Original Article
Aberrant RASSF5 gene transcribed region hypermethylation in pediatric hepatoblastomas

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Abstract: Background: Aberrant DNA methylation plays an important role in cancer and has been recognized to contribute to the activity of oncogenes and inactivity of tumor suppressor genes. RAS association domain family (RASSF) members have been shown to be epigenetically silenced by promoter methylation in cancers, including hepatoblastoma. Methods: We assessed the methylation patterns in the gene of RASSF5 from hepatoblastoma tissue samples harvested from patients using high-throughput mass spectrometry on a matrix-assisted laser desorption/ionization time-of-flight mass array. Results: Hypermethylation was found in the RASSF5 gene transcribed regionand was correlated with downregulation of RASSF5 RNA expression levels in the hepatoblastoma samples. Conclusions: The results indicate that aberrant methylation of RASSF5 may contribute to its downregulated mRNA expression in hepatoblastoma.

Keywords: RASSF5, methylation, hepatoblastoma

Introduction

Hepatoblastoma (HB) is the most common liver tumor in children with 0.5-1.5 cases per million children per year and an increasing incidence [1, 2]. Previous studies suggested an increases risk in patients with familial adenomatous polyposis coli, both low and high birth weights, maternal tobacco exposure, and constitutional trisomy [3-6]. However, the etiology of HB remains obscure. Currently, despite recent advances in the treatment of HB, the mortality rate is 35-50% in high-risk patients [7], and alpha-fetoprotein level, histological analysis, tumor resectability, and metastasis are the only prognosis factors for HB. To better understand the underlying pathophysiology and treatment of this disease, novel targets for early detection and improved therapies and prognosis are required.

DNA methylation is one of the key mechanisms of epigenetic alteration and the identification of DNA methylation is important for understanding cancer pathogenesis. Hypermethylation of DNA in promoters is recognized to silence genes, and many studies have focused on promoter methylation patterns [8]. Recently, researchers have shown that aberrant DNA methylation in transcribed regions of genes is also correlated with gene expression [9-11].

The RAS association domain family (RASSF), including 10 members (RASSF1-10), was recognized to be frequently inactivated by promoter hypermethylation in cancers including HB [12-14]. Previously, we performed a genome-wide analysis of DNA methylation in HB tissues to identify novel targets for further study of HB, and found distinctly higher levels of methylation in HB tissues compared with non-tumor tissue [15], including in regions around the RASSF5 gene. In this study, we used high-throughput mass spectrometry on a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass array to detected methylation changes in RASSF5 in HB tissues and investigated the possibility of RASSF5 becoming a therapeutic target in HB.

Material and methods

Sample collection and clinical information

Human HB and non-tumor liver tissues were obtained from patients who underwent resec-

Table 1. Clinical and pathological characteristics of the study subjects

Case	Age (months)	Sex	Diagnosis type	Alpha-fetoprotein (ng/ml)
1	7	Male	Mixed embryonal/fetal subtype	68490
2	23	Male	Mixed embryonal/fetal subtype	>121,000
3	11	Female	Mixed embryonal/fetal subtype	>121,000
4	10	Female	Mixed embryonal/fetal subtype	>121,000
5	20	Female	Mixed embryonal/fetal subtype	>121,000
6	7	Male	Mixed embryonal/fetal subtype	>121,000
7	30	Male	Epithelial type	>121,000
8	19	Male	Epithelial type	>121,000
9	7	Female	Epithelial type	>121,000

tion of HB in the Children's Hospital, Fudan University, Shanghai, China. Informed consent was obtained from the legal guardians of the patients. Clinical and pathologic data for these patients are listed in **Table 1**. The use of these human samples was approved by the Ethics Committee of the Children's Hospital of Fudan University. HB tumors and non-tumor tissue sections were stored at -80°C immediately after surgery until the time of analysis.

DNA/RNA extraction and quantitative reverse transcription-PCR (qRT-PCR)

DNA was extracted from nine HB primary tumors and adjacent non-tumor tissues and stored until subsequent use for mass spectrometry analysis. Total RNA was isolated from HB and normal tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions and then reverse transcribed using a PrimeScript RT reagent kit (Perfect Real Time) with Gdna Eraser (Takara Biotechnology Co., Ltd., Dalian, China). qRT-PCRs were conducted using qPCR with a SYBRGreen PCR kit. Gene expression was normalized to the GAPDH expression level and represented as fold-change by the $2^{-\Delta\Delta Ct}$ method and statistically analyzed [16]. The RASSF5 primers used for q-PCR were as follows: Forward, 5'-TGCTTGATCTCCT-GCAGTGT-3' and reverse, 5'-TCTCCAGAAAGCA-CCCTCAC-3' (length, 20 bp).

Mass spectrometry

RASSF5 gene primers were designed to cover the regions with the most CpG sites. The genome DNA was treated with bisulfite, and a T7-promoter tag was attached to the reverse primer for the subsequent PCR amplification. The DNA samples were treated with shrimp alkaline phosphatase (SAP) *in vitro* transcription and uracil-specific cleavage, then robotically dispensed onto silicon matrix preloaded chips (SpectroCHIP; Sequenom, San Diego, CA, USA). Mass spectra were collected with a MassARRAY Compact MALDI-TOF system (Sequenom), and the methylation ratios of the spectra were generated using EpiTYPER software v1.0 (Sequenom).

Statistical methods

All data are presented as mean ± standard error of the mean and comparisons between groups were evaluated using a two-tailed Student's t-test unless otherwise specified. Statistical analyses and graphical depiction of data were generated using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS 19.0 software (IBM SPSS, Armonk, NY, USA) for Windows. P<0.05 was considering to be statistically significant.

Results

RASSF5 gene transcribed region (body) methylation is elevated in HB

We collected nine pairs of HB and adjacent normal liver tissue samples and detected the methylation patterns of RASSF5 in 9 HB in these tissues. The primers covered most of the regions with the most CpG sites. The CpGs located in the promoter regions could not be detected, but the CpGs located in the gene body were typically methylated. Five sites in the RASSF5 body regions were suitable for analysis. Using two-way hierarchical cluster analysis, we found that all five CpG sites had a significantly higher degree of methylation in the HB tissues compared with the same sites in the non-tumor tissues. In addition, these sites had a significantly higher level of methylation of the RASSF5 gene between HB tissues and their non-tumor counterparts (0.6391±0.02630 vs. 0.4688±0.02728, respectively; P<0.01; **Figure 1**).
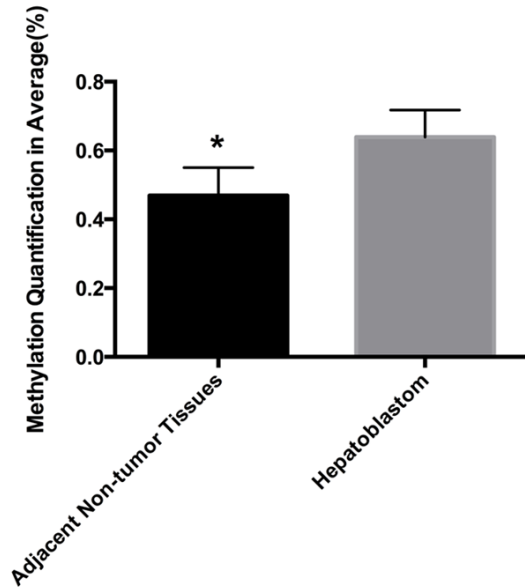
Figure 1. Methylation levels of RASSF5 body region were significantly elevated in the hepatoblastoma tissue samples (n=9) compared with adjacent non-tumor tissues (*P<0.01).
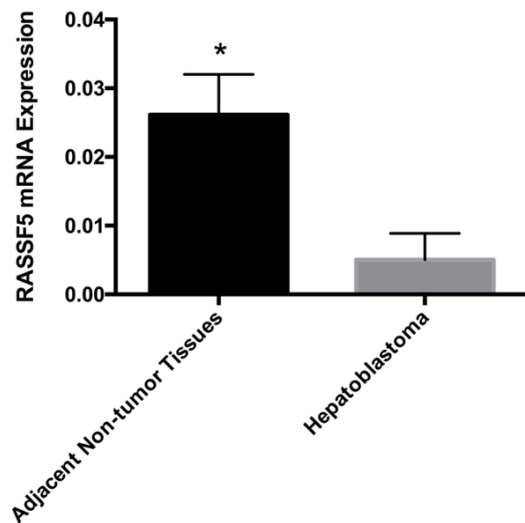

Figure 2. RASSF5 mRNA expression levels were significantly reduced in the HB tissue samples compared with the adjacent non-tumor tissues (*P<0.01).

RASSF5 mRNA expression levels are reduced in HB

To determine whether RASSF5 is involved in tumorigenesis, we detected RASSF5 mRNA expression levels using qPCR. We found the expression level of RASSF5 was significantly downregulated in the HB tissues compared with the matched non-tumor liver tissues
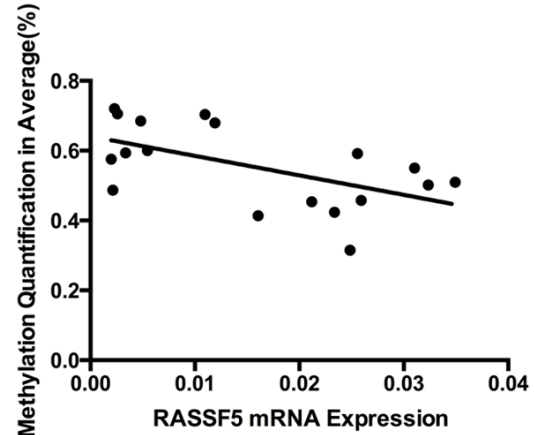

Figure 3. Correlation analysis of RASSF5 mRNA expression and the percentage of DNA methylation in nine hepatoblastoma primary tissues (two-tailed Pearson correlation: r=-0.05644; P=0.0147).

(0.0051±0.0013 vs. 0.0261±0.0020, respectively; P<0.01; **Figure 2**).

Correlation between RASSF5 mRNA expression and percentage of DNA methylation

Using linear Pearson's R correlation, we analyzed the correlations between RASSF5 mRNA expression and DNA methylation status of the CpG sites in the nine pairs of samples. Our data showed that RASSF5 mRNA expression was negatively correlated with its level of methylation (r=-0.5644; P=0.0147; **Figure 3**).

Discussion

DNA methylation and its effect on gene expression have been studied extensively for the last two decades [17]. Generally, methylation changes in CpG islands, CpG shores and CpG shelves [18], regulates several biological processes, including X chromosome inactivation, genomic imprinting, gene transcription and chomatin modification [19-21]. Most of these studies focused on gene promoter methylation [22, 23]. Hypermethylation or hypomethylation of gene promoter regions can result in transcriptional silencing or activation [17]. It is generally recognized that hypermethylation of suppressor gene promoters or hypomethylation of oncogene promoters contribute to tumorigenesis. Although several studies have investigated how gene body DNA methylation impacts gene expression [10, 24, 25], the function of gene body DNA methylation still remains obscure. It

has been suggested that gene body methylation may increase transcriptional activity by affecting the initiation of intragenic promoters or the activities of repetitive DNAs with the transcriptional unit [25]. Gene body DNA demethylation may lead to nucleosome destabilization in transcribed regions and reduced efficiencies of transcription elongation or slicing [11].

In our previous genome-wide analysis of DNA methylation in normal and HB liver tissues, which we performed using an Infinium Human Methylation 450 Beadchip, the data showed distinctively less methylation in positions near the RASSF5 gene [15]. To confirm the association between HB and RASSF5 methylation, in the present study, we performed a MALDI-TOF MS analysis of nine pair of tumor and adjacent normal liver tissues to specifically detect the RASSF5 methylation status.

RASSF5, also known as NORE1 (Novel Ras Effector 1), is the most commonly studied methylated gene in cancer so far. It is localized at 1q32.1 and has been reported as methylated and silenced in neuroblastoma, Wilms tumor, hepatocellular carcinoma and other cancer [26-30]. We detected five CpG sites in the gene body region of RASSF5 from each sample, and the degree of methylation of all five sites was significantly higher in the HB samples than in the normal liver tissues. In an independent cohort of nine adjacent HB-non-tumor tissue pairs, we investigated whether there was a correlation between RASSF5 methylation and mRNA expression. The RASSF5 mRNA expression levels in the nine paired samples was detected by qPCR, and the results showed that the RASSF5 mRNA expression levels in normal liver tissues were significantly higher than in HB tissues. Furthermore, the correlation analysis suggested that the methylation status of RASSF5 was significantly negatively correlated with its mRNA expression levels, which is in agreement with previously published data suggesting that RASSF5 may be an oncogene.

To our knowledge, this is the first report of hypermethylation of RASSF5 in HB. Although we failed to detect CpGs sites in the promoter regions of the RASSF5 gene, we found that DNA hypermethylation occurred in the RASSF5 gene body. Previous studies have suggested that gene body DNA methylation may be an intriguing additional target for therapy [11], so the results of the present study may help in understanding the role of DNA methylation in HB.

In conclusion, the current study indicated that aberrant body methylation of RASSF5 may contribute to its upregulated mRNA expression in HB. However, because HB is an uncommon disease of children, our sample size was limited. Therefore, further studies are required to fully understand whether aberrant methylation of RASSF5 is a consequence of gene expression or it can decrease gene expression levels.

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Disclosure of conflict of interest

None.

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