Hypermethylation of HIC-1 and 17p Allelic Loss in Medulloblastoma

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ABSTRACT

Medulloblastoma is the most common malignant brain tumor in children. Chromosome arm 17p13.3 is reduced to homozygosity in 35–50% of medulloblastomas, making it the most frequent genetic alteration in these tumors. HIC-1 (hypermethylated in cancer) is a putative tumor suppressor gene located in the area of common deletion. HIC-1 resides in a CpG island and is hypermethylated in many different tumor types. Therefore, we studied a series of tumor specimens for hypermethylation and deletion of the region containing the HIC-1 gene to determine whether these two mechanisms of gene inactivation play a complimentary role in medulloblastoma. Southern blotting was performed using the methylation-sensitive restriction endonuclease NotI. Methylation of NotI restriction sites located in HIC-1 was demonstrated in 26 (72%) of 36 tumors and 11 (92%) of 12 specimens of normal brain. Of these 26 tumors, 23 differed significantly from normal brain. A greater proportion of the cells from the tumors showed methylated alleles of the HIC-1 gene. A group of 15 (42%) of 36 tumors exhibited loss of heterozygosity (LOH) for DNA sequences located on chromosome arm 17p. There was no significant correlation between LOH and methylation status (P = 0.19). Methylation in tumors beyond that seen in normal brain predicted poor overall survival independent of clinical risk category (P = 0.014). The results of our study show that methylation of the CpG island that contains the HIC-1 gene is common in medulloblastoma and, together with LOH of 17p, may be a critical event in the formation and aggressiveness of this tumor.

INTRODUCTION

Brain tumors are the most common type of solid tumor in children. Medulloblastoma, the most common malignant childhood brain tumor, is classified as a primitive neuroectodermal tumor arising in the cerebellum. The most frequent genetic alteration in medulloblastoma is LOH6 of chromosome arm 17p13.3, occurring in 35–50% of tumors (1–4). The high incidence of 17p deletion supports the hypothesis that a tumor suppressor gene resides at this locus (5). This region of deletion on chromosome arm 17p is telomeric to the p53 gene (5) but includes the putative tumor suppressor gene HIC-1 (6, 7). Therefore, we studied the methylation status of the NotI sites in the CpG island containing the HIC-1 gene in medulloblastoma and normal brain controls. We also examined these specimens for LOH at 17p13.3 and clinical outcome to determine whether correlations exist between survival, chromosome arm 17p LOH, and methylation in the region of HIC-1.

MATERIALS AND METHODS

LOH Studies. DNA extracted from peripheral blood and tumor specimens was amplified by PCR using primers for the YNZ22.1 and D17S849 marker. These primers have been shown to have a polymorphic information content in 80 and 90%, respectively. Previous studies in our laboratory have shown that D17S849, a marker located distal to YNZ22.1 on 17p, is closely linked to YNZ22.1 (1). PCR amplification was performed in 40-µl reaction mixtures containing 50 ng of genomic DNA, 200 µM each of the four deoxynucleotides, 1 ng of each primer, and 1 unit of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, CT) in 1× PCR Buffer I (Perkin-Elmer). The reactions were run in a Perkin-Elmer thermal cycler under the following conditions: 95°C/10 min × 1 cycle; 95°C/1 min, 55°C/1 min, and 72°C/4 min × 30 cycles; and 72°C/7 min × 1 cycle. Amplification was performed using the D17S849 primer with the following conditions: 95°C/7 min × 1 cycle; 95°C/1 min, 55°C/1 min, and 72°C/2 min × 30 cycles; and 72°C/6 min × 1 cycle. PCR reaction products from each patient were run side by side on a 10% polyacrylamide gel (1). The gel was stained with ethidium bromide and viewed on a UV light box. When the bands were faintly visualized, the gel was fixed and silver stained for better resolution. The presence of two bands from the blood identified the primer as informative for that individual. If there was only one band from the blood, the subject had a constitutional homozygosity for that marker, and the primer was uninformative. If the tumor from an informative subject revealed two bands, there was no deletion. If the tumor had only one band, it was considered to have LOH for the targeted sequence.

Methylation Studies. Freshly frozen tumor, normal brain tissue, and peripheral blood leukocytes were obtained through a protocol approved previously by the Institutional Review Board. DNA extracted from these specimens was digested overnight with 100 units each of the restriction enzymes NotI and EcoRI at 37°C. The restriction enzyme NotI cuts DNA at sequences consisting of: GCGGCCGC. The enzyme will not cut, however, if the cytosine residues in the CpG dinucleotides are methylated, resulting in polymorphic restriction products. The DNA fragments were then resolved on a 1% agarose gel in 1× Tris-borate EDTA buffer. After Southern transfer was performed, the nylon membrane was hybridized with a P32-labeled YNZ22.1 probe using ExpressHyb Hybridization Solution (Clontech, Palo Alto, CA) at 63°C for 1 h (8). The YNZ22.1 probe is located 1.4 kb telomeric to HIC-1 and is a marker for the HIC-1 gene. The presence of 5–16 kb bands indicates methylation of some or all of the NotI sites, whereas 4–5 kb bands are present if the NotI sites are unmethylated (Fig. 1). To assure complete digestion of DNA by NotI, the same blots were also probed using a v-abl gene fragment, which contains two NotI sites. The expected 4-kb band was obtained for each sample indicating complete digestion.

Analysis of Methylation. To characterize the pattern of methylation, radiographs of Southern blots were scanned and analyzed on an Apple G4 computer using the public domain NIH Image program (developed at the United States NIH and available on the Internet). The absorbance of the bands in each lane was measured and expressed as a fraction of the total signal obtained from all of the bands in that lane. The x coordinate of each band was measured and compared with the migration of a marker to predict the size of each band using a linear regression forecasting model (Microsoft Excel). Correlative analyses between methylation, LOH, clinical risk category, and outcome were performed using 2×2 contingency tables and the two-tailed Fisher’s exact test. NotI restriction sites N1 or N2, reside within 800 bp of each other in the 5’ region of the HIC-1 gene (Fig. 1) and, therefore, constitute the sites most relevant to gene inactivation. A band resulting from methylation of all of these sites would be predicted to be 11.5–12.5 kb with the variability being attributable to the number of repeats in the variable number of tandem repeats region located between N1 and N2. If the N1 site is also methylated, the predicted band would be 16.6–17.1 kb. Contamination of tumor specimens with normal brain tissue was limited by the surgeon’s practice of obtaining specimens from areas identified as clearly neoplastic by frozen section. However, contamination from blood and infiltrating leukocytes could not be com-
RESULTS

LOH Studies. LOH for the YNZ22.1 marker was identified in 12 of 31 (39%) informative specimens. YNZ22.1 was noninformative in 5 of 36 (14%) specimens. LOH for the D17S849 marker was shown in 12 of 30 (40%) informative specimens studied. D17S849 was noninformative in 3 of 30 (10%) specimens and not determined in 3 specimens. There was complete correlation between the YNZ22.1 and D17S849 markers in all of the specimens in which both were informative. Using these correlative markers, we established chromosome arm 17p allelic loss in 15 of 36 (42%) medulloblastoma specimens (Table 1). This is consistent with the incidence reported previously of 17p deletions (35–50%) in medulloblastoma (1, 2, 4).

Methylation Studies. Using the YNZ22.1 marker, 35 of 36 tumor specimens (97%) showed methylation at some or all of the NorI restriction sites. No peripheral blood specimen showed any methylation. All 12 specimens of normal brain (5 from cerebellum and 7 from cerebral cortex) analyzed revealed a pattern consistent with methylation of some of the NorI restriction sites (Fig. 2). Eleven of 12 specimens had bands indicative of methylation that included sites N2–4 and N1 or N3. Methylation patterns differed between these 11 normal brain specimens and the majority of medulloblastoma specimens in the proportion of signal represented by the largest band, which varied from 3 to 33% (Fig. 3). Therefore, aberrant methylation was defined as having a pattern characterized by the presence of a band indicative of a methylation region containing NorI sites 2–4, whose signal accounted for >33% of the total signal from that sample. Of the 36 tumors analyzed, 23 (64%) were aberrantly methylated, and 13 (36%) were not (Table 1). In 15 (65%) of the aberrantly methylated tumors, all of the signal was attributable to the band representing methylation, inclusive of sites N2–4.

Correlation of Methylation and LOH. Of the 23 tumors with aberrant methylation in the region of the HIC-1 gene, 9 exhibited 17p LOH, and 16 did not. There was no statistically significant correlation between methylation status and LOH (P = 0.09).

Correlation between Methylation and Clinical Outcome. Available outcome data for the patients whose tumors were studied were limited to overall survival and clinical risk stratification. Treatment consisted of craniospinal irradiation with local boost to the tumor bed with or without chemotherapy, which varied by treatment protocol. With follow-up ranging from 2 to 10 years, 17 subjects are alive, and 19 are dead. Aberrant methylation correlated with a negative survival advantage with an odds ratio of 7.2 (P = 0.014). Clinical high-risk stratification, based on age, extent of resection, or presence of disease outside of the posterior fossa, was also significantly correlated with poor outcome (odds ratio 5.3, P = 0.041) in this data set. Methylation was not correlated with clinical risk category (P = 0.18) and is therefore an independent prognostic factor. LOH status was not correlated with overall survival (P = 1.0).

DISCUSSION

Medulloblastoma, the most common malignant brain tumor of childhood, is a primitive neuroectodermal tumor arising in the posterior fossa (cerebellum and brainstem) of primarily young children. Treatment includes resection, irradiation, and chemotherapy. Treatment is stratified according to clinical risk factors that predict poor outcome, including age <3 years, partial resection, and dissemination of disease outside of the posterior fossa. Although DNA ploidy (9), TrkC expression (10), mitotic index (11, 12), c-erbB-2 expression (13–15), histological subtype, and chromosome arm 17p deletion (2, 4, 7, 16, 17) have been posited as independent prognostic factors, no molecular genetic or biological marker has achieved widespread acceptance as a predictor of treatment outcome. Although survival is achieved for the majority of patients treated for medulloblastoma, the neuropsychological sequelae of therapy are significant.

HIC-1 is a putative tumor suppressor gene that was initially isolated from a CpG island that resides in a region reduced frequently to homozygosity in many different types of human tumors (18). The location of the HIC-1 gene in a commonly deleted region and studies

Table 1 Results from methylation and LOH studies in medulloblastoma a

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Top band</th>
<th>LOH YNZ</th>
<th>LOH 849</th>
<th>Outcome</th>
<th>Risk group</th>
</tr>
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<tr>
<td>M 01</td>
<td>Tumor 56%</td>
<td>--</td>
<td>ND</td>
<td>Dead</td>
<td>Poor</td>
</tr>
<tr>
<td>M 02</td>
<td>Tumor 66%</td>
<td>--</td>
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<td>Dead</td>
<td>Poor</td>
</tr>
<tr>
<td>M 04</td>
<td>Tumor 0</td>
<td>+</td>
<td>+</td>
<td>Dead</td>
<td>Good</td>
</tr>
<tr>
<td>M 07</td>
<td>Tumor 0</td>
<td>+</td>
<td>+</td>
<td>Dead</td>
<td>Good</td>
</tr>
<tr>
<td>M 08</td>
<td>Tumor 1%</td>
<td>+</td>
<td>+</td>
<td>Dead</td>
<td>Good</td>
</tr>
<tr>
<td>M 11</td>
<td>Tumor 100%</td>
<td>--</td>
<td>--</td>
<td>Dead</td>
<td>Poor</td>
</tr>
<tr>
<td>M 12</td>
<td>Tumor 62%</td>
<td>+</td>
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<tr>
<td>M 13</td>
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<td>Good</td>
</tr>
<tr>
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<td>--</td>
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<td>Good</td>
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<tr>
<td>M 20</td>
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<td>+</td>
<td>Dead</td>
<td>Poor</td>
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<tr>
<td>M 22</td>
<td>Tumor 100%</td>
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<td>--</td>
<td>Alive</td>
<td>Good</td>
</tr>
<tr>
<td>M 26</td>
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<td>--</td>
<td>--</td>
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<td>Poor</td>
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<tr>
<td>M 28</td>
<td>Tumor 62%</td>
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<td>Dead</td>
<td>Poor</td>
</tr>
<tr>
<td>M 31</td>
<td>Tumor 50%</td>
<td>--</td>
<td>NI</td>
<td>Alive</td>
<td>Poor</td>
</tr>
<tr>
<td>M 33</td>
<td>Tumor 100%</td>
<td>+</td>
<td>+</td>
<td>Dead</td>
<td>Poor</td>
</tr>
<tr>
<td>M 34</td>
<td>Tumor 67%</td>
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<td>Dead</td>
<td>Good</td>
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<tr>
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<td>NI</td>
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<tr>
<td>M 37</td>
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<td>--</td>
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<tr>
<td>M 38</td>
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<td>Poor</td>
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<tr>
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<tr>
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<tr>
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<td>--</td>
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<tr>
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<td>Tumor 100%</td>
<td>--</td>
<td>--</td>
<td>Dead</td>
<td>Poor</td>
</tr>
<tr>
<td>M 49</td>
<td>Tumor 49%</td>
<td>--</td>
<td>--</td>
<td>Dead</td>
<td>Poor</td>
</tr>
<tr>
<td>M 50</td>
<td>Tumor 0</td>
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<td>+</td>
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<td>Poor</td>
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<td>+</td>
<td>ND</td>
<td>Dead</td>
<td>Poor</td>
</tr>
<tr>
<td>M 54</td>
<td>Tumor 100%</td>
<td>--</td>
<td>--</td>
<td>Dead</td>
<td>Poor</td>
</tr>
<tr>
<td>M 55</td>
<td>Tumor 0</td>
<td>+</td>
<td>+</td>
<td>Alive</td>
<td>Good</td>
</tr>
<tr>
<td>M 56</td>
<td>Tumor 0</td>
<td>+</td>
<td>+</td>
<td>Dead</td>
<td>Poor</td>
</tr>
<tr>
<td>M 57</td>
<td>Tumor 18%</td>
<td>NI</td>
<td>--</td>
<td>Dead</td>
<td>Poor</td>
</tr>
<tr>
<td>M 58</td>
<td>Tumor 53%</td>
<td>--</td>
<td>--</td>
<td>Alive</td>
<td>Good</td>
</tr>
<tr>
<td>M 59</td>
<td>Tumor 100%</td>
<td>NI</td>
<td>+</td>
<td>Alive</td>
<td>Good</td>
</tr>
<tr>
<td>M 61</td>
<td>Tumor 0</td>
<td>NI</td>
<td>+</td>
<td>Alive</td>
<td>Good</td>
</tr>
<tr>
<td>M 63</td>
<td>Tumor 63%</td>
<td>--</td>
<td>--</td>
<td>Dead</td>
<td>Good</td>
</tr>
</tbody>
</table>

aM, medulloblastoma; NB, normal brain; +, LOH; −, retained heterozygosity; NI, noninformative because of constitutional homozygosity; ND, not determined.

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showing that transfection of HIC-1 into tumor cell lines will inhibit growth support its function as a tumor suppressor gene (18). The HIC-1 gene spans 4.6 kb, contains three exons, and codes for two transcrip- 
tional products (6, 18). There are two promoter elements: (a) the first containing a conserved GC box; and (b) the second containing a TATA box (6, 18, 19). The region upstream of the HIC-1 sequence has a p53-binding site, and the transcription of HIC-1 is activated by wild-type p53 (6, 18). The HIC-1 transcription product contains five Krueppel-like C2H2 zinc finger motifs and an NH2-terminal BTB/ 
POZ domain characteristic of a family of transcriptional repressors (6, 18). The density of CpG dinucleotides in the HIC-1 gene makes it a target for inactivation through methylation.

According to Knudsen’s hypothesis, two “hits” are required to inactivate both alleles of a tumor suppressor gene and promote tumori- 
genesis (20). These insults can occur by deletion, mutation, or methylation (21–23). Methylation of the cytosine residues in CpG dinucleotides of gene promoters results in the suppression of tran- 
scription (18, 21, 24, 25). Tumor suppressor genes inactivated by hypermethylation include retinoblastoma-1 (26), VHL (renal cancer; Ref. 27), CDKN2B (hematological malignancies; Ref. 28), and MLH1 and APC (colorectal cancer; Refs. 29–31). Studies suggest that this inhibition involves the packing of methylated DNA into a closed chromatin configuration mediated by MeCP2, a protein with a methylation-specific DNA binding site and a transcriptional repression site (32–35). MeCP2 recruits histone deacetylases, which change the conformation of core histones by deacetylating their lysine-rich tails. This conformational change causes histones to bind DNA more tightly, potentially blocking the access of transcription factors (34– 
36). Thus, the combination of deletion and methylation could inactiv- 
te both alleles of a tumor suppressor gene, resulting in oncogenesis.

In our series of medulloblastoma specimens, we were unable to show that medulloblastoma formation depends on methylation of every nondeleted allele of the HIC-1 gene, because 5 of 21 medullo-
blastosomas without LOH do not exhibit aberrant methylation. This is similar to published findings in breast cancer where the authors speculated that LOH might have affected the methylated allele (37). Our data suggest that methylation patterns vary from cell to cell within a given tumor, because tumors with LOH still exhibited multiple bands. An alternative explanation is that contaminating cells, such as neurons or glial cells, contribute to the heterogeneity of the methylation pattern. We feel that this is an unlikely explanation given the relatively small amount of DNA from contaminating stromal cells, compared with DNA from tumor cells. In colon carcinogenesis, density of methylation in the region of HIC-1 increases as benign adenomatous polyps progress toward carcinoma (38). Our data exhibit a baseline level of methylation in normal brain, which increases in the majority of medulloblastoma specimens. Furthermore, those tumors with the most methylation have the most aggressive phenotype as evidenced by the poor clinical outcome of these patients. A similar association between decreased HIC-1 expression and clinically aggres- 
sive phenotype has been shown in lung cancer (39). A model in 
which an increasing extent of methylation is selected for during medulloblastoma formation or progression would be supported by this data.

In addition to gene inactivation, methylation may predispose to chromatin instability and loss of genetic material (40–44). This may be attributable to a delay in DNA transcription or alterations in chromatin formation (41, 42, 45). Hypermethylation has been shown to be accompanied by LOH of chromosome 16 in hepatocellular carcinoma (40). LOH and hypermethylation of chromosome arm 17p has been identified as well in non-small cell lung cancer (43), hepa-
tocellular carcinoma (24), renal tumors (41), and neural tumors (42). Among these 36 medulloblastomas, no statistically significant corre- 
lation was found between LOH for chromosome arm 17p and pres- 
ence or degree of methylation in the region of HIC-1. In fact, the data trend toward demonstrating that LOH is associated with a decrease of aberrant methylation.

Our study shows that partial methylation is common in the CpG island containing the HIC-1 gene in normal brain tissues. We have also demonstrated that medulloblastomas exhibit a pattern of aberrant methylation, which differs in extent from normal brain, as represented by the increased proportion of signal in the largest band. The predis- 
position of methylation in the region of HIC-1 toward a poor outcome may indicate a functional importance for HIC-1 in determining tumor aggressiveness. Aberrant methylation of 17p13.3, when taken together with LOH, may be a critical event in the inactivation of both alleles of the putative tumor suppressor gene HIC-1, contributing to onco-
genesis.

REFERENCES


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