Aberrant Methylation of the HIC1 Promoter Is a Frequent Event in Specific Pediatric Neoplasms

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ABSTRACT
Purpose: To determine the role of methylation of HIC1, a candidate tumor suppressor gene on 17p13.3, in various types of pediatric tumors.

Experimental Design: We examined the methylation status of the HIC1 promoter by methylation specific PCR in 157 pediatric tumors and 27 nonmalignant tissues. We correlated methylation with mRNA expression by reverse transcription-PCR in eight tumor-derived cell lines.

Results: HIC1 methylation was frequent in medulloblastomas (80%, 12 of 15), retinoblastomas (67%, 6 of 9), rhabdomyosarcomas (59%, 13 of 22), germ cell tumors (55%, 6 of 11), and neuroblastomas (36%, 14 of 39); neuroblastomas (43%, 12 of 28), ganglioneuromas (17%, 1 of 6); and ganglioneuroblastomas (20%, 1 of 5). In contrast, a low incidence of methylation was observed in osteosarcomas (17%, 2 of 12), Ewing’s tumors (9%, 1 of 11), Wilms’ tumors (3%, 1 of 31), and hepatoblastomas (0%, 0 of 7). HIC1 methylation was more frequent in the aggressive alveolar subtype of rhabdomyosarcomas (100%, 8 of 8) than the embryonal subtype (33%, 4 of 12; P < 0.005) and was rare in the nonmalignant tissues examined. Methylation was also demonstrated by sequencing in nine randomly selected tumor samples. Seven of eight pediatric tumor cell lines examined were methylated and showed loss or reduced HIC1 mRNA. Expression was strongly induced in all cell lines by treatment with the demethylating agent 5-aza 2’-deoxycytidine.

Conclusions: Our data suggest that aberrant methylation of HIC1 may play a role in the pathogenesis of specific pediatric tumors.

INTRODUCTION

Cancer is the second leading cause of death in children <14 years of age. The tumors that develop in children are different from those of adults because they grow very rapidly. Several types of tumors have been commonly reported in children. However, the molecular genetic and epigenetic alterations in these tumors have not been thoroughly investigated.

DNA methylation changes are among the most common detectable abnormalities in human neoplasia. Several tumor suppressor genes and genes regulating growth and development (1) are silenced in cancer by aberrant methylation of CpG islands in their promoter regions. HIC1 is a candidate tumor suppressor gene located distal to p53 on chromosome 17p13.3 and encodes a zinc finger transcription factor (2). This chromosomal region is reduced to homozygosity in a large number of breast (3), ovarian (4), and lung cancers (5), suggesting that a tumor suppressor gene resides at this locus. Furthermore, the transfection of a sense construct (2) encoding HIC1 into cancerous cells resulted in a significant reduction in cell survival also supporting its role as a tumor suppressor gene.

HIC1 is hypermethylated in a large number of adult solid tumors, including those of the colon (6), lung (7), breast (8), brain, kidney (9, 10), liver (11), cervix (12), and ovary (13, 14). A few studies have demonstrated that methylation of HIC1 may also be important in childhood tumors. A high frequency of methylation was observed in neoplastic hematopoietic cells, particularly in acute and recurrent lymphocytic leukemias (15) and in medulloblastomas, the most common malignant brain tumor in children (16).

To determine the potential role of HIC1 in other types of pediatric tumors, we analyzed the methylation status of its promoter, by MSP, in different types of pediatric tumors and nonmalignant tissues. The pediatric tumors included medulloblastomas, rhabdomyosarcomas, retinoblastomas, neuroblastomas, hepatoblastomas, osteosarcomas, Ewing’s tumors, germ cell tumors, and Wilms’ tumors. We also examined pediatric tumor cell lines to determine the association between HIC1 promoter methylation and gene expression.
MATERIALS AND METHODS

Tissue Specimens. We obtained surgically resected specimens of 157 childhood tumors and 27 nonmalignant pediatric tissues. The tumors consisted of medulloblastomas (mean age, 5.2 years), retinoblastomas (mean age, 1.7 years), rhabdomyosarcomas (alveolar subtype, mean age, 9 years; embryonal subtype, mean age, 4.34 years; anaplastic subtype, mean age, 2 years), germ cell tumors (mean age, 8.62 years), neuroblastomas (neuroblastomas, mean age, 1.7 years; ganglioblastomas, mean age, 6.5 years; ganglioneuromablastomas, mean age, 5.96 years), osteosarcomas (mean age, 11.9 years), Ewing’s tumors (mean age, 9.8 years), Wilms’ tumors (mean age 3.16 years), and hepatoblastomas (mean age, 0.81 years). Most of the tumors were obtained from the Children’s Hospital Medical Center (Dallas, TX). The retinoblastomas were obtained from the University of Siena (Siena, Italy). Nonmalignant samples included corresponding histologically normal kidney (n = 8) from Wilms’ cancer patients and autopsy tissues from children without cancer (five muscle and four liver) and peripheral blood lymphocytes from 10 healthy adults. All samples were collected after obtaining appropriate Institutional Review Board approval and informed consent. Tissues were stored frozen at −80°C before use.

Pediatric Cancer Cell Lines. Eight pediatric cancer cell lines [SH-SY5Y, ATCC # CRL-2266 (neuroblastoma); BE(2)-M17, ATCC # CRL-2267, (neuroblastoma); SK-N-BE(2), ATCC # CRL-2271, (neuroblastoma); SK-N-FL, ATCC # CRL-2142 (neuroblastoma); SK-N-SH, ATCC # HTB-11, (neuroblastoma); D283, ATCC # HB-185, (medulloblastoma); SK-N-MC, ATCC # HTB-10, (neuroblastoma); and SK-NEP-1, ATCC # HTB-48, (Wilms’ tumor) were obtained from the ATCC (Manassas, VA) and grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD), supplemented with 10% fetal bovine serum and incubated in 5% CO2 at 37°C.

DNA Extraction. Genomic DNA was extracted from tumor and cell line samples by digestion with 200 μg/ml proteinase K (Life Technologies, Inc.) at 50°C for 1 day, followed by phenol:chloroform (1:1) extraction and ethanol precipitation (Life technologies, Inc.). About 100 mg of tissue were used for extraction.

Methylation Analysis. Methylation analysis was performed by MSP of sodium bisulfite-treated DNA as described previously (17). Briefly, 1 μg of DNA was denatured by incubation with 0.2 M NaOH for 10 min at 37°C in 50 μL. Aliquots of 10 mM hydroquinone (30 mL; Sigma Chemical Co., St. Louis, MO) and 3 mM sodium bisulfite (adjusted to pH 5.0, 520 mL; Sigma Chemical Co.) were added, and the reaction was incubated at 50°C for 16 h. Treated DNA was purified by use of a Wizard DNA Purification System (Promega Corp., Madison, WI), resuspended in 20 μL of sterile water, and stored at −70°C until used. One-tenth of bisulfite-treated DNA was used for PCR with specific primers for methylated and unmethylated sequences of $HIC1$ as described previously (12). The primer sequences of $HIC1$ gene promoter mapped to the downstream promoter and for the unmethylated reaction were 5’-TTGCGTTTGGTGGTTTTTGTGTTTTG-3’ (sense) and 5’-AACCGAAAACTATCAACCCTCG-3’ (antisense), which amplify a 118-bp product. The primer sequences of $HIC-1$ gene promoter for the methylated reaction were 5’-TCGTTTTTCGCTTTTGTTCGTT-3’ (sense) and 5’-AACCGAAAAACTATCAACCCTCG-3’ (antisense), which amplify a 95-bp product. The 5’ position of the sense unmethylated and methylated primers corresponds to bp 20 and 26 of GenBank accession no. L41919, respectively, and −617 and −611 relative to the $HIC1$ major transcription start site, respectively. Water blanks without added DNA were included as negative PCR controls in each assay. Bisulfite-modified lymphocyte DNA from healthy volunteers served as a positive control for the unmethylated allele. This DNA was methylated in vitro with Sss1 methyltransferase (New England Biolabs, Inc., Beverly, MA), subjected to bisulfite modification, and then used as a positive control for amplification of methylated alleles. PCR products were analyzed on 2% agarose gels containing ethidium bromide. All clearly visible bands were considered positive. Results were confirmed by repeating the bisulfite reaction and MSP for all samples.

DNA Sequencing. The MSP products of nine randomly selected tumor samples (four neuroblastomas, three rhabdomyosarcomas, one osteosarcoma, and one medulloblastoma) were isolated on 1% agarose gels. DNA was purified using ultrafree-DA filters (Millipore Corp., Bedford, MA) and ethanol precipitated. One-hundred ng of purified DNA were sequenced by the Applied Biosystems PRISM dye terminator cycle sequencing method (Perkin-Elmer Corp., Foster City, CA). PCR products were sequenced from both ends with the forward and reverse MSP primers, respectively.

RT-PCR Analysis. Expression of $HIC1$ RNA was analyzed by RT-PCR. Total RNA was extracted from the cell lines with TRIzol (Life Technologies, Inc.) following the manufacturer’s instructions. Reverse transcription was performed on 1 μg of total RNA with SuperScript II First-Strand Synthesis...
using an oligo (dT) primer (Life technologies, Inc.). The primer sequences and conditions used for RT-PCR were as described previously (11). The housekeeping gene GAPDH (18) was used as an internal control for reverse transcription in all samples. PCR products were analyzed on 2% agarose gels containing ethidium bromide. RNA transcribed from normal human brain (Clontech Laboratories, Inc., Palo Alto, CA) served as positive control for HIC1 expression.

**Aza-CdR Treatment.** Seven pediatric cancer cell lines [SH-SY5Y, BE (2)-M17, SK-N-BE (2), SK-N-Fl, SK-N-SH, D283, and SK-N-MC] either not expressing or expressing very low levels of HIC1 RNA were incubated in culture medium with Aza-CdR at a concentration of 2 μg/ml for 6 days, with medium changes on days 1, 3, and 5. Cells were harvested at the end of the 6th day for extraction of total RNA and tested for gene expression. Statistical Analysis. Statistical differences between groups were determined using χ² tests and by Fisher’s exact two-tailed test. Probability values \( P < 0.05 \) were considered to be statistically significant.

**RESULTS**

**Methylation of HIC1 in Pediatric Tumors and Nonmalignant Tissues.** Table 1 shows the frequency of HIC1 promoter methylation in the pediatric tumors examined. Differences in HIC1 methylation were seen in the various types of pediatric tumors. HIC1 promoter methylation was frequent (36–80%) in medulloblastomas, retinoblastomas, rhabdomyosarcomas, germ cell tumors, and neuroblastic tumors. An increased frequency of methylation was observed in the neuroblastomas (malignant tumors) compared with the less aggressive ganglioneuroblastomas and benign ganglieneuromas. However, the differences were not statistically significant. Among the rhabdomyosarcomas, methylation was significantly higher in the aggressive alveolar type compared with the embryonal tumor type \( (P < 0.005) \). A low frequency of methylation (0–17%) was seen in osteosarcomas, Ewing’s tumors, Wilms’ tumors, and hepatoblastomas. Methylation was absent in the DNA from peripheral blood lymphocytes of 10 healthy volunteers and in nonmalignant liver and muscle tissues, whereas it was rare in the nonmalignant kidney tissues. In the tumor samples, most of which consist of mixtures of tumor and normal cells, both methylated and unmethylated alleles for HIC1 were present in the samples that were methylated. The presence of the unmethylated form in all tumor tissues confirmed the integrity of the bisulfite-treated DNA. Representative examples of methylation analysis in tumor tissues are shown in Fig. 1.

**HIC1 Methylation and RNA Expression in Cell Lines.**

Eight pediatric cancer cell lines were examined for HIC1 methylation and gene expression. Methylation was seen in seven of the eight pediatric cell lines examined (Fig. 2). Only the methylated alleles were present in six of these cell lines, whereas one cell line had both methylated and unmethylated alleles. Loss or very low levels of HIC1 mRNA were seen in all seven methylated cell lines by RT-PCR, whereas the cell line SK-NEP-1 was not methylated and expressed the mRNA. RNA expression was strongly induced in all seven low/nonexpressing cell lines after treatment with the demethylating agent Aza-CdR, indicating that loss of expression was associated with gene methylation (Fig. 3).

**DNA Sequencing.** We sequenced the MSP products from nine tumor samples. In all samples, the methyl cytosine residues at all 12 CpG sites within the amplicon remained as cytosines, whereas the other cytosines at non-CpG sites were converted to thymines, confirming methylation and successful bisulfite modification (data not shown).

**DISCUSSION**

Recently, the role of epigenetics in cancer has gained considerable importance as a mechanism for silencing of tumor
suppressor genes. Although several genes have been examined in pediatric cancers, only a few, such as the \textit{p15INK4B} (17), \textit{ER} (9), \textit{RASSF1A} (19), caspase 8 (20), \textit{DcR1}, and \textit{DcR2} (21), were found to be frequently methylated. A high frequency of \textit{HIC1} methylation was reported in pediatric tumors, namely medulloblastomas (16) and leukemias (15). In the current study, we demonstrated that \textit{HIC1} promoter methylation was frequent in other types of pediatric neoplasms and that \textit{HIC1} methylation in pediatric tumor cell lines was associated with loss of RNA expression and could be restored by reversal of methylation with 5aza deoxycytidine treatment.

The high incidence of \textit{HIC1} methylation in several tumors, such as medulloblastomas, retinoblastomas, rhabdomyosarcomas, germ cell tumors, and neuroblastic tumors, and very low frequencies in other pediatric tumors, such as osteosarcomas, Ewing’s tumors, Wilms’ tumors, and hepatoblastomas, indicate that this is a tumor type-specific alteration and suggest that some of these tumors may share a common etiology. The high frequency of \textit{HIC1} methylation in medulloblastomas (80%) observed in our study is in agreement with the 72% incidence determined by restriction digestion and Southern blotting by Rood et al. (16). The low incidence of methylation in nonmalignant tissues in our study and absence of methylation in peripheral blood lymphocytes, also reported by Issa et al. (15), indicate that methylation is associated with neoplastic change. Methylation of \textit{HIC1} in these other types of pediatric tumors has not been described previously.

It was interesting to note that the same tumors, such as medulloblastomas, retinoblastomas, rhabdomyosarcomas, and neuroblasticomas, that exhibited high frequencies of \textit{HIC1} methylation were also reported earlier to be frequently methylated for the \textit{RASSF1A} and \textit{caspase 8} genes (19, 20), suggesting that these tumor types may be predisposed for epigenetic changes.

However, in the same subset of samples analyzed, we found no significant correlation between methylation of \textit{HIC1} and either the \textit{RASSF1A} or \textit{caspase 8} genes reported previously, indicating that \textit{HIC1} methylation was probably independent of these two genes.

We observed a significantly higher incidence of \textit{HIC1} methylation in the aggressive alveolar subtype of rhabdomyosarcomas compared with the embryonal subtype. Previous reports in other tumor types also attest to a similar association of \textit{HIC1} methylation with tumor aggressiveness. Thus, \textit{HIC1} methylation was found to be a progressive event in hematopoietic neoplasms with a 100% incidence in recurrent acute lymphocytic leukemias (15). Makos et al. (6) also reported that methylation in the region of \textit{HIC1} increased in extent as benign adenomatous polyps progressed toward carcinoma. These reports, and the demonstration by Nicolli et al. (24) that retained expression of \textit{HIC1} in breast cancer was associated with good outcome and by Hayashi et al. (25) that reduced expression of \textit{HIC1} was associated with short survival in lung cancers, may indicate a functional importance for \textit{HIC1}.

The association of \textit{HIC1} methylation with loss of transcript and restoration of expression by demethylation supports earlier studies of \textit{HIC1} gene regulation by promoter methylation (8). Absence or low levels of RNA expression were seen in all of the cell lines that were methylated for \textit{HIC1}. The lack of the unmethylated allele in all except one of the methylated cell lines suggests that inactivation may be caused by methylation of both alleles or by a combination of methylation and allelic loss. However, one cell line showed lack of expression despite the presence of both methylated and unmethylated alleles. It is possible that inactivation of the unmethylated allele in this cell line could be by some other mechanism, because according to Knudson’s hypothesis (26), both alleles of a gene must be inactivated (either by methylation, mutation, or deletion) for a tumor suppressor gene to be inactivated. Indeed, Eguchi et al. (7) and Fuji et al. (8) have demonstrated a significant correlation between methylation and loss of heterozygosity at the \textit{HIC1} locus in lung and breast cancers, indicating that genetic and epigenetic changes are involved in gene regulation, whereas Rood et al. (16) did not find any significant correlation in medulloblastomas.

In conclusion, our current findings indicate that \textit{HIC1} is
frequently methylated in select types of pediatric tumors and may play a role in the pathogenesis of specific types of pediatric cancers via epigenetic mechanisms.

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