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ORIGINAL ARTICLE

Aquaporin-1 Protein Expression of the Primary Tumor May Predict Cerebral Progression of Cutaneous Melanoma

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Abstract

Brain metastasis is a frequent complication of the progression of malignant melanoma. In a previous study aquaporin 1 (AQP1) protein expression was found to be associated with increased mortality and decreased progression free survival in cutaneous melanoma. To explore further the potential of this marker we studied the AQP1 protein expression in 67 metastatic melanoma patients using immunohistochemistry. Primary tumor samples were acquired from patients with brain (BR) ($n = 44$) and extracranial (EC) ($n = 23$) metastases, while brain metastatic samples were collected during neurosurgical resection ($n = 5$). Patients with brain metastases had shorter overall survival ($p = 0.02$) and significantly higher AQP1 expression in the primary tumors (median H-score = 250 vs. 140, $p = 0.044$) as compared to patients of the EC metastasis group. AQP1 expression was found to be significantly lower in the brain metastases compared to the corresponding primary tumors (median H-score = 35 vs. 300 $p = 0.01$). However, in brain metastases AQP1 expression was heterogenous, AQP1 protein was more abundant in the melanoma cells far away from the capillaries as compared to tumor cells adjacent to vessels indicating a hypoxia-driven expression of AQP1. We suggest that AQP1 expression could well be a prognostic marker of brain metastatic potential of human cutaneous melanoma.

Keywords AQP1 protein · Cutaneous melanoma · Melanoma brain metastases · Metastatic melanoma

Introduction

Malignant melanoma is the third most frequent cause of brain metastases, closely ranking behind pulmonary and breast cancers [1]. The incidence of brain metastases (BM) in cutaneous melanoma is considered very high: 20% of the metastatic melanoma patients already present with progression to the brain at the initial diagnosis, and BM is developed during the course of the disease in nearly 50% of the cases [2]. If we take into account the data coming from autopsies, the numbers are even more astonishing: 75% of all patients died of malignant melanoma were found to have metastases in the brain [3]. The prognosis of melanoma with brain metastasis is very poor: the median overall survival (OS) of the affected patients is 4–5 months in general, while in some selected cohorts treated with aggressive neurosurgical approaches or radiosurgery, the survival could be extended to 8–10 months [1].

Organ selectivity is the characteristics of the metastasization process and various cancer types have different metastatic patterns. Preclinical and clinical data suggest that brain metastasization requires unique potential to cross blood-brain-barrier (BBB), interact with the unique brain stroma, the glial tissue, and establish vasculature by using vessel cooption technology but not neo-angiogenesis [4]. Malignant melanoma is the most metastatic human cancer type where the smallest primary tumor may have organ metastatic potential. On the other hand, this metastatic potential has a clear organ selectivity for the brain. There were several studies which attempted to analyze the molecular background of this unique organ selectivity. Studies converged on the theme that melanoma cells apply "neurogenic mimicry" which helps to adapt to the unique microenvironment of the brain [5, 6]. Furthermore, melanoma cells similar to some other cancer types, are very efficient to cross the blood-brain-barrier, although the molecular mechanism of this is still not clear [7]. Finally, all the metastatic tumor

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cells must be able to provide their blood supply. Cutaneous melanoma is very efficient in the use of the preexisting blood vessels (vessel cooption-type of blood supply) [8] which is also the main type of blood supply of the brain metastases instead of the common neo-angiogenesis [9].

Experimental works suggested a link between Aquaporin 1 (AQP1) expression and the metastatic potential of rodent and human melanoma cell lines [10–12]. Studies on human melanoma gene expression signatures found repeatedly AQPs among the genes which were involved in shaping metastatic potential [13, 14]. Our group was the first to demonstrate an association between the primary tumor AQP1 protein expression and decreased progression free- and overall survival in melanoma patients [15]. While our clinical data supported the role of aquaporin water channels in melanoma progression, many questions remained unanswered regarding the underlying mechanisms.

The potential role of the APQ1 in the brain metastatic progression of cutaneous melanoma could be a significant contributing factor to the previously documented increased mortality characterizing the AQP1 positive melanoma patients. Our current study was designed to compare brain and non-brain metastatic primary melanomas for AQP1 protein expression with a special focus on the expressional changes during the cerebral progression of the disease.

Materials and Methods

Patients and Tumor Samples

The present retrospective study was based on two groups of consecutive patients altogether representing 67 cases of metastatic cutaneous melanoma. All patients were diagnosed and operated with a curative intent between 2003 and 2014 at the two largest Hungarian centers of dermato-oncology: the Department of Dermatology, Venerology and Dermatooncology of Semmelweis University, and the Department of Dermatology of the National Institute of Oncology in Budapest, Hungary. Every patient included in the study participated in close clinical follow up by regular staging, including the standardized use of state of the art intra-cranial imaging by computed tomography (CT) and magnetic resonance imaging (MRI). Consecutive patients in the extra-cranial metastasis group (EC) ($n = 22$) did not show any radiological or clinical signs of cerebral progression during their follow up, while patient included in the brain (BR) metastasis group ($n = 43$) developed intracranial lesions showing the typical MRI characteristics of brain metastasis on the contrast enhanced sequences. Staging was performed according to the latest guideline [16]. Patients' clinical history and tumor characteristics were obtained from the clinical databases of the participating institutions, while the outcome data of the patients lost to outpatient clinical follow up was controlled by telephone interviews. The tissue blocks containing the tumor samples were all processed at the 2nd Department of Pathology of Semmelweis University, including 5 specimens obtained during neurosurgical tumor removal of melanoma metastasis at the National Institute of Clinical Neuroscience in Budapest, Hungary. Our investigations were performed strictly according to the Declaration of Helsinki and they were approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics (IRB, SE TUKEB 32/2007).

Immunohistochemistry

Routinely formalin-fixed and paraffin-embedded tumor tissue blocks were cut into 5 μm thick sections and mounted on SuperFrost Plus slides (Gerhard Menzel GmbH, Braunschweig, Germany). Human AQP1 was detected by 7D11 anti-AQP1 primary antibody at 4 °C overnight (mouse monoclonal, used in 1:50 dilution, Abcam, Cambridge, MA, USA). Reaction was performed in the Benchmark Ultra immunostainer (Ventana Medial System INC. Tucson, AZ, USA) where the UltraView Universal AP-red detection system was used. Slides were counterstained with hematoxylin, washed in water, dehydrated, and cover-slipped before analysis.

Scoring

We used the H-score system for quantifying the immunoreaction according to previously published protocols [15]. The Stained sections were evaluated by independent expert readers blinded to the tumor characteristics and survival data of the subjects. Discordant cases were consulted until agreement was achieved.

Statistical Analysis

Statistical analysis was performed using Statistica 10 software (Statsoft INC Tulsa OK, USA). Mann-Whitney U test and Unpaired T-test was used to compare clinical and pathological findings of the two patient cohorts. Survival probabilities were estimated by the Kaplan–Meier method; differences assessed by the Log-rank test. All test were two-tailed with a confidence interval of 95%, significance was defined at p 0.05.

Results

Detailed clinical follow up was carried out for a mean time of 65.0 months (0.0; 180.0). In order to confirm the previously established concept of decreased overall survival in case of intra-cranial metastatic progression, we compared the survival of the patients with BR and EC metastatic progression. We found that the overall survival of the patients with intra-cranial progression (M1d) was indeed worse, compared to the patients developing extra-cranial metastasis exclusively (55,1

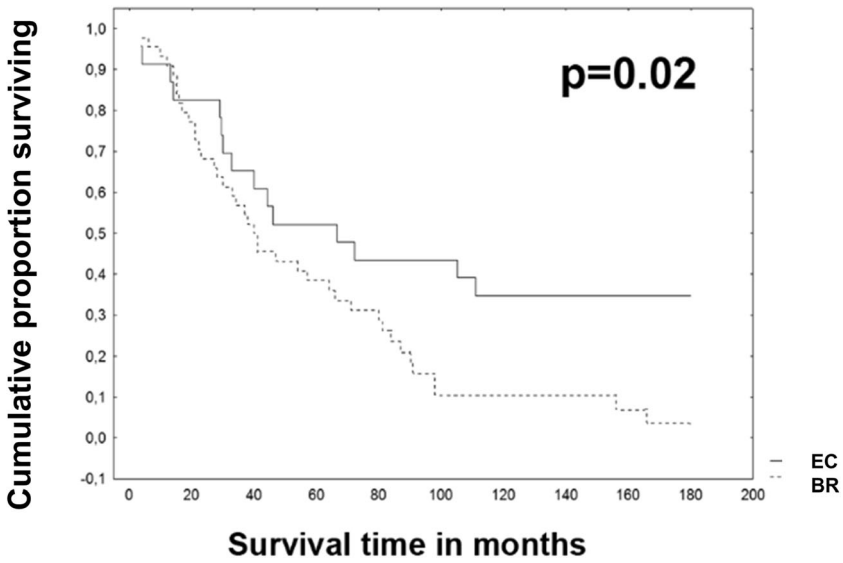

Fig. 1 Survival analysis of metastatic melanoma patients using Kaplan-Meier statistics. Data indicate the overall survival of cutaneous melanoma patients with brain (BR) and extra-cranial (EC) metastatic progression. Significant difference can be observed in the overall survival ($p = 0.02$) favoring the EC group

vs 83,0 months from the discovery of the primary tumor, $p = 0.03$) (Fig. 1b and Table 1).

AQP1 protein expression was determined and scored in the primary tumor samples of all 67 patients treated for metastatic melanoma. Positive AQP1 labeling appeared as a red membranous staining on endothelial cells and cytoplasmic and membrane labeling of tumor cells in the primary cutaneous samples (Fig. 2a), while the capillary endothelial cells in the brain tissue did not express the AQP1 protein, similarly to previous reports [15] (Fig. 2b).

AQP1 positive melanoma cells were found in 60 primary tumors (89.56%), with a mean H-score of 187.00 (range 0; 300) for the entire study population. While the two pre-specified patients groups (EC vs BR metastasis group) did no not show any significant difference in the standard prognostic Breslow index ($p = 0.054$), we found significantly higher AQP1 H-score in the "BR" group compared to the "EC" primary tumors ($p = 0.019$ on T-test, $p = 0.034$ on Mann Whitney U-test) (Fig. 3a). Since the EC group was heterogenous concerning visceral and LND metastasis we have compared LND metastatic cases to visceral metastatic cases as well as BR metastatic cases. Our statistical analysis indicated that there was no difference in AQP1 expression between the LND and visceral metastasis subgroups but the visceral (non-LND) metastatic group remained still statistically significantly different from the BR metastatic cases ($p = 0,048$) (Fig. 3b), suggesting the unique association with BR metastatic potential of the tumors.

Five out of the 67 patients, we were able to analyze the change in the AQP1 protein expression during the intracranial metastatic progression of the disease following the surgical removal of the metastatic lesions. We found lower AQP1 H-score both in the individual cases and by comparing the two groups ($p = 0.01$): while the primary tumors were characterized by a median AQP1 H-score of 300, in the brain metastatic group the median H-score was only 35. (Fig. 4) Looking for a potential cause of reduced expression in the brain metastases we have found that the AQP1 positive tumor

Table 1 Characteristics of patients included in the histological evaluation

Group	1. EC metastases patients	2. Brain metastases patients	p
Number of subjects	23	44	n. a.
Age	56,0(26,6;80,5)	56,3(24,8;82,9)	0,963
Male gender	73,9%	61,36%	0,304
Clark	3,97 (3,0;5,0)	3,52 (1,0;5,0)	0,058
Breslow thickness (mm)	6,62(0,8;52,0)	3,23 (0,1;10,0)	0,058
Follow up (months)	84,0 (3,8;186,7)	55,1 (4,0;227,0)	0,030

Comparison of the baseline characteristics of the patients included in the histological analyses and clinical follow up. Patients were assigned to the two risk groups based on the site of their metastatic progression. Descriptive statistics for continuous measures are given as the mean with upper and lower range, for discrete data, percentages are tabulated

n.a. stands for not applicable

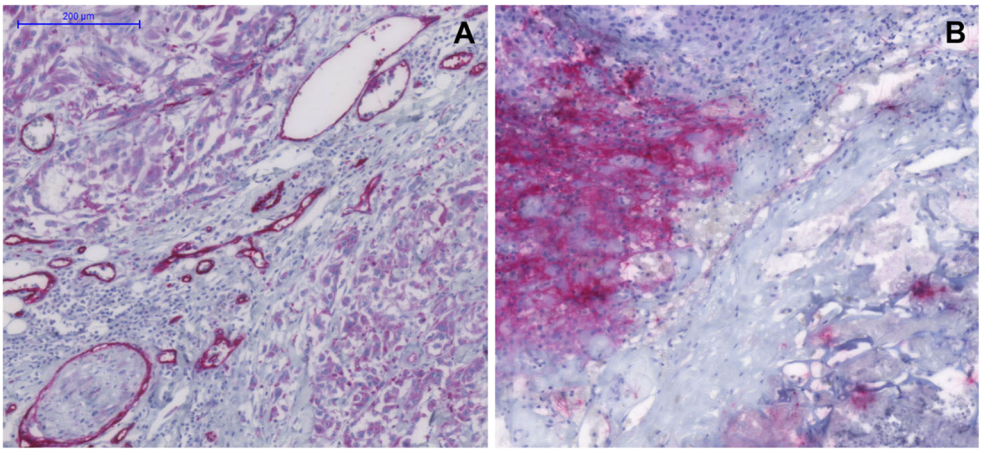

Fig. 2 AQP1 Protein expression in malignant melanoma. Low power view of AQP1 positive primary melanoma sample (**a**) and corresponding cerebral melanoma metastasis (**b**). **a**: Intratumoral capillaries in the primary sample show prominent positive reaction (red signal), while the AQP 1+ melanoma cells are characterized by distinct cytoplasmic and membrane associated red signal in an even distribution. **b**: APQ1+ melanoma cells in the metastatic sample show an uneven distribution, with a preference toward the regions distal to capillaries, while the capillaries themselves do not express AQP1. Bar = 200 um

cells mainly fund in areas distal to the micro-vessels, while the melanoma cells adjacent to capillaries were mostly negative for AQP1 (Fig. 2b).

Discussion

Melanoma is characterized by the highest frequency of intracranial progression among all tumor types, and the brain metastases are largely responsible for the high mortality associated with the disease [17]. Extravasation into the brain is the key element of cerebral metastasis formation, and due to the compound structure of the blood brain barrier, this process is more complex and requires a longer time for tumor cells, than the extravasation into other organs [18]. Transmigration of melanoma cells through BBB requires PLEKHA5 expression [19] and heparanase [20]. Extravasation of melanoma involves Cx26 and the chemokine receptor CCR4 [20] and its regulator miR146a [21]. In gene expression studies it was found that brain metastatic melanoma cells upregulate components of the glutamate receptor signaling pathway involving the receptors itself and their downstream targets such as CAMKII [6, 22]. It is of note that illegitime expression of CB receptor by melanoma cells may also contribute to the brain metastatic potential [23, 24]. Comparison of melanoma brain and extracranial metastases demonstrated specific activation of the PI3K/AKT signaling pathway in the brain metastases and overexpression of SGK3, SGSM2 and ELOVL2 genes [25].

Aquaporin water channels were shown to enhance the extravasation ability of tumor cells by potentially facilitating the rapid changes in cell volume, which accompany the changes in cell shape that occur as the migrating cell squeezes through the vessel wall [11]. In order to further explore the biological significance of APQ1 expression in relation to the progression to the brain, we have studied brain- and non-brain metastatic melanoma patients. The standard histologic features of the

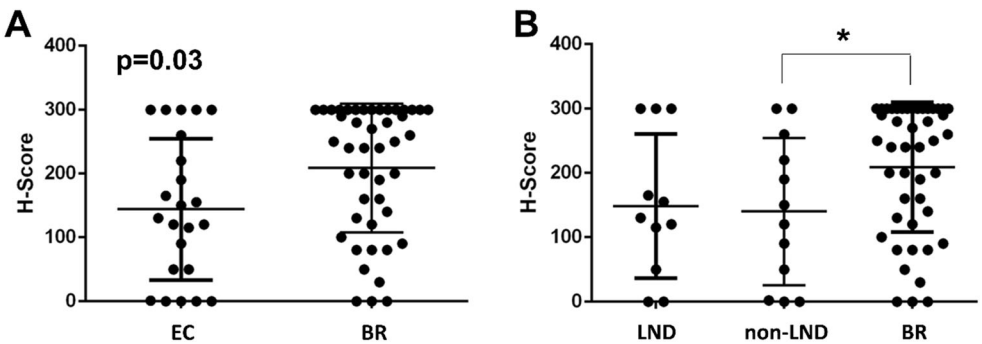

Fig. 3 Quantitative measurement of AQP1 expression in primary melanomas. AQP1 expression in the BR and EC metastatic patient groups. Each case is represented by a dot, while the bars represent the mean and standard deviation. On the left side (**a**) primary tumors are divided in two groups based on the extra-cranial vs. cerebral progression, while on the right side the extra-cranial group is further divided into lymph nodal (LND) and non-lymph nodal subgroups. Primary tumors with progression to the brain (BR) showed higher AQP1 expression compared to both extra-cranial subgroups (**b**)

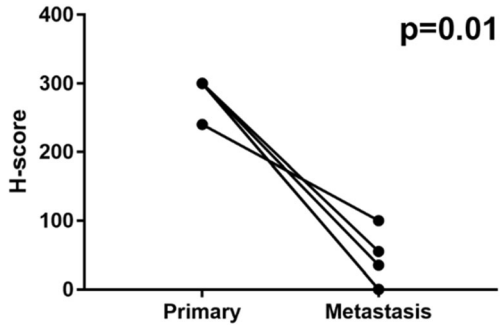

Fig. 4 Comparison of AQP1 expression in primary and corresponding brain metastatic tumors. ($n = 5$) The connected dots represent the H-score of corresponding primary and metastatic melanoma samples. A significant reduction of AQP1 expression during the cerebral progression of cutaneous primary melanoma can be demonstrated. ($p = 0.01$)

primary tumors in the two groups did not differ significantly, all of them were characterized by similarly high Breslow index. We have shown for the first time that the primary melanomas with metastatic potential to the central nervous system have a significantly higher APQ1 protein expression as compared to the primary tumors with extra-cranial progression. Overexpression of AQP1 can be due to increased copy number of this gene located on the chr7p14.3. Previous study indicated that although there was no amplification in that region, polysomy of the chr7 is a relatively frequent feature in malignant melanoma providing extra copies of AQP1 and several other genes including EGFR [26]. The so-called metastasis genes can be divided into metastasis initiating ones and metastasis maintenance genes. AQP1 could well be one of the metastasis initiating genes of human melanoma which are required for intra- and extravasation.

The comparative analysis of the corresponding primary and metastatic tumor samples indicated a significant reduction of the AQP1 expression during the intracranial progression of the disease. However, we have also noted a very characteristic staining pattern showing an increased expression of the AQP1 protein of melanoma cells in the less vascularized regions of the brain metastases. These observations seem to confirm the previously suggested mechanism of hypoxia induced AQP1 expression [27]. The homogenously upregulated expression of AQP1 in the primary melanomas with high Breslow index is most probably not due to local hypoxia-induced HIF activation rather than to the oncogenic activation such as BRAF mutation, as we have shown before [15]. As compared to the relatively small primary tumors (in mm ranges), the brain metastases are significantly larger where the vessel cooption process to provide oxygen is less effective leading to the development of significant hypoxia and necrosis. With the growth of the brain metastases significant areas of the tumor becoming distant from the feeding vessels, and according to our observations, melanoma cells in these less vascularized regions are likely to increase their AQP1 expression, most probably due to a secondary HIF-activated stimulus. In response to the intracellular lactic acidosis caused by hypoxia, tumor cells are required to shuttle H+ to the extracellular compartment; this may involve reaction of H^+ and HCO_3 catalyzed by the cytosolic carbonic anhydrases [28]. H_2O produced as a result of hypoxia could be subsequently transported by AQP1 to the extracellular compartment to decrease cytotoxic edema. The water channels may therefore not only facilitate the extravasation of the metastatic cells into the brain, but they may also make them more resistant to hypoxia during the expansion of the tumor. Altogether, our results suggest AQP1 protein expression as a prognostic factor of brain metastatic potential of malignant melanoma and may offer novel therapeutic avenues as well.

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Compliance with Ethical Standards

Ethical Statement This manuscript has not been published previously. This study is not part of another larger clinical study. We state that no data have been fabricated or manipulated. We also state that the Result section contains our own data. All authors expressed their consent to publish this manuscript in the final form. Authors whose names appear on the submission have contributed sufficiently to this work and therefore share collective responsibility and accountability for the demonstrated results.

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