Differential expression and prognostic significance of *SOX* genes in pediatric medulloblastoma and ependymoma identified by microarray analysis

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The objective of this study was to identify differentially expressed and prognostically important genes in pediatric medulloblastoma and pediatric ependymoma by Affymetrix microarray analysis. Among the most discriminative genes, three members of the *SOX* transcription factor family were differentially expressed. Both *SOX4* and *SOX11* were significantly overexpressed in medulloblastoma (median, 11-fold and 5-fold, respectively) compared with ependymoma and normal cerebellum. *SOX9* had greater expression in ependymoma (median, 16-fold) compared with normal cerebellum and medulloblastoma ($p < 0.001$ for all comparisons). The differential expression of the *SOX* genes was confirmed at the protein level by immunohistochemical analysis. Survival analysis of the most discriminative probe sets for each subgroup showed that 35 and 13 probe sets were predictive of survival in patients with medulloblastoma and ependymoma, respectively. There was a trend toward better survival with increasing *SOX4* expression in medulloblastoma. *SOX9* expression was predictive for favorable outcome in ependymoma. The mRNA levels of *BCAT1*, a mediator of amino acid breakdown, were higher (median, 15-fold) in medulloblastoma patients with metastases compared with those without metastasized disease ($p < 0.01$). However, the correlation between *BCAT1* expression and metastatic medulloblastoma could not be confirmed at the protein level. The potential prognostic effect of the genes associated with outcome should be evaluated in ongoing studies using larger groups of patients. Furthermore, our findings support further analysis of the functional properties of the selected genes, especially *SOX4* and *BCAT1* for medulloblastoma and *SOX9* for ependymoma, to evaluate the use of these genes as potential tumor markers, prognostic markers, and drug targets in pediatric brain tumors. *Neuro-Oncology 10, 648–660, 2008 (Posted to Neuro-Oncology [serial online], Doc. D07-00109, June 24, 2008. URL http://neuro-oncology.dukejournals.org; DOI: 10.1215/15228517-2008-032)*

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Tumors of the CNS are the second most common malignancy of childhood and are generally associated with a worse prognosis than many other pediatric malignancies.[1] Medulloblastoma and ependymoma are frequently observed malignant brain tumors in children.[2] Medulloblastomas arise in the infratentorial pos-

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terior fossa and have a tendency to metastasize within the CNS.[3,4] Ependymomas most frequently occur in the posterior fossa or spinal cord.[3,5]

Most currently known prognostic factors for pediatric brain tumors are based on clinical and histological criteria, such as age, extent of tumor resection, and histological grade. However, insight into molecular abnormalities (genetic and/or posttranscriptional) underlying these tumors is limited. Hence, information on markers that can be used to characterize these tumors better and potentially serve as new targets for therapy is lacking. Studies based on molecular analyses and gene expression profiling are now evolving and may provide possible clues about pathogenesis and prognostic factors in pediatric brain tumors.[6–8]

In this study, we sought to identify aberrantly expressed and prognostically important genes in pediatric medulloblastoma and ependymoma. By analyzing microarray data, we identified genes that may provide new insights into the biological behavior of these brain tumors. We validated the aberrant expression of these genes on the protein level by immunohistochemical analysis in a larger group of pediatric brain tumor samples. Further characterization may clarify whether these genes can serve as new tumor markers, prognostic factors, or therapeutic targets in pediatric brain tumors.

Materials and Methods

Study Population and Samples

In this study we analyzed 40 fresh-frozen tumor samples from 25 newly diagnosed medulloblastomas (19 without and 6 with radiological visible leptomeningeal metastases), 2 medulloblastomas at relapse (1 without and 1 with radiological visible leptomeningeal metastases), 11 newly diagnosed ependymomas (4 cellular ependymomas and 7 anaplastic ependymomas), and 2 ependymomas at relapse (1 cellular ependymoma and 1 anaplastic ependymoma). Most of these tumor samples were also analyzed by two-dimensional difference in gel analysis, which resulted in the identification of stathmin, annexin A1, and calcyphosine as differentially expressed proteins in medulloblastoma and ependymoma.[9] Mean patient age at the time of tumor sample collection was 7.0 years (range, 0.98–14.87 years) for the medulloblastoma patients and 5.6 years (range, 0.66–15.57 years) for the ependymoma patients ($p > 0.05$). All samples were collected at the Erasmus MC Sophia Children's Hospital, University Medical Center Rotterdam (Rotterdam, The Netherlands) between 1990 and 2004. Each patient or patient's relatives gave informed consent prior to enrollment. After surgery, tumor samples were immediately placed in liquid nitrogen and stored at –80°C until processing. Control cerebellar tissue was obtained postmortem from five adult patients without a history of brain tumor. Before RNA extraction, 4–μm cryosections were prepared from the same tissue blocks as used for RNA extraction and stained with hematoxylin and eosin to confirm tumor histology.

Paraffin-embedded tissue for immunohistochemical validation of the differentially expressed genes was available for 23 (19 nonmetastatic and 4 metastatic) of the 27 medulloblastomas and 10 (3 cellular ependymomas and 7 anaplastic ependymomas) of the 13 ependymomas studied by microarray. To enlarge the number of patients for these immunohistochemical validation experiments, we also included paraffin-embedded material from patients with newly diagnosed medulloblastoma and ependymoma from whom no fresh-frozen tissue was available for microarray analysis. Additionally, paraffin-embedded tissues from normal cerebellum, normal cortex, normal ependyma, and normal plexus choroideus were included as normal controls.

Microarray

RNA extraction, labeling, and hybridization. Total RNA was isolated by homogenizing tissue samples in Trizol reagent (Invitrogen, Breda, The Netherlands) using a tissue homogenizer (B. Braun, Spangenberg, Germany). RNA was isolated according to the manufacturer's protocol with minor modifications.[10] RNA integrity was checked using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Production of biotinylated antisense complementary RNA (cRNA) and hybridization of the labeled cRNA to Human Genome U133 (HGU133) plus 2.0 GeneChip oligonucleotide microarrays (Affymetrix, Santa Clara, CA, USA) were performed according to the manufacturer's protocols. All arrays had a 3' to 5' GAPDH ratio of <3.0.

Statistical analysis of microarray data. We analyzed expression data using R version 2.4.0 (http://www.r-project.org/). Raw data were normalized using the variance stabilization procedure (vsn).[11] Expression profiles of medulloblastoma, ependymoma, and cerebellum were statistically compared using the Wilcoxon test and corrected for multiple testing errors by applying the false discovery rate (FDR) described by Benjamini et al.[12] (MULTTEST package). An FDR of <1% was considered statistically significant, meaning that less than 1% of the significant genes are false positives. We calculated the fold up-regulation or down-regulation using the formula $e^{(\text{vsn value } A - \text{vsn value } B)}$, in which A and B are the groups to be compared.

We estimated survival probabilities by the Kaplan-Meier method using the SPSS software package, version 11.0 (SPSS Inc., Chicago, IL, USA). Overall survival was defined as time from diagnosis until death or last contact. Event-free survival was defined as time from diagnosis until disease progression, relapse, second malignancy, death, or last contact. We performed a Cox regression analysis using SPSS 11.0 to identify genes whose expression was predictive for survival. For each gene, we calculated a hazard ratio and its 95% confidence interval. An effect was considered statistically significant if p was ≤ 0.05. A hazard ratio of <1 indicated a favorable influence on survival, and a hazard ratio of >1 indicated an unfavorable influence on survival.

Functional Gene Ontology Analysis

Functional properties of the differentially expressed genes were analyzed by Ontologizer, an XML-based Java application (www.charite.de/ch/medgen/ontologizer),[13] using annotations from the Gene Ontology (GO) database (www.geneontology.org). Probe sets with the same gene symbol were counted as one. Overrepresented genes in brain tumor samples were identified by comparing the percentage of genes in each annotation from the total number of genes on the Affymetrix HGU133 plus 2.0 microarray to the percentage of genes in each annotation from the selected subsets of genes using the parent–child method implemented in Ontologizer.[13] The parent–child method was preferred to the term-to-term method, because it takes into account the dependencies between different GO annotations. Overrepresentation of a GO annotation was thus measured with respect to the presence of its parental terms in the selected set of genes, instead of measuring individual GO terms. To correct for multiple testing errors, we used the Westfall-Young correction.[14] An FDR of <1% was considered statistically significant.

Immunohistochemistry

For the selected discriminative genes, data were validated at the protein level by immunohistochemical analysis. For this purpose, we collected formalin-fixed paraffin-embedded tissues for the medulloblastoma, ependymoma, control cerebella, cortex, normal ependyma, and plexus choroideus. Tissue sections (4 μm) were deparaffinized and rehydrated through a graded xylene-ethanol series and incubated for 30 min in 3% hydrogen peroxide in phosphate-buffered saline (PBS) to inhibit endogenous peroxidases. Antigen retrieval was performed by boiling the slides for 15 min in 0.01 mol/liter citric acid (pH 6.0). Six percent goat serum (Vector Laboratories, Burlingame, CA, USA) in PBS was used as a protein block for 1 h at room temperature. Tissue sections were incubated with antibodies against SOX4 (1:500 in 1% goat serum; Sigma Aldrich, Zwijndrecht, The Netherlands), SOX9 (1:2,000 in 1% goat serum; U.S. Biological, Swampscott, MA, USA), SOX11 (1:50 in 1% goat serum; Sigma Aldrich), and BCAT1 (1:200 in 1% goat serum; BD Biosciences, Alphen aan den Rijn, The Netherlands) at 4°C overnight. The sections were then incubated with biotinylated goat antirabbit IgG (Vector Laboratories) at a dilution of 1:1,000 in PBS for 1 h at room temperature. This was followed by a 1–h incubation with avidin-biotin peroxidase complex (1:400 dilution; Vectastain ABC kit, Vector Laboratories). Staining was performed using 0.5 mg/ml 3,3'-diaminobenzidine, 0.03% (vol/vol) hydrogen peroxide in 30 mM imidazole, 1 mM EDTA (pH 7.0). Tissue sections were slightly counterstained with hematoxylin, dehydrated, and mounted. As a negative control, the primary antibodies were omitted.

Immunohistochemistry was scored according to the percentage of cells that were positively stained in each slide: weak, 0%–25% of the tumor cells stained positive; moderate, 25%–50%; strong, 50%–75%; and very strong, 75%–100%. Differences in the results for immunohistochemistry for the analyzed subgroups were tested for significance using the chi-square test in SPSS 11.0.

Results

Microarray Gene Expression Analysis

Gene expression profiles were generated for 27 medulloblastoma, 13 ependymoma, and 5 control cerebellum samples. A comparison between medulloblastoma and normal cerebellum samples showed that 3,447 probe sets were differentially expressed, with an FDR of <1%. Between ependymoma and normal cerebellum samples, we found 3,708 differentially expressed probe sets, and between medulloblastoma and ependymoma, 4,720 differentially expressed probe sets, with an FDR of <1% (Fig. 1).

We found that 394 probe sets were most discriminative for medulloblastoma, because these were significantly differentially expressed both between medulloblastoma and cerebellum and between medulloblastoma and ependymoma (Fig. 1). Functional analysis (GO) revealed that the GO annotations involved in the regulation of the cell cycle, DNA replication, and response to stimuli were overrepresented in this set of 394 differentially expressed probe sets (corresponding to 253 genes with annotations in the GO database) (Fig. 2). The number of most discriminative probe sets for ependymoma was 582 (Fig. 1). Functional GO analysis did not reveal any significant overrepresentation of GO annotations in the 582 probe sets specific for ependymoma. We found that 1,541 probe sets were aberrantly expressed in both medulloblastoma and ependymoma compared with normal cerebellum, but the expression of these did not differ significantly between medulloblastoma and ependymoma (Fig. 1). Thus, these probe sets are likely to be related to the presence of a tumor but not specifically discriminative for either medulloblastoma or ependymoma. Of all probe sets, 133 probe sets were statistically differentially expressed among all subgroups (medulloblastoma, ependymoma, and cerebellum) (Fig. 1).

As previously found by others,[15,16] *OTX2* was differentially expressed in medulloblastoma with the highest fold-change ratio (median, 158-fold) compared with cerebellum. Several members of the *SOX* gene family were differentially expressed among medulloblastoma, ependymoma, and control cerebellum. *SOX4* expression was higher in medulloblastoma (median, 16-fold) compared with normal cerebellum and in medulloblastoma (median, 8-fold) compared with ependymoma (Fig. 3A). *SOX11* was highly overexpressed in both brain tumor types but especially in medulloblastoma, with a median 84-fold increase in medulloblastoma and median 21-fold increase in ependymoma compared with normal cerebellum (Fig. 3B). *SOX9* was significantly overexpressed in ependymoma, with a median 10-fold increase in ependymoma compared with normal

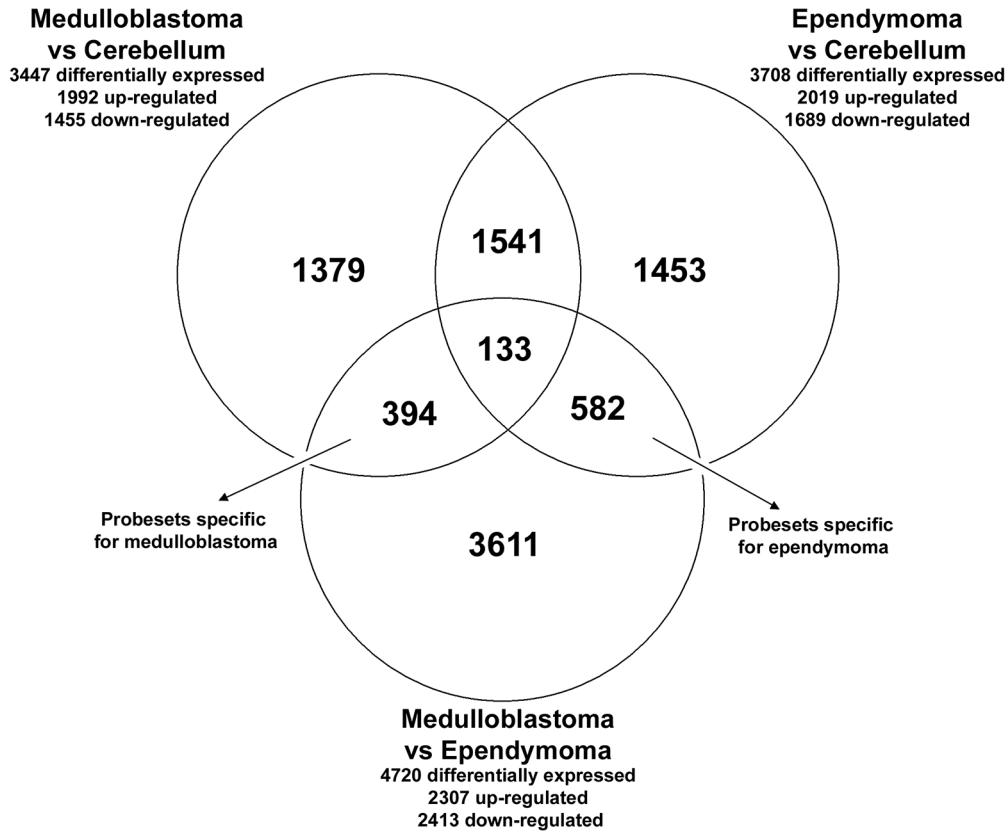

Fig. 1. Venn diagram indicating the number of differentially expressed probe sets from the comparisons of medulloblastoma, ependymoma, and normal cerebellum. All probe sets that were differentially expressed with a false discovery rate <1% between medulloblastoma, ependymoma, and control cerebellum were used to create a Venn diagram: 394 probe sets were most discriminative for medulloblastoma, being differentially expressed both between medulloblastoma and cerebellum and between medulloblastoma and ependymoma; 582 probe sets were found to be most discriminative for ependymoma, being significantly different between ependymoma and cerebellum and between ependymoma and medulloblastoma; 1,541 were differentially expressed in both medulloblastoma and ependymoma when compared with cerebellum, but they were not found to discriminate between medulloblastoma and ependymoma; 133 probe sets were differentially expressed among all subgroups.

cerebellum and median 17-fold increase in ependymoma compared with medulloblastoma (Fig. 3C) ($p < 0.001$ for all comparisons).

No significant probe sets were found that could explain differences between low- and high-grade ependymoma or between medulloblastoma with and without radiological metastases at diagnosis. The lack of significant probe sets after multiple testing may be due to the limited sample size. To obtain an impression of potentially metastasis-related probe sets in medulloblastoma, we also investigated which probe sets were significantly differentially expressed at $p < 0.01$, without correction for multiple testing, and had a fold-change ratio higher than 5. Remarkably, 3 of 241 probe sets with $p < 0.01$ encoded the *BCAT1* gene. This gene had a median 15-fold increased expression in medulloblastoma patients with radiological metastases than in medulloblastoma patients without radiological metastases (Fig. 4). Between low- and high-grade ependymoma, no probe sets were differentially expressed ($p < 0.01$) with a fold-change ratio higher than 5.

Survival Analysis

Overall survival and event-free survival for medulloblastoma patients were 31.7 ± 9.0% and 29.6 ± 9.0%, respectively. For patients with ependymoma, overall survival was 9.7 ± 9.2% and event-free survival was 8.3 ± 8.0%. In patients with medulloblastoma, increasing age was significantly associated with unfavorable survival ($p = 0.0040$; hazard ratio = 0.82). Metastatic disease was predictive only for unfavorable event-free survival and not for overall survival. For patients with ependymoma, increasing age was not associated with unfavorable outcome.

Using the most discriminative probe sets for either medulloblastoma or ependymoma (394 and 582, respectively, as described above), we performed a Cox regression analysis to identify the probe sets whose expression was predictive for outcome. In patients with medulloblastoma, 14 probe sets were significantly correlated with a favorable overall survival (hazard ratio <1.0), and 21 probe sets were predictive of unfavorable overall

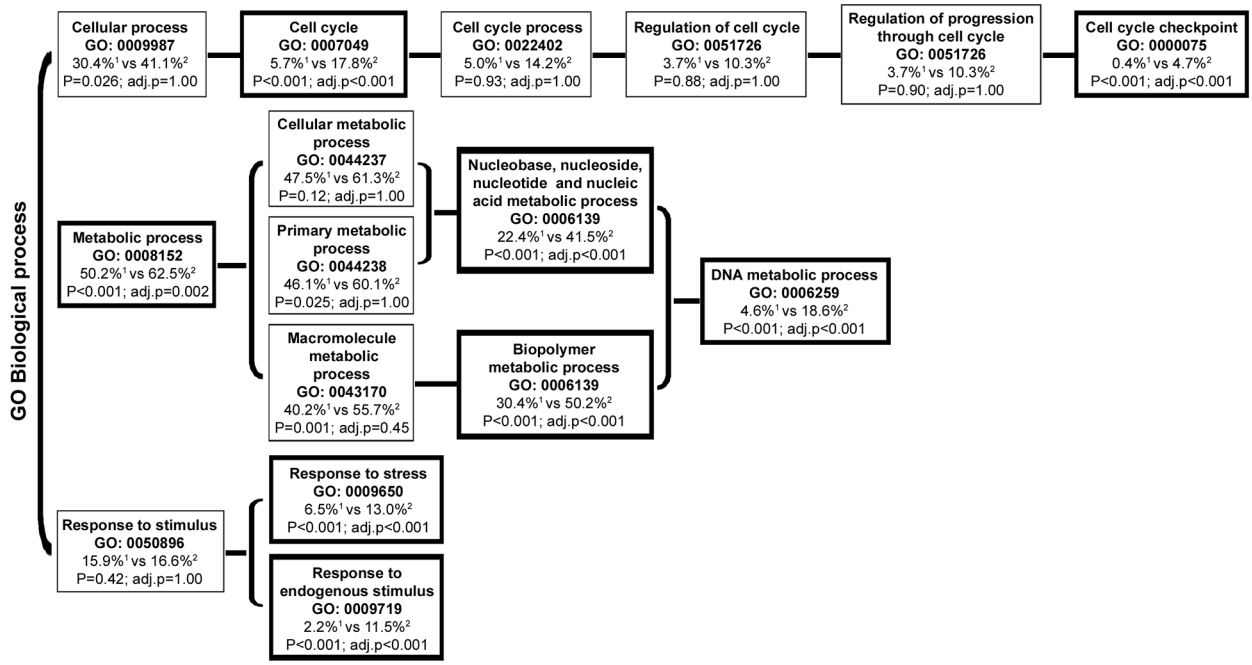

Fig. 2. Functional Gene Ontology database (GO) analysis. The figure displays the GO annotations that were overrepresented with an FDR <1% within the 394 probe sets that were discriminative for medulloblastoma. The 394 probe sets corresponded to 253 genes for which a biological GO annotation was available. Functional categories that are proportionally overrepresented in the sets of selected probe sets compared with all probe sets present on the array are shown in boxes with bold outlining. 1: Percentage of genes from the total number of genes present on the Affymetrix Human Genome U133 plus 2.0 microarrays per GO annotation. 2: Percentage of genes from the selected differentially expressed genes in medulloblastoma per GO annotation. adj.p, *p*-value after correction for multiple testing.

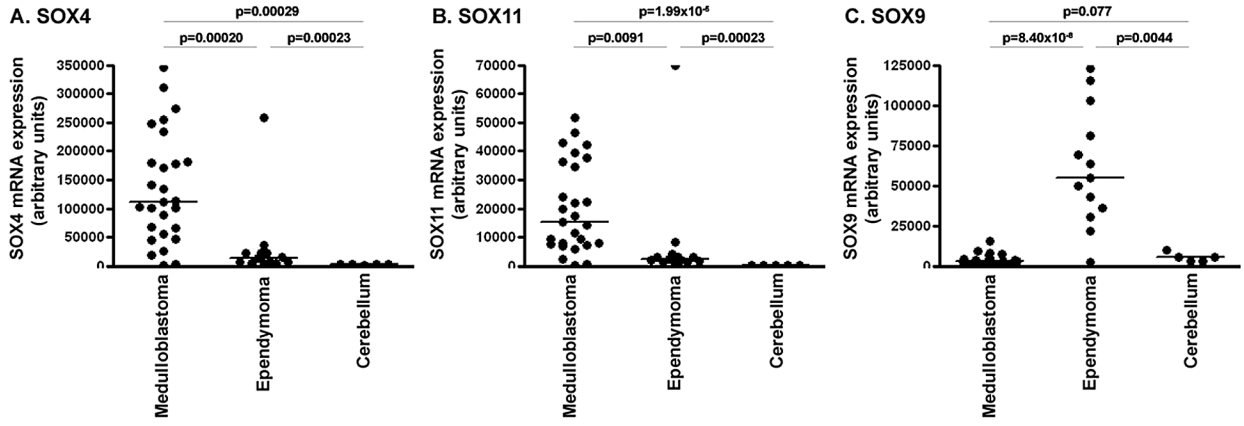

Fig. 3. Differential expression of *SOX4*, *SOX11*, and *SOX9* in medulloblastoma, ependymoma, and control cerebellum: variance stabilization procedure normalized mRNA expression levels of *SOX4* (A), *SOX11* (B), and *SOX9* (C) in medulloblastoma, ependymoma, and cerebellum obtained by Affymetrix Human Genome U133 plus 2.0 microarray analysis. *SOX4* and *SOX11* were significantly overexpressed in medulloblastoma, whereas *SOX9* was significantly overexpressed in ependymoma.

survival (hazard ratio >1.0; Table 1). In patients with ependymoma, six probe sets were associated with favorable overall survival, and seven probe sets were indicative of unfavorable outcome (Table 2).

We also evaluated the aberrantly expressed genes chosen for further analysis (*SOX4*, *SOX9*, *SOX11*, and *BCAT1*) for their predictive effect on survival. There was a trend toward favorable prognosis with increasing *SOX4* expression levels in patients with medulloblastoma ($p = 0.065$; hazard ratio = 0.78). Expression of *SOX9*, *SOX11*, or *BCAT1* was not significantly associated with the outcome of medulloblastoma. *SOX9* expression was significantly associated with favorable overall survival in patients with ependymoma ($p = 0.027$; hazard ratio = 0.44).

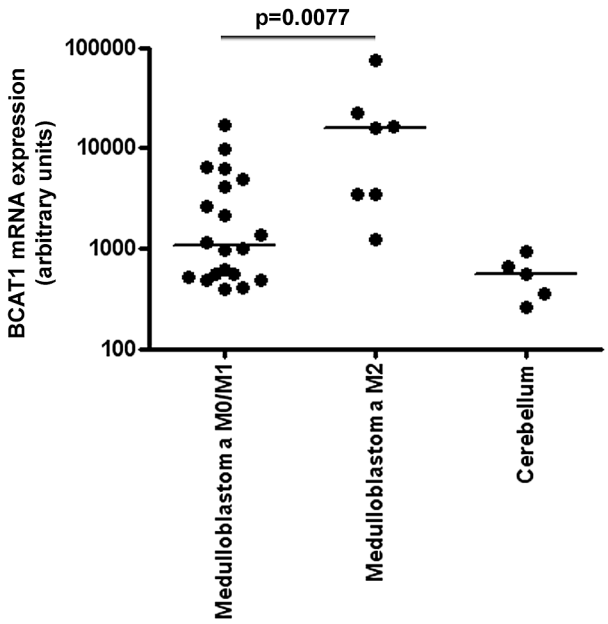

Fig. 4. Differential *BCAT1* expression in metastatic medulloblastoma: variance stabilization procedure normalized mRNA expression levels of *BCAT1* in medulloblastoma without (M0/M1) and with (M2) radiological leptomeningeal metastases at diagnosis obtained by Affymetrix Human Genome U133 plus 2.0 microarray analysis.

Validation of Potential Tumor Markers by Immunohistochemistry

The striking difference in mRNA expression of the three *SOX* genes among medulloblastoma, ependymoma, and cerebellum, and the potential prognostic effect of *SOX4* and *SOX9* expression, prompted us to validate these genes with a different technique in an extended set of patient samples.

Immunohistochemistry confirmed the differential expression of *SOX4* and *SOX11*, showing stronger *SOX4* and *SOX11* staining in medulloblastoma compared with ependymoma (Table 3; $p < 0.001$ for both *SOX4* and *SOX11*). We observed strong to very strong *SOX4* and *SOX11* nuclear positivity in almost 75% of the medulloblastomas. Representative images of intense *SOX4* and *SOX11* staining in medulloblastomas are shown in Fig. 5, A and C, respectively. *SOX4* staining was weak to moderate in all ependymoma cases except one. *SOX11* expression was limited (<50% of the cells) in more than 75% of the ependymomas. Representative images of *SOX4* and *SOX11* staining in ependymomas are shown in Fig. 5, B and D, respectively. *SOX4* and *SOX11* positivity was not observed in normal cerebellum, cortex, normal ependyma, or plexus choroideus.

The high expression of *SOX9* in ependymomas was also confirmed by immunohistochemistry. *SOX9* staining was nuclear. *SOX9* nuclear positivity was strong to very strong in approximately 75% of the ependymomas (Table 3, Fig. 5F) and weak to moderate in more than 90% of the medulloblastomas ($p < 0.001$; Table 3, Fig. 5E). Cerebellum, cortex, normal ependyma, and plexus choroideus were negative for *SOX9*.

Expression of *BCAT1*, which was differentially expressed at the mRNA level between medulloblastoma with and without radiological metastases at diagnosis, was also studied by immunohistochemical analysis (Table 3). At the protein level, the difference in *BCAT1* expression between metastatic and nonmetastatic medulloblastoma was not evident (Table 3). Three medulloblastomas showed very strong homogeneous cytoplasmic *BCAT1* staining (Fig. 5G). Two of these had radiological metastases at diagnosis; one patient did not have radiological metastases, although tumor cells were found in the diagnostic cerebrospinal fluid of this case. Six medulloblastomas also showed very strong staining, but only within distinct areas of the tumor (50%–75% of the cells) (Fig. 5H). None of these patients had radiologically visible metastases at diagnosis, and one of these patients had tumor cells in the diagnostic cerebrospinal fluid. Four medulloblastomas showed intense *BCAT1* staining in less than 50% of the cells (one of these cases had radiological visible metastases at diagnosis, and in another case tumor cells were found in the diagnostic cerebrospinal fluid). In the other medulloblastomas, ependymomas, cerebellum, cortex, normal ependyma, and plexus choroideus, *BCAT1* positivity was absent.

Discussion

In this study we compared gene expression profiles of 27 pediatric medulloblastomas, 13 pediatric ependymomas, and 5 normal cerebella. Because at least parts of all medulloblastomas are thought to arise from the external granular layer of the cerebellum, cerebellum was used as a normal control. For each tumor subgroup, we identified probe sets that were most discriminative and that could be used for functional (GO) and survival analysis. Functional analysis (GO) of the most discriminative probe sets for medulloblastoma mainly showed overrepresentation of genes involved in regulation of the cell cycle, replication, and response to stimuli, but among them there were also many transcription factors. For example, expression of *OTX2* was found to be 140-fold higher in medulloblastoma than in normal cerebellum, which validates earlier findings using serial analysis of gene expression (SAGE).[15]

In addition to *OTX2*, several members of the *SOX* gene transcription factor family were differentially expressed in either medulloblastoma or ependymoma. *SOX4* and *SOX11* were highly overexpressed in medulloblastoma, whereas *SOX9* was highly overexpressed in ependymoma. The differential mRNA expression of *SOX4*, *SOX11*, and *SOX9* was confirmed at the protein level by immunohistochemistry. In addition, survival analysis showed that both *SOX4* and *SOX9* were predictive of outcome in medulloblastoma and ependymoma, respectively. The SOX proteins compose a family of more than 20 transcription factors characterized by the presence of a high-mobility-group (HMG)

Table 1. Probe sets predictive for overall survival in patients with medulloblastoma

Probe ID	Gene Name	Gene Symbol	Chromosomal Location	*p* Value	Hazard Ratio (95% CI)
236852_at	F-box protein 43	FBXO43	8q22.2	0.030	0.096 (0.012-0.80)
209884_s_at	Solute carrier family 4, sodium bicarbonate cotransporter, member 7	SLC4A7	3p22	0.045	0.33 (0.11-0.97)
206142_at	Zinc finger protein 135	ZNF135	19q13.4	0.022	0.36 (0.15-0.87)
1554101_a_at	Transmembrane and tetratricopeptide repeat containing 4	TMTC4	13q32.3	0.022	0.38 (0.16-0.87)
225982_at	Upstream binding transcription factor, RNA polymerase I	UBTF	17q21.3	0.024	0.39 (0.18-0.89)
235422_at	Full-length cDNA clone CS0DB008YK14 of Neuroblastoma Cot 10-normalized of Homo sapiens	—	—	0.036	0.41 (0.17-0.94)
242736_at	—	—	—	0.048	0.43 (0.19-0.99)
244387_at	Transcribed locus	—	—	0.0020	0.46 (0.28-0.75)
204061_at	Protein kinase, X-linked	PRKX	Xp22.3	0.028	0.52 (0.29-0.93)
223627_at	Ring finger and KH domain containing 3	RKHD3	15q25.2	0.045	0.54 (0.30-0.99)
219740_at	Vasohibin 2	VASH2	1q32.3	0.045	0.57 (0.33-0.99)
230596_at	CDNA FLJ39261 fis, clone OCBBF2009391	—	—	0.0060	0.60 (0.42-0.87)
208497_x_at	Neurogenin 1	NEUROG1	5q23-q31	0.0050	0.64 (0.47-0.88)
237007_at	—	—	—	0.028	0.66 (0.46-0.96)
227911_at	Rho GTPase activating protein 28	ARHGAP28	18p11.31	0.052	1.62 (1.00-2.63)
228109_at	Ras protein-specific guanine nucleotide-releasing factor 2	RASGRF2	5q13	0.015	1.63 (1.10-2.41)
213056_at	FERM domain containing 4B	FRMD4B	3p14.1	0.0010	1.81 (1.29-2.54)
204023_at	Replication factor C (activator 1) 4, 37kDa	RFC4	3q27	0.036	2.24 (1.06-4.77)
205393_s_at	CHK1 checkpoint homolog (S. pombe)	CHEK1	11q24-q24	0.051	2.36 (1.00-5.56)
205394_at	CHK1 checkpoint homolog (S. pombe)	CHEK1	11q24-q24	0.043	2.36 (1.028-5.42)
204407_at	Transcription termination factor, RNA polymerase II	TTF2	1p22	0.029	2.80 (1.11-7.05)
201833_at	Histone deacetylase 2	HDAC2	6q21	0.036	2.89 (1.07-7.79)
234863_x_at	F-box protein 5	FBXO5	6q25-q26	0.017	3.39 (1.24-9.23)
211450_s_at	MutS homolog 6 (E. coli)	MSH6	2p16	0.011	3.66 (1.34-10.01)
203207_s_at	Mitochondrial fission regulator 1	MTFR1	8q13.1	0.0050	3.69 (1.48-9.17)
230503_at	Transcribed locus	—	—	0.011	3.89 (1.36-11.17)
234992_x_at	Epithelial cell transforming sequence 2 oncogene	ECT2	3q26.1-q26.2	0.030	3.92 (1.15-13.44)
235295_at	Transcribed locus	—	—	0.023	3.92 (1.20-12.79)
227928_at	Chromosome 12 open reading frame 48	C12orf48	12q23.2	0.027	4.33 (1.18-15.86)
216559_x_at	Heterogeneous nuclear ribonucleoprotein A1	HNRPA1	10q11.22	0.010	4.72 (1.44-15.41)
223225_s_at	SEH1-like (S. cerevisiae)	SEH1L	18p11.21	0.021	6.49 (1.33-31.68)
218433_at	Pantothenate kinase 3	PANK3	5q34	0.024	12.48 (1.40-111.51)
236030_at	REST corepressor 2	RCOR2	11q13.1	0.0010	12.73 (2.78-58.20)
231380_at	Chromosome 8 open reading frame 34	C8orf34	8q13	0.018	14.1 (1.58-125-87)
205953_at	Leucine-rich repeats and immunoglobulin-like domains 2	LRIG2	1p13.1	0.033	35.0 (1.32-927.42)

DNA-binding domain.[17] *SOX4* and *SOX11* belong to subgroup C of the *SOX* gene family. *SOX4* is known to play a role in normal embryonic development of, for example, the heart, CNS, lungs, and thymus.[18–20] *SOX4* has been shown to be overexpressed in various malignancies,[21–23] including medulloblastoma.[16,24] Interestingly, *SOX4* is one of the most frequently targeted genes by retroviral insertional mutagenesis.[25,26] Down-regulation of *SOX4* expression in prostate cancer cell lines resulted in a strong decrease in cell viability and a corresponding increase in apoptosis.[23] However, *SOX4* induction has also been shown to impair cell viability and induce apop-

Table 2. Probe sets predictive for overall survival in patients with ependymoma

Probe ID	Gene Name	Gene Symbol	Chromosomal Location	*p* Value	Hazard Ratio (95% CI)
243929_at	—	—	—	0.022	0.0010 (0.00010-0.29)
238479_at	Full length insert cDNA clone ZC34E11	—	—	0.024	0.0040 (0.00010-0.47)
227313_at	Protein Associated with Tlr4	MGC40499	7q22.1	0.040	0.024 (0.0010-0.85)
233223_at	CDNA FLJ20843 fis, clone ADKA01954	—	—	0.025	0.052 (0.0040-0.69)
201324_at	Epithelial membrane protein 1	EMP1	12p12.3	0.047	0.26 (0.068-0.98)
201426_s_at	Vimentin	VIM	10p13	0.040	0.42 (0.18-0.96)
215321_at	Rap2-binding protein 9	RPIB9	7q21.12	0.027	2.26 (1.10-4.67)
217478_s_at	Major histocompatibility complex, class II, DM alpha	HLA-DMA	6p21.3	0.048	3.53 (1.01-12.39)
229823_at	Transcribed locus	—	—	0.046	10.81 (1.04-112.08)
225792_at	Hook homolog 1 (Drosophila)	HOOK1	1p32.1	0.039	11.72 (1.14-121.01)
211742_s_at	Ecotropic viral integration site 2B	EVI2B	17q11.2	0.030	37.41 (1.43-979.18)
203998_s_at	Synaptotagmin I	SYT1	12cen-q21	0.032	550.73 (1.743-174045.00)
206137_at	Regulating synaptic membrane exocytosis 2	RIMS2	8q22.3	0.0080	1508.40 (6.73-338057.40)

tosis.[27] The contradictory effect of *SOX4* expression on apoptosis in different cell types suggests that *SOX4*, like *c-Myc*, has both anti- and proapoptotic activities. The balance between anti- and proapoptotic signals induced by *SOX4* expression might thus be tissue specific and dependent on external signals. Therefore, and because the biological role of high *SOX4* expression in medulloblastoma is unknown, further research should focus on the functional characteristics of *SOX4*, for example, by modulating *SOX4* expression by RNA interference. In our study, *SOX4* expression was associated with a more favorable outcome in medulloblastoma. Because these findings are in contrast with those of Neben et al.,[28] who found *SOX4* to be a poor prognostic factor in medulloblastoma, prospective studies with large numbers of patients should be conducted to determine the exact influence of *SOX4* on the outcome in medulloblastoma.

SOX11 is expressed in neural precursors throughout the neuroepithelium and is also expressed in areas of the brain in which neurons undergo differentiation during later stages of neural development.[29] *SOX11* was found to be overexpressed in fetal brain tissue and malignant gliomas.[30] Expression of *SOX11* in malignant glioma may be the result of a dedifferentiation process during tumorigenesis, because *SOX11* is normally down-regulated after maturation of the brain.[30] Because we observed medulloblastoma to be characterized by a high expression level of *SOX11*, our data may underline the embryonal origin of these tumors.

In contrast to *SOX4* and *SOX11*, *SOX9* mRNA and protein expression levels were up-regulated in ependymoma. *SOX9* belongs to the subgroup of *SOX* E genes, which control different aspects of differentiation of astrocytes, oligodendrocytes, and Schwann cells.[31] Outside the CNS, *SOX9* is required for chondrogenesis and the development of the male gonads.[32] *SOX9* is likely to be of importance in ependymomas, because, in concordance with our results, strong nuclear *SOX9* expression has also been described in pediatric and adult high-grade neuroepithelial tumors.[33] Moreover, data from the study of Modena et al.[34] (see their supplementary table 3 at pierotti.group.ifom-ieo-campus.it/suppl/epd.html) also confirm *SOX9* overexpression in ependymoma, showing an approximately 6-fold higher *SOX9* expression in ependymomas compared with other tissues in the study. In the intestinal epithelium, *SOX9* was found to be expressed in a pattern characteristic of Wnt targets.[35] Overexpression of *SOX9* in cultured colon carcinoma cells resulted in decreased expression of the tumor-suppressor gene *CDX2*, suggesting *SOX9* to be a potential important contributor to cancer progression.[35] In contrast, *SOX9* expression was significantly associated with a more favorable outcome in ependymoma in our study. Because *SOX9* does not seem to result in potentiation of disease progression of ependymoma, as was suggested with colon carcinoma, larger prospective studies should determine the exact role of *SOX9* as a prognostic factor in ependymoma.

The presence of radiological leptomeningeal metastases at diagnosis is an important adverse prognostic factor in medulloblastoma patients.[36] We found *BCAT1* mRNA expression levels to be median 15-fold increased in medulloblastoma with radiological leptomeningeal metastases. *BCAT1* is a cytosolic branched-chain amino acid aminotransferase reported to be involved in the control of the cell cycle by suppressing the G1-to-S transition of normal cells.[37] *BCAT1* is involved in various malignancies. The mouse homologue of *BCAT1* has shown to be amplified and overexpressed in a teratocarcinoma cell line.[38] Retroviral transduction of fetal rat brain

Table 3. Immunohistochemical results for *SOX4*, *SOX11*, *SOX9*, and *BCAT1* immunoreactivity in medulloblastoma, ependymoma, and control tissues

Gene Expression	*n*	0%–25% Weak	25%–50% Moderate	50%–75% Strong	75%–100% Very Strong
SOX4					
Medulloblastoma	29	7%	21%	31%	41%
Nonmetastatic	25	8%	24%	28%	36%
Metastatic	4	0%	0%	50%	50%
Ependymoma	15	87%	13%	0%	0%
Cellulare	7	86%	14%	0%	0%
Anaplastic	8	88%	13%	0%	0%
Cerebellum	5	100%	0%	0%	0%
Cortex	5	100%	0%	0%	0%
Normal ependyma	5	100%	0%	0%	0%
Plexus choroideus	5	100%	0%	0%	0%
SOX11					
Medulloblastoma	30	7%	10%	30%	53%
Nonmetastatic	24	8%	8%	29%	54%
Metastatic	6	0%	17%	33%	50%
Ependymoma	16	56%	19%	19%	6%
Cellulare	7	57%	29%	0%	14%
Anaplastic	9	56%	11%	33%	0%
Cerebellum	5	100%	0%	0%	0%
Cortex	5	100%	0%	0%	0%
Normal ependyma	5	100%	0%	0%	0%
Plexus choroideus	5	100%	0%	0%	0%
SOX9					
Medulloblastoma	30	63%	30%	7%	0%
Nonmetastatic	25	60%	32%	8%	0%
Metastatic	5	80%	20%	0%	0%
Ependymoma	16	13%	13%	31%	44%
Cellulare	7	0%	14%	14%	71%
Anaplastic	9	22%	11%	44%	22%
Cerebellum	5	100%	0%	0%	0%
Cortex	5	100%	0%	0%	0%
Normal ependyma	5	100%	0%	0%	0%
Plexus choroideus	5	100%	0%	0%	0%
BCAT1					
Medulloblastoma	34	62%	12%	18%	9%
Nonmetastatic	28	64%	11%	21%	4%
Metastatic	6	50%	17%	0%	33%
Ependymoma	5	100%	0%	0%	0%
Cerebellum	5	100%	0%	0%	0%
Cortex	5	100%	0%	0%	0%
Normal ependyma	5	100%	0%	0%	0%
Plexus choroideus	5	100%	0%	0%	0%

cells with SV40 large T-antigen induced tumors with characteristic features of medulloblastoma that showed amplification of *BCAT1*.[39,40] In colorectal adenocarcinomas, high *BCAT1* expression was associated with a greater tendency to metastasize and a decreased disease-free survival rate.[41] Because *BCAT1* was found to be a direct target for *myc* activation in oncogenesis,[37,42] the overexpression of *BCAT1* in malignancies might be a result of *myc* activation. In our study, *BCAT1* mRNA expression was not significantly correlated with *c*-, *N*-,

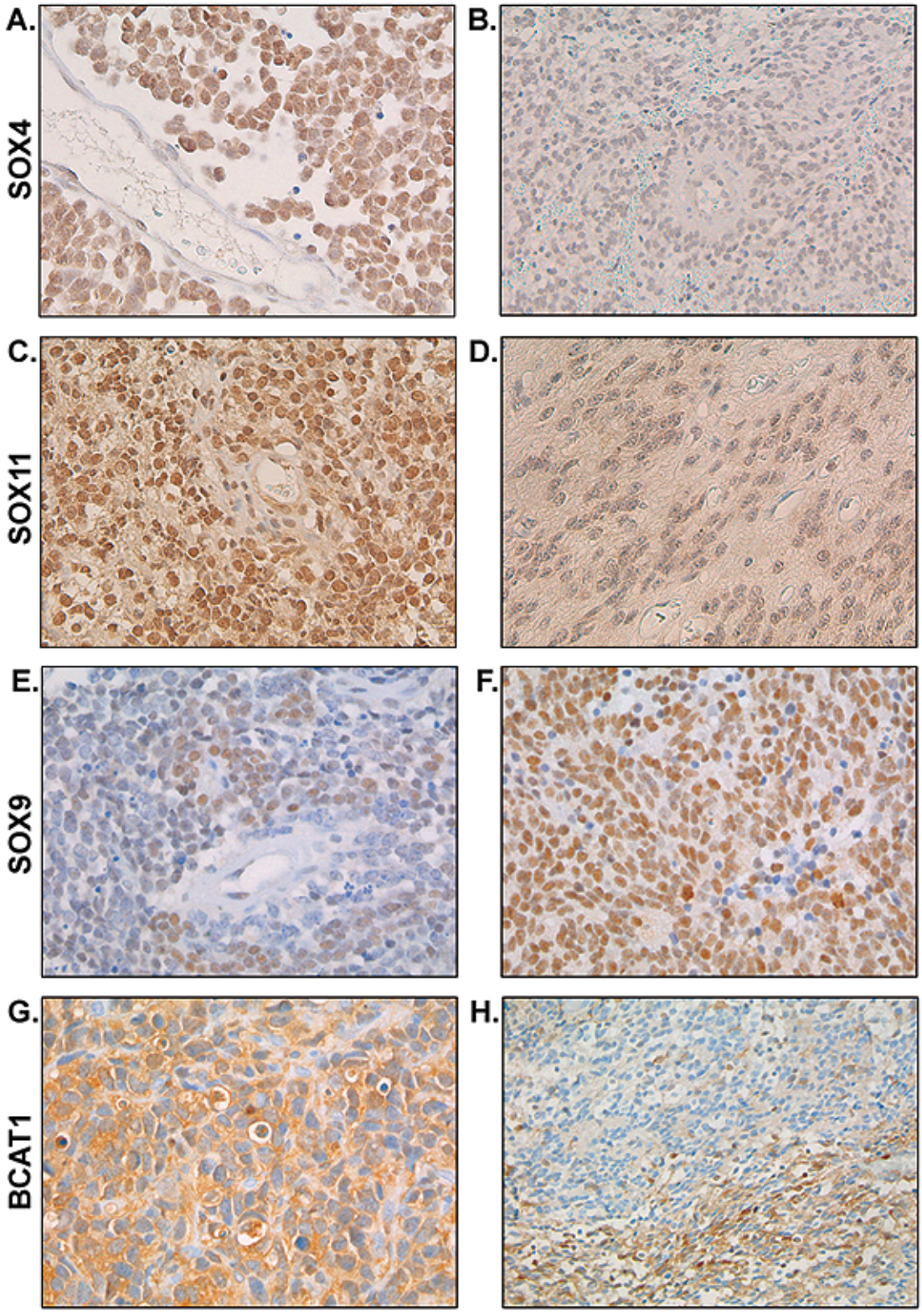

Fig. 5. Immunohistochemistry for *SOX4*, *SOX11*, *SOX9*, and *BCAT1* in medulloblastoma and ependymoma. Immunohistochemistry confirmed the overexpression of *SOX4* in medulloblastoma (A) compared with ependymoma (B). *SOX11* protein expression was also shown to be higher in medulloblastoma (C) compared with ependymoma (D). In contrast, *SOX9* expression was confirmed to be higher in ependymoma (F) compared with medulloblastoma (E). (G and H) Representative images of strong homogeneous cytoplasmic staining of *BCAT1* and focal *BCAT1* staining, respectively, in predominantly metastasized medulloblastoma.

or *L-myc* mRNA expression (data not shown), which suggests that other factors are responsible for the upregulation of *BCAT1* expression in medulloblastoma, as was also suggested for other neuronal tumors.[43]

Evaluation of *BCAT1* expression in the data set of Thompson et al.[7] did not show a significant difference in expression between medulloblastomas with and those without evident metastasized disease (⩾M2 stage). In our study, the correlation between *BCAT1* expression and metastatic disease in medulloblastoma was not as clear at the protein level, because several nonmetastatic medulloblastomas also showed strong *BCAT1* immunoreactivity. The lack of correlation between mRNA and protein expression levels can be caused by the instability of mRNA or protein, high turnover of protein, or translational repression by microRNAs.[44] Moreover, the fact that the pattern of *BCAT1* staining was mainly focal and not homogeneous (Fig. 5H) might also explain why positive *BCAT1* immunohistochemistry did not correlate with high *BCAT1* mRNA expression. Because *BCAT1* positivity has been shown to be very variable within the tumor, overall *BCAT1* expression as detected by microarray analysis might depend on which part of the tumor is analyzed. The focal pattern of staining might suggest the presence of a subset of *BCAT1*–positive tumor cells in some medulloblastomas that may have a higher tendency to metastasize. It would be interesting to specifically study *BCAT1* expression in the cells already metastasized within the CNS. Because these isolated cells were not available, we evaluated *BCAT1* protein expression (Western blot) in the cerebrospinal fluid of several medulloblastoma patients with metastasized disease and a number of control patients. Interestingly, *BCAT1* protein expression was very low in the cerebrospinal fluid of all control patients and high in the cerebrospinal fluid of the medulloblastoma patients (data not shown). Therefore, *BCAT1* might still be an interesting new marker for metastasis, and more extensive studies should be performed. In addition, because *BCAT1* can be inhibited by gabapentin,[45] its use as a therapeutic target should also be investigated.

In addition to evaluating the differential expression of genes in medulloblastoma and ependymoma, we performed a Cox regression analysis to determine which genes were predictive of survival in these tumors. Interestingly, several of these genes have already been shown to be of importance in the biology of malignancies and may well be new interesting therapeutic targets in pediatric brain tumors. In medulloblastoma, one of the genes associated with adverse outcome was *CHEK1*, which codes for a serine/threonine kinase implicated in the DNA damage checkpoint response and the replication checkpoint.[46] Inhibition of *CHEK1* has been used to sensitize tumor cells to DNA antimetabolite chemotherapeutic drugs.[47] Because increasing *CHEK1* expression resulted in worse overall survival for patients with medulloblastoma, inhibition of *CHEK1* expression might also result in higher chemosensitivity of these tumors and consequently higher survival rates.

HDAC2, a histone deacetylase negatively influencing prognosis in medulloblastoma in our study, was overexpressed in colorectal cancer and was required to maintain the transformed phenotype of colon carcinoma cells.[48] In addition, HDAC2 was found to be capable of regulating p53 activity by inhibiting p53 DNA binding.[49]

Interestingly, *ECT2*, another poor prognostic gene for medulloblastoma in our study, was indicative of an unfavorable outcome in glioma.[50]

A gene associated with more favorable prognosis in medulloblastoma was *NEUROG1*, a basic helix-loop-helix transcription factor that is important during neurogenesis. *NEUROG1* has been suggested to be a marker for a subgroup of medulloblastomas deriving from progenitor cells of the cerebellar ventricular zone.[51] *NEUROG1* may thus be of interest in the search for the cells of origin of medulloblastoma. In contrast to our data, Rostomily et al.[52] found a correlation between *NEUROG1* expression and the presence of distant metastases in medulloblastoma. However, they evaluated *NEUROG1* expression in only three medulloblastoma patients without distant metastases.

In conclusion, this study shows that gene expression profiling, using Affymetrix HGU133 plus 2.0 microarrays, is a tool to identify genes that are aberrantly expressed and of prognostic importance in pediatric brain tumors. *SOX4* and *SOX9*, which are overexpressed in medulloblastoma and ependymoma, respectively, also had an influence on outcome. Prospective studies involving a larger number of patients will be needed to confirm the prognostic effect of the genes identified in our study, and analysis of the functional characteristics of the selected genes should reveal the possibilities of using these genes as new tumor markers and therapeutic targets in pediatric medulloblastoma and ependymoma.

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