Original Paper

Pathobiology

Pathobiology 2015;82:84–89
DOI: 10.1159/000430956

Received: December 16, 2014
Accepted after revision: April 27, 2015
Published online: June 18, 2015

Expression of Methylthioadenosine Phosphorylase (MTAP) in Pilocytic Astrocytomas

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Key Words
Brain tumors · Glioma · Immunohistochemistry · Pilocytic astrocytoma

Abstract
Background/Objectives: Pilocytic astrocytomas (PAs) are the most frequent astrocytomas in children and adolescents. *Methilthioadenosine phosphorylase (MTAP)* is a tumor-suppressor gene, the loss of expression of which is associated with a poor prognosis and better response to specific chemotherapy in leukemia and non-small-cell lung cancer. The expression of MTAP in brain tumors remains largely unknown and its biological role in PA is still unexplored. Our aims were to describe the immunohistochemical MTAP expression in a series of PAs and relate it to the clinicopathological features of the patients. ***Methods:*** We assessed MTAP expression on immunohistochemistry in 69 pediatric and adult patients with PA in a tissue microarray platform. ***Results:*** Retained expression of MTAP was seen in >85% of the tumors compared to in the nonneoplastic adjacent tissue. Only 3 supratentorial tumors showed a complete loss of MTAP expression. No significant association with clinicopathological features or overall survival of the patients was found. ***Conclusions:*** MTAP expression is retained in PAs and is not an outcome predictor for these tumors. Nevertheless, a subset of patients with PAs exhibiting a loss of MTAP could potentially benefit from treatment with specific chemotherapy, especially when lesions are recurrent or surgical resection is not recommended.

Introduction

Pilocytic astrocytoma (PA) is the commonest brain tumor in children and teenagers in the USA [1] and Brazil [2, 3]. PAs are considered benign, exhibiting an indolent nature. PAs in adults are rare and usually more aggressive [4]. Although the majority of cases occur as sporadic disease, PAs can arise in the heredity context of type 1 neurofibromatosis (Nf1) [5–7]. Nf1-PAs are usually less aggressive and located at extracerebellar sites [8]. The overall prognosis for PAs is good, but in some cases they are aggressive, leading to death [9–11]. Extension of resection and high mitotic activity are key factors related to a poor prognosis in PAs [10, 11].

1015–2008/15/0822–0084$39.50/0

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The molecular pathogenesis of PAs has been extensively studied, particularly the constitutive activation of the mitogen-activated protein kinase (MAPK) pathway through *BRAF* proto-oncogene alterations [9, 12–21], such as *KIAA1549-BRAF (K:B)* fusion (in up to 80% of cases, mainly in cerebellar lesions) and V600E *BRAF* point mutation (in 10% of cases, more frequent in supratentorial lesions) [9, 22, 23]. Recently, point mutations in the *fibroblast growth factor receptor 1 (FGFR1)* gene, a MAPK upstream receptor, were described in extracerebellar tumors as an alternative mechanism for MAPK activation [20].

Canonically, the MAPK pathway leads to increased cell proliferation; however, it may also cause oncogene-induced senescence in cells [9]. Oncogene-induced senescence restricts the progression of benign tumors, such as melanocytic nevi and PAs, in response to V600E *BRAF* mutation [19, 24]. In PAs, the ectopic expression of the V600E *BRAF* mutation results in induction of the INK4a/ARF locus, at 9p21, with subsequent overexpression of *p16*, a known senescence marker [19, 25], the loss of expression of which correlates with a shorter overall survival (OS) in sporadic PAs [19] and a more aggressive course in some Nf1-PAs [26].

Additionally, the 9p21 locus harbors the tumor suppressor gene *methylthioadenosine phosphorylase (MTAP)* [27–29], which is coexpressed with $p16^{INK4a}$ in various malignant tumors [29–33]. Homozygous deletion of *MTAP* upregulates de novo synthesis of purine (DNSP) and increases the proliferation of cancer cells [29, 30]. Interestingly, *MTAP* deletion increases the sensitivity of neoplastic cells to DNSP inhibitors such as methotrexate, L-alanosine and pemetrexed [27, 30], particularly in leukemia [29, 30] and other solid tumors, e.g. in the lung, liver and breast [30, 33–35]. In tumors of the central nervous system, deletion and gene copy-number breakpoints of *MTAP* have been reported in glioblastomas [36, 37], and in pediatric high-grade gliomas [38], respectively. However, none of these studies assessed MTAP expression by immunohistochemistry.

The biological role of *MTAP* in PAs is still unexplored. Our aims were to describe the immunohistochemical MTAP expression in a series of PAs and relate it to the clinicopathological features of the patients.

Materials and Methods

Patients

From 1993 to 2013, 69 patients with PA were retrieved from the Pathology Department of the Barretos Cancer Hospital (HCB) and the Hospital Clinics of the Ribeirão Preto School of Medicine, University of São Paulo (HCRP), Brazil. Recurrent lesions from 5 patients, 1 with 2 relapsed lesions, were also analyzed, totalling 75 samples. The patients were grouped according to gender, age (pediatric: ≤19 years old and adult: ≥20 years old) and tumor location, i.e. cerebellar or extracerebellar. Patients with Nf1-PAs (n = 5) had confirmed clinical diagnosis of Nf1 by standardized criteria. Surgical resection was classified as gross total resection or partial resection, measured by immediate postsurgical CT scan [39]. The outcome of patients was categorized as favorable (stable, partially resected lesions and totally resected lesions) or unfavorable (progressive or recurrent lesions, patients with a Karnofsky index <70 and/or death). An event was defined as the growth of a residual lesion or the recurrence of a totally resected lesion, detected clinically or by radiological exam. This study was approved by the Ethics Committees of both institutions (HCB/87362 and HCRP/212313).

Tissue Microarray Construction

After review of the cases by two independent neuropathologists, two blocks of tissue microarray (TMA) were constructed from the formalin-fixed, paraffin-embedded samples, using the Beecher Instruments™ TMA platform, with 1.0-mm (HCB cases) and 1.5-mm (HCRP cases) needles. To represent the heterogeneity of the tumors, we obtained 1–8 cores from each case (average: 3.6 cores/case). In 9 cases, adjacent nonneoplastic cerebellum was represented in the samples and included in the TMA.

Western Blot Analysis

Cells from the SNB19 and MDA-MB231 cell lines, known to, respectively, express and not express MTAP [40, 41] were grown in 25-cm^2 flasks (3×10^6 cells) to 80% cell confluence, scraped in standardized lysis buffer and then centrifuged to extract total protein. After electrophoresis, the blots were performed and incubated with 5% nonfat dry milk in TBS-T for 1 h at room temperature, with MTAP polyclonal antibody for 15 h at 4°C (Proteintech, Chicago, Ill., USA; 1:800) and β-actin (Cell Signaling, Boston, Mass., USA; 1:5,000) as a control for 1 h at 4°C. Ultimately, the blots were washed with TBS-T and incubated with anti-rabbit IgG (for MTAP) or anti-mouse IgG (for β-actin) HRP-linked antibody (Cell Signaling; 1:5,000 for both antibodies). Immunodetection was done with Amersham ECL Western blotting detection reagents in automatic ImageQuant LAS 4000 mini (GE Healthcare).

Immunohistochemical Analysis

To confirm the diagnosis of PA, we initially performed immunohistochemical analysis of galectin-3 [42] and mutated IDH-1 [43] from the TMA slides. The reactions were accomplished using the established protocols for Ventana Benchmark Ultra platform (Ventana-Roche, Tucson, Ariz., USA) and the monoclonal antibodies anti-galectin 3 (Diagnostic BioSystem, Pleasanton, Calif., USA; clone 9C4, dilution 1:50), and anti-mutated IDH-1 (R132H; DIANOVA, Hamburg, Germany; clone H09, dilution 1:50). The results were classified as negative or positive, when at least 10% of the tumor cells exhibited cytoplasmic positivity for each marker.

For MTAP evaluation, we performed a manual immunohistochemical assay on the TMA slides. Briefly, following sample dehydration and antigen retrieval with a mixed buffer (citrate 10 mM/EDTA 1 mM, pH 6.0) for 4 min at 125°C and 20 min at 90°C, the slides were incubated with the MTAP polyclonal antibody (Pro-

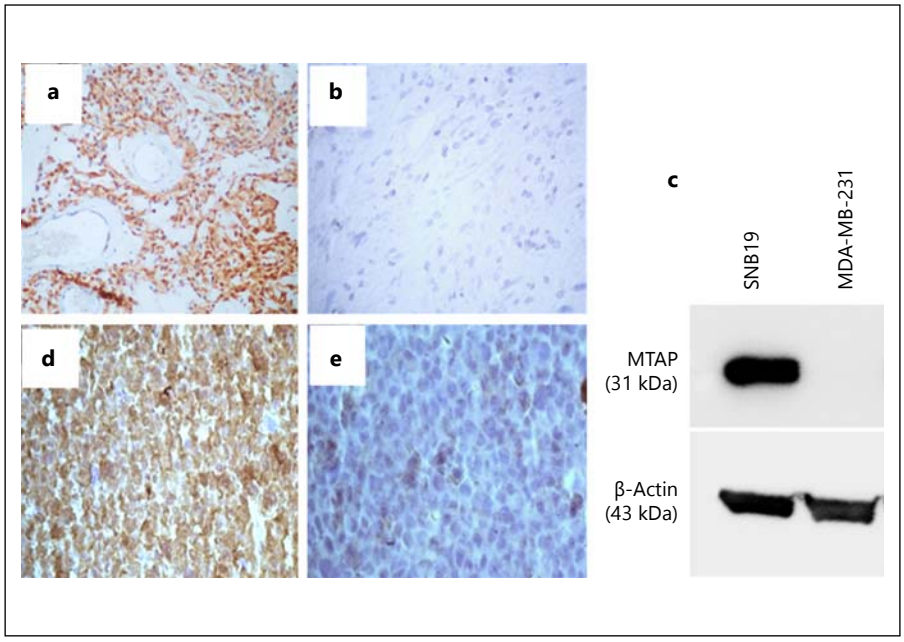

Fig. 1. a Immunohistochemical cytoplasmic expression of galectin-3. ×100. **b** A sample of PA, negative for mutated IDH-1 (400×). **c** Western blot assay showing expression of MTAP in the SNB19 cell culture and no expression in the MDA-MB-231 cell culture. Immunocytochemistry shows cytoplasmic expression of MTAP in the SNB19 cell lineage (**d**) and a lack of MTAP expression in the MDA-MB-231 cell line (**e**). ×400.

Table 1. Clinicopathological features of patients with PA

	Patients, n	MTAP expression			
		0	≤3	≥4	p value
Patients (total)	69				
Male	38	3	2	33	0.127
Female	31	0	5	26	
Age group					
Pediatric	60	3	6	51	1.0
Adult	9	0	1	8	
Nf1					
Yes	5	1	0	4	0.271
No	64	2	7	55	
Location					
Cerebellar	36	0	5	31	0.163
Extracerebellar	33	3	2	28	
Extension of resection					
Total	31	1	3	27	1.0
Partial	38	2	4	32	
Outcome					
Favorable	41	2	5	34	0.865
Unfavorable	28	1	2	25	
Recurrence					
Yes	5	0	1	4	0.525
No	26	1	2	23	
Progression					
Yes	17	1	0	16	0.145
No	21	1	4	16	
Death					
Yes	4	0	1	3	0.474
No	65	3	6	56	

teintech; 1:300) at room temperature overnight. Then slides were incubated with secondary antibody and streptavidin peroxidase, stained with 3,3′ diaminobenzidine chromogen and counterstained with hematoxylin.

The subcellular localization (cytoplasmic and/or nuclear), extension and intensity of the reaction were further evaluated and scored. The extension of reaction was measured as: 0 (negative), 1 (≤25% of positive cells), 2 (25–50% of positive cells) or 3 (≥50% of positive cells). Intensity was measured as: 0 (negative), 1 (weak), 2 (moderate) or 3 (strong). The sum of these parameters resulted in scores assumed as reduced expression (0–3) or retained expression (4–6). Endothelial positivity was the internal positive control. External controls for both TMAs were normal liver and prostate, in addition to the cell-blocks of SNB19 and MDA-MB231 (fig. 1c–e). Average values were considered in cases with >1 core on the TMA.

Statistical Analysis

The statistical analysis was performed using SPSS version 20 for Windows™ (IBM, Chicago, Ill., USA) with $p < 0.05$ considered statistically significant for the Fisher exact test, Pearson χ^2 test and McNemar test. Event-free survival (EFS) and OS were calculated by the Kaplan-Meier method.

Results

The clinicopathological features of the series are summarized in table 1. Patients' age ranged from 0.3 to 53.4 years old (median: 9.1 years old). Four pediatric patients died of disease (3 females and 1 male), 1 due to a recurrent tumor and 3 due to the progressive growth of lesions, within 1.7, 2.6, 6.5 and 10.7 years after the first surgery.

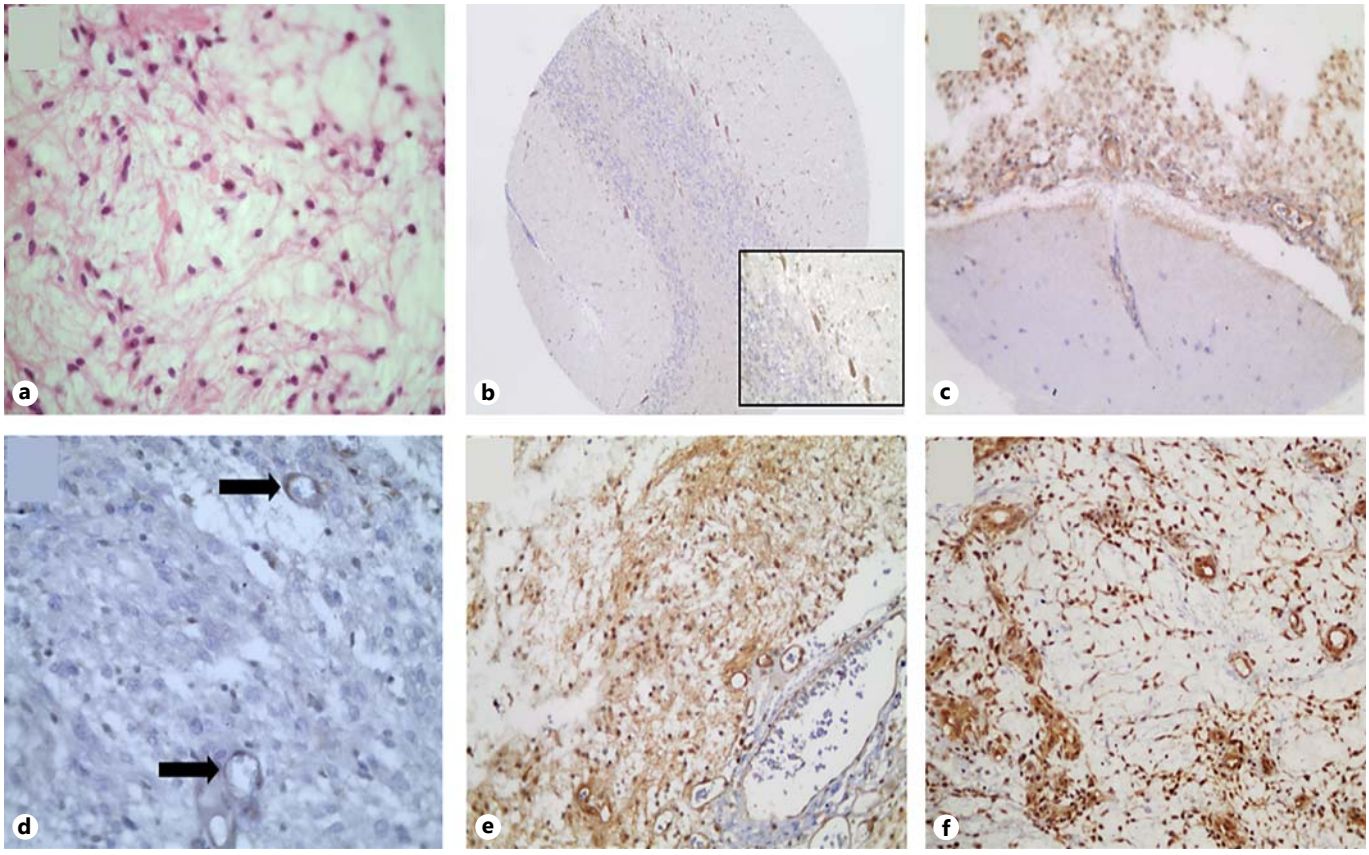

Fig. 2. Patterns of immunohistochemical MTAP expression in adjacent normal tissue and in PAs. **a** A PA with piloid and round cells in a loose background with Rosenthal fibers. HE. ×400. **b** Normal cerebellum, depicting strong reaction in Purkinje cells and astrocytes lacking MTAP expression. ×20. **Inset**: strong cytoplasmic reaction of Purkinje cells. ×200. **c** Normal cerebellum-tumor transition – the tumor showed a moderate reaction. ×100. **d** Tumor with loss of MTAP expression and positive endothelial reaction (arrows). ×400. **e** Moderate cytoplasmic MTAP staining. ×100. **f** Strong cytoplasmic MTAP reaction in piloid area of a PA. ×100.

The median EFS and OS for the entire cohort was 3.5 (0.1–16.3) and 4.0 (0.6–16.6) years, respectively. Patients with relapsed tumors had a shorter EFS (0.1–10.7 years, median 0.9) and OS (0.7–16.2 years, median 2.7). All the relapsed lesions maintained the histopathological criteria for PA.

Galectin-3 was expressed in 95.7% of the series and all cases lacked mutated IDH-1 (R132H) expression (fig. 1a, b), confirming the diagnosis of PA.

Following MTAP antibody validation by Western blot and immunocytochemistry in the SNB19 and MDA-MB231 tumor cell lines (fig. 1), we achieved adequate results in 67/69 of the primary lesions and in 5/6 of the relapsed lesions. We observed cytoplasmic MTAP expression in 59/67 primary tumors (88.1%; fig. 2). Ten cases (14.5%) presented reduced expression, including 3 that lost MTAP expression completely (fig. 2d).

In the nonneoplastic cerebellum, Purkinje cells showed strong cytoplasmic positivity for MTAP, while the nonneoplastic astrocytes were negative (score: 0) in 8/9 cases (fig. 2b, c) and faintly positive in 1 case (score: ≤3). The difference between neoplastic and nonneoplastic astrocytes was statistically significant in the Wilcoxon test ($p = 0.005$). In contrast, there were no significant differences in MTAP expression between groups (table 1).

PAs in the optic pathways showed strongest MTAP expression (4/4 lesions, score: 6). The 3 tumors with a complete loss of MTAP expression were located in the cerebral hemispheres (table 2). Among these patients, 1 showed an Nf1-PA context and 1 had multiple recurrences (not analyzed in this study).

The relapsed lesions displayed similar expression to their primary counterparts ($p = 0.5$, McNemar test). One patient had stronger expression in the relapsed lesion (score: 4 and 6) and the patient with 3 available samples presented decreasing MTAP expression over time (score: 6, 5 and 4, respectively).

Table 2. MTAP expression according to the location of the tumor

	MTAP score			
	negative	≤3	≥4	total
Cerebellum	0	5	31	36
Brain stem	0	1	3	4
Medullary	0	0	5	5
Suprasellar	0	0	6	6
Optic pathways	0	0	4	4
Cerebral hemispheres	3	1	10	14
Total	3	7	59	69

Finally, MTAP expression did not influenced the OS or EFS, as seen in the Kaplan-Meier curves ($p = 0.645$, $p = 0.736$).

Discussion

To the best of our knowledge, this is the first study to assess MTAP expression on immunohistochemistry in central nervous system tumors. We report that MTAP expression is retained in the majority of PAs. The few studies that have analyzed MTAP in gliomas reported *MTAP* deletions and reduced mRNA expression in pediatric [38] and adult glioblastomas [36, 37, 40, 44]. We also observed reduced MTAP tumor expression in a small number of infiltrative diffuse astrocytomas (i.e. WHO grades II–IV), with a trend towards lower expression in high-grade tumors (data not published), which suggests that the loss of MTAP expression is associated with increased glioma malignancy.

In this series of PAs, the loss of MTAP expression observed in a few cases did not influence prognosis, and we could not confirm that MTAP is a predictor of outcome. In contrast, the loss of expression of the neighbor gene *p16* has a prognostic impact on PAs [19]. Recently, it was described that the simultaneous loss of expression of *p16* and MTAP determines shorter survival of patients with non-small-cell lung cancer [45].

The normal distribution of MTAP in the central nervous system is not properly defined [46]. We describe that nonneoplastic astrocytes had virtually a complete loss of MTAP expression as opposed to the adjacent neoplastic astrocytes. In experimental studies on dementia, MTAP overexpression has been related to senescence and loss of neuroprotective function compared to normal astrocytes [47, 48]. We therefore hypothesized that the adjacent cells play an essential role in the establishment of boundaries to the growth of PAs by avoiding these cells from senescence, thereby maintaining their neuroprotective role. This, associated with the oncogene-induced senescence in the tumor cells, would help to explain the indolent behavior of PAs. Finally, the increased expression in specific cell subtypes, such as in the astrocytes of the optic nerve and retina, suggested by previous investigations [46], may explain why the tumors located in the optic pathways displayed stronger expression of MTAP in our series.

The great interest in assessing the expression of MTAP in tumors arises from the therapeutic potential of DNSP inhibitors, such as methotrexate, L-alanosine and pemetrexed [27]. It has been reported that a loss of MTAP expression sensitizes cancer cells to DNSP inhibitors, as shown in studies on leukemia and lung cell lines [30] and various malignant neoplasms [27]. This is particularly relevant for lymphomas, leukemias [27, 29] and non-small-cell lung carcinoma [34, 45]. Nevertheless, chemotherapy regimens with pemetrexed were not effective for pediatric patients with medulloblastomas, ependymomas and high-grade gliomas [49], and the addition of methotrexate in the chemotherapeutic treatment of infratentorial ependymomas did not improve the response to these drugs [50]. However, none of these studies assessed MTAP expression in the tumors. In the light of the knowledge about MTAP expression, further clinical studies could help to elucidate the poor results of DNSP inhibitors in pediatric brain tumors. Although it is a small subset, patients with PAs that exhibit a loss of MTAP expression could potentially benefit from treatment with DNSP inhibitors, especially when lesions are recurrent or unachievable to surgical resection.

To conclude, understanding the role of MTAP in an indolent neoplasm, such as PA, may help to uncover the mechanisms by which the activation of MAPK leads to senescence, instead of stimulating cell proliferation in these tumors. Additional studies, with larger cohorts, comparisons with other glioma subtypes and correlations with different molecular mechanisms of MAPK activation are needed to confirm and extend our findings and to elucidate the role of MTAP in gliomagenesis.

Acknowledgments

This study was partially supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (2012/19590-0) and an HCB program of financial support (Programa de Apoio e Incentivo à Pesquisa).

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