Molecular Genetic Analysis of Ependymal Tumors

NF2 Mutations and Chromosome 22q Loss Occur Preferentially in Intramedullary Spinal Ependymomas

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Ependymal tumors are heterogeneous with regard to morphology, localization, age at first clinical manifestation, and prognosis. Several molecular alterations have been reported in these tumors, including allelic losses on chromosomes 10, 17, and 22 and mutations in the NF2 gene. However, in contrast to astrocytic gliomas, no consistent molecular alterations have been associated with distinct types of ependymal tumors. To evaluate whether morphological subsets of ependymomas are characterized by specific genetic lesions, we analyzed a series of 62 ependymal tumors, including myxopapillary ependymomas, subependymomas, ependymomas, and anaplastic ependymomas, for allelic losses on chromosome arms 10q and 22q and mutations in the PTEN and NF2 genes. Allelic losses on 10q and 22q were detected in 5 of 56 and 12 of 54 tumors, respectively. Six ependymomas carried somatic NF2 mutations, whereas no mutations were detected in the PTEN gene. All six of the NF2 mutations occurred in ependymomas of WHO grade II and were exclusively observed in tumors with a spinal localization (P = 0.0063). These findings suggest that a considerable fraction of spinal ependymomas are associated with molecular events involving chromosome 22 and that mutations in the NF2 gene may be of primary importance for their genesis. Furthermore, our data suggest that the more favorable clinical course of spinal ependymomas may relate to a distinct pattern of genetic alterations different from that of intracerebral ependymomas. (Am J Pathol 1999, 155:627–632)

Ependymal tumors are histologically classified into distinct entities. The World Health Organization (WHO) classification of tumors of the central nervous system distinguishes four major types of ependymal tumors: the myxopapillary ependymoma (WHO grade I), the subependymoma (WHO grade I), the ependymoma (WHO grade II), and the anaplastic ependymoma (WHO grade III).1 However, histological parameters alone appear to be of limited significance for the prognosis of patients with ependymomas and anaplastic ependymomas.2 The postoperative survival time of these patients also depends on clinical factors, such as age of onset, tumor location, and extent of tumor resection.3–5 For example, adult patients with ependymoma fare better than children with histopathologically comparable tumors.3 Moreover, spinal ependymomas are associated with a more favorable prognosis than intracranial ependymomas of the respective WHO grade.6

Recent studies have demonstrated the feasibility of molecular parameters for subdividing glioblastomas (GBM), oligodendrogliomas, and oligoastrocytomas into genetically distinct tumor groups that may be associated with a different prognosis and response to therapy.7–9 In contrast, no consistent alterations suitable for a molecular subdivision of ependymomas have been forwarded so far. Mutations in those genes frequently affected in gliomas such as TP53, CDKN2A, and EGFR have been shown to be rare.10–13 On the other hand, cytogenetic and molecular genetic analyses of ependymal tumors have indicated that loss of the long arm of chromosome 22 is a common aberration in these tumors.14,15 The gene for neurofibromatosis type 2 (NF2) maps to 22q12,16 and represents an interesting candidate gene for ependymomas, because patients with neurofibromatosis type 2 are predisposed to the development of ependymomas.18 However, previous analyses of NF2 on limited numbers of ependymal tumors produced conflicting results.11,19–22

Another interesting candidate tumor suppressor gene for ependymomas is the PTEN gene from 10q23, which is mutated in a significant fraction of glioblastomas.23–28

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Individual ependymomas with allelic losses on the chromosomal arm 10q have been reported. However, so far PTEN has not been investigated for mutations in these tumors. In the present study, we have therefore examined a series of 62 ependymal tumors of all major histological types for allelic losses on the chromosomal arms 22q and 10p, as well as for mutations in the entire coding regions of the \(NF2\) and \(PTEN\) genes.

**Materials and Methods**

**Tumor Specimens, Histopathology, and Control DNA**

Native tumor specimens and corresponding blood samples were obtained from patients treated at the University Hospital Bonn, the University Hospital Zurich, and the University Hospital Duesseldorf between 1990 and 1998. DNA from 10 tumors came from the University of Chicago Medical Center and was previously examined for mutations in seven of the 17 \(NF2\) gene exons. All tumors were classified according to the WHO guidelines. The tumor specimens were examined microscopically before phenolic DNA extraction to exclude contamination by nontumorous tissue. Our series of 62 tumor specimens included six myxopapillary ependymomas \(WHO\) grade I (E myx I; mean age 31 years), three subependymomas \(WHO\) grade I (SE; mean age 62 years), 29 ependymomas \(WHO\) grade II (E II; mean age 37 years), 23 anaplastic ependymomas \(WHO\) grade III (E III; mean age 20 years), and one ependymoblastoma \(WHO\) grade IV (EB; age 5 years). Seven of the tumors were located in the lateral ventricles, six in the third ventricle, 26 in the fourth ventricle, and 23 in the spinal cord. Tumor types and locations are summarized in Table 1.

**Microsatellite Analysis for Loss of Heterozygosity**

To identify allelic losses on the chromosomal arms 10q and 22q, the following primer pairs were used for nonradioactive microsatellite analysis: D10S676, D10S677, D22S684, D22S258, and NF2-CA3. Polymerase chain reaction (PCR) was performed in a final volume of 10 \(\mu\)l containing 10 ng of DNA, 50 mmol/L KCl, 20 mmol/L Tris-HCl (pH 8.4), 200 \(\mu\)mol/L of each deoxynucleoside triphosphate, 0.1% gelatin, 10 pmol of each primer, 1.5–2.5 mmol/L \(MgCl_2\), and 0.25 U \(Taq\) polymerase (Gibco BRL, Gaithersburg). Initial denaturation at 94°C for 5 minutes was followed by 30 cycles on an automated thermal cycler (Hybaid; Omnigene). These included denaturation at 94°C for 40 seconds, annealing at temperatures from 50°C to 62°C, depending on the primer pair for 40 seconds and extension at 72°C for 60 seconds. A final extension step at 72°C for 10 minutes was added. PCR products were separated on 8% denaturing acrylamide gels and visualized by silver staining. Loss of heterozygosity (LOH) was scored as previously described.

**Single-Strand Conformation Polymorphism Analysis and Direct Sequencing**

For analysis of the \(PTEN\) and \(NF2\) genes two sets of previously published primers were used. \(PTEN\) PCR was performed in a final volume of 10 \(\mu\)l containing 10 ng of DNA, 50 mmol/L KCl, 20 mmol/L Tris-HCl (pH 8.4), 200 \(\mu\)mol/L of each deoxynucleoside triphosphate, 0.1% gelatin, 10 pmol of each primer, 1.0–2.0 mmol/L \(MgCl_2\), and 0.25 U \(Taq\) polymerase (Gibco BRL). Initial denaturation at 94°C for 3 minutes was followed by 35 cycles on an automated thermal cycler (Biometra UNO Thermoblock, Goettingen, Germany). These included denaturation at
94°C for 40 seconds, annealing at temperatures ranging from 50°C to 62°C (depending on the primer pair) for 40 seconds, and extension at 72°C for 40 seconds. A final extension step at 72°C for 10 minutes was added. Single-strand conformation polymorphism (SSCP) analysis was performed on a sequencing apparatus (Pokerface II; Hoefer, San Francisco, CA), using 8%, 10%, 12%, and 14% acrylamide gels. Electrophoresis was run at 3–40 W and variable temperatures for 15 h. Silver staining of the gels was performed as previously described. Aberrantly migrating SSCP bands were excised and the DNA was extracted, followed by reamplification with the same set of primers and sequencing on a semiautomatic sequencer (Applied Biosystems; model 373A), using a Taq cycle sequencing kit (Applied Biosystems). Each amplicon was sequenced bidirectionally.

**Table 2. NF2 Mutations in Six Patients with Ependymomas**

<table>
<thead>
<tr>
<th>Tumor no.</th>
<th>WHO</th>
<th>Age</th>
<th>Exon</th>
<th>Alteration</th>
<th>Consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>3546</td>
<td>E II</td>
<td>54</td>
<td>10</td>
<td>907/908 del 1bp (A)</td>
<td>303fs→308X</td>
</tr>
<tr>
<td>3640</td>
<td>E II</td>
<td>43</td>
<td>13</td>
<td>1396 C→T</td>
<td>Arg466X</td>
</tr>
<tr>
<td>4314</td>
<td>E II</td>
<td>52</td>
<td>7</td>
<td>675 + 5 g→a</td>
<td>Splice donor</td>
</tr>
<tr>
<td>7336</td>
<td>E II</td>
<td>67</td>
<td>1</td>
<td>99/100/101 ins 1bp (G)</td>
<td>33fs→48X</td>
</tr>
<tr>
<td>7882</td>
<td>E II</td>
<td>55</td>
<td>7</td>
<td>675 + 1 g→a</td>
<td>Splice donor</td>
</tr>
<tr>
<td>9582</td>
<td>E III</td>
<td>34</td>
<td>5</td>
<td>465 C→T</td>
<td>Polymorphism Pro156Pro</td>
</tr>
<tr>
<td>9606</td>
<td>E II</td>
<td>15</td>
<td>5</td>
<td>448–464 ins 7bp</td>
<td>152fs→153X</td>
</tr>
</tbody>
</table>

Abbreviations: fs = frameshift; X = stop codon.

Statistical Analysis

The correlations of age versus location and age versus NF2 mutations were evaluated by the unpaired t-test. The associations of NF2 mutations with tumor location and NF2 mutations with LOH 22 were analyzed by the Fisher Exact Test.

**Results**

**Loss of Heterozygosity on Chromosomal Arms 10q and 22q**

LOH at microsatellite markers from 22q was detected in tumors from 12 of 54 informative ependymoma patients (22%). Among these, nine were diagnosed as E II, two as E III, and one as EB IV. LOH at one or more loci on 10q was detected in tumors from five of 56 informative ependymoma patients (9%). Among these, one was diagnosed as E II and four as E III. The data are summarized in Table 1.

SSCP and Sequencing of the NF2 and PTEN Genes

SSCP analysis was performed for all 17 exons of the NF2 gene, including the splice sites. PCR-amplified exons showed an aberrant migration pattern in seven of the 62 ependymomas. One of these seven patients exhibited the respective alteration in constitutional DNA from peripheral blood leukocytes. DNA sequencing of this case revealed a silent mutation that was interpreted as a sequence polymorphism.

In the remaining six patients, no SSCP shifts were obtained for constitutional DNA, indicating that the aberrant bands obtained from the tumor DNA represent somatic mutations. This assumption was confirmed by DNA sequencing, which revealed mutations affecting splice sites in two tumors, frameshift mutations (two deletions, one insertion) with the introduction of premature stop codons in three tumors, and a nonsense mutation creating an immediate stop codon in one tumor. The positions and types of sequence alterations are compiled in Table 2. Representative data are shown in Figure 1. All tumors with NF2 mutations were intramedullary spinal E II.

![Figure 1](image-url)
SSCP analysis of all coding exons of the PTEN gene did not reveal aberrantly migrating bands in any region of the 62 ependymal tumors investigated. However, DNA controls from GBMs with known PTEN mutations showed the expected alterations with the assay conditions applied.

Association with and Correlation of Clinical, Morphological, and Molecular Features

Four of the six patients with somatic NF2 mutations were informative for at least one of the microsatellite markers from 22q analyzed. All four tumors displayed LOH at these loci, a finding indicating complete loss of wild-type NF2 in the tumor cells. LOH at loci from 22q was found in tumors from eight of 50 informative patients without a detectable NF2 mutation, including five of 21 informative E II. There was a significant association between NF2 mutation and LOH on 22q for the group of WHO grade II ependymomas (P = 0.01) as well as all ependymal tumors evaluated (P = 0.0016). NF2 mutations were detected in six of 14 intramedullary spinal E II, but in 0 of 15 intracranial E II. Thus NF2 mutations were closely associated with an intramedullary spinal localization of the tumor (P = 0.0063). The six patients (mean age 48 years) with tumors carrying a NF2 mutation were significantly older than the remaining 56 patients (mean age 29 years) of our series (P = 0.0439). In addition, the 23 patients with spinal ependymomas (mean age 41 years) were significantly older than the 39 patients with intracranial ependymomas (mean age 24 years; P = 0.0037). This association between age and tumor location (intramedullary spinal; mean age 47 years versus intracranial; mean age 27 years) remained significant, when only the 29 patients with an E II were considered (P = 0.0093).

Discussion

The present study reveals NF2 mutations in a high percentage (43%) of intramedullary spinal E II but fails to identify any NF2 mutations in intracranial E II as well as all E I myx, SE I, and E III studied. The number of NF2 mutations in intramedullary spinal E II may even be higher, given the limited sensitivity of SSCP analysis. Our findings confirm and explain previous data from independent studies that have yielded contradictory results. Whereas four studies11,20–22 analyzing a total of 38 ependymomas, four of which were of intramedullary spinal location, detected only one NF2 mutation in an intramedullary ependymoma, another report found five tumors with NF2 mutations among seven intramedullary spinal ependymomas.19 Our series of 62 ependymomas included 29 E II, 14 of which were intramedullary spinal and 15 were intracranial lesions. NF2 mutations were detected in six of 14 intramedullary but none of 15 intracranial E II (P = 0.0063). This nonrandom distribution suggests the existence of genetically distinct subsets among E II.

Cytogenetic alterations and allelic losses involving the long arm of chromosome 22 represent well-known aberrations in ependymal tumors.14,15,33–36 In our study, LOH 22 was most prevalent in intramedullary spinal E II and was seen in six of 11 informative cases. All informative intramedullary spinal E II patients with an NF2 mutation exhibited LOH 22. Although LOH on chromosome 22 occurred more frequently in patients with intramedullary than with intracranial ependymoma WHO grade II, this association failed to reach significance (P = 0.2). The association of LOH 22 with the presence of NF2 mutations suggests a close relationship between these alterations in E II. LOH 22 was also observed in three of 14 intracranial E II, in one of 17 anaplastic intracranial E III, and in one of two anaplastic intramedullary spinal E III. Because there was no association between LOH 22 and NF2 mutations among intracranial ependymomas, these data may either suggest the presence of another tumor suppressor gene on chromosome 22 or result from a chromosomal instability causing random deletion of genomic material. A putative chromosome 22 tumor suppressor gene other than NF2 has been proposed in cytogenetic studies.36 However, our data neither support nor exclude the existence of a further ependymoma-associated tumor suppressor gene on chromosome 22. It shall be interesting to await the analysis of the INI1/hSNF5/SMARCB1 tumor suppressor gene on chromosome 22, which has been shown to be mutated in pediatric rhabdoid tumors.37

Clinical data from previous reports support the hypothesis of distinct biological subsets of ependymoma. Intramedullary spinal ependymomas differ from intracranial ependymomas with respect to age at diagnosis and to prognosis. Patients with intramedullary spinal ependymoma are, on average, approximately 40 years of age.6,38–40 These spinal tumors are associated with a significantly better prognosis than their intracranial counterparts.6,41 Thus ependymomas are likely to include genetically different tumor entities, as has already been shown for glioblastomas and oligoastrocytomas. GBMs carrying TP53 mutations (termed GBM type 1) predominantly occur in younger patients and constitute the majority of secondary GBMs (ie, GBMs with a previous history of lower grade astrocytoma) and giant cell GBMs, whereas those without TP53 mutations (termed GBM type 2) usually develop as primary GBMs in patients of advanced age.7 Oligoastrocytomas fall into groups genetically resembling astrocytomas or oligodendrogliomas, depending on the presence of TP53 mutations or allelic losses on the chromosomal arms 1p and 19q.8 In the case of oligoastrocytomas this distinction may be of major clinical relevance because allelic losses on 1p and 19q have been shown to be a highly predictive indicator for the chemosensitivity of anaplastic oligodendrogliomas.9 Likewise, molecular subdivision of ependymomas may prove to be of importance for future therapeutic procedures.

One study presented results hinting at the involvement of a chromosome 10 tumor suppressor gene in ependymomas.29 The PTEN gene is a likely tumor suppressor candidate for gliomas with allelic deletions on chromo-
some 10. None of the 62 ependyomomas of our series was identified as carrying a mutation in this gene. These data probably exclude PTEN as an ependymoma relevant tumor suppressor gene. On the other hand, chromosome 10 may harbor other tumor-associated genes. An interesting candidate is the DMBT1 gene, which has been shown to be homozygously deleted in up to 38% of GBMs. Among 56 ependymomas informative for markers on the long arm of chromosome 10, LOH was observed in four E III and one E II. The higher fraction of LOH in E III may reflect an increase of genomic instability in these tumors or indicate an association with malignant progression. In the present series only two markers on 10q were examined. However, allelic losses on chromosome 10 in gliomas predominantly include both arms and usually correspond to the loss of an entire chromosome. We therefore feel that our data do not support a prominent role for a tumor suppressor gene on chromosome 10 in the pathogenesis of ependymomas.

In conclusion, the present study of a large series of intramedullary spinal and intracranial ependymomas strongly supports the hypothesis that intramedullary spinal E II constitutes a molecularly distinct tumor entity characterized by a high incidence of LOH on 22q and NF2 gene mutations. Mutations in the PTEN gene on chromosome 10 seem not to play a significant role in ependymomas. The involvement of other putative tumor suppressor genes on chromosomal arms 22q and 10p/q merits further investigation.

**Acknowledgment**

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**References**

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