Extensive Genomic Abnormalities in Childhood Medulloblastoma by Comparative Genomic Hybridization

David A. Reardon, Edson Michalkiewicz, James M. Boyett, Jack E. Sublett, Ruth E. Entrekin, Susan T. Ragsdale, Marcus B. Valentine, Frederick G. Behm, Hao Li, Richard L. Heideman, Larry E. Kun, David N. Shapiro, and A. Thomas Look


ABSTRACT

We analyzed 27 samples of primary medulloblastoma, using comparative genomic hybridization and a novel statistical approach to evaluate chromosomal regions for significant gain or loss of genomic DNA. An array of nonrandom changes was found in most samples. Two discrete regions of high-level DNA amplification of chromosome bands 5p15.3 and 11q22.3 were observed in 3 of 27 tumors. Nonrandom genomic losses were most frequent in regions on chromosomes 10q (41% of samples), 11 (41%), 16q (37%), 17p (37%), and 8p (33%). Regions of DNA gain most often involved chromosomes 17q (48%) and 7 (44%). These findings suggest a greater degree of genomic imbalance in medulloblastoma than has been recognized previously and highlight chromosomal loci likely to contain oncogenes or tumor suppressor genes that may contribute to the molecular pathogenesis of this tumor.

INTRODUCTION

Medulloblastoma is the most common embryonal central nervous system tumor in children, and accounts for approximately 20% of childhood brain tumors overall. Cytogenetic studies have demonstrated loss of the distal end of chromosome 17p, usually with an i(17q), in 30–50% of cases (1). Additional findings in small subsets of tumors, either by cytogenetic analysis or by LOH allelotyping, include deletions on chromosomes 5q, 6, 9, 10, 11, 16q, and 22 (1–6). Despite these observations, the genetic changes that contribute to the pathogenesis of medulloblastoma remain largely undefined. In the present analysis, we applied CGH and a rigorous statistical evaluation of fluorescence intensities to a large series of primary medulloblastoma samples. The results indicate numerous regions of nonrandom gains and losses, many of which represent new findings in this tumor, including two novel amplicons at 5p15.3 and 11q22.3.

MATERIALS AND METHODS

Patients and Tumor Samples. The clinical features of the 27 patients included in this study are summarized in Table 1. The diagnosis of medulloblastoma was established according to criteria of the WHO (7), and clinical staging followed the criteria of Chang et al. (8) based on systematic postoperative neuraxis evaluation. All samples were obtained during initial surgery after informed consent and before any further therapeutic intervention. Samples were frozen immediately in liquid nitrogen after resection and stored at −80°C until the time of analysis.

CGH and Digital Imaging. CGH, digital image acquisition, and fluorescence intensity measurements were performed as described previously (9–11). Briefly, modified nick-translation reactions, incorporating biotin-14-dUTP and digoxigenin 11-dUTP into 10–50 ng of reference and tumor DNA, respectively, yielded fragments of 700–2000 bp in length. The labeled DNA probes (300 ng) were mixed with 30 μg of Cot1 human DNA and hybridized to normal metaphases for 72 h at 37°C. Bound DNA fragments were stained with avidin-FITC (green) and antidigoxigenin-rhodamine (red) prior to counterstaining with 4',6-diamidino-2-phenylindole (40 ng/ml) in antifade medium (Vectorshield, Vector Laboratories, Inc., Burlingame, CA). In 23 of the 27 cases, there was sufficient tumor material to permit a second hybridization using rhodamine-labeled tumor and FITC-labeled normal DNA (“reverse labeling CGH”) to confirm the results of standard hybridization with FITC-labeled tumor. Two negative control CGH experiments, in which aliquots of normal DNA were labeled differentially with green and red fluorochromes, respectively, were also performed to identify chromosomal regions with artifically abnormal fluorescence profiles. Metaphase images that were smooth and of high quality were captured with an epifluorescence microscope (Zeiss Axiopt, Oberkochen, Germany) and a cooled charge-coupled device camera (Photometrics, Tuscon, AZ). Mean ratios of FITC (green)-to-rhodamine (red) signal intensities were calculated with dedicated software (SmartCapture, Vyvis, Inc., Downers Grove, IL), using 8–30 homologues of each chromosome per tumor sample. Regions immediately adjacent to and including heterochromatic segments were excluded from analysis, because they typically showed very low FITC and rhodamine fluorescence intensities due to partial hybridization suppression with Cot1 DNA.

FISH. Two-color FISH analysis was used to verify distal 10q loss determined by CGH. Interphase nuclei from 18 of the samples analyzed by CGH were available for hybridization to P1 clone 6G6 (12), which encompasses the MXII gene locus from 10q24–25 (13) together with the a-satellite probe from chromosome 10, as described previously (14). A minimum of 100 nuclei per hybridization was studied, with a deletion defined as the presence of both one centromeric and P1 signal (indicating monosomy) or the presence of fewer P1 signals than centromeric signals in >50% of the tumor cells. The presence of more than two signals from both the P1 and centromeric probes defined a gain in copy number by FISH. DNA indices were determined with fresh tumor cell suspensions collected at initial surgery according to standard techniques (15).

Statistical Considerations. In contrast to most other CGH studies, we attempted to control for the adverse effects of both chromosomal and nonchromosomal variability, using a mixed model with random effects (16) to construct 99% simultaneous CI estimates of mean green:red ratios. Simultaneous CIs adjust for possible false positive conclusions resulting from multiple comparisons. In this model, tumor DNA sampling and metaphase selection were considered random effects, whereas chromosome segments for which fluorescence intensities were measured were considered fixed effects. Data were analyzed using the mixed procedure of the SAS/STAT software (17). Significant gains and losses of genomic regions were defined by CIs that were either above or below a mean ratio value of 1.0. Amplifications were identified by direct microscopic visualization of a discrete region of genomic gain. For the 23 tumors studied by reverse labeling as well as standard CGH, significant gains and losses had to meet the above-described CI criteria in both types of experiments. Among the four tumors studied exclusively by standard labeling, the analysis specifically excluded abnormalities that occurred on chromosomal regions known to be evaluated unreliably by CGH; these include telomeric segments and the regions of chromosomes distal 1p, 16p, 19, and 22 (10).

Fisher’s exact test (18) was used to test for significant associations between CGH findings occurring in at least one-third of the samples and clinical
RESULTS

The clinical features of the study group (Table 1) were representative of childhood medulloblastoma patients overall (23). The median age was 7.9 years (range, 1.7–19.9), with a male:female ratio of 1:7.1. There were eight cases with metastatic tumor at diagnosis and 19 in which the disease was confined locally to the posterior fossa. Twenty patients were alive, 16 without disease progression, at the time of the follow-up (median, 5.8 years; range, 0.2–10.3 years). Treatment consisted of gross total resection (n = 18) or subtotal resection (n = 9) followed by different combinations of chemotherapy and radiotherapy, depending on the age and clinical stage of the patient as well as the extent of residual tumor.

Fig. 1 and Table 1 summarize the cumulative frequency of all chromosomal gains and deletions. Most samples had multiple CGH abnormalities (mean no. per tumor, 7.4; range, 0–22). By contrast, two control experiments involving differentially labeled normal DNA samples revealed fluorescence profiles within the 99% simultaneous confidence intervals for all chromosomal regions, except chromosome 19, a region known to be not reliable using CGH (data not shown). The samples revealed fluorescence profiles within the 99% simultaneous confidence intervals for all chromosomal regions, except chromosome 19, a region known to be not reliable using CGH (data not shown). The mean number of abnormalities in amplicon-containing tumors was significantly less than the number identified in tumors without amplicons (2.0 versus 8.1; P = 0.04). Of note, all three tumors containing amplicons also demonstrated losses involving chromosome 9q (Fig. 1). By contrast, loss involving 9q was noted in only one tumor lacking an amplicon.

FISH analysis with an MXJ11 probe from chromosome band 10q24–25 yielded results that agreed with CGH findings in 15 of 18 cases (Table 2). Seven cases showed loss by both techniques (Fig. 3), whereas eight were normal. Three cases (nos. 4, 14, and 17) with DNA indices indicative of near-tetraploid cell populations, had loss of the entire chromosome 10 by CGH, but did not show loss by FISH. Direct comparison of CGH and FISH analyses in such cases may not be valid. This is because CGH identifies regions of loss or gain relative to the DNA content of the entire genome (10) and therefore is definitive evidence of their significance will require a prospective CGH analysis of additional childhood medulloblastoma samples.

Statistical comparison of the chromosomal distributions of genomic abnormalities that occurred in at least one-third of the tumors studied revealed that losses in regions 8p and 16q were more likely to occur together than in a random pattern (P = 0.04). Similar analysis revealed a significant univariate association (P = 0.03) between the presence of metastatic disease at diagnosis and losses involving chromosomes 10q and 11. However, neither of these associations retained statistical significance after adjustment for multiple comparisons, and definitive evidence of their significance will require a prospective CGH analysis of additional childhood medulloblastoma samples.

DISCUSSION

In this study, we used CGH and a stringent statistical evaluation of green:red ratio values to identify significant regions of genomic gain or loss in childhood medulloblastoma. Most of the samples showed...
multiple regions of abnormality, suggesting a much higher degree of genomic imbalance in this tumor than has been reported previously (1–6, 24). In the single earlier CGH study of medulloblastoma, Schütz et al. (24) observed a frequency and pattern of CGH abnormalities that differed strikingly from the findings described here. For example, we identified an average of 7.4 regions of imbalance per tumor, whereas Schütz et al. reported only 2. Most of the regions demonstrating frequent loss in our series, including chromosomes 10q (41%), 11 (41%), 16q (37%), and 8p (33%), were reported to be either normal or only sporadically deleted by Schütz et al. Loss involving chromosome 17p, which was the sole region of recurrent loss identified in the Schütz et al. study and noted in 22% of their cases, in agreement with the 30–55% frequency reported by others (1). Finally, the regions of high-level amplification we report were not those observed by Schütz and co-workers.

Although the reason for these discrepancies is not entirely clear, at least three factors may have contributed. First, all of our samples were taken exclusively from primary tumors; Schütz et al. included both primary and pretreated tumors, the latter accounting for nearly 50% of their observed abnormalities. Secondly, universal amplification of tumor genomic DNA by degenerate oligonucleotide-primed PCR was required for five of the samples analyzed by Schütz et al., whereas sufficient tumor material was available to permit direct analysis of genomic DNA in all of our cases. Finally, we pursued a fundamentally different approach to defining regions of CGH abnormality, relying on a rigorous method of statistical analysis (16) that adjusted for both chromosomal and nonchromosomal variability (e.g., normal DNA contamination of tumor samples) to produce simultaneous 99% CIs for each mean green:red ratio. By contrast, Schütz et al. defined regions of CGH abnormality based on threshold values from historical data (24). Of note, 124 (62%) of the abnormalities identified by our model’s simultaneous 99% CIs and confirmed by reverse-labeling experiments had average ratio profiles that deviated less than the 0.25 threshold used by the Schütz group.

Losses involving chromosomes 10q, 11, and 16q have been identified in medulloblastoma by LOH (4, 6, 25) and cytogenetic analyses (1) but at significantly lower frequencies than those observed in the present study. The potential importance of genomic loss in these regions is highlighted by the rarity with which they showed gain by CGH (Fig. 1). We also observed frequent genomic loss on chromosomes 17p (37%) and 8 (33%), including a pattern consistent with an isochromosome q in two of nine cases involving 8p and five of nine cases involving 17p. Loss involving chromosome 8 has not been reported previously in medulloblastoma. A similar frequency of 17p loss/i(17q) has been observed by others (1, 2, 4–6, 26, 27). In contrast to prior cytogenetic studies, in which i(17q) was often observed as an isolated abnormality (1), all of the tumors in our series with an apparent i(17q) had multiple additional genomic alterations.

The pattern of loss observed in all of these regions was consistent with deletion of an entire chromosome or arm, although a consensus...
region of loss affecting the distal aspect of 10q was identified. In this regard, loss of distal 10q is one of the most common abnormalities in high-grade gliomas (28–30). The likelihood that one or more tumor suppressor genes reside in this region is strengthened by the demonstration that microcell-mediated transfer of chromosome 10 fragments into a human glioma cell line suppressed phenotypic transformation both in vitro and in vivo (31). Recently, a candidate tumor suppressor gene has been identified that maps to chromosome 10q23 and is mutated frequently in high-grade gliomas and other tumors (32, 33). Another candidate tumor suppressor on chromosome 10q is the MXII gene, which maps to 10q24–25 (13). Its product forms heterodimers with MAX that suppress transcription and compete with MYC for binding site occupancy, thereby antagonizing transcriptional activation of gene expression by MAX-MYC oncoproteins. The overall concordance of our FISH and CGH data reinforces the candidacy of MXII as a tumor suppressor in medulloblastoma.

In addition to chromosome 17q, overrepresentation of tumor genomic material most frequently involved chromosome 7. Of note, genomic loss involving chromosome 7 was not observed in this study. The mechanism by which low-level gain may contribute to tumorigenesis is difficult to define, because an increase in DNA copy number presumably leads to increased expression of multiple genes on these chromosomes. Gain of chromosome 7 has been noted frequently in medulloblastoma (1) as well as in other central nervous system (34–37) and extraneural tumors (38–40). The potential oncogenic contribution of this abnormality remains uncertain, inasmuch as trisomy 7 has also been detected in normal tissue adjacent to neoplasms lacking this change (41).

Our results corroborate previous studies demonstrating the rarity of oncogene amplification in medulloblastoma (3, 27, 42). Nonetheless, three of our samples contained amplicons that appear similar to those detected with CGH at 11q22–23 (35) and SplS (43) in patients with glioblastoma multiforme. The 11q22.3 site in the present study maps distal to the cyclin D1 locus on 11q13, which is known to be amplified in a variety of tumors (44). The fact that tumors containing amplicons in this study had significantly fewer abnormalities compared to tumors without amplicons (P = 0.04) suggests a major pathogenetic role for amplicons at 5p15.1 and 11q22–23 in some cases of medulloblastoma. All three cases with amplicons also demonstrated loss involving chromosome 9q, which contains the human PTC locus and is known to be mutated in sporadic medulloblastoma (45) as well as in Gorlin syndrome, an autosomal dominant condition that predisposes patients to medulloblastoma (46, 47). The pattern of chromosomal loss and amplification observed in these cases of medulloblastoma is analogous to the pattern of distal 1p loss and N-myc amplification observed in another childhood embryonal tumor, neuroblastoma (48).

Table 2 Summary of chromosome 10 CGH and MXII FISH results

<table>
<thead>
<tr>
<th>Case no.</th>
<th>DNA index</th>
<th>CGH result</th>
<th>FISH result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0; 1.82</td>
<td>Distal q arm loss</td>
<td>Loss (93%)</td>
</tr>
<tr>
<td>2</td>
<td>1.64; 2.62</td>
<td>Whole chromosome loss</td>
<td>Loss (57)</td>
</tr>
<tr>
<td>3</td>
<td>1.43</td>
<td>Whole chromosome loss</td>
<td>Disomy</td>
</tr>
<tr>
<td>4</td>
<td>1.0; 1.92</td>
<td>q arm loss</td>
<td>Loss (81)</td>
</tr>
<tr>
<td>5</td>
<td>1.26</td>
<td>Whole chromosome loss</td>
<td>Loss (70)</td>
</tr>
<tr>
<td>6</td>
<td>Not done</td>
<td>Normal</td>
<td>Gain</td>
</tr>
<tr>
<td>7</td>
<td>1.28; 2.44</td>
<td>Normal</td>
<td>Gain</td>
</tr>
<tr>
<td>8</td>
<td>1.0</td>
<td>Distal q arm loss</td>
<td>Loss (86)</td>
</tr>
<tr>
<td>9</td>
<td>1.59; 2.11</td>
<td>Whole chromosome loss</td>
<td>Disomy</td>
</tr>
<tr>
<td>10</td>
<td>1.0; 1.84; 3.5</td>
<td>Whole chromosome loss</td>
<td>Disomy</td>
</tr>
<tr>
<td>11</td>
<td>2.57; 2.23</td>
<td>Normal</td>
<td>Gain</td>
</tr>
<tr>
<td>12</td>
<td>1.0; 2.0</td>
<td>Normal</td>
<td>Disomy</td>
</tr>
<tr>
<td>13</td>
<td>1.46</td>
<td>Whole chromosome loss</td>
<td>Loss (52)</td>
</tr>
<tr>
<td>14</td>
<td>2.01</td>
<td>Normal</td>
<td>Gain</td>
</tr>
<tr>
<td>15</td>
<td>Not done</td>
<td>Normal</td>
<td>Disomy</td>
</tr>
<tr>
<td>16</td>
<td>1.0; 1.53; 1.86; 2.01</td>
<td>Normal</td>
<td>Gain</td>
</tr>
<tr>
<td>17</td>
<td>Not done</td>
<td>Normal</td>
<td>Disomy</td>
</tr>
<tr>
<td>18</td>
<td>1.0</td>
<td>Normal</td>
<td>Disomy</td>
</tr>
</tbody>
</table>

a Some tumors contained more than one population of malignant cells by flow-cytometric analysis of DNA content.

b Loss is defined as the presence of single centromeric and PI signals (indicating monosomy) or fewer PI signals than centromeric signals in >50% of the tumor cells. Disomy indicates the presence of two PI and centromeric signals in the majority of cells, whereas the presence of more than two signals from both the PI and centromeric probes defined copy number gain.

Fig. 2. Representative "standard" (top) and "reverse" (bottom) labeled CGH results, demonstrating an example of a tumor containing the amplicons that mapped to chromosomes 5p (A) and 11q (B) and an i(17q) (C). Each panel depicts a fluorescent micrograph and plot of the green:red fluorescence ratio (Y-axis) along the normalized chromosome length (X-axis), including the mean (middle line) with upper and lower 99% CIs.© 1997 American Association for Cancer Research.
Chromosome 10

Fig. 3. Representative medulloblastoma sample (sample 15) showing concordant distal 10q loss by CGH and FISH. A (CGH), mean fluorescence intensity (blue line) and simultaneous 99% CI (red lines) for chromosome 10. B (FISH), microphotograph demonstrating two copies of the chromosome 10 α satellite control signal (red) and one copy of the MXII probe signal (green) per interphase nucleus.

Our statistical analysis demonstrated a univariate association of genomic loss involving chromosomes 10q and 11 with the presence of metastatic disease at diagnosis, a known adverse prognostic feature of patients with medulloblastoma (49, 50). A similar analysis revealed that losses involving chromosomes 8p and 16q were more likely to occur together than in a random pattern, suggesting that lack of gene expression from these two regions may act in a complementary fashion in a multistep molecular pathway to medulloblastoma development. However, these findings must be regarded as preliminary, because they did not retain statistical significance upon adjustment for multiple comparisons. Additional study of larger numbers of patients will be needed to clarify the clinical importance of such genetic abnormalities in medulloblastoma.

In conclusion, the application of a mixed statistical model to CGH analysis has identified a more extensive complement of genomic aberrations in childhood medulloblastoma than has been reported previously. Learning how these changes contribute to tumorigenesis may prove to be an arduous task. It will be necessary, for example, to distinguish primary (tumor-initiating) abnormalities from secondary changes that do not play an early role in pathogenesis but are important for perpetuation of the malignant phenotype. Additional studies employing larger sample sizes and locus-specific molecular techniques are needed to confirm and extend these findings, determine their prognostic utility, and ultimately identify the target genes. Finally, future CGH studies would likely benefit from use of continuous 99% CIs, rather than fixed thresholds, to determine the importance of genomic imbalances.

ACKNOWLEDGMENTS

We thank John Gilbert for editing this manuscript and Dr. Edward Prochownik for the MXII PI FISH probe. The first author dedicates this work to the memory of his friend and mentor Mark S. Roth, M. D.

REFERENCES


Extensive Genomic Abnormalities in Childhood Medulloblastoma by Comparative Genomic Hybridization

David A. Reardon, Edson Michalkiewicz, James M. Boyett, et al.

*Cancer Res* 1997;57:4042-4047.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/57/18/4042

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/57/18/4042. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.