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Prognostic implications of chromosome 17p deletions in human medulloblastomas

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Abstract

DNA derived from medulloblastoma biopsies was analyzed to determine if deletions of the 17p region, mutations of the *TP53* gene, or amplification of the c-*myc*, N-*myc*, *EGFR* (epidermal growth factor receptor), or *MDM2* (murine double-minute-2) genes was indicative of a poor prognosis. Loss of heterozygosity for 17p, observed in 8/28 (29%) paired samples, was associated with a shortened survival period ($p = 0.045$ by the logrank test). *TP53* mutations occurred in 2/46 (4.3%) tumor samples. c-*myc* Amplification was seen in 3/43 (6.9%) cases, while none of the tumors contained amplified N-*myc, EGFR,* or *MDM2* genes. These results demonstrate that, while only rare medulloblastomas contain *TP53* gene mutations or amplification of the c-*myc* gene, loss of heterozygosity on chromosome 17p is indicative of a significantly worse prognosis among patients with these tumors. Further, these results provide a strong impetus for a prospective analysis of loss of heterozygosity in a cooperative group setting, which would include tumor staging, a selection of treatment modalities, and multivariate analyses.

Introduction

Medulloblastoma is a highly malignant tumor of the central nervous system with a marked propensity for both local parenchymal invasion and metastatic spread, particularly through the subarachnoid space. Treatment with maximal surgical resection plus neuraxis radiotherapy produces a 60–70% disease-free survival five years from diagnosis in the minority (30%) of children with completeley resected nonmetastatic tumors. Unfortunately, the majority (70%) of children display more advanced disease with only a 30–40% five-year disease-free survival. Features that are associated with a worse prognosis include extensive residual tumor following initial surgical intervention or a young age (< 2 years old). The predictive value of tumor ploidy or evidence for glial or neuronal differential remains speculative, and to date, no molecular or cytogenetic features have been identified, with data that is statistically significant, as being prognostic [1, 2].

Cytogenetic analysis has demonstrated abnormalities of chromosome 17 in 30 to 50% of these tumors, the most frequent abnormality being isochromosome 17q [i(17q)], resulting in 17p monosomy [3–5]. Loss of 17p has been confirmed by restriction fragment length polymorphism analysis [1, 6, 7]. Although tumor suppressor gene *TP53* is located on 17p, mutations of this gene are rare in medulloblastomas [7–10]. Double-minute chromosomes,

indicating gene amplification [4, 5], are seen in a subset of cases, and the gene most commonly amplified is c-*myc* [11]. In the present study, 17p deletions as determined by loss of heterozygosity (LOH) studies using microsatellite and minisatellite probes, *TP53* gene mutations, and amplification of the c-*myc,* N-*myc, EGFR* (epidermal growth factor receptor), and *MDM2* (murine double-minute-2) genes were evaluated in a series of medulloblastomas to determine their prognostic significance. *TP53* mutations were uncommon, occurring in 2 of 46 cases, as were examples of gene amplification, which consisted of c-*myc* amplification in 3 tumors. LOH for 17p, however, occurred in 8 of 28 (29%) paired samples, and the cases with 17p loss had a worse survival rate that was statistically significant as compared with the patients in whom 17p was retained.

Materials and methods

Tumor samples

A total of 56 (38 male and 18 female) tumor samples were obtained for the present study; 48 patients were 1 to 18 years old and 6 were greater than 18 years old. The diagnosis of medulloblastoma was confirmed histologically in each case. Frozen tissue was available for DNA extraction in 47 cases, and 9 samples were prepared from formalin-fixed, paraffin-embedded tumor tissues. Cryostat sections of the frozen tissues and stained sections of the paraffin-embedded tissues were examined to select the region containing a maximum number of malignant cells. Genomic DNA was extracted from the frozen tumor and blood as described earlier [12] and from paraffin sections by the method of Louis *et al.* [13].

LOH

Normal control genomic DNA was available in 28 cases. These paired medulloblastoma biopsies were analyzed for LOH via microsatellite probes, minisatellite probes, or both. Standard polymerase chain reaction (PCR) was carried out using simple sequence repeat microsatellite probes, D17S849, D17S796, D17S786, D17S804, D17S799, D17S520, D17S122, D17S261, D17S7805, and D17S798 obtained from Research Genetics (Huntsville, AL) and paired samples digested with appropriate restriction enzymes were evaluated by Southern analysis with minisatellite probes 144D6, YNZ 22.1, HRP5.5, HP53b, EW503, MYH2, YNM67, ERB, and THH59 obtained from American Type Culture Collection (Rockville, MD). LOH was scored if one of the two alleles in the tumor DNA was either absent or reduced in intensity by 80% as compared with normal DNA.

Gene amplification

Forty-three primary medulloblastomas were analyzed for gene amplification by either Southern or slot blot analysis or both, depending upon the availability of DNA, using a 1.6-kilobase (kb) *Sst*I fragment of pHSR-1 for c-*myc,* a 1-kb *Eco*Ri-*Bam*HI fragment of pE7 for *EGFR,* and a 0.9-kb *Xho*I fragment of c14-2 (a gift from Bert Vogelstein, Johns Hopkins University, Baltimore, MD) for the *MDM2* gene. Amplification was defined as a signal of greater than five times the reference signal.

PCR-SSCP analysis

Forty-six primary biopsies were analyzed for *TP53* alterations as determined by single-strand conformation polymorphism (SSCP) analysis of PCR products. Based on published *TP53* sequence data, oligonucleotide primers with 18 bases complementary to the sequence flanking the exon/intron junction were designed [14].

Nucleotide sequencing

Genomic DNA (1 μg) from tumors that exhibited SSCP band-shift, LOH on 17p, or elevated *TP53* immunostaining was amplified. The resulting 2.9-kb fragment (from exons 4 to 9) was subcloned in pBS+ vector (Stratagene, CA) and sequenced using Sequenase T4 DNA polymerase (U.S. Biochemicals,

Cleveland, OH). In tumors where SSCP band-shift was observed, only the relevant exon was sequenced. In other cases, exons 5–8 were sequenced. Sequencing was done at least three times on each sample.

Immunohistochemistry

Primary antibody (anti-*TP53* clone PAb 1801, Ab-2, Oncogene Sciences, Uniondale, NY) or a control isotype-matched monoclonal antibody at the same concentration was applied to frozen sections using the streptavidin-biotin-horseradish peroxidase system (Zymed Laboratories, San Francisco, CA).

Statistical analysis

The method of Kaplan-Meier [15] was employed to construct life tables for survival, and the logrank chi-square statistic was used to compare them [16]. Since the question is whether LOH is an adverse prognostic factor, statistical tests (p values) were one-tailed.

Results

LOH

LOH for 17p was observed in 8 of 28 samples (29%) as shown in Table 1. The PCR-based restriction fragment length polymorphism analysis using microsatellite probes showed that most of the samples were informative for heterozygosity. A representative gel showing an elevated degree of heterozygosity and LOH at the locus D17S796 is shown in Fig. 1.

TP53 alterations

A total of 46 medulloblastoma biopsies (16 paired plus 30 unpaired) were analyzed by SSCP analysis for hot exons 5 to 8 and by immunohistochemical staining to screen for point mutations in the *TP53* gene. A significant electrophoretic mobility shift

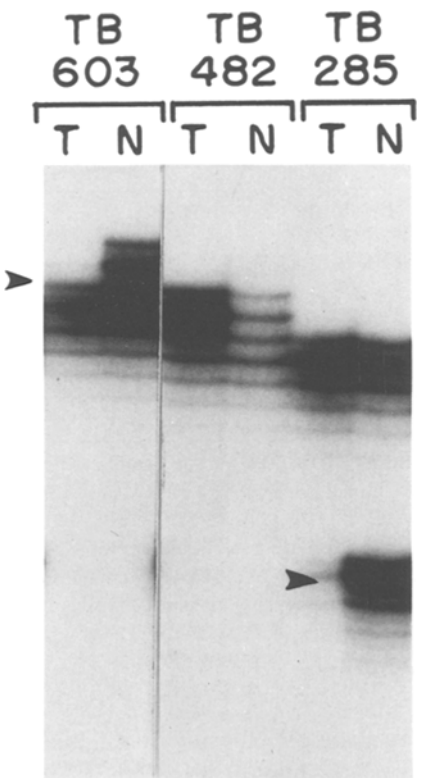

Fig. 1. A representative (CA)n repeat assay of three medulloblastoma tissue bank (TB) samples. Paired tumor (T) and blood (B) samples were amplified by polymerase chain reaction in the presence of [α-^{32}P]dCTP using matched pair primers specific to the locus D17S796, and the products were analyzed on denaturing 6% polyacrylamide gel electrophoresis. The arrows indicate loss of heterozygosity.

was detected in 1 of the 46 primary tumor samples: TB558 showed a mobility shift in exon 7. Three samples – TB285, TB346, and TB518 – showed positive reactivity on immunohistochemical staining. These samples were again screened on SSCP for other exons and for direct nucleotide sequencing as shown in Table 2. One tumor (TB558) with loss of the 17p region and a band-shift on SSCP analysis, however, was found to be mutated in exon 7 at codon 248. Another unpaired medulloblastoma, TB346, which showed a band-shift in exon 10, had a mutation at codon 342 resulting in termination of the *TP53* protein chain. Two of the biopsies that were found positive for immunostaining were normal for the *TP53* gene sequence in the hot exons and exon 10.

Table 1. Loss or retention of heterozygosity in chromosome 17 regions of 8 medulloblastoma biopsies as observed by microsatellite probes. A total of twenty-eight paired samples were analyzed; the 8 samples that demonstrated loss of regions on 17p are shown here.

17p							17p[b]	Around centromere		17q		17q[c]
TB#[a]	D17S849	D17S796	D17S786	D17S804	D17S799	D17S520		D17S122	D17S261	D17S805	D17S798	
208[d]	ND	ND	ND	ND	ND	ND	–	ND	ND	ND	ND	ND
219	–	–	+	+	+	–	–	ND	+	+	+	+
285	–	–	–	–	+	+	–	+	+	+	+	+
401	–	–	–	+	–	+	–	+	–	+	+	+
525	–	–	–	+	+	+	–	+	+	ND	+	+
558[e]	–	–	–	+	+	+	–	+	+	+	+	+
603[f]	–	–	–	–	+	–	–	–	–	–	+	+
656[f]	ND	–	–	+	ND	+	–	–	–	ND	ND	ND

[a] Abbreviations: TB, tissue bank; +, retention of heterozygosity; –, loss of heterozygosity; ND, not done.
[b] Cumulative 17p.
[c] Cumulative 17q.
[d] TB208 showed LOH using minisatellite probes only. A sample was not available for microsatellite analysis.
[e] TB558 was not informative for LOH with minisatellite probes.
[f] TB603 and TB656 samples were analyzed using microsatellite probes only.

Gene amplification

Forty-three primary biopsies of medulloblastoma were screened for amplification of the c-*myc*, N-*myc*, *EGFR*, and *MDM2* genes. Amplification of c-*myc* was found in 3 cases (TB172, TB173, and TB401), while none of the tumors demonstrated amplification of the N-*myc*, *EGFR*, or *MDM2* genes.

Prognostic significance

The Kaplan-Meier survival curve showing LOH for 17p in 8 of the 28 patients is shown in Fig. 2. The patients showing LOH for 17p survived for a shorter period of time as compared with the patients who had no LOH for 17p. A potentially significant trend was observed between the loss of 17p and a short-

Table 2. TP53 mutations in medulloblastomas as confirming by nucleotide sequencing. A total of 46 samples were screened for *TP53* alterations. The samples that showed LOH, band-shift on SSCP, or were positive for mutation are shown.

TB#[a]	LOH for 17p	Band-shift on SSCP	Immunostaining	Specific mutation in nucleotide sequence
208	Y	N	ND	N
219	Y	N	ND	N
285	Y	N	Y	N
401	Y	N	N	N
525	Y	N	N	N
558	Y[b]	Y, exon 7	Y	248, CGG → CCG ARG → PRO[c]
346	ND	Y, exon 10	Y	342, CGA → TGA ARG → STOP[c]
518	ND	N	Y	N
603[d]	Y	ND	ND	ND
656[d]	Y	ND	ND	ND

[a] Abbreviations: TB, tissue bank; Y, yes; N, no; ND, not done.
[b] LOH observed only by microsatellite probes.
[c] Number indicates the codon number of *TP53* protein.
[d] These samples could not be analyzed for *TP53* alterations and LOH via minisatellite due to nonavailability of sufficient DNA.

ened survival period. A logrank test showed a significant difference between the two groups ($p = 0.045$).

Tumors showing *TP53* mutations (2 out of 46) could not be analyzed for statistical significance due to the very small sample size. However, those patients whose biopsies (TB558 and TB346) showed *TP53* mutations also had shorter survival periods (6 and 9 months, respectively). Similarly, tumors having amplification of c-*myc* (TB172, TB173, and TB401) survived for 3.5, 2, and 9 months, respectively.

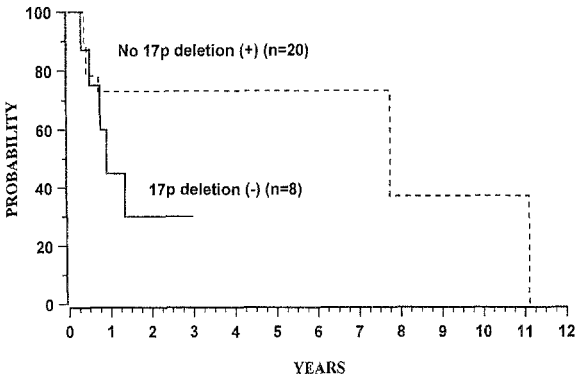

Fig. 2. Kaplan-Meier survival curve based on 17p deletion comparing patients whose tumors retained (n = 20) or lost (n = 8) heterozygosity. Fewer than 5 patients were followed for more than 2 years (5 of 8 deaths occurred within 2 years) of those who lost heterozygosity. Among those who retained heterozygosity, fewer than 5 were followed greater than 3 years (5 of the 7 deaths were within 1 year). A logrank test showed a significant difference between the two groups ($p = 0.045$).

Discussion

Cytogenetic work published earlier by this laboratory [3, 4] and other [5] has shown the most common abnormalities in medulloblastomas to be the occurrence of isochromosome 17q [i(17q)] and double-minutes. The present study was undertaken to examine the frequency of LOH for DNA sequences located on the short arm of chromosome 17; of molecular alterations in tumor suppressor gene *TP53* located within the deleted region on 17p; and of amplification of the c-*myc,* N-*myc,* and *EGFR* genes in a large number of medulloblastomas and to determine if these genetic alterations could be used to identify a subset of patients with worse prognoses.

LOH was found in 8 of 28 (29%) biopsies. The most commonly deleted region in this study was 17p13.1–13.3, harboring the tumor suppressor gene *TP53,* which has been implicated in the pathogenesis of divergent tumor types including cancer of the breast, lung, colon, esophagus, bladder, and liver; leukemias; and glial brain tumors. Loss of heterozygosity for regions located at 17p12–13.1 in many of these tumors is frequently associated with mutation in the *TP53* gene. The majority of *TP53* mutations in human cancers, including brain tumors, are located within the hot exons 5 to 8 of the *TP53* gene [17]. Among the medulloblastomas showing LOH for 17p, only one sample showed mutation of the *TP53* gene, and this occurred in exon 7 at codon 248 (Arg → Pro). The remaining cases did not show any mutations in hot exons 5 to 8. One unpaired tumor, TB346, showed a mutation in exon 10 at codon 342 (Arg → Stop), which resulted in the termination of the *TP53* peptide chain. This detection of a *TP53* mutation in exon 10, which lies outside the evolutionary conserved regions, is very infrequent and has been reported recently in astrocytoma [18]. The incidence of *TP53* mutations in our series was 2 of 46. A similar low incidence of *TP53* mutations in primitive neuroectodermal tumors or medulloblastomas has been reported by others for sample groups of 14 [7], 20 [1], 12 [10], and 19 [9]. These observations support the idea that, while mutations in this gene are uncommon in medulloblastomas, at least one third contain LOH for 17p. This discrepancy between frequent LOH for 17p and infrequent mutations of the *TP53* gene suggests the involvement of a previously undescribed tumor suppressor gene located distal to *TP53* on the short arm of chromosome 17.

Inactivation of the *TP53* gene is a common mechanism of progression in a large variety of neoplasms. Furthermore, in addition to intragenic mutations in *TP53,* other proteins involved in the regulation of its function can be targeted and produce the same physiological consequences as direct *TP53* alterations. Recently, *MDM2* has been shown to be a potential regulator of *TP53* activity and is amplified in sarcomas where *TP53* mutations are

uncommon [19]. Overexpression of *MDM2* protein can abolish the transactivating activity of *TP53*. Cytogenetic analysis of medulloblastomas has shown the presence of double-minute chromosomes in a subset of cases [3–5]. In the present series, none of the tumors with or without *TP53* mutations showed amplification of the MDM2 gene, suggesting that amplification of MDM2 is not an alternative molecular mechanism by which medulloblastoma escapes *TP53*-regulated growth control.

We also analyzed these tumors for amplification of oncogenes c-*myc*, N-*myc*, and *EGFR*, which are commonly overexpressed in pediatric tumors [20]. We detected amplification of the c-*myc* gene in 3 of 43 tumors. None of the tumors showed any amplification of the N-*myc* or the *EGFR* gene. These results indicate that oncogene amplification, as detected by Southern or slot blot analysis, is infrequent in medulloblastomas, and it represents the principle mechanism of oncogene activation only in a small subset of medulloblastomas.

Infrequent *TP53* Mutations (4.3%) and c-*myc* gene amplification (6.9%) could not be used for statistical analysis in the studies of survival. However, LOH for 17p, with a frequency of 29%, demonstrated a trend toward a shorter survival period in patients. A logrank test showed significant difference between the two groups ($p = 0.045$), with a trend towards a clear prognostic value. This study is the first to report that deletion of the 17p region is associated with a shortened survival period that is statistically significant in medulloblastomas. Loss of the 17p locus has been correlated without statistical significance with poor response to treatment in a limited number of medulloblastoma cases in an independent study [1, 2]. The demonstration by two independent investigations of loss of 17p as a prognostic indicator in medulloblastoma provides strong impetus for a prospective analysis of this parameter in a cooperative group setting, which would include tumor staging, a selection of treatment modalities, and multivariate analyses. The definition of a molecular variable capable of identifying medulloblastoma cases that are likely to progress rapidly would provide a powerful tool for the management of these patients.

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