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Human hedgehog interacting protein expression and promoter methylation in medulloblastoma cell lines and primary tumor samples

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Abstract

Medulloblastoma is the most common pediatric brain tumor and its development is affected by genetic and epigenetic factors. In this study we found there is low or no expression of the hedgehog interacting protein (HHIP), a negative regulator of the sonic hedgehog pathway, in most medulloblastoma cell lines and primary samples explored. We proceeded to promoter methylation assays of this gene by MCA-Meth, and found that HHIP was hypermethylated in all medulloblastoma cell lines, but only in 2 out of 14 (14%) primary tumor samples. Methylation correlated with low or unexpressed HHIP in cell lines but not in primary tumor samples. These results suggest the possibility of epigenetic regulation of HHIP in medulloblastoma, similarly to gastric, hepatic and pancreatic cancer. However, HHIP seems to be not only under regulation of

promoter methylation, but under other factors involved in the control of its low levels of expression in medulloblastoma.

Keywords

Human hedgehog interacting protein (HHIP); Medulloblastoma; Methylation; Melting curve analysis-methylation (MCA-Meth); GLI1

Introduction

Medulloblastoma is the most common and malignant pediatric brain tumor. Survival of medulloblastoma patients is still a challenge for neuro-oncologists. It has been reported that the sonic hedgehog pathway regulates the growth and patterning of the cerebellum [1]. Sonic hedgehog signaling has both mitogenic and morphogenic character [2, 3]. This signaling pathway starts by binding of the *N*-terminal cholesterol-modified Shh protein to the 12 transmembrane PTCH1 receptor. Before this binding, PTCH1 inhibits a seven transmembrane receptor, smoothened (SMO). After binding of the Shh ligand to PTCH1, SMO is relieved, enters the cytoplasm, and activates the main transcriptional factor zinc finger protein, GLI1, with the help of a serine/threonine protein [4, 5]. Activated GLI1 moves from the cytoplasm into the nucleus and regulates Shh signaling target genes including PTCH1, Cyclin D2, and plakoglobin, among others [6].

PTCH1 is one of the down-stream target genes of Shh signaling, but also acts as a negative regulator of Shh. Similarly, another receptor named hedgehog interacting protein (HHIP) binds the three hedgehog proteins (Shh, Ihh, and Dhh) and inhibits their downstream signaling activation. The binding affinity of HHIP to Shh is not less than that of PTCH1. Therefore, both HHIP and PTCH1 act as competitors for binding to Shh ligands [7]. HHIP is also one of the downstream target genes of Shh signaling and acts as a tumor suppressor gene (TSG) [8]. Because HHIP is a negative regulator of Shh signaling activation and a TSG, the possibility of genetic and epigenetic alterations of this gene in malignant transformation should be tested. There are a few reports of the absence or low expression of HHIP in Shh-activated cell lines and samples from pancreatic [9], hepatic [10], and gastric cancer [11]. This low HHIP expression was restored after treatment of the cell lines with the demethylating agent 5-aza-2'-deoxycytidine. These results confirm promoter methylation of HHIP in those tumors.

In this study we have checked for HHIP mRNA expression in six medulloblastoma cell lines and 14 primary tumor samples. Our expression results motivated us to further analyze the HHIP promoter for methylation. Our technique for promoter methylation analysis was different from conventional methylation-specific PCR (MSP); we used the melting curve analysis–methylation assay (MCA-Meth) method, which not only gives an accurate percentage of methylation in the promoter region assessed, but also enables very easy study of methylation in large cohorts of patients in a short time.

Materials and methods

Cell lines

We used four medulloblastoma cell lines: PFSK-1, Daoy, D283Med, and SK-PN-DW. Cell lines PFSK-1, Daoy, D283Med, and SK-PN-DW were purchased from the American Type Culture Collection, Manassas, VA, USA. The cell lines were cultured in RPMI 1-glutamax medium (Gibco–BRL, Gaithersburg MD, USA), supplemented with 10% fetal bovine serum, 10% non essential amino acids, 1% penicillin, and 0.1% amphotericin B, and maintained in an incubator at 37°C in an atmosphere with 5% CO₂ to expand the cells. Sub-culture of cells were performed after 80% confluence with the help of trypsin/EDTA.

Primary tumor samples

We used 14 medulloblastoma primary tumor samples (RNA and DNA) collected from the Department of Pathology, Johns Hopkins Medical Institute, Baltimore, MD, USA. Permission for the use of these tumors for research purposes was granted by the Johns Hopkins Medical Institute Institutional Review Board, under protocol #99-12-29-05.

Real time RT–PCR

RNA was extracted from the six cell lines by using the Quick Prep Total RNA extraction Kit (Amersham Bioscience, UK). We purchased normal brain tissue RNA (Stratagene, Cedar Creek, TX, USA) as normal control of expression. The control came from a single donor, female, 66 years old (Stratagene, catalog number 54005, product name: MVP™ total RNA, human brain). Total extracted RNA of both cell lines and primary tumor samples (1 µg of each) was converted into cDNA by use of the Superscript II RNase H reverse transcriptase kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). cDNA was used for study of the expression of HHIP and GLI1 [12] with the help of the Bio-Rad iQ 5 real time PCR machine. All samples were run in triplicate and their expression results were normalized with the housekeeping gene GAPDH. Primer sequences for the HHIP gene were: forward 5'ATGGTGGGTTGTGCTTTCC3' and reverse 5'AGTTGTGTTTGTGCTTTCTGCT3'; for the GLI1 gene, primers were taken from a previously published article [12]. Conditions for real time RT–PCR were: denaturation at 95°C for 5 min; and 35 cycles of 95°C for 1 min, annealing temperature 59.5°C for 40 s, and extension at 72°C for 50 s. A final extension at 72°C for 10 min was added. Melting curve analysis was done at 72°C for 1 min and finally 95°C for 10 min.

HHIP promoter analysis

We selected HHIP promoter sequences from a previously published article [13]. We analyzed 1,500 promoter bp for possible CpG islands with the help of Methprimers [14]. The promoter has two CpG rich regions, of 373 bp and 224 bp. They were considered as first and second CpG islands, respectively (Fig. 2a). We designed primers for methylation analysis in the first CpG island. The amplified region spanned 227 bp, with 14 CpGs.

DNA extraction and bisulfite modification

DNA was extracted from cell lines and primary tumor samples by the Wizard genomic DNA purification kit (Promega Biosciences, Madison, WI, USA) according to manufacturer's procedure. To ensure the purity of the DNA extracted, we confirmed the ratio OD260/OD280 of the DNA was approximately 1.8 and the ratio OD260/OD230 was between 2.0 and 2.2. In addition, the DNA concentration was confirmed to be between 0.1 and 0.9 µg/µl. Approximately 1 µg extracted DNA was used for bisulfate modification with the CpGenome DNA modification kit (Chemicon International, Temecula, CA, USA). Normal blood DNA and in-vitro methylated DNA (IMD) were regarded as negative and positive controls, respectively, of promoter methylation analysis.

MCA-Meth

MCA-Meth, developed in our laboratory [15], makes use of primers free from CpGs that amplify the target region irrespective of its methylation status. This high-throughput technique is suitable and efficient for analysis of heterogeneous pools of methylation patterns in cell lines and primary tumor samples. Methylated and unmethylated DNA promoter sequences are distinguished on the basis of melting curve peaks and differences in melting temperature. For MCA-Meth, 5 ng bisulfite DNA, 2×IQ SYBR green supermix (Bio-Rad) and 2.5 pmol forward and reverse primers were mixed up to 25 µl of final reaction volume. The PCR reaction was 94°C for 10 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 40 s, and extension at 72°C for 30 s; and a final extension step at 72°C for 10 min. After amplification, melting curve analysis was performed between 70 and 90°C with the temperature increased by 0.5° every 30 s. DNA amplification and melting curve analysis were done in an iQ5 Multicolor real time PCR detection system (Bio-Rad).

Statistical analysis

All the expression study was performed in triplicate, including SD calculations. We obtained *P*-values after applying two-tailed analysis with the help of Graph-Pad Prism4 (San Diego, CA, USA).

Results

Expression of HHIP in medulloblastoma cell lines and tumor samples

When HHIP mRNA expression was assessed in the four medulloblastoma cell lines it was found that expression was usually either low (Daoy) or absent (PFSK-1, D283 and SK-PN-DW) in comparison with normal brain tissue (Fig. 1a; Table 1). Similarly, we checked 14 primary medulloblastoma samples and found expression was either low or absent except for one sample, P13 (Fig. 1b; Table 2). We also found no correlation between HHIP mRNA expression and GLI1 expression [12] in medulloblastoma cell lines (Fig. 1c), although HHIP and GLI1 mRNA expression correlated in primary tumor samples (Fig. 1d). We also compared expression of GLI1 and HHIP in anaplastic, classical, and nodular medulloblastomas, and found a statistical correlation in the anaplastic subgroup only (Fig. 1e–g).

HHIP promoter methylation analysis of medulloblastoma cell lines

The low levels of HHIP expression among cell lines and tumors motivated us to further explore the possibility of HHIP promoter methylation (Fig. 2b–e; Table 1). We performed methylation analysis by MCA-Meth. Hemi/partial methylation was 80–85% for one cell line (D283) and 55–60% for the other three cell lines. For all the cell lines expression of HHIP was lower than for normal brain tissue. Our amplified product for the MCA-Meth study contains a total of 14 CpGs. We consider either complete methylation or hemi/partial methylation on the basis of the number of methylated CpGs detected (Fig. 3).

HHIP promoter methylation analysis of medulloblastoma primary tumor samples

MCA-Meth revealed hemi/partial HHIP methylation in two of 14 samples (14%) (P20 and P29); no methylation was observed in the remaining samples (Figs. 4a–f and 4g–l) (Table 2). Surprisingly, there was no correlation of promoter methylation with mRNA expression among primary tumor samples. For most of the samples no HHIP expression or methylation was observed. No melting peaks were observed for three samples (P10, P17, and P23) (Fig. 4g), possibly because of DNA degradation that prevented a good amplification after bisulfite treatment.

Discussion

Medulloblastoma is a pediatric brain tumor and, in general, its prognosis is bad because of a lack of specific biomarkers in its early development. Therapies remain ineffective. Neuro-oncologists and tumor-biologists are trying to minimize the side effects of any kind of therapy and attempting to enhance the life span of tumor patients. The role of the sonic hedgehog signaling pathway is well characterized as the major signaling pathway with both epigenetic and genetic alterations leading to medulloblastoma development. Mutations in SMO and PTCH1 promote basal cell carcinoma and medulloblastoma; also, GLI1 over-expression suggests the possibility of Shh activation [12, 16–19].

HHIP has been defined as an antagonist of Shh signaling, and has similar affinity to PTCH1 for binding to three hedgehog proteins Shh, Ihh, and Dhh. Low expression of HHIP has been reported in tumors such as pancreatic [9], gastrointestinal [11], and hepatic cancer [10, 20]. It is well known that epigenetic modifications of TSGs affects cancer progression. The HHIP gene is also regarded as a TSG assisting in cancer progression. As far as we are aware, there is no report on HHIP expression and promoter methylation analysis in medulloblastoma.

Our results show low levels of expression or no expression of HHIP in cell lines, associated with HHIP promoter methylation. It has been reported that the HHIP promoter has 11 consensus sequences for binding of the bHLA transcription factor, which is indirectly down-regulated by Shh signaling in vascular remodeling [13]. As far as we know from the literature, not only promoter methylation affects silencing of HHIP gene expression in pancreatic cancer, but other factors which need to be explored [9]. Other genetic alterations, for example LOH, somatic cell mutation, or regulation of microRNA, have been reported to cause silencing of the HHIP gene in hepatocellular carcinoma [10]. Interestingly, besides the

HHIP promoter, H3–K4 and H3–K9 methylation also contribute to silencing HHIP expression in gastrointestinal cancer [11].

On the basis of melting curve analysis of tumor samples, hemi/partial methylation was observed in only two (P20 and P29) of 14 (17%) and no methylation was observed for the other samples. Except for samples P13, P20, and P29 there was no expression of HHIP among tumor samples. This discrepancy suggests two possibilities: either there is methylation in the second promoter region or other possible factors may be involved in HHIP expression, including signaling pathways such as sonic hedgehog, notch, or both, suppressing the expression of this gene [8, 13]. Experiments to prove those hypotheses and further explore the involvement of HHIP or other Hedgehog pathway factors in medulloblastoma might include methylation detection in the second HHIP promoter region, HHIP demethylation treatments and further expression tests at the level of mRNA and protein, and HHIP transfection and expression in medulloblastoma cell lines to examine its effect on survival, proliferation, etc.

A discrepancy of methylation between tumor samples and cell lines has been reported for tumor suppressor gene RASSF1A in head and neck cancer [21]—26% methylation of the RASSF1A gene was observed in cell lines and 0% in primary tumors. Another study also suggested a methylation discrepancy in head and neck cancer [22]. They considered this discrepancy might be because of different distribution of methylated cytosines at CpG islands between cell lines and primary tumors. On the basis of analysis of 70 widely used cancer cell lines from 12 different tumor types, Paz et al. [23] suggested that cancer cell lines generally have higher levels of CpG island hypermethylation than tumor samples. Another possible cause of low expression of HHIP is the promotion of angiogenesis, because it has been demonstrated that reduced expression of HHIP in tumor neo-vasculature may contribute to increased Hh signaling within the tumor and possibly promote angiogenesis [24].

Taken together, our results for HHIP expression and methylation analysis in medulloblastoma cell lines and primary tumors suggest not only epigenetic regulation of HHIP but also the possibility of an activated Shh signal that might down-regulate this gene, at least in this specific childhood brain tumor. This possibility is also supported by previous studies which might partially explain the contribution of Shh signaling to medulloblastoma and other tumors [16–18, 25].

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Fig. 1.

Comparative HHIP mRNA expression in medulloblastoma cell lines and primary tumor samples. **a** HHIP expression in medulloblastoma cell lines. **b** HHIP expression in primary tumor samples. **c** Comparative mRNA expression of HHIP and GLI1 in medulloblastoma cell lines, without significant correlation. **d** Comparative mRNA expression of HHIP and GLI1, with significant correlation. **e** Comparative mRNA expression of HHIP and GLI1 in anaplastic medulloblastomas, with significant correlation. **f** Comparative mRNA expression of HHIP and GLI1 in classical medulloblastoma, without significant correlation. **g**

Comparative mRNA expression of HHIP and GLI1 in nodular medulloblastoma, without significant correlation. We performed Pearson's coefficient correlation of Gli1 and HHIP expression in the cell lines and primary tumor samples, and in the different subgroups of medulloblastomas

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Fig. 2.

HHIP promoter methylation analysis by MCA-Meth in medulloblastoma cell lines. **a** Two CpG-rich regions in the HHIP promoter in a span of 1,500 bases. **b** Melting curve shows hemi/partial methylation in PFSK-1 cell line. **c** Melting curve shows hemi/partial methylation in Daoy cell line. **d** Melting curve shows hemi/partial methylation in D283 cell line. **e** Melting curve shows hemi/partial methylation in SK-PN-DW cell line

Fig. 3. HHIP promoter with 14 CpGs, and methylation in medulloblastoma cell lines and samples. **a** For most cell lines methylation is observed in the amplified promoter region; percentages vary from 80–85% methylation (12/14 CpGs) in D283 to 55–60% CpGs methylated (8/14) in PFSK-1, Daoy, and SK-PN-DW. **b** Primary medulloblastoma samples not methylated at any the 14 selected CpGs of the HHIP promoter. **c** For one sample (P20) 36% (5/14 CpGs) methylation is observed in the selected HHIP promoter region. **d** For one sample (P29) 43% (6/14 CpGs) methylation is observed in the selected HHIP promoter region

Fig. 4. HHIP promoter methylation analysis by MCA-Meth in medulloblastoma samples. **a–f, i, k,** and **l** Melting curves indicative of no methylation in tumor samples. **g** No melting curve in tumor sample. **h** and **j** Melting curves indicative of hemi/partial methylation in tumor samples

Table 1

HHIP promoter methylation status in medulloblastoma cell lines

Cell lines	HHIP mRNA expression	HHIP promoter methylation status	Percentage methylation
PFSK-1	–	Hemi/partial methylation	55–60
Daoy	±	Hemi/partial methylation	55–60
D283	–	Complete methylation	85–90
SK-PN-DW	–	Hemi/partial methylation	60–65

HHIP human hedgehog interacting protein; “+”, expression; “–”, no expression; “±”, no detectable expression

Table 2

HHIP expression and promoter methylation profile in the medulloblastoma samples

Samples ID	Sex	Birth date	Date of surgery	Age (years)	Diagnosis	HHIP mRNA	HHIP promoter methylation
P3 (T1003)	M	5/18/1969	5/6/1992	23.0	Classic medulloblastoma	–	No methylation
P4 (T1004)	M	3/9/1987	5/20/1993	6.2	Anaplastic medulloblastoma (moderate)	–	No methylation
P7 (T1008)	M	11/27/1987	9/16/1997	9.8	Anaplastic medulloblastoma (severe)	–	No methylation
P10 (T1007)	M	4/15/1971	6/19/1995	24.2	Classic medulloblastoma	–	No methylation
P11 (T1012)	F	4/30/1997	8/31/1999	2.3	Anaplastic medulloblastoma (focal moderate)	–	No methylation
P13 (T1014)	F	8/21/1995	11/20/2000	5.3	Anaplastic medulloblastoma (focal moderate)	+++	No methylation
P14 (T1016)	F	9/9/1991	10/25/2000	9.1	Anaplastic medulloblastoma (focal moderate)	–	No methylation
P17 (T1022)	M	11/30/1998	10/14/1999	10.9	Anaplastic medulloblastoma (moderate)	–	No methylation
P20 (T1015)	M	11/22/1978	11/20/2000	22.0	Classic medulloblastoma	+	Hemi/partial methylation (30–35%)
P21 (T1023)	M	11/15/1987	12/27/1999	12.1	Anaplastic medulloblastoma (severe)	–	No methylation
P29 (T1019)	M	3/9/1974	11/16/2000	26.7	Nodular medulloblastoma	+	Hemi/partial methylation (35–40%)
P30 (T1026)	M	6/24/1991	2/21/2001	9.7	Classic medulloblastoma	–	No methylation
P23 (T1021)	F	8/15/1984	8/23/2000	16.0	Anaplastic medulloblastoma (focal moderate)	–	No methylation
P52 (T325)	M	10/5/1991	10/22/1997	6.1	Anaplastic medulloblastoma (focal moderate)	–	No methylation

HHIP human hedgehog interacting protein; “–”, no expression; “+”, low expression; “+++”, high expression