Identification of Gains on 1q and Epidermal Growth Factor Receptor Overexpression as Independent Prognostic Markers in Intracranial Ependymoma

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Abstract

Pathogenesis of ependymomas is still poorly understood and molecular markers for risk-adapted patient stratification are not available. Our aim was to screen for novel genomic imbalances and prognostic markers in ependymal tumors.

Experimental Design: We analyzed 68 sporadic tumors by matrix-based comparative genomic hybridization using DNA microarrays containing 6,400 genomic DNA fragments. Novel recurrent genomic gains were validated by fluorescence in situ hybridization using a tissue microarray consisting of 170 intracranial ependymomas. Candidate genes were also tested for mRNA expression by quantitative real-time PCR, and protein expression was determined by immunohistochemistry on the tissue microarray.

Results: Chromosomal gain of 1q correlated with pediatric patients (P = 0.004), intracranial ependymomas (P = 0.05), and tumors of grade III (P = 0.002). Gain of 1q21.1-32.1 was associated with tumor recurrence in intracranial ependymomas (P < 0.001). Furthermore, gain of 1q25 as determined by fluorescence in situ hybridization represented an independent prognostic marker for either recurrence-free survival (P < 0.001) or overall survival (P = 0.003). Recurrent gains at 5p15.33 covering hTERT were validated by immunohistochemistry, and elevated protein levels correlated with adverse prognosis (P = 0.01). In addition to frequent gains and high-level amplification of epidermal growth factor receptor (EGFR) at 7p11.2, immunohistochemistry revealed protein overexpression to be correlated with poor prognosis (P = 0.002), EGFR protein status subdivides intracranial grade II ependymomas into two different risk groups (P = 0.03) as shown by multivariate analysis.

Conclusions: Thus, the states of 1q25 and EGFR represent independent prognostic markers for intracranial ependymomas to identify patient subgroups with different risk profiles in further clinical investigations. Moreover, EGFR might serve as therapeutic target for more specific chemotherapy applications.

Ependymomas originate from the ependymal monolayer of the cerebral ventricles or from the central canal of the spinal cord. These tumors represent the third most common brain tumors of childhood accounting for ~10% of cases but may also occur in adults (1, 2). Intracranial tumors are predominant in pediatric patients (90%), whereas most adults show spinal manifestation (60%; refs. 3, 4). Spinal tumor localization is usually associated with a favorable prognosis after gross total resection and does not need to be treated with adjuvant therapy (5, 6). In contrast, intracranial ependymomas tend to local tumor progression, which is reflected by a 5-year overall survival of only ~60% (7, 8). The influence of the recent histologic ependymoma classification (WHO 2000) on clinical outcome, and the relationship between ependymoma grade and specific chromosomal aberrations is controversially discussed (2, 4, 9–12). Further important aspects of intracranial tumors remain challenges that urgently need to be addressed, including tremendous clinical heterogeneity (13) and tumor recurrence, which is associated with poor survival (14).

Little is known about the genetic mechanisms underlying ependymal neoplasms and no molecular marker of clinical relevance has been identified thus far. Cytogenetic studies by conventional comparative genomic hybridization (CGH) revealed numerous chromosomal aberrations in these heterogenous tumors, such as a 30% to 50% incidence of aberrations involving chromosome 22, including monosomy 22 and deletions of 22q (9, 12, 15). Neurofibromatosis type 2 is associated with spinal ependymomas in ~5% of cases (16),
indicating a role of the NF2 tumor suppressor gene at 22q12.2 in these tumors (17, 18). RNA expression profiling using DNA microarrays identified differences in ependymoma subgroups and potential candidate genes on chromosome 22q (13, 19). About 40% of pediatric ependymomas are reported to display balanced profiles by conventional CGH in comparison with only 10% in adults (9, 10, 12). Therefore, it has been suggested that the development of ependymomas at younger age might often be independent of chromosomal instability.

Because no genetic factors to predict patients’ outcome are available thus far, our aims were to identify novel genomic imbalances by means of high-resolution genomic profiling and to validate their prognostic potential in a large series of ependymal tumors. Accordingly, we did matrix-based CGH of 68 sporadic tumors using a DNA microarray consisting of 6,400 genomic fragments (6K array), which cover the human genome with an average resolution of 0.5 Mb (20–24).Selected candidate genes of frequently aberrant genomic regions were additionally tested for mRNA expression by quantitative real-time PCR (QRT-PCR) in 10 representative tumor samples. Confirmation of gained regions by fluorescence in situ hybridization (FISH) and validation of array-based data by immunohistochemistry were done using a tissue microarray consisting of 170 clinically well-characterized intracranial ependymomas. Potential markers were correlated with clinicopathologic data in univariate and multivariate analyses.

Materials and Methods

Tumor material and patient characteristics. All samples used in this study were randomly collected at the Department of Neuropathology, Burdenko Neurosurgical Institute (Moscow, Russia) between 1987 and 2003. All diagnoses were confirmed by histologic assessment of specimens obtained at surgery by at least two neuropathologists according to the criteria of the 2000 WHO classification (2). These tumors were classified by histology as myxopapillary (grade I), classic (grade II), or anaplastic (grade III) ependymomas, considering the criteria for nonmyxopapillary tumors presented by Merchant et al. (25).

Approval to link laboratory data to clinicopathologic variables was obtained by the institutional review board. Tumor and patient characteristics are summarized in Table 1. Evaluation of the extent of resection is based on postoperative contrast computed tomography or magnetic resonance imaging. Only patients with intracranial ependymomas received radiotherapy or chemotherapy. Megavoltage external beam irradiation was applied to 105 patients represented on the tissue microarray and to 32 patients of the tumors subjected to matrix-based CGH. Radiotherapy was initiated 2 to 3 weeks after surgery. Total radiation doses varied from 52 to 64 Gy and conventional fractioning was used for all irradiated patients with 1.6 to 2.0 Gy per fraction. Adjuvant chemotherapy using lomustine, cisplatin, and vincristine was administered to 36 patients of the tissue microarray study and to 18 patients analyzed by matrix-based CGH (26).

On June 1, 2005, the 170 ependymoma patients included in the tissue microarray study had a median follow-up time of 62 months, a median overall survival of 112 months (range, 3–140 months), and a median recurrence-free survival of 72 months (range, 3–137 months), estimated according to Korn (27). One hundred ninety patients survived, whereas 51 patients died during follow-up. All 51 deceased patients displayed local tumor recurrence.

Microarray production and hybridizations. Selection of genomic clones, isolation of BAC DNA, performance of degenerate oligonucleotide primer-PCR, and preparation of microarrays were done as described previously (21, 28, 29). Genomic DNA from tumor tissue and blood from healthy donors was isolated using the Blood and Cell Culture kit (Qiagen, Hilden, Germany) following the instructions of the suppliers. Labeling, hybridization, and washing procedures were done as reported previously (21).

Microarray data analysis. Raw data processing and normalization was done as reported previously (21), except for the filter settings (intensity/local background >3; mean/median intensity <1.3; SD of clone log 0.153 (log2 ratio) for scoring gains or losses. A region was scored as imbalanced if at least two adjacent clones reached the threshold ratios. Imbalances with log2 ratios of less than –1 were scored as homozygous deletions because this value corresponds to an average copy number of <1, indicating the presence of at least a subpopulation of cells with a homozygous deletion. Copy number gains with log2 ratios higher than 1 were scored as amplifications. The chromosomal mapping information is based on the Ensembl database (version 28) and the University of California at Santa Cruz genome database (May 2004). All data sets are accessible via the public National Center for Biotechnology Information Gene Expression Omnibus (GSE3435 and GPL2920).

RNA isolation and QRT-PCR. Total RNA isolation, QRT-PCR, and relative quantification was carried out as published previously (22). As a standard for normalization, a reference of pooled total RNA was used (Stratagene, La Jolla, CA). Primer sequences for each target gene [HOXC4, epidermal growth factor receptor (EGFR), dual-specificity phosphatase 12 locus (DUSP12), CDKN2A, ARHGEF5, hTERT, transforming, acidic coiled-coil containing protein 2 (TACC2), and MINPP1] and the housekeeping genes (PGK1 and LMB) are available upon request.

Preparation of tissue microarrays. H&E-stained sections from each paraffin block were prepared to define representative tumor regions. Microarray preparation was done as described previously (31). After preparation, the tissue sections were again H&E stained and reevaluated by pathologists.

Immunohistochemistry. Antibodies against the following antigens were used: EGFR (rabbit polyclonal, clone sc-03, 1:250; Santa Cruz Biotechnology, Santa Cruz, CA), p16 (mouse monoclonal, clone DCS-50, 1:20; Progen, Heidelberg, Germany), hTERT (mouse monoclonal, clone 44F12, 1:50; Novocastra, Newcastle, United Kingdom), and ARHGEF5 (mouse monoclonal, clone 3A12-B5, 1:100; Abnova Corp., Jhongli City, Taiwan). Immunostaining, evaluation, and semiquantitative categorization of nuclear (hTERT and p16) or cytoplasmic/membranous (EGFR and ARHGEF5) protein expression was done as described recently (22).

Fluorescence in situ hybridization. Two-color FISH to the tissue microarrays was done using a Spectrum Orange–labeled probe for 1p36 and a Spectrum Green–labeled 1q25 probe (Vysis, Downers Grove, IL). Pretreatment of slides, hybridization, posthybridization processing, and signal detection were done as outlined previously (32).

Statistical analysis. Estimation of survival time distributions was done according to the method of Kaplan and Meier. For pairwise comparisons of survival time distributions, the log-rank test was used. Pairwise comparisons of other patient characteristics were done by the Mann-Whitney test for continuous variables and by Fisher’s exact test for categorical variables.

For the matrix-based CGH study, the copy number data of 5,708 autosomal clones were discretized to –2 (biallelic loss), –1 (loss),

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0 (balanced), +1 (gain), and +2 (amplification). To identify regions of clones that best characterize ependymoma samples according to patients' age, tumor localization, and tumor grade, we used the method of nearest shrunken centroid classification as proposed by Tibshirani et al. (33), available as software for prediction analysis of microarrays. This approach shrinks each class centroid (mean) toward the overall centroid (mean). Classification is then made to the nearest shrunken centroid or prototype. We used 10-fold cross-validation to select the classifiers defined by a subset of clones. To identify prognostically relevant regions of clones, we applied a new method of penalized Cox regression called smoothly clipped absolute deviation (SCAD; ref. 34). This method selects variables and estimates coefficients simultaneously. The implementation of the method includes singular value decomposition of the design matrix as preliminary step (35). In addition to BACs, patients' age and level of resection were included as possible prognostic factors.

In the tissue microarray study, multivariate analysis of the dependence of survival times on candidate protein levels or chromosomal gain of 1p36 and 1q25 together with relevant clinicopathologic variables was done by Cox proportional hazards regression. Missing data were estimated with a multiple-imputation technique using predictive mean matching with n = 50 imputations.

To provide quantitative information about the relevance of results of the statistical analyses, hazard ratios and their corresponding 95% confidence intervals were computed. All statistical computations were done using the statistical software environment R version 2.1.1 (R Development Core Team, 2004) together with R packages multtest and pamr from the Bioconductor project (36).

Results

Chromosomal imbalances in ependymomas. In this study, 68 primary tumors were analyzed by high-resolution matrix-based CGH. With respect to tumor localization, frequency plots of all detected chromosomal imbalances are shown in Fig. 1. The most recurrent aberrations affecting large chromosomal regions in spinal tumors were gains of 7q11.23-22.1 [11 of 19 (58%)] and 9p24.3-qter (58%) as well as losses of 22q (53%) and 14q (32%). Intracranial tumors showed recurrent gross gains of 12q13.13-13.3 [17 of 49 (35%)] and losses of 22q (38%) or 6q25.3-qter (33%). Independent from tumor localization, common chromosomal imbalances included gains of chromosomes 7, 9, 15q, and 18 as well as losses of 22q (53%) and 14q (32%). Intracranial tumors showed recurrent gross gains of 12q13.13-13.3 [17 of 49 (35%)] and losses of 22q (38%) or 6q25.3-qter (33%). Independent from tumor localization, common chromosomal imbalances included gains of chromosomes 7, 9, 15q, and 18 as well as losses of 22q. In contrast, typical aberrations for spinal tumors were gains of chromosome 16 and losses of 14q, whereas intracranial tumors specifically showed gains of 1q and losses of 6q. With exception of two pediatric cases [2 of 29 (7%)], all tumors displayed DNA copy number imbalances in at least one chromosomal region.
No significant differences were found between clinicopathologic subgroups, including patients’ age, tumor localization, and tumor grade (Table 1), and number of chromosomal aberrations. We detected three qualities of chromosomal profiles in ependymal tumors: (a) almost balanced tumors displaying up to two gross chromosomal imbalances [10 of 68 (15%)], (b) tumors that show more than two aberrations and nearly diploid state [36 of 68 (53%)], and (c) tumors with more than two aberrations that seem to be aneuploid [22 of 68 (32%)].

**Hotspots of DNA copy number aberrations.** By superimposing all genomic profiles, minimally overlapping regions of DNA copy number imbalances were identified. Regions smaller than 3 Mb, which were imbalanced in at least five tumors (termed “genomic hotspots”), and all loci exhibiting amplification or homozygous deletion are listed in Table 2. A single homozygous deletion spanning the CDKN2A gene at 9p21.3 was detected in a grade III ependymoma with intracranial tumor localization obtained from a pediatric patient. At 7p11.2, an amplification covering the EGFR locus was found in one occasion in a grade II ependymoma with spinal manifestation of an adult patient. The most frequent genomic hotspots for losses were detected on chromosome arm 10q (e.g., MINPP1 and TACC2) and at the distal short arm of chromosome 9 at 9p24.31 (FOXD4). Highly recurrent gains were found at 7q34 (ARHGEF5), 12q13.13 (HOXC4), and 5p15.33 (hTERT).

**Correlations between genomic imbalances and ependymoma subgroups.** We applied prediction analysis of microarrays to the data sets to identify chromosomal imbalances, which are associated with clinicopathologic variables (Table 1). The prognostic signatures consisted of the following clone sets: patients’ age, 1,252 clones; tumor localization, 955 clones; and tumor grade, 1,685 clones. Subsequently, we correlated gains and losses derived from the prognostic signature to certain patient subgroups by Fisher’s exact test and identified numerous significant associations (Fig. 2A). Notably, gains of 1q were significantly correlated with pediatric patients, intracranial tumor localization, and grade III tumors.
**Table 2. DNA copy number aberrations in 68 ependymomas**

<table>
<thead>
<tr>
<th>Localization</th>
<th>Count</th>
<th>Start clone</th>
<th>End clone</th>
<th>Size (Mb)</th>
<th>Candidate gene</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gains</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1q23.3</td>
<td>11</td>
<td>RP11-5K23</td>
<td>RP11-47H16</td>
<td>0.14</td>
<td>DUSP12</td>
</tr>
<tr>
<td>5p15.33</td>
<td>16</td>
<td>RP11-117B23</td>
<td>RP11-325I22</td>
<td>0.30</td>
<td>hTERT</td>
</tr>
<tr>
<td>7q34</td>
<td>26</td>
<td>RP11-811J9</td>
<td>RP11-298A10</td>
<td>0.14</td>
<td>EGFR</td>
</tr>
<tr>
<td>10q26.3</td>
<td>10</td>
<td>RP11-140A10</td>
<td>RP13-449N13</td>
<td>0.18</td>
<td>DUSP12</td>
</tr>
<tr>
<td>11p12.1</td>
<td>14</td>
<td>RP11-164L18</td>
<td>RP11-411D10</td>
<td>0.73</td>
<td>MDK</td>
</tr>
<tr>
<td>12q13.3</td>
<td>23</td>
<td>RP11-657H18</td>
<td>RP11-834C11</td>
<td>0.33</td>
<td>HOXC4</td>
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<tr>
<td>14q32.33</td>
<td>7</td>
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<td>RP11-521B24</td>
<td>0.39</td>
<td>MTA1</td>
</tr>
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<td>16p11.2</td>
<td>11</td>
<td>RP11-408D2</td>
<td>RP11-261D10</td>
<td>2.30</td>
<td>SLC6A10</td>
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<td><strong>Losses</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>RP11-244H3</td>
<td>RP4-765A10</td>
<td>0.59</td>
<td>ZNF262</td>
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<tr>
<td>9p24.31</td>
<td>11</td>
<td>GS1-41L13</td>
<td>GS1-77L23</td>
<td>0.34</td>
<td>FOXD4</td>
</tr>
<tr>
<td>10q23.2</td>
<td>11</td>
<td>RP11-351D13</td>
<td>RP11-539A10</td>
<td>0.10</td>
<td>PRKCA</td>
</tr>
<tr>
<td>10q23.31</td>
<td>13</td>
<td>RP11-157H10</td>
<td>RP11-399L7</td>
<td>0.20</td>
<td>MINPP1</td>
</tr>
<tr>
<td>10q26.13</td>
<td>11</td>
<td>RP11-500G22</td>
<td>RP11-105F10</td>
<td>0.30</td>
<td>TACC2</td>
</tr>
<tr>
<td>10q26.3</td>
<td>12</td>
<td>RP13-491I5</td>
<td>RP11-122K13</td>
<td>0.17</td>
<td>TUBGCP2</td>
</tr>
<tr>
<td>11q22.2</td>
<td>5</td>
<td>RP11-864G5</td>
<td>RP11-817J15</td>
<td>0.50</td>
<td>BIRC2</td>
</tr>
<tr>
<td>17q24.2</td>
<td>7</td>
<td>RP11-115N5</td>
<td>RP11-4F22</td>
<td>0.67</td>
<td>PRKCA</td>
</tr>
<tr>
<td><strong>Amplifications</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7p11.2</td>
<td>1</td>
<td>RP5-1091E12</td>
<td>RP11-339F13</td>
<td>0.34</td>
<td>EGFR</td>
</tr>
<tr>
<td><strong>Homozygous deletions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9p21.3</td>
<td>1</td>
<td>RP11-113D19</td>
<td>RP11-33K8</td>
<td>3.01</td>
<td>CDKN2A</td>
</tr>
</tbody>
</table>

*Genomic hotspots. Defined as imbalanced in at least five tumors (gains or losses) and smaller than 3 Mb.
suppressor p16 in addition to ARHGEF5, hTERT, and EGFR. Regarding p16, we found a severe deficiency of protein expression. Unexpectedly, detectable protein expression of p16 showed a tendency to correlate with poor prognosis [21 of 152 (13%); \( P = 0.05 \)]. With respect to ARHGEF5, overexpression was observed [52 of 155 (34%)] but did not correlate with overall survival (data not shown). Analysis of hTERT revealed overexpression in a subset of tumors and was found to be a significant factor for adverse outcome [22 of 163 (59%); \( P = 0.01 \); Fig. 3B]. EGFR overexpression was frequently detected and was correlated with adverse outcome in intracranial tumors [96 of 163 (59%); \( P = 0.002 \); Fig. 3C]. We additionally tested protein expression in a set of spinal ependymomas and found similar levels of protein overexpression [22 of 36 (61%)] compared with intracranial tumors. No correlation between EGFR overexpression and overall survival was observed in spinal tumors (data not shown).

Gain of 1q25 and EGFR protein overexpression represent independent prognostic markers. Cox proportional hazards analysis included clinicopathologic factors like patients’ age, gender, chemotherapy, radiotherapy, level of resection, and tumor grade as well as the chromosomal status of 1p36 and 1q25 in addition to the protein levels of p16, ARHGEF5, hTERT, and EGFR. Estimation of variables influencing recurrence-free survival resulted in a final model containing the level of resection, tumor grade, and gain of 1q25. Gross total resection was significantly associated with a good prognosis (\( P = 0.002 \)), whereas gains of 1q25 (\( P < 0.001 \)) and grade III tumors (\( P < 0.001 \)) showed a much worse prognosis. Testing variables that correlate with overall survival lead to a final model, including patients’ age, radiotherapy, tumor grade, and gain of 1q25. A significant association with good prognosis was observed for application of radiotherapy (\( P = 0.008 \)), whereas gain of 1q25 (\( P = 0.003 \)) and grade III (\( P < 0.001 \)) were correlated with a poor outcome. In contrast, patients’ age did

### Table 3. QRT-PCR of selected candidate genes frequently imbalanced in ependymomas

<table>
<thead>
<tr>
<th>Gene</th>
<th>1E3</th>
<th>1E7</th>
<th>1E9</th>
<th>1E10</th>
<th>1E11</th>
<th>1E12</th>
<th>1E13</th>
<th>1E14</th>
<th>1E21</th>
<th>1E36</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOXC4</td>
<td>1.94</td>
<td>1.75</td>
<td>2.63</td>
<td>3.76</td>
<td>6.73</td>
<td>2.16</td>
<td>−0.30</td>
<td>6.28</td>
<td>1.76</td>
<td>3.51</td>
</tr>
<tr>
<td>EGFR</td>
<td>3.23</td>
<td>0.77</td>
<td>2.86</td>
<td>3.15</td>
<td>1.96</td>
<td>3.15</td>
<td>5.53</td>
<td>1.11</td>
<td>5.13</td>
<td>2.25</td>
</tr>
<tr>
<td>DUSP12</td>
<td>2.40</td>
<td>1.08</td>
<td>1.95</td>
<td>2.00</td>
<td>1.87</td>
<td>3.27</td>
<td>2.81</td>
<td>3.30</td>
<td>1.77</td>
<td>2.13</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>−5.08</td>
<td>−5.49</td>
<td>1.41</td>
<td>1.04</td>
<td>−5.75</td>
<td>−4.90</td>
<td>−4.17</td>
<td>−1.73</td>
<td>−4.93</td>
<td>−4.40</td>
</tr>
<tr>
<td>ARHGEF5</td>
<td>2.28</td>
<td>0.55</td>
<td>1.41</td>
<td>−2.62</td>
<td>−2.56</td>
<td>−0.56</td>
<td>3.48</td>
<td>5.94</td>
<td>−1.73</td>
<td>3.59</td>
</tr>
<tr>
<td>TACC2</td>
<td>1.67</td>
<td>0.70</td>
<td>−2.32</td>
<td>3.85</td>
<td>5.30</td>
<td>5.47</td>
<td>4.90</td>
<td>5.94</td>
<td>4.83</td>
<td>6.39</td>
</tr>
<tr>
<td>MINPP1</td>
<td>0.08</td>
<td>−0.41</td>
<td>4.00</td>
<td>0.76</td>
<td>0.57</td>
<td>1.63</td>
<td>−0.65</td>
<td>1.02</td>
<td>0.40</td>
<td>0.12</td>
</tr>
<tr>
<td>hTERT</td>
<td>5.97</td>
<td>0.24</td>
<td>1.27</td>
<td>−0.70</td>
<td>−3.50</td>
<td>0.21</td>
<td>−3.50</td>
<td>2.15</td>
<td>−6.65</td>
<td>−3.51</td>
</tr>
</tbody>
</table>

**NOTE:** Normalized log2 ratios corresponding to relative mRNA expression of eight candidate genes analyzed in 10 representative tumor samples (1E3-1E36).
not reach significance ($P = 0.1$). Thus, gain of 1q25 represented the strongest independent molecular marker for either tumor recurrence or overall survival in this study.

When only considering intracranial tumors of grade II ($n = 83$) with respect to overall survival, the final model contained chemotherapy, radiotherapy, gender, and EGFR expression. Notably, EGFR overexpression was the only statistically significant variable for a poor prognosis in this ependymoma subgroup ($P = 0.03$).

**Discussion**

**Novel candidate genes in ependymal tumors.** Matrix-based CGH analysis identified recurrent DNA copy number alterations, indicating several novel candidate genes that may play a role in ependymoma pathogenesis (Table 2). The ARHGEF5 gene at 7q34 showed a copy number gain in 26 ependymal neoplasms representing the most frequent small genomic aberration detected in our tumor series (38%). This gene encodes a guanine nucleotide exchange factor for Rho GTPases and showed either a differential mRNA or protein expression. It is involved in control of cytoskeletal organization and progression of primary breast carcinoma (37). Another frequently gained genomic hotspot includes HOXC4 at 12q13.13 (34%). The homeobox genes HOXC encode a highly conserved family of transcription factors and play an important role in morphogenesis or development of neurons, which have been suggested to be involved in malignant transformation of prostate cells (38). By QRT-PCR, we showed that HOXC4 was strongly overexpressed in 9 of 10 tumors analyzed.
Candidate tumor suppressor genes located in the deleted regions on chromosome arm 10q included MINPP1 at 10q23.31 (19%), which codes for an inositol phosphatase with homologies to PTEN. It has been implicated in the pathogenesis of follicular thyroid carcinomas (39). However, relative mRNA expression has been shown to be unaffected in our tumor series. Additionally, frequent deletions of TACC2 at 10q26.13 (16%), which is required for mitotic spindle maintenance and is involved in breast cancer (40), may play a role in ependymomas. QRT-PCR revealed a strong expression of TACC2 mRNA in all tested samples, suggesting dosage overcompensation by the remaining allele in these tumors. Considering losses of the telomeric band of chromosome 9 at 9p24.31 (16%), FOXD4, as a member of the forkhead box family of transcription factors, represents an interesting candidate gene, which is expressed in human embryonic stem cells and is associated with several cancers (41). Detailed functional studies will be required to elucidate the role of each of these candidate genes in ependymoma pathogenesis.

Existence of distinct ependymoma subgroups. Several studies using conventional CGH reported on a higher frequency of balanced profiles in tumors from children compared with those obtained from adult patients (9, 10, 12). However, we did not find significant differences between the number of genomic imbalances and certain clinical subgroups, including patients’ age. This can be explained by the superior resolution of our 6K array compared with studies that used conventional CGH with limited resolution (~5-10 Mb). Although some genomic imbalances are common in tumors with different localization (Fig. 1), several chromosomal abnormalities were significantly associated with clinicopathologically relevant subgroups (Fig. 2A), indicating that ependymal tumors may develop along substantially different pathways. There is an overlap with findings published by Hirose et al. (9), including gain of 1q and loss of 6q in intracranial tumors. According to our matrix-based CGH profiles, we confirmed the existence of three different chromosomal patterns in ependymomas as reported previously (10). Strikingly, gain of 1q was correlated with pediatric patients, intracranial location, and grade III and therefore seems to play an important role in “high-risk” ependymomas. In summary, our data imply the existence of clear genetic differences in ependymoma subgroups.

Role of chromosomal gains on 1q in intracranial ependymomas. Gains and amplifications on 1q were reported to be a common feature in pediatric intracranial ependymomas (10, 14, 42). The minimal overlapping region was mapped to 1q24-31 by conventional CGH (14). We identified two large, commonly gained regions on 1q (>3 Mb), one at 1q21.3-23.1 and another at 1q31.1-31.3. Although the “driver oncogene(s)” on 1q remains to be identified, DUSP12 at 1q23.3 is a candidate gene located in a frequently gained genomic hotspot. It is overexpressed in 10 of 10 tested samples and might be involved in the tumorigenesis of more aggressive ependymomas. This gene is located in a region often amplified in liposarcomas (43), and mRNA levels correlate with that of cyclin D1 throughout the cell cycle (44), suggesting a role in regulating cell division and potentially in neoplastic transformation.

By a multivariate approach (SCAD), gains of 1q21.1-32.1 were correlated with tumor recurrence in intracranial tumors (Fig. 2B). Two of 164 clones of the significant SCAD predictor map to 22q12.3-13.1 rather than 1q, representing a minimally overlapping region for gains on 22q. These overlapping PACs cover 0.233 Mb and contain seven protein coding genes, including IL2RB, RACC2, or SSTR3. Although we could not define a minimally overlapping region for losses on 22q, this small gain indicated genomic instability in an ependymoma-associated chromosomal segment. However, a role of gains on 22q for tumor recurrence in intracranial ependymomas seems to be an artifact of the model resulting from only 4 of 49 tumors.

Based on our findings by SCAD, we further investigated the influence of 1q gains to overall survival by FISH to the tissue microarray. Log-rank test (Fig. 3A) and Cox proportional hazards analysis identified gain of 1q25 as an independent prognostic marker in intracranial ependymomas. Moreover, gains of 1q25 represented the strongest molecular marker for either recurrence-free or overall survival in this study, thereby validating and confirming the results obtained by SCAD.

Being a controversially discussed issue, we found tumor grade as the strongest clinicopathologic variable for recurrence-free and overall survival followed by the novel molecular marker of 1q25 in a large set of tumors using Cox proportional hazards method. The grading of our series was based on the WHO 2000 classification, additionally considering the criteria proposed by Merchant et al. (25) to distinguish between well-differentiated (grade II) and anaplastic tumors (grade III). Applying this model for tumor grading was recently confirmed to be a useful prognostic variable (26). For this reasons, we support the usefulness of this combined grading system.

Delineation of p16, hTERT, and EGFR by genomic, mRNA, and protein expression profiling. The only biallelic deletion reported in ependymomas thus far affected CDKN2A, detected by a homozygous deletion assay (45). In our series, we also found a homozygous deletion of 3.01 Mb at 9p21.3 covering the CDKN2A locus, which was also lost in 4 (7%) additional tumors. Furthermore, a difference in site-specific mRNA expression was found with higher levels in spinal tumors than in intracranial tumors supporting the results of a previously published study (13). Notably, expression of p16 was correlated to unfavorable prognosis regarding intracranial tumors in univariate analysis but had no influence in multivariate analysis. It might be possible that the physiologic state shows such low protein concentrations that detection of loss of p16 expression is impossible and therefore only higher protein levels due to deregulated expression result in immunopositivity (45). In summary, our results showed fundamental changes of this tumor suppressor at several levels, but the role of p16 in ependymal tumors remains unclear.

As hTERT at 5p15.33 represents another prominent oncogene frequently gained in our study that has not been described in ependymomas thus far (24%), we further analyzed mRNA and protein expression levels. Usually, hTERT expression is repressed in normal human somatic cells but is reactivated in many tumors. Overexpression was detected in a subset of tumors on mRNA or protein level, and its expression in intracranial tumors was correlated with a poor prognosis in univariate analysis (Fig. 3B). In many neoplasms, increased telomerase activity is associated with poor clinical outcome (46), suggesting also a role in ependymomas.

Although a few amplifications were reported in ependymomas (e.g., of MYCN or CDK4; ref. 11), amplifications in general seem to be rare events. Here, we presented for the first...
time a 0.34 Mb high-level amplification of EGFR in ependymomas. Notably, the region at 7p11.2 containing EGFR was also gained at low copy level in 24 (37%) other tumors. As amplifications often indicate genes important for tumorigenesis, it is not surprising to detect frequent and strong overexpression on mRNA and protein levels, independent of the tumor localization or DNA copy number status. This finding implies other mechanisms in addition to genomic amplification for gene deregulation of EGFR. In contrast to spinal tumors, protein overexpression correlated significantly with an adverse outcome in intracranial tumors using univariate analysis (Fig. 3C) and thereby suggests that other thus far unknown effectors might accompany EGFR overexpression in this tumor subgroup. However, clinicopathologic variables, predominantly tumor grade, turned out to be better variables for overall and recurrence-free survival in multivariate analysis. For patients with intracranial grade II tumors, only EGFR overexpression was a significant factor for a poor prognosis and therefore provides useful information for risk stratification and patients’ treatment. Accordingly, screening for EGFR immunopositivity, especially in intracranial grade II tumors, might help to identify more malignant lesions in a pool of relatively benign tumors. The status of EGFR in ependymoma determined by immunohistochemistry was published previously to be a helpful covariable in low-grade tumors (47). However, another study reported on more frequent coexpression of ERBB2 and ERBB4 compared with EGFR, although the authors showed EGFR to be highly expressed on mRNA level in some samples (48). Based on our results, recurrent genomic gains and amplification together with frequent EGFR overexpression on mRNA and protein levels indicate a role in the pathogenesis of ependymoma.

Conclusions

The combination of DNA copy number profiling by high-resolution matrix-based CGH and FISH with protein expression analyses using immunohistochemistry to large series of tumors on tissue microarrays has been shown previously to represent a powerful tool in pediatric brain cancer research (22, 24). In this study, the application of these methods led to the identification of two independent prognostic markers in intracranial ependymoma. The states of 1q and EGFR might help to subdivide patients into different risk groups in further clinical investigations. Moreover, and because no reliably effective chemotherapy for ependymoma patients is available thus far, EGFR provides a potential target for therapeutic intervention as proposed for gliomas (49).

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Identification of Gains on 1q and Epidermal Growth Factor Receptor Overexpression as Independent Prognostic Markers in Intracranial Ependymoma

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