The Transcription Factor Evi-1 Is Overexpressed, Promotes Proliferation, and Is Prognostically Unfavorable in Infratentorial Ependymomas

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Abstract

Purpose: Ependymomas are glial tumors of presumably radial glial origin that share morphologic similarities with ependymal cells. The molecular genetics of ependymomas of supratentorial, infratentorial, and spinal location is heterogeneous. We aimed at identifying pathways operative in the development of infratentorial ependymomas.

Experimental Design: To do so, gene expression profiles of tumor cells laser microdissected from infratentorial ependymomas (n = 15) were compared with that of nonneoplastic ependymal cells laser microdissected from autopsy tissue (n = 7).

Results: Among 31 genes significantly overexpressed (>5-fold) in ependymomas, transcription factor EVI1 (ecotropic viral integration site 1) showed the highest overexpression (35-fold). Evi-1 protein expression could be confirmed in formalin-fixed, paraffin-embedded samples of 26 of 28 infratentorial ependymomas but only in 7 of 47 nonependymal glial tumors (P < 0.001). Furthermore, MDS1/EVI1 fusion transcripts were detectable in 17 of 28 infratentorial ependymomas and significantly correlated with MGMT (O6-methylguanine-DNA-methyltransferase) promoter hypermethylation (P < 0.05). In primary infratentorial ependymoma cells, transfection with EVII-specific siRNAs resulted in significant growth inhibition [48 hours: 87% ± 2% and 74% ± 10% as compared with control (mean ± SD; P < 0.001)]. The prognostic role of EVII could further be validated in an independent cohort of 39 infratentorial and 26 supratentorial ependymomas on the basis of mRNA expression profiling. Although in supratentorial ependymomas EVII expression status had no prognostic impact, in infratentorial ependymomas, high EVII expression was associated with shorter overall survival and progression-free survival.

Conclusions: To conclude, the transcription factor Evi-1 is overexpressed in infratentorial ependymomas, promotes proliferation of ependymal tumor cells, and is prognostically unfavorable. Clin Cancer Res; 17(11); 3631–7. ©2011 AACR.

Introduction

Ependymomas are glial tumors showing morphologic features resembling that of ependymal cells lining the inner surfaces of the central nervous system. Located in proximity to the ventricular system or the spinal canal, ependymomas may develop supra- and infratentorially as well as in the spinal cord (1). These tumors occur in all age groups, but intracranial ependymomas are most common in children, where they are most frequently encountered infratentorially within the posterior fossa (2). Whereas in adult cases of infratentorial ependymomas, 5-year survival rates as high as 70.8% have been reported (3), prognosis is substantially worse in children (2, 4) because gross total resection, which represents an important prognostic factor, is less frequently achieved (5) and adjuvant radiotherapy often cannot be employed because of the young age of the patients. Furthermore, ependymomas are largely chemo-resistant (6–8), raising the need for novel therapeutic approaches and a better understanding of pathways involved in their tumorigenesis.

It is now evident that ependymomas of infratentorial, supratentorial, and spinal cord location are genetically distinct and may in fact represent distinct tumor entities (9–12). In recent years, a number of gene expression profiling studies have been conducted on whole tumor tissue, to identify genes differentially expressed in ependymomas of different location (11–16). Given the high vascularity of anaplastic ependymomas, it is not surprising...
Materials and Methods

Materials and patients

For laser microdissection and subsequent gene expression profiling, snap-frozen surgical samples of 15 infratentorial ependymomas [7 ependymomas (WHO grade II) and 8 anaplastic ependymomas (WHO grade III)] were retrieved from the archives of the Institute of Neuropathology Münster, the Institute of Neuropathology Düsseldorf, and the Institute of Brain Research Tübingen. Median age of 7 males and 8 females was 13 years (range: 2–38 years). For protein expression of identified genes, genetic analyses and methylation studies, formalin-fixed, paraffin-embedded (FFPE) samples from 28 infratentorial ependymomas [18 ependymomas (WHO grade II) and 10 anaplastic ependymomas (WHO grade III)] were retrieved from the archives of the Institute of Neuropathology Münster. Median age of these patients was 25 years (range: 0–76 years). Sixteen of the patients were male and 12 female. In all cases, the diagnosis was confirmed neuropathologically according to the current WHO classification (18). For comparison, FFPE tissues from 47 nonependymal gli tumors [5 pilocytic astrocytomas (WHO grade I), 5 oligodendrogliomas (WHO grade II), 7 fibrillary astrocytomas (WHO grade II), 7 anaplastic astrocytomas (WHO grade III), 4 anaplastic oligodendrogliomas (WHO grade III), and 19 glioblastomas (WHO grade IV)] were employed.

Cell culture

Primary cell cultures of an infratentorial ependymoma (WHO grade II) were grown in culture medium [DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 mg/mL and 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 mg/mL]. Sections were crysyl violet stained and air dried. Laser microdissection was conducted using a Zeiss system equipped with an UVA-laser. A total of 10,000 cells were microdissected from each sample and collected in microcentrifuge tubes containing Buffer RLT (RNeasy Micro kit; Qiagen). Total RNA from these samples was then extracted following manufacturer’s instructions. All steps were carried out under RNase-free conditions.

Gene expression profiling

RNA was amplified using the GeneChip Two-Cycle cDNA Synthesis Kit (Affymetrix) and subsequently transcribed to cRNA using the GeneChip IVT Labeling Kit (Affymetrix). After confirmation of cRNA integrity, fragmented cRNA was hybridized for 16 hours under constant rotation to U133A 2.0 gene chips (Affymetrix) representing 18,400 transcripts and variants of 14,500 human genes. After washing and staining (Fluidics Station 450; Affymetrix), fluorescence was scanned on a GeneChip Scanner 3000 (Affymetrix). Data files were evaluated using the GeneSpring Software (Agilent). After global chip quality control and fluorescence gradient correction, differentially expressed probe sets were identified. To screen for differentially expressed candidate genes, normalized data were filtered for probe sets displaying expression levels at least 5-fold higher or lower in ependymoma tumor cells compared with microarray data sets from 7 samples representing laser-microdissected normal ependymal cells from human autopsy tissues that had been generated under comparable conditions.

Quantitative reverse-transcription PCR

On RNA isolation from human tissue samples and cell cultures using the RNeasy Kit (Qiagen) and reverse transcription of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), cDNA was used for quantitative reverse-transcription PCR (qRT-PCR) measurement in duplicates using the TaqMan Gene Expression assay EVI1 (ecotropic viral integration site 1; HS01115415_m1). Data were normalized for glyceraldehyde-3-phosphate dehydrogenase expression using the comparative threshold cycle method.

RT-PCR for MDS1/EVI1 fusion transcript in FFPE tissues

RNA was isolated in FFPE tissue of 28 infratentorial ependymomas and cDNA was generated using the High Capacity Reverse Transcription Kit (Applied Biosystems). Expression of the MDS1/EVI1 fusion transcript was evaluated using 2 specific primers (MDS1/EVI1 fwd: TCAACAACGACGGCCATGA and MDS1/EVI1 rev: GCATCTATGCAGAACTTCACATTGT).

Translational Relevance

Here, we show that the transcription factor Evi-1 (ecotropic viral integration site 1) is highly expressed in infratentorial ependymomas and promotes proliferation. Furthermore, high EVI1 expression was associated with significantly shorter overall and progression-free survival in ependymoma patients, suggesting that EVI1 not only plays a role in the biology of ependymomas but also is of prognostic relevance.
streptomycin, at 37°C in 5% CO₂. All cell culture reagents were purchased from PAA Linz.

**Immunohistochemistry and immunocytochemistry**

Immunohistochemistry and immunocytochemistry were carried out using a rabbit polyclonal antibody directed against Evi-1 (Ab28457; Abcam; 1:200) and the avidin–biotin method on an automated staining system (TechMate; DAKO). Expression of Evi-1 was scored as present (1) or absent (0).

**Western blot**

Protein lysates of snap-frozen ependymomas and primary cell cultures were separated on 7.5% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schüll). Membranes were blocked with 5% bovine serum albumin (BSA) in TST buffer for 30 minutes and incubated with a primary rabbit polyclonal antibody against Evi-1 (1:500 in TST; Abcam) at 4°C overnight. Detection was carried out with a peroxidase-conjugated secondary antibody and the ECL plus Kit (GE Healthcare). After stripping, membranes were reprobed with an antibody against β-actin (A5441; 1:10,000; Sigma).

**RNA interference**

Two different siRNA oligonucleotides for EVI1 inhibition as well as a scrambled (Scr) siRNA for negative control (All Star Negative siRNA) were purchased from Qiagen. A total of 40,000 cells per mL were seeded the day before siRNA transfection in 24-well plates containing 500 μL culture medium. Cells were then transfected by adding 3 μL HiPerFect Transfection Reagent (Qiagen) and 3 μL siRNA dissolved in culture medium without FCS (5 nmol/L) per well according to manufacturer’s recommendations. Cells were maintained in full culture medium for 24, 48, and 72 hours before lysis and subsequent RNA isolation. Extent of residual expression of EVI1 was evaluated using qRT-PCR.

**Proliferation**

Cellular growth was assessed by a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium; (CCVK-I)] assay (PromoCell). Briefly, ependymoma cells were seeded at a density of 40,000 cells per mL in a 24-well plate. Forty-eight hours after siRNA transfection, 50 μL assay solution was added to the culture medium and incubated for 1.5 hours. Supernatant was transferred to a 96-well plate and measurement was done at

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**Figure 1.** Expression of Evi-1 in infratentorial ependymoma. Microarray analysis of tumor cells from 15 infratentorial ependymomas compared with laser-microdissected ependymal cells revealed a total of 143 differentially expressed genes (more than 5-fold up- or downregulation). EVI1 was the gene most upregulated (55-fold, highlighted in red; A). Expression of EVI1 was validated in 12 infratentorial ependymomas using qRT-PCR. Eleven of 12 tumors overexpressed Evi-1 compared with normal non-LMPC (sub-)ependymal tissue (B). Expression of Evi-1 protein was found in 26 of 28 FFPE samples using immunohistochemistry (C), whereas only 7 of 47 nonependymal glial tumors expressed the protein (bottom left inset). Staining could be confirmed by Western blot (bottom right inset). Furthermore, cells of the ependymal lining did not show distinct Evi-1 protein expression (top right inset). MDS1/ Evi1 fusion transcript could be found in 17 of 28 infratentorial ependymomas using RT-PCR (D; lane 1: negative; lanes 2–8: ependymomas with positive expression of MDS1/EVI1; lane 9: water control).
450 nm using an ELISA reader. Experiments were independently carried out twice, and 3 wells were evaluated for each experimental condition.

Validation of expression data and prognostic role
Expression of EVII was validated in a large set of ependymomas, for which microarray gene expression profiles as well as detailed clinical data were available. Patients of this cohort were part of a larger study that has already been published (19). This set comprised 65 intracranial ependymomas of which 39 were of infratentorial location. Of the infratentorial ependymomas, 17 were ependymoma (WHO grade II) and 22 were anaplastic ependymoma (WHO grade III). In the 39 patients harboring infratentorial ependymomas [median age: 13 years (range: 1–63 years)], mean progression-free survival (PFS) and overall survival (OS) times accounted for 4.75 and 5.9 years, respectively. Sixteen recurrences and 8 deaths had occurred (19). To evaluate the impact of EVII mRNA expression status on PFS and OS, EVII microarray expression data were dichotomized into high (EVI1 expression higher than mean EVI1 expression of all tumors) and low (EVI1 expression lower than mean EVI1 expression of all tumors).

Statistics
Effects of siRNA treatment on proliferation were evaluated by ANOVA followed by LSD (least significant difference) test, whereas the effect of EVII expression and other variables on OS and PFS was evaluated using log-rank test and Cox regression. All statistical analyses were conducted using PASW Statistics version 18.0 (SPSS Inc.).

Results
Gene expression profiling identifies EVI1 to be highly overexpressed in tumor cells of infratentorial ependymomas
Analysis of microarray data showed a total of 31 genes to be significantly overexpressed more than 5-fold (Fig. 1A) in tumor cells of infratentorial ependymomas as compared with nonneoplastic ependymal cells, whereas 112 genes were downregulated more than 5-fold (Supplementary Table S1). Among those, EVII coding for the transcription factor Evi-1 showed the highest overexpression (35-fold). On validation of microarray data using qRT-PCR, which could be carried out in fresh frozen tumor tissue of 12 of the 15 ependymomas used for microarray analysis, a significant overexpression of EVII mRNA as compared with nonneoplastic ependymal and subependymal brain tissue could be confirmed (95- fold; mean ± SD; P = 0.009 Mann–Whitney U Test; Fig. 1B).

Evi-1 protein is overexpressed in infratentorial ependymomas
On examination of 28 infratentorial ependymomas using immunohistochemistry, 26 of 28 cases (93%) showed tumoral Evi-1 protein expression (Fig. 1C). Expression of Evi-1 protein was not correlated with clinical factors such as age or WHO grade (data not shown). As shown in Table 1, the proportion of tumors showing Evi-1 protein expression was significantly lower in nonependymal gliomas: only 7 of 47 tumors (15%) showed Evi-1 protein expression (χ² = 43.285, P < 0.001).

Table 1. Evi-1 protein expression in 47 non-ependymal gliomas

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Expression (%)</th>
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<tbody>
<tr>
<td>Pilocytic astrocytoma (WHO I)</td>
<td>5 2 (40)</td>
</tr>
<tr>
<td>Oligodendroglioma (WHO II)</td>
<td>5 0 (0)</td>
</tr>
<tr>
<td>Fibrillary astrocytoma (WHO II)</td>
<td>7 0 (0)</td>
</tr>
<tr>
<td>Anaplastic astrocytoma (WHO III)</td>
<td>7 2 (29)</td>
</tr>
<tr>
<td>Anaplastic oligodendroglioma</td>
<td>4 1 (25)</td>
</tr>
<tr>
<td>(WHO III)</td>
<td></td>
</tr>
<tr>
<td>Glioblastoma multiforme (WHO IV)</td>
<td>19 2 (11)</td>
</tr>
<tr>
<td>Total</td>
<td>47 7 (15)</td>
</tr>
</tbody>
</table>

Figure 2. Expression and functional relevance of Evi-1 in infratentorial ependymoma cells. Primary cultures of an infratentorial ependymoma show strong expression of Evi-1 protein (A). Immunocytochemical staining was also validated by Western blot (top right inset) and immunofluorescence (bottom right inset). Downregulation of EVII by 2 different siRNAs (Evi1_2 and Evi1_5) resulted in significantly reduced proliferation 48 hours after transfection as compared with control (Scr). *** P < 0.001 (B).
The *MDS1/EVI1* fusion transcript is expressed in infratentorial ependymomas and associated with MGMT promoter methylation status

The expression array probe set (221884_at) as well as the RT-PCR primers recognize all known *EVI1* transcripts. However, *EVI1* and *MDS1* may form a fusion transcript, which is common in both neoplastic as well as nonneoplastic tissues (20). We therefore also examined the expression pattern of *MDS1/EVI1* fusion transcripts in ependymomas. On RT-PCR, the presence of a *MDS1/EVI1* fusion transcript was detectable in 17 of 28 infratentorial ependymomas (61%; Fig. 1D). No association between *EVI1* overexpression and *MDS1/EVI1* fusion transcript status was observed. Because the newly introduced PR domain of the fusion protein has the potential to act as a methyltransferase (21), *MDS1/EVI1* expression status was correlated with MGMT (O6-methylguanine-DNA-methyltransferase) promoter methylation status, which had been already determined in these tumors (22). Interestingly, a significant association of MGMT promoter methylation and *MDS1/EVI1* expression status was observed ($P < 0.05$).

**Evi-1 plays a functional role in ependymal tumor cells**

To investigate the functional role of Evi-1 in infratentorial ependymomas, the effect of *EVI1* knockdown was investigated in primary cultures derived from an infratentorial ependymoma expressing Evi-1 protein (Fig. 2A). Transfection of the primary cultures with 2 different siRNAs directed against *EVI1* resulted in a significant decrease of *EVI1* mRNA with a residual expression of about 30% as compared with Scr control (data not shown). Under these conditions, a significant inhibition of cellular growth was observed [48 hours of incubation: 87% ± 2% and 74% ± 10% of Scr control (mean ± SD; $P < 0.001$; Fig. 2B)].

**EVI1 has a prognostic role in infratentorial ependymomas**

To determine the prognostic role of *EVI1* and validate our data, a different microaarray data set comprising 39 infratentorial ependymomas and 26 supratentorial ependymomas was examined. Infratentorial ependymomas showed a 7-fold upregulation of *EVI1* mRNA expression as compared with supratentorial ependymomas. Although in supratentorial ependymomas *EVI1* mRNA expression status had no prognostic impact (data not shown), in infratentorial ependymomas, shorter OS was associated not only with incomplete resection and WHO grade but also with high *EVI1* mRNA expression [82 months (68–96) vs. 104 months (99–109); $P = 0.029$; Table 2]. Comparably, shorter PFS was associated with incomplete resection, WHO grade, and high *EVI1* mRNA expression [52 months (35–68) vs. 99 months (88–110); $P = 0.001$; Table 3]. Age and gender did not affect OS or PFS significantly. On multivariate analysis taking into account all of the above factors in addition to incomplete resection (HR: 10.282 (2.251–46.966), $P = 0.003$), high *EVI1* mRNA expression [HR: 5.948 (1.325–26.701), $P = 0.02$] was associated with shorter PFS (Fig. 3).

**Discussion**

Using gene expression profiling, *EVI1* was found to be highly overexpressed in tumor cells laser microdissected from infratentorial ependymomas, suggesting a role of this transcription factor in the tumorigenesis of infratentorial ependymomas. Evi-1 had been originally discovered

### Table 2. Effect of clinical and molecular factors on OS

<table>
<thead>
<tr>
<th>Factor</th>
<th>OS [mean mos (95% CI)]</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>EVI1</em> low vs. high</td>
<td>104 (99–109) vs. 82 (68–96)</td>
<td>0.029</td>
</tr>
<tr>
<td>Age (&lt;18 vs. &gt;18 years)</td>
<td>84 (72–96) vs. 101 (90–112)</td>
<td>0.072</td>
</tr>
<tr>
<td>WHO grade (II vs. III)</td>
<td>104 (98–110) vs. 81 (68–94)</td>
<td>0.045</td>
</tr>
<tr>
<td>Gender (male vs. female)</td>
<td>89 (76–102) vs. 94 (84–104)</td>
<td>0.303</td>
</tr>
<tr>
<td>Resection (incomplete vs. gross total)</td>
<td>81 (65–96) vs. 100 (92–108)</td>
<td>0.009</td>
</tr>
</tbody>
</table>

### Table 3. Effect of clinical and molecular factors on PFS

<table>
<thead>
<tr>
<th>Factor</th>
<th>PFS [mean mos (95% CI)]</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>EVI1</em> low vs. high</td>
<td>99 (88–110) vs. 52 (35–68)</td>
<td>0.001</td>
</tr>
<tr>
<td>Age (&lt;18 vs. &gt;18 years)</td>
<td>63 (47–79) vs. 85 (67–104)</td>
<td>0.149</td>
</tr>
<tr>
<td>WHO grade (II vs. III)</td>
<td>89 (74–105) vs. 58 (41–75)</td>
<td>0.040</td>
</tr>
<tr>
<td>Gender (male vs. female)</td>
<td>68 (50–85) vs. 78 (60–96)</td>
<td>0.313</td>
</tr>
<tr>
<td>Resection (incomplete vs. gross total)</td>
<td>42 (26–59) vs. 97 (87–107)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
because its activation through retroviral insertion caused leukemia in a mouse model (23). Transcriptional activation of Evi-1, however, is not only caused by retroviral insertion but also by a variety of genetic aberrations (24). A biological role of Evi-1, commonly found to be overexpressed in acute myeloid leukemia (25), to our knowledge has not yet been described in central nervous system tumors. On evaluation of functional effects of Evi-1 in ependymal tumor cells, we observed that downregulation of EVI1 resulted in decreased cellular growth, suggesting an involvement of Evi-1 in proliferation and the tumorigenesis of infratentorial ependymomas. When interpreting the relative moderate extent of decrease in cellular growth, the lower transfection efficiency and proliferative activity in primary cultures as compared with cell lines has to be taken into account. In leukemic cells, Evi-1 has been shown to be a critical regulator (26) and overexpression of Evi-1 in ovarian carcinoma cell lines promotes cellular growth in vitro (21). Interestingly, Evi-1 has also been shown to be involved in neuroectodermal differentiation (27).

EVI1 and MDS1 can form a fusion transcript, which is common in both neoplastic as well as nonneoplastic tissues (20). The MDS1/EVI1 fusion transcript contains a putative methyltransferase domain (21) and has been associated with differential promoter methylation in acute myeloid leukemia (28), suggesting that the observed correlation of MDS1/EVI1 fusion transcript status and MGMT promoter methylation might represent more than an epiphenomenon. Because MGMT promoter methylation status is less frequently encountered in ependymomas as compared with malignant astrocytic tumors (22) and has no proven prognostic role (29), the clinical implications of these findings remain uncertain.

An important finding of the present study is the observation that EVI1 mRNA overexpression was associated with shorter OS and PFS. These findings are well in line with the dismal prognostic role of Evi-1 in acute myeloid leukemia (25) and suggest that Evi-1 expression status might serve as a valuable prognostic marker. On multivariate analysis, in addition to known important prognostic factors (i.e., WHO grade and extent of resection), EVI1 overexpression showed to be significantly associated with PFS. Nevertheless, because of the relatively small number of recurrences and the retrospective nature of this study, the prognostic role of EVI1 expression status will need to be validated in future larger prospective trials. Further studies on the function of EVI1 and other differentially expressed genes will hopefully contribute to a better understanding of the complex molecular pathogenesis of infratentorial ependymomas. To conclude, the transcription factor Evi-1 is highly overexpressed in infratentorial ependymomas, promotes proliferation of ependymal tumor cells, and has an unfavorable prognostic role in patients harboring infratentorial ependymomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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