vessels with strong CD34 expression (Figures 5-7).

CD20 expression – B lymphocytes

CD20 positive cells were observed only in 8 out of 35 cases (22.8%). The intensity of the B lymphocytes infiltration was estimated as (+) (Figure 8).

There was a statistically significant correlation between staging and CD20 expression ($p=0.013$) - none of the choroidal melanoma specimens in pT1 and pT2 stage contained B lymphocytes infiltration within the tumor, and all melanomas in pT3 stage presented B cells infiltration.

Staging	CD3 Negative	CD3 Positive	P value
pT1	0	2	P=0.204
	0%	5.7%	
pT2	2	12	
	5.7%	34.3%	
pT3	0	19	
	0%	54.3%	
pT1	CD20 Negative	CD20 Positive	P value
	2	0	P=0.013
pT2	5.7%	0%	
	14	0	
pT3	40%	0%	
	11	8	
	31.5%	22.8%	
pT1	CD34 Negative	CD34 Positive	P value
	2	0	P=0.026
pT2	5.7%	0%	
	3	11	
pT3	8.6%	31.4%	
	3	16	
	8.6%	45.7%	

Table 2: CD3, CD20 and CD34 expression in choroidal melanoma of various staging.

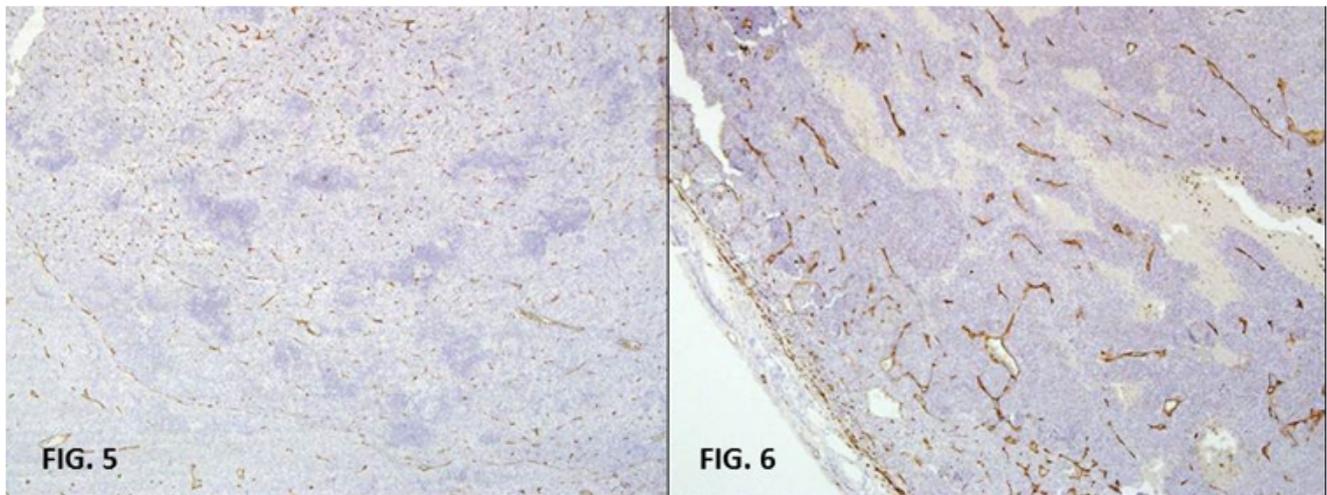
There was no statistically significant correlation between CD20 and CD34 expression ($p=0.080$). There was also no statistically significant correlation between CD20 and CD3 expression ($p=0.428$). Although in all advanced melanomas (which were in pT3 stage) both T and B lymphocytes were present.

There was also no statistically significant correlation between CD3 and CD34 expression, moreover 26 out of 33 choroidal melanomas with dense T cells infiltration (78.8%) were reached in CD34 positive blood vessels within the tumor ($p=0.346$).

Discussion

Intraocular melanoma of the ciliary body and choroid, together called the posterior uvea, is the most common primary ocular malignant tumor in adults. However still the disease leads to enucleation and in some cases to the distinct metastases and death. New data suggested that known histopathological factors like: tumor location, thickness, and recurrence are predictors of mortality. Some data indicate that depletion and antibody blocking experiments confirm, that MHC class II-restricted, endogenously synthesized epitopes are presented to CD4(+) T cells. Therefore, the MHC class II vaccines are efficient antigen presenting cells that activate tumor-specific MHC class II-restricted, CD4(+) T lymphocytes, and they are a novel and potential immunotherapeutic for metastatic cancers [14-19].

In our study we presented inflammatory cells and markers of vascularization within the choroidal melanomas. It is a very important task according to skin melanoma, although there are only few data associated with the choroidal melanomas. Some data indicate, that inflammation, and especially macrophage infiltrations are associated with a poor prognosis for the patient [20]. Macrophages play a role in promoting angiogenesis, and thus may stimulate tumor growth; in addition, macrophages have also been found to suppress antimelanoma immune responses. Others observed in choroidal melanoma specimens an immune infiltration with suppressive T cells [21,22]. In our present study T cells were observed in almost all (94.3%) choroidal melanomas. Also Staibano et al. [23] observed a significance T cells infiltration within the choroidal melanomas, mostly cytotoxic



Figures 5 and 6: CD34 expression- endothelial cells marker in the new small blood vessels within the melanoma. Magn. 40X, 100X

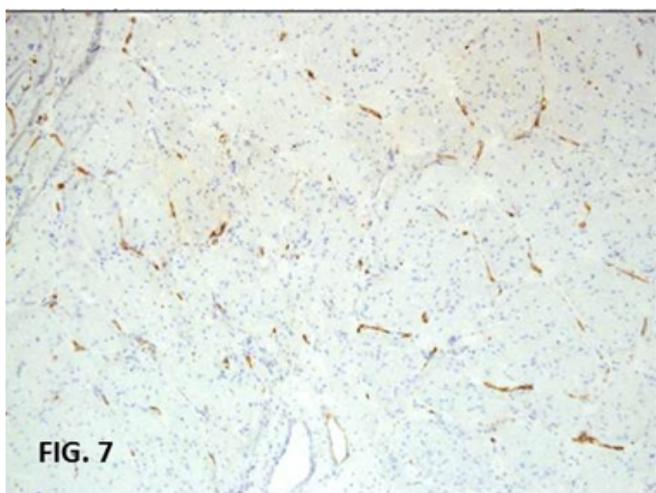


Figure 7: Optic nerve axons with melanoma estimated as pT3. CD34 expression in small blood vessels. Magn. 100X.

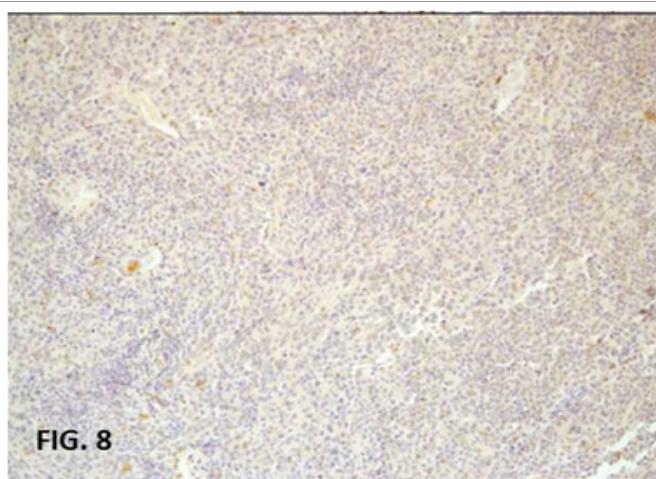


Figure 8: CD20 negative expression in pT1 melanoma epithelioid type. Magn. 100X.

T cells, suggesting, that elimination of tumor cells may be possible by activation of cytotoxic cells present within ocular melanomas. In our study all of more advanced melanomas (pT2 and pT3) contained T lymphocytes infiltration. Also Niederkorn et al. [21] revealed that the presence of tumor infiltrating lymphocytes (TIL) or tumor-infiltrating macrophages (TIM) is associated with poor prognosis in choroidal melanoma patients, suggested that some immune responses to intraocular tumors might exacerbate, rather than mitigate, tumor progression. Vu et al. [24] observed that epithelioid melanomas contained more inflammatory cell, including macrophages and T lymphocytes, than spindle cell type. Lagouros et al. [25] presented lymphocytic infiltration within 40.5% enucleated choroidal melanomas and it was related to worse prognosis. What is also surprised, no data concerning B lymphocytes infiltration within the choroidal melanoma were performed. In our study only 8 specimens were positive for CD20, however all of them were most advanced melanoma cases in pT3 stage.

CD34 is a fine endothelial cells marker, allowing to estimate blood vessels proliferation [12]. In many types of neoplasms the angiogenesis is associated with tumor progression and poor prognosis, that is so the antiangiogenesis therapy is used effectively in lung, gastrointestinal tract or sometimes brain tumors [26]. However in choroidal melanoma still this subject is not taken. That is so we used CD34 marker to evaluate endothelium dependent vessels proliferation within the choroidal melanomas. CD34 expression was observed in 77.1% cases, all were more advanced tumors in pT2 and pT3 stage. It was a statistically significant result, indicating that proliferation of the blood vessels is directly related to the tumor progression and worse prognosis. Also Chen L et al. [26] investigated the pattern and distribution of microcirculation in an intraocular animal model by immunostaining with anti- CD34 antibodies, finding that the number of endothelium-dependent vessels significantly correlated with the tumor size and worse prognosis. Chen X et al. [27] found that the labeling of tumor cells by CD34 was associated with an elevated calculation of MVD (microvascular density), which was found to be associated with death from metastatic melanoma. Kivelä T et al. [28] also showed the importance of angiogenesis within ocular melanoma. In their study the presence of blood vessels networks was clinically associated with growth of small uveal melanocytic tumors and with the rate of regression of uveal melanoma after brachytherapy. Those

networks were also associated with development of exudative retinal detachment from uveal melanoma. Mäkitie T et al. [29] observed new vessels proliferation within 134 out of 167 melanomas (80%), contributing to prognosis in uveal melanoma.

Conclusion

Our study presents that CD34 expression might be valuable marker to predict choroidal melanoma growth. It may support the hypothesis that angiogenesis is associated with tumor choroidal melanoma progression and worse clinical course. Also evaluation of the presence of B lymphocytes within the tumor may be related to more advanced choroidal melanomas, however this hypothesis still needs more detailed studies. CD3 is observed in almost of all the evaluated choroidal melanomas and therefore it is worth to determinate the subgroups of the T lymphocytes within the choroidal melanoma in association with staging.

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