MT1G Hypermethylation: A Potential Prognostic Marker for Hepatoblastoma

LUIS H. T. SAKAMOTO, BEATRIZ DE CAMARGO, MARIANA CAJAIBA, FERNANDO A. SOARES, AND ANDRÉ L. VETTORE

Ludwig Institute for Cancer Research, São Paulo Branch [L.H.T.S., A.L.V.], São Paulo 01323-903, Brazil; Departments of Pediatrics [B.D.C.] and Pathology [M.C., F.A.S.], A. C. Camargo Hospital, São Paulo 01509-010, Brazil; Department of Science Biology [A.L.V.], Federal University of São Paulo (UNIFESP), São Paulo 04039-032, Brazil

ABSTRACT: Hepatoblastoma comprises only 1% of all cancers in childhood. Because of its low frequency, a small number of prognostic factors are described in hepatoblastoma and most of them are related to resectability. Microarray studies showed a large number of underexpressed genes in hepatoblastoma. Because aberrant DNA methylation has been recognized as an alternative mechanism for tumor suppressor gene inactivation, this could be involved with gene downregulation in these tumors. Despite the rarity of hepatoblastoma, this study evaluated the methylation pattern of 25 genes in 20 paraffin-embedded tumor specimens and five non-neoplastic liver samples (normal control) by quantitative methylation-specific PCR (QMSP). The examination of the methylation profile of hepatoblastoma samples and normal liver specimens revealed a high tumor-specific DNA hypermethylation in the promoter regions of five genes (*APC*, *CDH1*, *MT1G*, *RASSF1A*, and *SOCS1*). Furthermore, *MT1G* hypermethylation showed a significant correlation with poor prognosis of patients with hepatoblastoma. This study represents the first quantitative evaluation of promoter hypermethylation in hepatoblastoma and demonstrated that aberrant methylation is a frequent event in this malignancy. Furthermore, our data provide evidence that *MT1G* hypermethylation may be useful as prognostic indicator for this disease and suggest that patients with hepatoblastoma may benefit from demethylating drug treatments. (*Pediatr Res* 67: 387–393, 2010)

Hepatoblastoma is the most common malignant liver tumor in childhood. Data from Surveillance Epidemiology and End Results (SEER) estimate an incidence of 0.5–1.5 cases per million children per year, which accounts for 79% of hepatic neoplasia in children younger than 15-y and for 1% of all cancers in this age group (1).

Few prognostic factors are available for hepatoblastoma. The most important features appear to be low alpha-fetoprotein level, pure fetal histology, gross resectability of the tumor, and the presence of metastases in the lung and small cell undifferentiated tumor type. In fact, complete surgical resection remains the most important intervention required for long-term survival (2,3).

Although some chromosomal alterations have been found in hepatoblastoma (1q, 2q, 7q, 8, 17q, and 20 amplifications), no etiological relationship or prognostic value could be attributed to these findings. The majority of tumors from patients with hepatoblastoma do not show any detectable chromosomal anomaly (2,4).

DNA methylation, catalyzed by DNA methyltransferases, involves the addition of a methyl group to the carbon 5 position of the cytosine ring in CpG dinucleotides (5). This is associated with several changes in chromatin structure, including the regulation of histone methylation and acetylation and the recruitment of proteins to the methylated sites, which usually leads to the obstruction of the promoter, hindering gene transcription and subsequently silencing the gene (6). There is increasing evidence that in addition to genetic aberrations, the epigenetic processes play a major role in carcinogenesis. Aberrant methylation (hypermethylation) of gene promoter regions is the most widely studied epigenetic abnormality in human malignancies. This epigenetic event acts as an alternative to mutations and deletions to disrupt tumor suppressor gene function (7).

Aberrant promoter methylation has been described for several genes in various malignant diseases, and each tumor type may have its own distinct pattern of methylation (8,9). It was reported previously that gene hypermethylation may occur in patients with hepatoblastoma. Harada *et al.* (10), analyzing aberrant promoter hypermethylation in pediatric tumors and cell lines, observed *RASSF1A* hypermethylation in 19% of hepatoblastoma speci-

Abbreviations: *ACTB*, beta actin; *AIM1*, absent in melanoma 1; *APC*, adenomatous polyposis coli; *CALCA*, calcitonin-related polypeptide alpha; *CCNA1*, cyclin A1; *CCND2*, cyclin D2; *CDH1*, cadherin 1, type 1, E-cadherin (epithelial); *CDKN2A*, cyclin-dependent kinase inhibitor 2A (p16); *CDKN2B*, cyclin-dependent kinase inhibitor 2B (p15); *DAPK*, death-associated protein kinase 1; *ESR1*, estrogen receptor 1; *GSTP1*, glutathione S-transferase pi; *MGMT*, O-6-methylguanine-DNA methyltransferase; *MINT31*, methylated in tumors 31; *MLH1*, mutL homolog 1, colon cancer, nonpolyposis type 2; *MT1G*, metallothionein 1G; *MSP*, methylation-specific PCR; *P14ARF*, cyclin-dependent kinase inhibitor 2A; *PMR*, percent of methylated reference; *PTGS2*, prostaglandin-endoperoxide synthase 2; *QMSP*, quantitative MSP; *RARβ*, retinoic acid receptor, beta; *RASSF1A*, Ras association (RalGDS/AF-6) domain family 1; *RB1*, retinoblastoma 1; *SCGB3A1*, secretoglobin, family 3A, member 1; *SFRP1*, secreted frizzled-related protein 1; *SIOPEL*, The International Childhood Liver Tumour Strategy Group; *SOCS1*, suppressor of cytokine signaling 1; *THBS1*, thrombospondin 1; *TIMP3*, TIMP metallopeptidase inhibitor 3

mens. Shim *et al.* (11) showed that 50% of 24 patients with hepatoblastoma had hypermethylated *CDKN2A*. Nagai *et al.* (12) found that CpG islands in promoter region of *SOCS1* gene were hypermethylated in seven of 15 hepatoblastoma samples examined. It is important to note that all of these studies focused on few genes and were conducted using conventional methylation-specific PCR (MSP) technique.

Therefore, the aims of this study were a) to determine the promoter methylation status of 25 genes in hepatoblastoma using the real-time quantitative MSP (QMSP) technology, which is more sensitive than conventional MSP, b) to compare the methylation status with clinical characteristics of the patients, and c) to evaluate if this kind of molecular analysis could be useful as a prognostic marker.

MATERIALS AND METHODS

Patients, sample collection, and DNA preparation. The study involved 20 patients with hepatoblastoma treated between 1984 and 2005 at Hospital do Cancer A. C. Camargo, São Paulo, Brazil. A majority of the patients (95%) were submitted to a neoadjuvant chemotherapy scheme. Formalin-fixed and paraffin-embedded (FFPE) tumor samples were obtained from pathology archives. Tissue sections were stained with hematoxylin/eosin according to standard procedures and all cases were revised and the diagnosis was confirmed. Five FFPE specimens of fetal liver from miscarriages (25–36 wk) were included in the study as normal controls.

The study was approved by the Ethics Committee of the Hospital do Câncer A. C. Camargo and performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

Microdissection was applied to paraffin-embedded sections to enrich tumor cell content to >90%. After microdissection, genomic DNA was extract from 10 tissue sections using the DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's recommendations.

Bisulfite treatment. Bisulfite treatment of DNA converts unmethylated cytosines to uracil, but the methylated ones remain as cytosines. Sodium-bisulfite conversion of 2 μg of DNA was performed by the modification of a previously described method (13). In brief, 2 μg of DNA from each sample was denatured in 0.2 M NaOH for 20 min at 50°C (in a total volume of 20 μL). The denatured DNA was diluted in 500 μL of a freshly made bisulfite solution (2.5 M sodium metabisulfite, 125 mM hydroquinone, 350 mM sodium chloride, pH 5.0) and incubated for 3 h at 70°C in the dark. Bisulfite-modified DNA was purified using the Wizard DNA Clean-Up System (Promega, San Luis Obispo, CA) according to the manufacture's instructions and eluted in 45 μL of water at 80°C. After treatment with NaOH (final concentration 0.3 M) for 10 min at room temperature, the treated DNA was precipitated by the addition of 75 μL of ammonium acetate (5M), 2.5 volumes of ethanol, and 2 μL of glycogen (5 mg/mL). Each resulting DNA pellet was washed with 70% ethanol, dried, dissolved in 110 μL of water, and stored at −80°C.

Gene selection. A total of 25 genes were selected for QMSP-based examination of methylation abnormalities. All the genes evaluated in this study showed tumor suppressor activities and their silencing could contribute to tumorigenesis process. *CCNA1, CCND2, CDKN2A, CDKN2B, P14ARF, RB1,* and *SOCS1* are involved in cell cycle control, *CDH1* in cell adhesion, *PTGS2* in the regulation of inflammatory response, *ESR1, APC, DAPK, RASSF1A, RARβ, TIMP3,* and *THBS1* in signal transduction processes, *GSTP1* in cell detoxification, *MGMT* and *MLH1* in DNA repair, *CALCA* and *MT1G* in cell-cell signaling processes, and *SFRP1* and *SCGB3A1* in cell differentiation and proliferation. The function of *AIM1* is not yet well understood. *MINT31* is a chromosomal region frequently methylated in human cancers. It has been shown that these genes are affected by aberrant promoter methylation in association with transcription silencing in different types of human malignancies.

Quantitative methylation-specific PCR analyses. Bisulfite-modified DNA was used as a template in fluorogenic QMSP assays carried out in a final volume of 25 μL in 96-well plates in a ABI Prism SDS 7000 (Applied Biosystems, Foster City, CA). PCR was performed in separate wells for each primer/probe set and each sample was run in triplicate. The final reaction mixture contained 3 μL of bisulfite-modified DNA, 1.2 μM of forward and reverse primers, 200 nM probe, 0.5 U of platinum *Taq* polymerase (Invitrogen, Frederick, MD), 200 μM dNTPs, 16.6 mM ammonium sulfate, 67 mM Trizma, 6.7 mM magnesium chloride (2.5 mM for *CDKN2A*), 10 mM

mercaptoethanol, 0.1% DMSO, and 1 × ROX dye (Invitrogen, Frederick, MD). PCR was conducted with the following conditions: 95°C for 2 min, followed by 45 cycles at 95°C for 15 s and 60°C for 40 s.

Each plate included patient DNA samples, multiple water blanks, a negative control (normal leukocyte DNA), and serial dilutions (90–0.009 ng) of a positive control for constructing the calibration curves on each plate. Leukocyte DNA from a healthy individual was methylated *in vitro* with SssI methyltransferase (New England Biolabs Inc., Beverly, MA) to generate completely methylated DNA at all CpG and used as positive control.

Primers and probes were designed to specifically amplify the promoter regions of the 25 genes of interest and the internal control gene, *ACTB* (Table 1) (14–23). The percent of methylated reference (PMR) was calculated according to previously reported equation (methylated target-gene quantification in the sample/ACTB quantification in the sample)/(methylated target-gene quantification in the reference/ACTB quantification in the reference) (20). The reference sample consisted of SssI methyltransferase-treated DNA used for the standard curve and was amplified as a reaction control in all PCR plates.

Cases were scored as positive if a PMR value of ≥0.05% was obtained. This cutoff was chosen for being clinically relevant and also to exclude very low-level background readings that can occur in some samples.

Statistical analysis. SPSS 10.0 (Statistical Package for Social Science) for Windows was used for all statistical analyses. Descriptive statistics were used to summarize study data. Statistical significance was defined as a two-tailed p value ≤0.05. Comparisons between clinical-demographic variables and methylation patterns were performed using the χ^2 test or Fisher's exact test.

Survival curves were estimated using the Kaplan-Meier method. Survival data were censored for patients alive at the last observation. The log-rank test was used to compare survival outcomes. The univariate Cox regression model was used to evaluate the methylation level influence on the overall survival (OS).

RESULTS

Patient characteristics and clinical predictors. The patient clinical characteristics are summarized in the Table 2. The age at diagnosis ranges from 0.36 to 83.68 mo (mean of 23.89 mo). Boys (65%) were more affected. Time of symptoms ranges from 0 to 12 mo (mean of 3 mo).

Eight-five percent of patients had The International Childhood Liver Tumour Strategy Group (SIOPEL) PRETEXT III and IV disease (24). Metastasis at diagnosis was detectable in six of the 20 patients (30%) and the lung was the most frequent site. Alpha-fetoprotein level at diagnosis was obtained from 16 patients and two of them showed <100 ng/dL and died of disease. In 19 of the 20 patients (95%), the treatment protocol was based on neoadjuvant chemotherapy followed by tumor resection and adjuvant chemotherapy. The chemotherapy schema was cisplatin/carboplatin/doxorubicin in 50% of the cases, whereas cisplatin/doxorubicin was used in 45% of patients. Only one (5%) patient was submitted to cisplatin monotherapy. According to Radiologic WHO criteria, 60% of the cases exhibited partial response to the chemotherapy treatment, whereas 20% showed stable disease. Surgical resectability included partial hepatectomy (70%), segmentectomy (10%), and liver transplantation (20%). Mixed histologic subtype was the most frequent (75%). The fetal component was present in 85% of the cases.

Quantitative methylation-specific PCR in hepatoblastoma. The first approach was to verify the methylation pattern of the 25 target genes in 10 tumor samples (pilot group). There was no methylation of *AIM1, CCNA1, CDKN2A, DAPK, MGMT, MINT31, MLH1, P14ARF, RB1,* and *THBS1* in the samples tested and hypermethylation of *CALCA* (20%), *CDKN2B* (20%), *ESR1* (20%), *GSTP1* (10%), *RARβ* (30%), *SFRP1* (20%), and *TIMP3* (20%) were rare events. Conversely, *APC* (60%), *CCND2* (50%), *CDH1* (50%), *MT1G* (70%), *PTGS2*

Table 1. *Primers and probes used in the QMSP assays*

Gene	Reference	Forward 5–3′	Probe 6FAM 5–3′TAMRA	Reverse 5–3′
ACTB	14	TGGTGATGGAGGAGGTTTAGTAAGT	ACCACCACCCAACACACAATAACAAACACA	AACCAATAAAACCTACTCCTCCCTTAA
AIM1	15	CGCGGGTATTGGATGTTAGT	GGGAGCGTTGCGGATTATTCGTAG	CCGACCCACCTATACGAAAA
APC	14	GAACCAAAACGCTCCCCAT	CCCGTCGAAAACCCGCCGATTA	TTATATGTCGGTTACGTGCGTTTATAT
CALCA	14	GTTTTGGAAGTATGAGGGTGACG	ATTCCGCCAATACACAACAACCAATAAACG	TTCCCGCCGCTATAAATCG
CCNA1	15	TCGCGGCGAGTTTATTCG	CGTTATGGCGATGCGGTTTCGG	CCGACCGCGACAAACG
CCND2	16	TTTGATTTAAGGATGCGTTAGAGTACG	AATCCGCCAACACGATCGACCCTA	ACTTTCTCCCTAAAAACCGACTACG
CDH1	14	AATTTTAGGTTAGAGGGTTATCGCGT	CGCCCACCCGACCTCGCAT	TCCCCAAAACGAAACTAACGAC
CDKN2A	17	TTATTAGAGGGTGGGGCGGATCGC	AGTAGTATGGAGTCGGCGGCGGG	GACCCCGAACCGCGACCGTAA
CDKN2B	14	AGGAAGGAGAGAGTGCGTCG	TTAACGACACTCTTCCCTTCTTTCCCACG	CGAATAATCCACCGTTAACCG
DAPK	17	GGATAGTCGGATCGAGTTAACGTC	TTCGGTAATTCGTAGCGGTAGGGTTGG	CCCTCCCAAACGCCGA
ESR1	14	GGCGTTCGTTTTGGGATTG	CGATAAAACCGAACGACCCGACGA	GCCGACACGCGAACTCTAA
GSTP1	18	AGTTGCGCGGCGATTTC	CGGTCGACGTTCGGGGTGTAGCG	GCCCCAATACTAAATCACGACG
HIN1	19	TAGGGAAGGGGGTACGGGTTT	ACTTCCTACTACGACCGACGAACC	CGCTCACGACCGTACCCTAA
MGMT	17	CGAATATACTAAAACAACCCGCG	AATCCTCGCGATACGCACCGTTTACG	GTATTTTTTCGGGGAGCGAGGC
MINT31	15	GAGTCGATTTATTAGGTTTCGTC	ACGCCGAAAAACACTTCCCCAAC	CGAAAACGAAACGCCGCGA
MLH1	14	CGTTATATATCGTTCGTAGTATTCGTGTTT	CGCGACGTCAAACGCCACTACG	CTATCGCCGCCTCATCGT
MT1G	20	CGTTTAAGGGATTTTGTATTTGGTTTAT	CGCGATCCCGACCTAAACTATACGCA	CCGCTAAATCCGCACCG
P14ARF	14	ACGGGCGTTTTCGGTAGTT	CGACTCTAAACCCTACGCACGCGAAA	CCGAACCTCCAAAATCTCGA
PTGS2	14	CGGAAGCGTTCGGGTAAAG	TTTCCGCCAAATATCTTTTCTTCTTCGCA	AATTCCACCGCCCCAAAC
RARB	21	GGGATTAGAATTTTTTATGCGAGTTGT	TGTCGAGAACGCGAGCGATTCG	TACCCCGACGATACCCAAAC
RASSF1A	16	GCGTTGAAGTCGGGGTTC	ACAAACGCGAACCGAACGAAACCA	CCCGTACTTCGCTAACTTTAAACG
RB1	14	TTAGTTCGCGTATCGATTAGCG	TCACGTCCGCGAAACTCCCGA	ACTAAACGCCGCGTCCAA
SFRP1	20	GAATTCGTTCGCGAGGGA	CCGTCACCGACGCGAAAACCAAT	AAACGAACCGCACTCGTTACC
SOCS1	22	GCGTCGAGTTCGTGGGTATTT	ACAATTCCGCTAACGACTATCGCGCA	CCGAAACCATCTTCACGCTAA
THBS1	23	CGACGCACCAACCTACCG	ACGCCGCGCTCACCTCCCT	GTTTTGAGTTGGTTTTACGTTCGTT
TIMP3	23	GCGTCGGAGGTTAAGGTTGTT	AACTCGCTCGCCCGCCGAA	CTCTCCAAAATTACCGTACGCG

(70%), *RASSF1A* (70%), *SCGB3A1* (40%), and *SOCS1* (60%) were frequently methylated (Table 3). Those genes that showed a high methylation frequency in hepatoblastoma (namely *APC*, *CCND2*, *CDH1*, *SCGB3A1*, *MT1G*, *PTGS2*, *RASSF1A*, and *SOCS1*) were tested in 10 additional cases and five normal controls.

We found that *RASSF1A* was methylated in 80% of all analyzed cases (16 of 20), *PTGS2* and *MT1G* in 55% (11 of 20), *SOCS1* in 40% (8 of 20), *APC*, *CCND2*, and *CDH1* in 30% (6 of 20), and *SCGB3A1* in 25% (5 of 20) (Table 3 and Fig. 1). We also observed that 95% of neoplastic samples presented methylation for at least one of these eight genes.

Although *CCND2*, *SCGB3A1*, and *PTGS2* genes are methylated in hepatoblastoma, they are also methylated in the control fetal normal liver samples and, for this reason, are not good candidates for tumor markers. Moreover, the methylation of *APC*, *CDH1*, *MT1G*, *RASSF1A*, and *SOCS1* seems to be specific to tumor samples because no hypermethylation for these genes was detected in the control samples (Table 3 and Fig. 1).

Methylation levels and clinical-pathologic correlations. The methylation pattern of *APC*, *CDH1*, *MT1G*, *RASSF1A*, and *SOCS1* was analyzed for potential correlations with clinical characteristics of patients with hepatoblastoma, including age, histologic subtype, PRETEXT stage, SIOPEL risk group

(25), and presence of metastasis at diagnosis. No significant correlation was observed between these clinical features and methylation status of the genes tested.

The 5-y OS was 74.67%. There was no significant difference in OS by age, histologic subtype and PRETEXT stage. But, SIOPEL risk-based stratification (55.5% high risk *versus* 100% standard risk; $p = 0.0217$) and presence of metastasis at diagnosis (92.8% localized *versus* 33.3% metastatic; $p = 0.0066$) influenced the OS. Furthermore, the log-rank test comparing OSs showed that *MT1G* hypermethylation was also correlated with poor prognosis ($p = 0.0431$; Fig. 2).

In addition, a Cox regression univariate model analysis showed a significant positive association between the increase of *MT1G* methylation and bad prognosis for patients with hepatoblastoma ($p = 0.033$; Table 4).

DISCUSSION

The analysis of the clinical and epidemiologic characteristics of the cases analyzed here showed that they are similar to those included in earlier studies (3).

Many studies have described the importance of DNA methylation in the extinction of tumor suppressor gene activity in many human cancers. Considerable variations exist in promoter methylation profiles of different cancers, such that

Table 2. *Clinical characteristics of patients with hepatoblastoma at diagnosis*

Patient characteristics	Number of patients (%)
Total	20 (100)
Gender	
Male	13 (65.0)
Female	7 (35.0)
Age (mo)	
Median age	23.89
Range	0.36–83.68
≥24	7 (35.0)
<24	13 (65.0)
Time of symptoms (mo)	
Median	3
Range	0–12
SIOPEL PRETEXT	
I	0 (0.0)
II	3 (15.0)
III	9 (45.0)
IV	8 (40.0)
Metastasis at diagnosis	
Yes	6 (30.0)
No	14 (70.0)
Alpha-fetoprotein level at diagnosis	
>100 ng/dL	14 (70.0)
<100 ng/dL	2 (10.0)
Not available	4 (20.0)
Treatment protocol	
Cisplatin/carboplatin/doxorubicin	10 (50.0)
Cisplatin/carboplatin	9 (45.0)
Cisplatin	1 (5.0)
Response to chemotherapy*	
Complete response	0 (0.0)
Partial response	12 (60.0)
Stable disease	4 (20.0)
Progressive disease	0 (0.0)
Not available	4 (20.0)
Resectability	
Partial hepatectomy	14 (70.0)
Segmentectomy	2 (10.0)
Liver transplantation	4 (20.0)
Histology	
Mixed fetal and embrionary	6 (30.0)
Mixed embrionary	2 (10.0)
Mixed fetal	7 (35.0)
Pure fetal and embrionary	2 (10.0)
Pure fetal	2 (10.0)
Anaplastic	1 (5.0)

* According to radiological WHO criteria.

Table 3. *Promoter methylation frequency for the 25 genes analyzed in hepatoblastoma samples and in the normal control group (n = 5), pilot group (n = 10), and total group (n = 20)*

Genes	Hepatoblastoma samples, n (%)		Normal control samples, n (%)
	Pilot group	Total group	
MT1G	70 (7)	55 (11)	0 (0)
PTGS2	70 (7)	55 (11)	100 (5)
RASSF1A	70 (7)	80 (16)	0 (0)
APC	60 (6)	30 (6)	0 (0)
SOCS1	60 (6)	40 (8)	0 (0)
CCND2	50 (5)	30 (6)	20 (1)
CDH1	50 (5)	30 (6)	0 (0)
SCGB3A1	40 (4)	25 (5)	80 (4)
RARβ	30 (3)		
CALCA	20 (2)		
CDKN2B	20 (2)		
ESR1	20 (2)		
SFRP1	20 (2)		
TIMP3	20 (2)		
GSTP1	10 (1)		
AIM1	0 (0)		
CCNA1	0 (0)		
CDKN2A	0 (0)		
DAPK	0 (0)		
MGMT	0 (0)		
MINT31	0 (0)		
MLH1	0 (0)		
P14ARF	0 (0)		
RB1	0 (0)		
THBS1	0 (0)		

examined in this tumor (*AIM1*, *CALCA*, *CCNA1*, *CCND2*, *CDKN2B*, *ESR1*, *MINT31*, *MLH1*, *MT1G*, *P14 ARF*, *PTGS2*, *RB1*, *SCGB3A1*, *SFRP1*, *SOCS1*, *THBS1*, and *TIMP3*).

Because of restrictions in the available amount of DNA isolated from paraffin-embedded tumor samples, we first investigated the panel of 25 genes in 10 tumor cases. As a next step, we analyzed the eight most frequently methylated genes in a second series of cases (*n* = 10). According to the QMSP results, the genes most frequently affected by aberrant methylation in hepatoblastoma are *APC*, *CCND2*, *CDH1*, *SCGB3A1*, *MT1G*, *PTGS2*, *RASSF1A*, and *SOCS1*. However, the hypermethylation of *CCND2, PTGS2*, and *SCGB3A1* is not specific (these genes were also found methylated in the normal fetal liver samples).

Except for *MGMT*, *DAPK*, and *SOCS1*, the methylation frequencies found for the genes evaluated in our study are higher than previously reported. This fact could be explained by the higher sensibility of the QMSP method in relation to conventional MSP. However, it is important to note that precautions should be taken when comparing results of different studies concerning aberrant promoter methylation. Although the same tumor type could be evaluated, different methodologies and the use of different primers may yield varied results.

RASSF1A, the most frequently hypermethylated gene in our samples (80%), encodes a protein similar to the RAS effector protein (Nore1). This protein seems to interact with the DNA repair protein XPA and inhibits cyclin D1 accumulation, stopping the cell cycle. There are evidences that the lost or
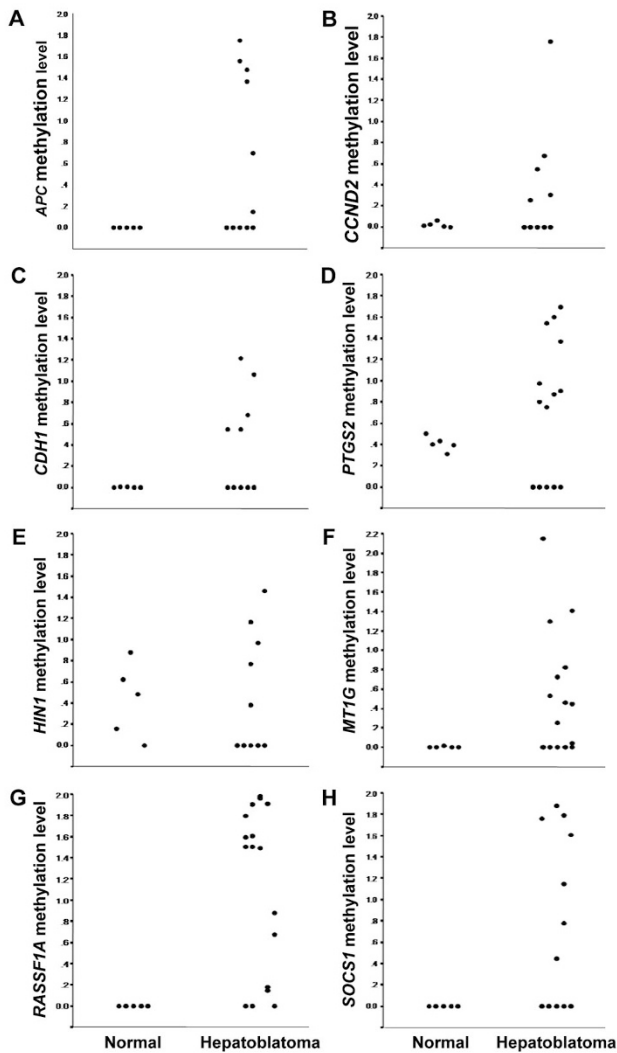
individual tumor types have characteristic methylation profiles (9). To date, few studies evaluated gene hypermethylation in hepatoblastoma (10–12,26,27). All of these studies relied on conventional MSP. Therefore, this study is the first to use QMSP to examine the methylation profile of this pediatric hepatic malignancy. QMSP is a real-time PCR-based assay that is more sensitive (it is capable of detecting methylated alleles in a background of normal at a threshold of 1 of 1,000 to 1 of 10,000) and more specific than conventional MSP since, in addition to the two PCR primers, the fluorescent-labeled hybridization probe must anneal correctly (23). We used QMSP to test a panel of 25 genes that include eight genes already reported to be methylated in this malignancy by conventional MSP assay (*APC*, *CDH1*, *CDKN2A*, *DAPK GSTP1*, *MGMT*, *RARβ* e *RASSF1A*) and 17 genes never

Figure 1. Patterns of hypermethylation observed for the eight selected genes in hepatoblastoma (cases) and fetal normal liver (control). X axis, proportion of methylated cases/tested cases for each sample type. Y axis, quantity of hypermethylation as log (PMR + 1) where PMR was calculated by the equation (methylated target gene in the sample/ACTB in the sample)/ (methylated target gene in the reference/ACTB in the reference) and the reference was a *in vitro* methylated DNA sample. (A) APC, (B) CCND2, (C) CDH1, (D) PTGS2, (E) SCGB3A1, (F) MT1G, (G) RASSF1A, (H) SOCS1.

Figure 2. Kaplan-Meier survival curve according to *MT1G* hypermethylation. The 5-y overall survival for the nonmethylated group (*solid line*) was 90%, whereas the 5-y overall survival for the hypermethylated group (*broken line*) was 60% (p = 0.0431).

Table 4. *Cox regression model comparing methylation level and overall survival*

Gene	β risk	p*
APC	1.654	0.476
CDH1	−15.816	0.395
MT1G	2.196	0.033
RASSF1A	0.007	0.995
SOCS1	−5.222	0.271

* Cox regression univariate model.

altered expression of this gene is associated with lung, colorectal, breast, and hepatocarcinoma pathogenesis (28–30). Harada *et al.* (10), using conventional MSP to analyze 27 hepatoblastoma samples, described the hypermethylation of *RASSF1A* (19%), *RARβ* (4%), and *GSTP1* (4%) and they could not detect methylation in *CDKN2A*, *MGMT*, *APC*, *DAPK*, *CDH1*, and *CDH13*. In another study, Sugawara *et al.,* (27) using MSP and bisulfite-modified DNA sequencing, detected *RASSF1A* hypermethylation in 38.5% of 39 patients with hepatoblastoma. It is important to note that there is a relevant difference between the study of Sugawara *et al.* and ours. In the former, all samples were obtained from patients without neoadjuvant chemotherapy, whereas ours were collected after chemotherapy exposure. Therefore, one can ask if the chemotherapy effect on the tumor could be related to the difference in *RASSF1A* methylation profile observed in two
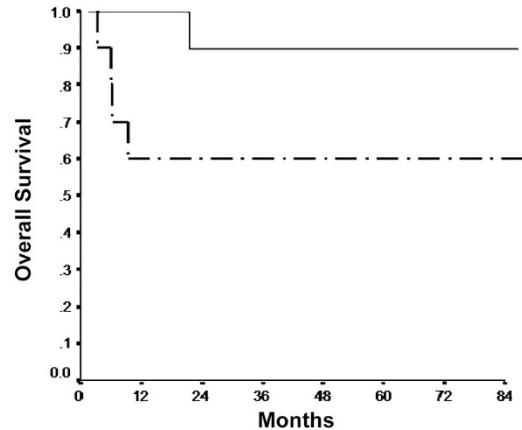
studies (38.5 *versus* 80%). In fact, Koul *et al.* (31) related high frequency of *RASSF1A* hypermethylation in cisplatin-resistant germ cell tumors. Therefore, it is possible that 1) cisplatin treatment is able to stimulate *RASSF1A* hypermethylation or 2) the exposure to this drug allows the selection of chemoresistant clones (making these clones more abundant in the selected specimen). If this second hypothesis is true, a high *RASSF1A* hypermethylation in hepatoblastoma samples before cisplatin-based therapy should indicate cisplatin resistance and could be useful as a drug-resistance predictor. This hypothesis remains to be confirmed by additional studies.

APC protein is evolved with the *CTNNB1* degradation and *Wnt* signaling pathway. Germ-line defects *APC* gene are related with familial adenomatous polyposis (FAP), Gardner and Turcot syndromes (32). As previously mentioned, patients with FAP have increased risk for development of hepatoblastoma (2). So, *APC* inactivation seems to be a mechanism associated with the hepatoblastoma pathogenesis and we found 30% of hypermethylation in this gene in our cases.

CDH1 encodes the adhesion glycoprotein cadherin. Mutations in this gene are associated with gastric, breast, colorectal, thyroid, and ovarian cancers. It is thought that the loss of function of cadherin may be related with cancer progression by increasing proliferation, invasion, and metastasis (33–35). Some studies have demonstrated the correlation between *Wnt*-pathway alterations and genesis and/or prognosis of hepatoblastoma (36,37). Considering that *CDH1* is a member of the *Wnt*-pathway maybe its silencing by aberrant methylation could contribute to the genesis of hepatoblastoma. We detected 60% of tumors with hypermethylated *CDH1*.

SOCS1 encodes a JAK-ligand protein, which regulates the cytosine-dependent signal transduction in the JAK/STAT pathway. The absence of SOCS could stimulate the kinase activity of JAK, promoting cell proliferation (38). The association between hypermethylation and transcriptional silencing of *SOCS1* has already been reported in hepatoblastoma (12). In this study, they analyzed 15 patients with hepatoblastoma and found 46.6% of *SOCS1* hypermethylation. This frequency is similar to our findings (40%) and suggests a participation of *SOCS1* in hepatoblastoma carcinogenesis.

MT1G encodes the metallothionein subtype 1G, a cysteine-rich residues protein that binds several heavy metals. Apparently, its biologic function is related to zinc and copper homeostasis and with cadmium toxicity protection. Jahroudi *et al.* (39) described a higher gene transcriptional activity of *MT1G* associated with heavy metals and dexamethasone exposure in cell line. Nagata *et al.* (40) compared the expression pattern of hepatoblastoma and normal liver tissue by microarray and found that *MT1G* was hypoexpressed in the tumor. Henrique *et al.* (41) demonstrated that *MT1G* hypermethylation was associated with advanced stages of prostate cancer. In addition, *MT1G* hypermethylation was not found in any normal prostate tissue in this study, which indicates that this molecular alteration should be associated with pathologic states. In our study, *MT1G* presented a high-specific hypermethylation frequency (55%) in the hepatoblastoma samples.

Another objective of this study was to investigate whether gene hypermethylation could be associated with clinical parameters, and particularly OS. Previous studies have failed to demonstrate association between methylation profile and clinical features of patients with hepatoblastoma (10–12,26,27). This study, for the first time, demonstrated a strong association between *MT1G* methylation level and poor outcome of patients with hepatoblastoma. According to our results, it seems that the higher the *MT1G* hypermethylation level in the tumor, the lower the patient OS.

In this study, despite the rarity of hepatoblastoma, we evaluate the hypermethylation pattern of 25 genes in 20 cases. We report for the first time that *APC*, *CDH1*, *MT1G*, and *SOCS1* are frequent targets of aberrant methylation in this malignancy and also demonstrate that *MT1G* hypermethylation correlates significantly to bad prognosis of patients with hepatoblastoma.

Our results, together with other findings, demonstrate that aberrant promoter methylation is a frequent event also in hepatoblastoma and suggest that patients with hepatoblastoma may benefit from demethylating drug treatments.

In summary, this study represents the first quantitative evaluation of the methylation profile of hepatoblastoma and our data provide further evidence that the methylation pattern may be useful as a prognostic indicator in this malignancy.

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