ORIGINAL PAPER

ASPM gene expression in medulloblastoma

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Abstract

Purpose Medulloblastomas are the most common malignant tumors of the central nervous system in childhood. The incidence is about 19–20% between children younger than 16 years old with peak incidence between 4 and 7 years. Despite its sensibility to no specific therapeutic means like chemotherapy and radiotherapy, the treatment is very aggressive and frequently results in regression, growth deficit, and endocrine dysfunction. From this point of view, new treatment approaches are needed such as molecular targeted therapies. Studies in glioblastoma demonstrated that *ASPM* gene was overexpressed when compared to normal brain and *ASPM* inhibition by siRNA-mediated inhibits tumor cell proliferation and neural stem cell proliferation, supporting *ASPM* gene as a potential molecular target in glioblastoma. The aim of this work was to evaluate *ASPM* expression in medulloblastoma fragment samples, and to compare the results with the patient clinical features.

Methods Analysis of gene expression was performed by quantitative PCR real time using SYBR Green system in tumor samples from 37 children. The *t* test was used to analyze the gene expression, and Mann–Whitney test was performed to analyze the relationship between gene expressions and clinical characteristics. Kaplan–Meier test evaluated curve survival.

Results All samples overexpressed *ASPM* gene more than 40-fold. However, we did not find any association between the overexpressed samples and the clinical parameters.

Conclusion *ASPM* overexpression may modify the ability of stem cells to differentiate during the development of the central nervous system, contributing to the development of medulloblastoma, a tumor of embryonic origin from cerebellar progenitor cells.

Keywords *ASPM* · Gene expression · Brain development · Medulloblastoma

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Introduction

Medulloblastoma (MB) is an embryonal brain tumor that arises in the cerebellum and comprises the most common malignant brain tumor in children [5, 8, 13], accounting for 15–20% of central nervous system tumors [11, 13]. This tumor occurs predominantly in the first decade of life, with a peak of incidence between 4 and 7 years of age [13]. Currently, therapeutic regimens of MB involving surgery, radiotherapy, and chemotherapy [5] can cure 60% of the patients with an average 5-year progression-free survival,

even so, about 10–15% of the patients die from disease within 2 years after diagnosis [5, 8]. Despite of considerable advances, the treatment still is very aggressive, and a lot of patients suffer severe lifelong neurocognitive deficiencies due to radiation-induced damage of the developing brain and endocrine dysfunction [5, 12, 13].

Looking for molecular markers, recent work in a different malignant brain tumor, glioblastoma, showed abnormal spindle-like microcephaly-associated (*ASPM*) gene as a potential molecular target. This study showed that *ASPM* expression is increased in glioblastoma when compared with normal brain, and its inhibition by siRNA-mediated knockdown inhibits tumor cell proliferation and neural stem cell proliferation [7]. *ASPM* gene maps to chromosome 1q31 and is essential for normal mitotic spindle function in embryonic neuroblasts [1]. The product of *ASPM* gene is a spindle pole/centrosome protein which promotes neuroblast proliferation and manages the orientation of mitotic cleavage, allowing symmetric and proliferative division of neuropithelial cells during brain development, as well as brain size expansion [4]. Hence, centrosome defects cause chromosome failure of segregation and, accordingly, lead to aneuploidy [16] and genetic instability, and modifications within this gene as mutation produces cause primary microcephaly [1, 2]. Moreover, due to high expression of *ASPM* in many tumor cells, studies have been suggested an important involvement of this gene in malignant transformation and tumor progression [9, 10].

Since *ASPM* gene fulfills an important role in brain size and is actively involved in cell proliferation in embryonic development and tumorigenesis, the purpose of the present study was to evaluate *ASPM* expression in MB samples and related the molecular results with the patient clinical features.

Material and methods

Patients

We selected 37 MB samples from patients attending the Pediatric Oncology Institute (IOP)/Grupo de Apoio ao Adolescente e a Criança com Câncer (GRAACC)—Federal University of São Paulo (UNIFESP)—Brazil. Informed consent was obtained from all patients/guardians according to the University's IRB (CEP/UNIFESP Nº1120/01). All MB patients were classified according to Chang et al. (1969) [3], as follows: M stage include include M0 (no evidence of disseminated disease), M1 (tumor cells identified by cerebrospinal fluid cytology only), M2 (intracranial metastatic tumor detectable by computed tomography (CT) or magnetic resonance imaging (MRI), and M3 (spinal metastatic tumor detectable by CT myelography or spine MRI).

RNA extraction and cDNA synthesis

Total RNA was isolated from frozen samples pulverized under nitrogen liquid and extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). To reduce the risk of genomic DNA contamination, DNase treatment was performed using deoxyribonuclease I Amplification Grade (Invitrogen, Carlsbad, CA, USA). The concentration of RNA was determined by spectrophotometry, and total RNA integrity was monitored by visualization of ribosomal RNAs (28S and 18S) on 1.0% agarose gel. One microgram of total RNA derived from 37 MB samples was reverse-transcribed using the SuperScript III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Quantitative real-time RT-PCR

Oligonucleotides for *ASPM* and hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) were designed using Primer Express Software from Applied Biosystems (Foster City, CA, USA) warranting that forward and reverse sequences were in different exons. We conducted a BLAST search to confirm the total gene specificity of the nucleotide sequences chosen for primers. Real-time polymerase chain reaction (PCR) amplification and data analysis were performed using the ABI Prism 7500 Sequence Detector System Applied Biosystems (Foster City, CA, USA). Each cDNA sample was mixed with 12 µl of Mastermix (SYBR® Green PCR Master Mix, Applied Biosystems). Cycling conditions consisted of two singles steps at 50°C and 95°C for 2 min and 10 min, respectively, followed by 40 cycles of amplification at 95°C for 15 s and at 60°C for 1 min for annealing and elongation. Experiments were performed in triplicate for both target genes and the endogenous gene. For each sequence, a standard curve was constructed to determine the assay sensitivity. The data were averaged from the values obtained in each reaction. Relative expression was automatically calculated and analyzed according to $2^{-\Delta\Delta Ct}$ method described previously [10]. *HPRT* was used as a housekeeping gene in order to standardize its relative expression levels with relative expression levels of two genes studied. A no-template control was included in each amplification reaction, as well as a reference RNA. The PCR efficiencies of the two genes were comparable (≥95%).

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 4 Program Software (San Diego, CA, USA). The clinical variables correlated with gene expression were: histological

MB subtype, metastatic status at diagnosis, risk, surgical resection, follow-up, and relapse. Overall survival was defined as the time from diagnosis until date of either the more recent follow-up or death. Overall survival was generated by applying the Kaplan–Meier method. The t test was used to analyze the gene expression of each gene studied. The Kruskal–Wallis and Mann–Whitney tests were used to analyze the relationship between the gene expressions and the clinical characteristics. Categorical data were studied using chi-square and Fisher's exact test applying software VassarStats (Website for Statistical Computation). In all cases, $p<0.05$ was considered statistically significant.

Results

All clinical and pathological variables of patients with tumors used in this study are summarized in Table 1. The median age of patients was 8 years (range 5 months to 18 years old). In our samples we found: stage M0, 67% ($n=$ 25), stage M1, 6% ($n=2$), stage M2, 13% ($n=5$), and stage M3, 13% ($n=5$). Stages M1, M2, and M3 were included in just one group named M+, 32,4% ($n=12$). The median expression value of each M stage is summarized in Table 2. Twenty-one of 37 patients died from disease, and 16 patients were alive disease-free or in treatment. Most of our samples had classic MB (92%).

The *ASPM* gene was overexpressed (cut off ≥2.0) in all 37 samples ($p<0.0001$; Fig. 1). By analyzing the clinical characteristics of these samples, we observed that 21 of 37 patients (57%) were high risk, three (0.08) belonged to the anaplastic histology or anaplastic/large cell, 21 (57%) died due to illness or from complications of treatment, 17 (46%)

Table 1 Clinical variables evaluated

Clinical variable	Patients (%)
Classification	
High risk	57
Low risk	43
Stage	
M0	67
M+	32
Surgery	
Total	54
Partial	46
Follow-up	
Death	57
Out or in treatment	43
Relapse	
Relapsed	26
Non relapsed	74

Table 2 Median expression values of each M stage

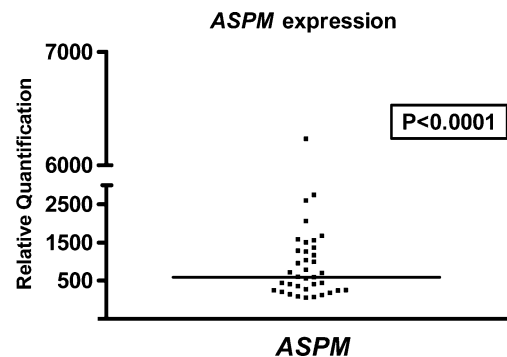| M stage	Median expression value
M0	684
M1	866
M2	423
M3	586
M+	422

M+ include the stages M1, M2, and M3

had partial resection of the tumor, and nine of 37 patients (24%) relapsed. Additionally, 12 of 37 patients (32%) were metastatic (Table 1). There was no significant correlation between gene expressions and clinical and survival parameters.

Discussion

Recent studies have shown that *ASPM* plays an important role in cytokinesis and cell proliferation in transformed cell lines, fetal tissue, and tumor cells [1, 15]. Despite these characteristics, few studies have been conducted to investigate its role in human cancers. During embryonic development of mice, *ASPM* is expressed in the cerebral cortical ventricular zone, and its expression is very low close to birth when neurogenesis in the cortical ventricular zone is complete. This suggests that *ASPM* is preferentially expressed in progenitor cells that produce neurons [1].

The reduced expression of *ASPM* inhibits proliferation of neural progenitor cells, thereby reducing the number of neurons formed, resulting in a small brain that is characteristic of autosomal recessive primary microcephaly (MCPH) [4]. In adults, *ASPM* mRNA is expressed in breast, lung, pancreas, uterus, colon, thyroid, liver, bladder, kidney, ovary, testis, stomach, lymph node, cervix, and esophagus, but is not found in the brain and skeletal muscle [9, 14]. Its

Fig. 1 Results representative of *ASPM* expression for all samples studied. The expression of *ASPM* was significantly higher in medulloblastoma fragments than in reference RNA ($p<0.0001$). The *black line* demonstrates the value of the median

expression is also increased in transformed cells and some types of cancers such as uterus, ovary, breast, colon, thyroid, testis, lymph node, stomach, and more recently, was found in glioblastoma and hepatocellular carcinoma [7, 9, 10]. Finally, besides *ASPM* overexpression as an important factor associated with hepatocellular carcinoma progression, it is a determinant for vascular invasion, lowest 5-year survival, and hence, poorer prognosis [10].

In our study, all 37 samples analyzed MBs showed high expression of *ASPM* ($p<0.0001$). Although some studies have found an association between the expression of *ASPM* and the degree of malignancy of some tumors, we found no correlation between the increased expression of this gene and clinical parameters studied here. This suggests that *ASPM* may be involved only in the origin of MB as part of abnormal neurogenesis during embryonic development. Our suggestion is that overexpression of this gene may modify the ability of stem cells to differentiate during the development of the central nervous system, contributing to the development of MB, a tumor of embryonic origin from cerebellar progenitor cells. However, this is a pilot study and we suggest that further investigation is warranted. More studies, especially functional ones, should be performed in order to confirm this hypothesis once investigation on glioma confirms that *ASPM* can be regulated at a translational level [6].

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