CASE REPORT

A rare case of double recurrent choroidal melanoma, with distinctive immunohistochemical features

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Abstract

A 57-year-old woman, with left choroidal melanoma treated by laser photocoagulation and a history of repeated vitrectomies, checked for left eye acute pain and foreign body-like sensation, symptoms that occurred after three years since the primary tumor treatment. The left eyeball was enucleated and the tissues were investigated by immunohistochemistry for markers associated with cell differentiation, proliferation and adhesion, cell cycle regulation, apoptosis control, vascularization, invasiveness and local immune response. We identified, in fact, two independent tumors, with different localization and sharing some common features, markers of a highly aggressive potential: loss of cell differentiation markers and cell cycle regulators, ability to avoid death by suppressing Fas antigen expression and important invasive capacity by down regulation of E-cadherin expression. However, only in the posterior tumor, we found cells with high proliferation rate, Fas ligand molecule expression and MMP-9 secretion, acquisitions associated with a much more aggressive behavior. These particular phenotypes allowed the posterior cells to grow and to invade the surrounding tissues more rapidly than the anterior ones, leading to the development of a large size tumoral mass, responsible for the clinical symptoms. Photocoagulation, by destroying the tissues, makes impossible the evaluation of the primary tumor's biological features, important for the tumor evolution. The absence of these data stresses the importance of patient monitoring, eventually addressing a panel of soluble markers associated with recurrence or metastasis development.

***Keywords*:** aggressiveness, choroidal melanoma, double recurrence.

Introduction

Choroidal melanoma, a subtype of uveal melanoma, is the most common primary malignant intraocular tumor that usually occurs in adult, white persons [1], with a higher incidence in males [1] and, exceptionally, in children [2]. The tumor rarely affects the optic nerve [3] but, depending on the tumor cell aggressiveness, it frequently metastasizes at distance and, sometimes, the metastases are already developed even before the diagnosis of the primary tumor [4].

In the past, the main treatment consisted in the enucleation of the eyeball, but since the 80's, rather conservative therapies were considered such as laser photocoagulation, thermotherapy, plaque radiotherapy, local tumor resection [5]. Despite the development of numerous treatments, none proved ideal for this life threatening malignancy in preventing the metastases or tumor recurrence, which occurred sometimes, after many years since the primary tumor treatment [5–7].

We report a rare case of double recurrence in a female patient with choroidal malignant melanoma treated in 2004 by laser photocoagulation. The short period of time between the treatment of the primary tumor and the occurrence of acute symptoms leading to the diagnosis of the double recurrence suggested that some of the tumor cells remained viable after photocoagulation and had a high potential of growth and local invasiveness.

The aim of this study was to assess this potential, by investigating the expression of several molecules known as important markers associated with malignancy features.

Patient

Our study presents the case of a 57-year-old woman, with an insidious beginning of the disease in 2004, materialized in the upper left side visual field loss. The visual test, the echography and the computed tomography (CT), confirmed the presence of a left eye choroidal melanoma, with regmatogen retinal detachment. In July 2004, a left vitrectomy was performed, the tumor was treated by laser photocoagulation and silicone was filled in the eye. In November 2004, due to low visual accuracy, a new vitrectomy was performed and the silicone was replaced by an expansive gas. The remnants of the silicone gel required a new intervention in 2005 for its removal. Starting with August 2007, the patient accused acute pain and foreign

body-like sensation in the left eyeball, and severe left hemicranias, symptoms that became, in time, permanent and refractory to the medication. In 2008, the patient checked in the "Nicolae Oblu" Emergency Hospital in Iassy and the examination of the left eye anterior pole revealed bulb conjunctiva postoperative scars, non-modified sclera and cornea preserved transparency. The anterior chamber had a significant depth, completely occupied by a silicone bubble blocking the anterior chamber angle. The iris was atrophic, with a round, centered, fixed and miotic pupil. The enucleation was accepted by the patient and the sectioned eyeball macroscopic examination evidenced a big white tumor in the posterior chamber, near the optic nerve, causing retinal detachment and a protrusion in the vitreous body.

Material and Methods

Microscopic investigation

The enucleated eye was formalin-fixed and paraffin-embedded. Various staining were performed, in order to identify the melanin pigment (Fontana), the cellular elements (Hematoxylin–Eosin and Van Gieson), and the extracellular matrix (Gordon Sweat).

Immunohistochemistry

Tissue sections (4 μm) were deparaffinized in xylene and rehydrated in a series of graded ethanol solutions. Antigen retrieval was performed by heating the sections, 20 minutes in Antigen Retrieval Solution (Dako, Denmark), pH 6.1, at 98^0C, followed by 5 minutes incubation with 3% H_2O_2/dH_2O or with Dual Endogenous Enzyme Block (EnVision G/2 Doublestain System, Rb/Mo, DAB+/Permanent Red, Dako, Denmark). The slides were incubated O/N at 4^0C with the recommended work dilutions of the following primary antibodies: mouse anti-human Melanosome (tyrosinosome matrix-related protein gp100 – clone HMB-45), polyclonal rabbit anti-S100a (N1519), mouse anti-human cytokeratin (clone AE1/AE3), mouse anti-human cytokeratin (clone MNF 116), mouse anti-human cytokeratin 18 (clone DC10), mouse anti-human vimentin (clone Vim3B4), mouse anti-human proliferating cell nuclear antigen (PCNA, clone PC 10), mouse anti-human HLA-DP, DQ, DR antigen (clone CR3/43), mouse anti-human p53 (clone DO-7), mouse anti-human $p21^{WAF1/Cip1}$ (clone SX118), mouse anti-human $p27^{Kip1}$ (clone SX53G8), mouse anti-human bcl-2 (clone 124), polyclonal rabbit anti-human bax (A3533), mouse anti-human Fas (clone DX2), mouse anti-human CD45 (clone T29/33), mouse anti-human CD68 (clone KP1), polyclonal rabbit anti-human CD3 (N1580), mouse anti-human CD20 (clone L26), mouse anti-human CD34 class II (clone QBEnd 10), all from Dako, Denmark and with mouse anti-human Fas ligand (clone Alf2.1, Sigma, Austria), mouse anti-human MMP-2 (clone 2C1), mouse anti-human MMP-9 (clone 2C3), both from Santa Cruz Biotechnology, USA.

For antigens with different localizations in the same cells (nucleus versus cytoplasm or membrane), or for antigens associated with different cell types (melanocyte versus leukocyte/macrophage/T- or B-lymphocyte), we performed double staining. The reactions were detected using EnVision+ Dual Link System-HRP kit or EnVision G/2 Doublestain System, Rabbit/Mouse (DAB+/Permanent Red kit, Dako, Denmark), followed by counterstaining in Mayer's Hematoxylin (Dako, Denmark).

As negative controls, we used Universal Negative Control for N-Series Mouse Primary Antibodies (Dako, Denmark) or Universal Negative Control for N-Series Rabbit Primary Antibodies (Dako, Denmark).

Positive reactions were visualized in light microscopy as brown or red precipitates, corresponding to the antigens localization, nuclear for PCNA, p53, p21, p27, cytoplasmic for gp100 protein, vimentin, cytokeratins, HLA class II antigens, bcl-2, bax, Fas, FasL, CD34 II, MMP-2, MMP-9, nuclear/cytoplasmic for S100a protein, membrane/cytoplasmic for CD45, CD68, CD3 and CD20. The tumors were considered positive if at least 5% of cells were labeled for an antigen and the percentages of positive cells were estimated in order to compare the two tumors.

Results

Histological examination

The microscopic investigation of the choroidal melanoma showed the presence of two independent tumors, one placed at the posterior pole of the eyeball, and the second one near the ciliary body. Both tumors had a significant cellular density, with a predominance of epithelioid cells showing important atypia, large eosinophilic cytoplasm, little or no melanin and round, vesicular nuclei with prominent nucleoli. The second morphological cell type was the elongated spindle melanocytes, with irregular shapes and long nuclei, diffused in both tumor masses, surrounding the nests of epithelioid elements. As compared with the anterior tumor, the posterior one had a larger number of mitoses, 4–5 *vs.* 0–1, in a high power field (×400), and a higher density of reticular fibers (data not shown). It penetrated the sclera and the optic nerve, expanding to the soft tissues in the eye socket and around the ciliary nerves, causing the retinal detachment, with cystic degeneration (data not shown).

Immunohistochemistry

The investigation for melanocyte differentiation markers has shown that S100 protein was expressed by both tumors, almost ubiquitously (Figure 1), approximately 60% of the cells in the posterior tumor expressed vimentin and gp100, respectively, while in the anterior mass, less than 0.1% cells expressed gp100 (Figure 2) and none was vimentin-positive (data not shown). For each of these two molecules, the majority of the positive cells were concentrated in the periphery of the posterior tumor (Figure 3), their number significantly decreasing in the deeper areas (Figure 4). Both tumors were negative for all the investigated cytokeratins: 1–8, 10, 14–19 (data not shown).

The higher percentage of PCNA-positive cells in the posterior tumor, ~30% (range between 10% and 50%),

as compared with the anterior one (<1%), correlated with the histological findings (Figure 5) and double labeling for PCNA/gp100 or PCNA/vimentin showed a relatively inverse association between these molecules expressions (Figure 6) – most of the PCNA-positive cells were negative for the differentiation markers.

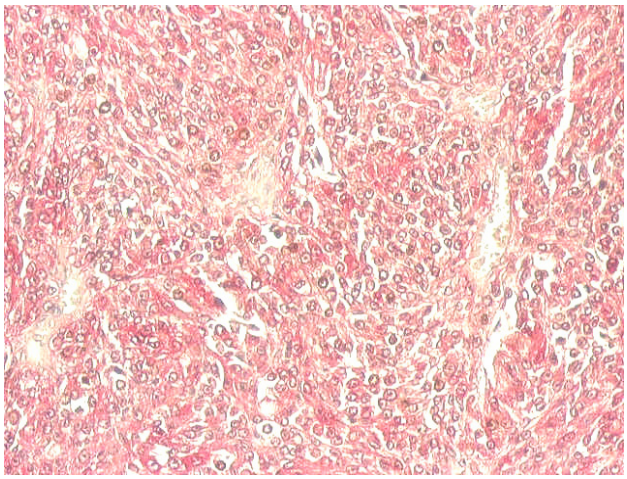

Figure 1 – ***S100a is expressed by the majority of cells in both tumors (posterior tumor, AP/Fast Red, ×100).***

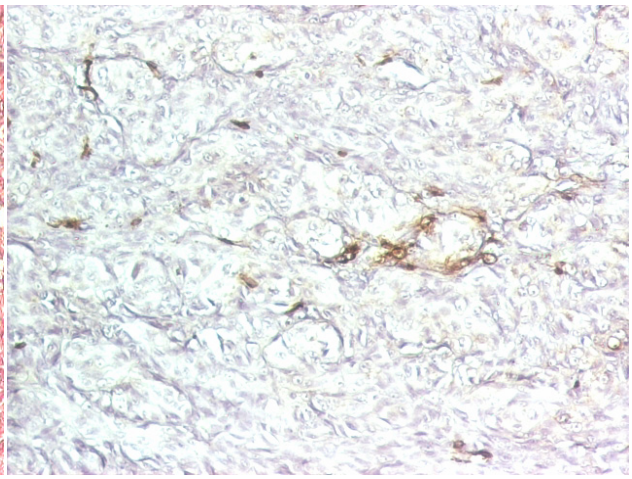

Figure 2 – ***The HMB-45 antibody identifies gp100, a melanocyte specific marker. The anterior tumor presents a very small number of cells (arrow) positive for this molecule (peroxidase/DAB, ×200).***

Figure 3 – ***Posterior tumor staining with the HMB-45 antibody. The positive cells are concentrated in the periphery (peroxidase/DAB, ×40).***

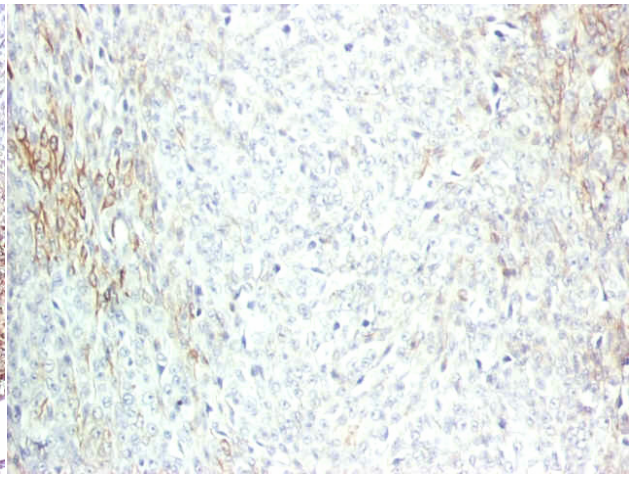

Figure 4 – ***Posterior tumor. Staining for vimentin shows that in the deeper areas, most cells have become negative for this marker (peroxidase/DAB, ×100).***

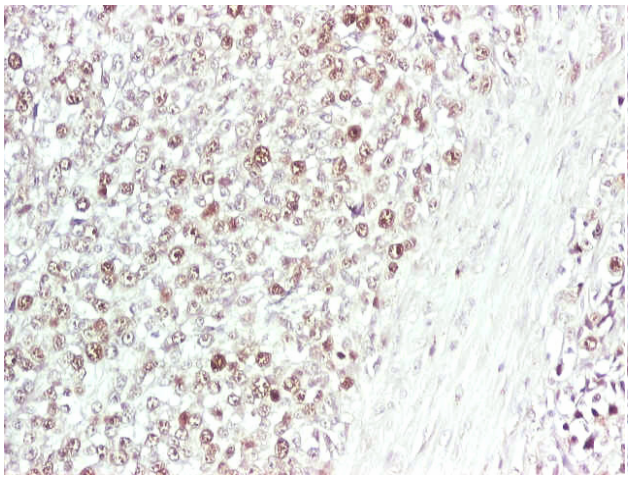

Figure 5 – ***PCNA nuclear expression in the posterior tumor suggests a high cellular proliferation rate (peroxidase/DAB, ×100).***

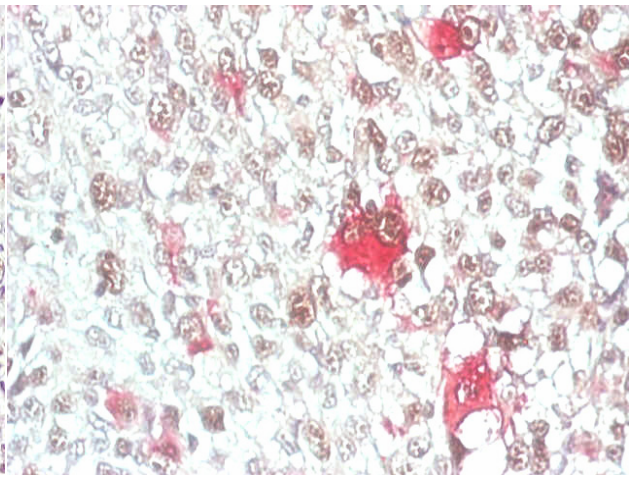

Figure 6 – ***Double labeling for PCNA and gp100. Many PCNA+ cells (brown precipitate, peroxidase/ DAB) have lost the gp100 expression (red precipitate, AP/Fast Red, ×200).***

Tumors were negative for Fas, p21 and p27 molecules, p53 and bcl-2 expressions were absent, but bax was present in more than 60% of the cells (Figure 7). Fas ligand was expressed only in the posterior tumor, by approximately 10% of malignant cells. The small number (<0.1%) of leukocytes (CD45+ cells), infiltrating both tumors, was FasL-negative (data not shown). We found 2% HLA-II-positive cells (Figure 8), no cell labeled for CD3 or CD20 and less than 0.1% cells, positive for CD68 (data not shown). Both tumors were well vascularized (Figure 9), with 4–5 CD34 II-positive vessels in high power field (×400). MMP-9 was expressed by 50% (range between 80% and 20%, Figure 10) of cells only in the posterior tumor, while for MMP-2 all cells were negative in both tumors (data not shown).

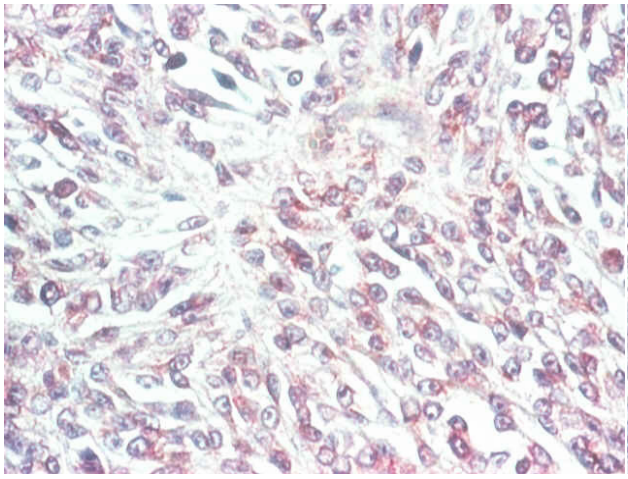

Figure 7 – ***Bax expression in the posterior tumor may support the hypothesis that the cells are still sensitive to apoptosis (AP/Fast Red, ×200).***

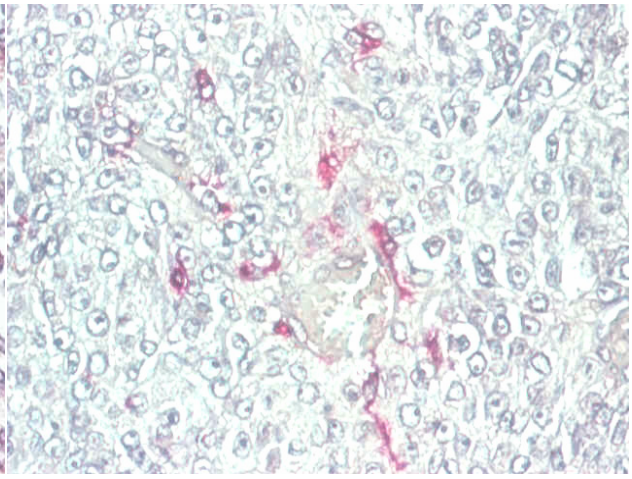

Figure 8 – ***Approximately 2% of the posterior tumor cells are HLA-II-positive, possibly dendritic cells or activated endothelial cells (AP/Fast Red, ×200).***

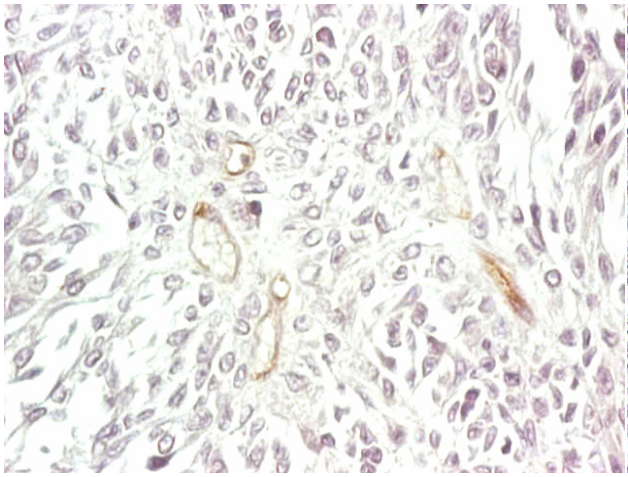

Figure 9 – ***Vessels in the posterior tumor, identified with an antibody specific for CD34 II molecule, marker for endothelium (peroxidase/DAB, ×200).***

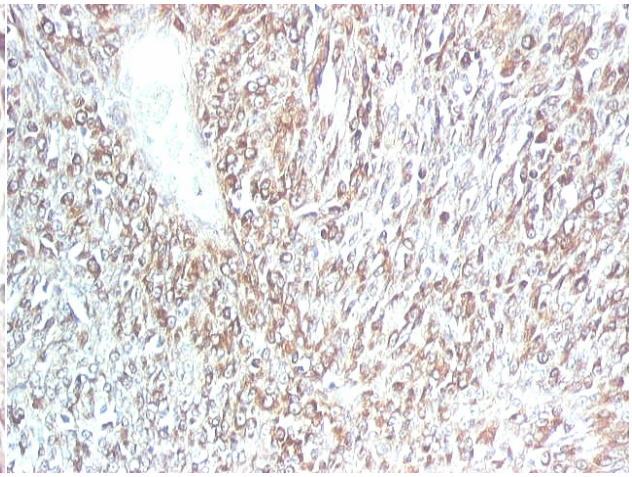

Figure 10 – ***MMP-9 expression in the posterior tumor suggests a high invasive potential (peroxidase/DAB, ×100).***

Discussion

A double recurrence occurring short time after the primary choroidal melanoma photocoagulation suggested a high potential of growth and local invasiveness of the remaining malignant cells. Hence, we were interested to evaluate this aggressive behavior.

The microscopic investigation of this case showed the presence of two independent tumors, with significant cellular density and with the predominance of epithelioid cells, a cell type, known as marker for high aggressiveness [4, 8].

The expression patterns of melanocyte differentiation markers, gp100 and vimentin, concentrated in the periphery of the posterior tumor mass and decreasing in the deeper areas, suggested a tendency of losing the expression of antigens associated with cell differentiation and were reported also by other authors in skin melanomas [9, 10]. We investigated, also, the presence of some epithelial cells markers, since their co-expression with vimentin seems to be associated with high invasive and metastatic potential in melanoma [11, 12], but both tumors were negative for all the investigated cytokeratins.

We found a high proliferation rate of the posterior tumor cells and an inverse association between the expressions of PCNA, important prognostic factor in human cancers, including uveal melanoma [13] and gp100 and vimentin, respectively. Taken together,

it seems that the majority of the proliferating cells have lost the differentiation markers expression, suggesting that, becoming less differentiated, these cells are thus escaping the mechanisms of cell cycle control.

To check this hypothesis, we investigated the expression of p21 and p27, cyclin-dependent kinase inhibittory proteins (CKIs), known to play a critical role in the control of the cell cycle progression [14]. Together with mutations in the p53 gene, alterations of CKIs expressions or functions were found to be involved in cell transformation and tumorigenesis [15, 16]. In our study, tumor cells were negative for p21 and p27, results that could explain the relationship between the loss of differentiation antigens and regulators of the cell cycle progression and the high cellular proliferation potential.

Tumor's development is the result of an imbalance between cellular proliferation and death. Apoptosis is the major type of physiological cell death, involved in the deletion of potentially harmful or unnecessary cells in various tissues [17], but malignant cells develop strategies to avoid it [18] and these mechanisms include an imbalance between the secretion and function of molecules promoting or inhibiting cell death. Bcl-2 and bax are involved in cell death control, with opposite effects. Bcl-2 is an anti-apoptotic and bax functions as an apoptosis inducer [19]. p53, the "guardian of the genome" is the most important regulator of these two molecules [20]. In normal cells, wild-type p53 protein has a very short half-life and thus is present in only minute amounts, below the detection level of immunohistochemical methods [21]. In situations associated with DNA damages, the protein is produced in large amounts; it blocks the cell cycle by stimulating the secretion of p21 and is able to induce apoptosis by inhibiting bcl-2 production and by stimulating bax secretion [19, 20]. Many human malignancies are frequently associated with p53 gene mutations and secretion of much more stable mutant proteins, unable to function as tumor suppressors. Consequently, many tumor cells overexpress bcl-2 and avoid death [20]. In our study, p53 and bcl-2 were absent, but bax was present in more than 60% of the cells. The negativity for p53 could be an argument that the gene was not mutated; the protein was secreted in reduced amounts and functioned normally, stimulating bax expression and supporting the idea that the tumor cells were still sensitive to apoptosis.

Fas (CD95/Apo-1), member of tumor necrosis factor receptor (TNFR) and nerve growth factor receptor (NGFR) families [22], is expressed by a large variety of cells, including epithelial and mesenchymal cells [23] and its interaction with Fas ligand activates a proteolytic cascade, mediated by proteases of the caspases family, leading to protein cleavage, DNA fragmentation and cell death [22]. Many cells are down-regulating Fas expression, during their malignant transformation as a strategy of immune response evasion [18] and this behavior characterized both tumors investigated in our study.

FasL is a type II membrane protein that belongs to the tumor necrosis factor family, able to induce cell death in Fas-bearing cells [17]. It is expressed by immune system effectors and is also present in the so called "immune privileged sites", such as the eye [24, 25]. Many mechanisms proved to be involved in the ocular tissues protection and homeostasis and one of the most important is the induction of apoptosis in the immune system cells, Fas ligand being constitutively expressed by several eye components [25]. Using similar mechanisms, different tumors, including melanomas [17], acquire the FasL expression and, consequently, are able to escape immunologic rejection by suppressing the immune response. Many studies have shown that, *in vitro*, FasL-positive tumor cells kill activated, Fas-bearing lymphocytes [26, 27], while *in vivo*, the expression of FasL by human tumors is associated with apoptotic depletion of tumor-infiltrating lymphocytes (TILs) [27]. In our study, only 10% of cells in the posterior tumor expressed FasL, an acquisition that possibly explained the reduced number of leukocytes infiltrating the tumor mass and the absence of T- and B-lymphocytes. We found, also, a small number of HLA-II-positive cells, which were macrophages and, most of them, probably, dendritic or activated endothelial cells so, further, we were interested in the vascular density evaluation, since tumor vascularization is a well known prognostic factor in many malignancies [28]. Both tumors were well vascularized and this rich network of vessels seems able to support the high proliferation rate of the posterior tumor cells, since no necrotic areas were found. The absence of a local efficient immune response, demonstrated by our results, completed this very advantageous situation for the tumor development.

Invasiveness is another feature of malignancies. Normally, cells are adherent one to another and this contact, mediated by different types of molecules, is responsible for tissue homeostasis [29]. Malignant transformation and melanoma development is associated with the loss of cadherins expression, notably, the E-cadherin [30]. More, the expression of VE-cadherin, found by Hendrix et al. in aggressive melanoma cells, correlated with their ability to form vascular channels [31]. In our case, the expression of E-cadherin was absent in both tumors, suggesting a high cellular invasive potential. The capacity of the tumor cells to invade the surrounding microenvironment is, also, associated with their ability to secrete enzymes involved in tissue destruction, such as metalloproteinases [32]. Only in the posterior tumor, we found a large number of cells expressing MMP-9, while for MMP-2 all cells were negative in both tumors. The absence of E-cadherin expression and the ability to secrete MMP-9 are important acquisitions for the posterior tumor cells, conferring them the ability to migrate, invade the surrounding tissues and form large tumoral mass.

Conclusions

Tumors aggressive potential is a sum of features related with their capacity to grow, invade tissues, migrate and metastasize and the assessment of this potential is important for the prediction of tumors' behavior and evolution.

We evaluated the aggressiveness of a double

choroidal melanoma recurrence and we found that both tumors had different percentages of cells that lost the melanocytic markers, escaped the cell cycle regulation and host immune response and acquired the ability of local invasiveness, features that could be considered markers of an aggressive behavior. Furthermore, the posterior tumor cells presented particular phenotypes associated with a higher aggressiveness. This tumoral profile is probably inherited from the primary tumor, but we can only speculate this, since its treatment, by destroying the tumor mass, prevented the evaluation of the tumor cells aggressive potential. We consider that, in such particular cases, in order to evidence, as soon as possible, a local recurrence or a metastasis at distance, the patient's periodical ophthalmologic examination is extremely important and computed tomography is essential. Also, it would be of great interest to identify serological markers that would allow monitoring these patients.

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