OTX1 and OTX2 Expression Correlates With the Clinicopathologic Classification of Medulloblastomas

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Abstract

OTX1 and OTX2 are transcription factors with an essential role in the development of the cerebellum. We previously described a high OTX2 expression in medulloblastoma. Here, we analyzed amplification and mRNA expression of OTX1 and OTX2 in a series of human medulloblastomas. In addition, OTX2 protein expression was analyzed on tissue arrays. The OTX2 gene was amplified in the medulloblastoma cell line D425 and mRNA and protein data showed expression in 114 of 152 medulloblastomas (75%), but not in postnatal cerebellum. Northern blot (n = 10) and reverse transcriptase-polymerase chain reaction (n = 45) analyses demonstrated that virtually all medulloblastomas expressed OTX1, OTX2, or both. OTX2 mRNA expression correlated with a classic medulloblastoma histology (29 of 34 cases), whereas expression of OTX1 mRNA only was correlated with a nodular/desmoplastic histology (9 of 11 cases). Immunohistochemical analysis of a series of classic medulloblastomas detected OTX2 protein expression in 83 of 107 (78%) cases. The OTX2-positive tumors of this series were preferentially localized in the vermis of the cerebellum, whereas OTX2-negative tumors more frequently occurred in the hemispheres of the cerebellum. In addition, OTX2-positive tumors were mainly found in children, but OTX2-negative tumors occurred in 2 patient groups: very young patients (<5 years) and adults (>20 years). Nodular/desmoplastic medulloblastomas are thought to arise from the external granular layer (EGL). However, it is unclear whether classic medulloblastomas also originate from the EGL or from the ventricular matrix. Analysis of human fetal brain showed OTX2 protein expression in a small number of presumptive neuronal precursor cells of the EGL, but not in precursor cells of the ventricular matrix. Combined with data from rodents, our results therefore suggest that both nodular/desmoplastic and at least part of the classic medulloblastomas originate from cells of the EGL, albeit from different regions.

Key Words: Brain tumor, Cerebellum, Medulloblastoma, OTX1, OTX2, P75NTR.

INTRODUCTION

Medulloblastomas are malignant embryonal tumors of the cerebellum (1). They are predominantly diagnosed in the first 2 decades of life (2), but they also occur in adults. The tumor accounts for approximately 20% of all central nervous system (CNS) tumors in children. We previously reported that the OTX2 gene can be highly expressed in medulloblastoma (3). The transcription factors OTX1 and OTX2, which have bicoid-like homeodomains, are the human orthologs of Drosophila orthodenticle (otd) (4). In Drosophila as well as in rodents, they are expressed in the developing head and are essential for correct brain development. Very recently, it has been found that the OTX2 gene can be amplified in medulloblastoma (5, 6). Although this further supports a major role for OTX2 in medulloblastoma pathogenesis, it is unknown how OTX2 relates to the clinical and histopathologic features of medulloblastoma.

Two main histopathologic subtypes are recognized in medulloblastoma. Classic medulloblastomas preferably arise in the vermis of the cerebellum and have the propensity to spread through the cerebrospinal fluid to the spinal canal (7–9). Nodular/desmoplastic medulloblastomas, in contrast, more often arise in the hemispheres and often infiltrate in the overlying meninges. They account for approximately 10% to 20% of childhood medulloblastomas (1). The cell type of origin of medulloblastomas is not established. Two types of precursors have been proposed: 1) progenitor cells of the ventricular matrix, which are also present in other parts of the CNS (9); and 2) progenitor cells of the external granule cell layer (EGL), which give rise to cerebellum development (10, 11). Expression analyses of the low-affinity nerve growth factor receptor (P75NTR or NGFR) and MATH1 indicated that nodular/desmoplastic medulloblastomas originate from the EGL. Based on...
expression analysis of calbindin-D28K, it has been proposed that classic medulloblastomas originate from subventricular matrix progenitor cells (7, 12–14).

In this study, we analyzed DNA copy number and mRNA and protein expression of OTX1 and OTX2 in human medulloblastomas. OTX2 was found to be amplified in a medulloblastoma cell line and was highly expressed in many tumors. Virtually all medulloblastomas expressed OTX1, OTX2, or both. OTX2 expression strongly correlated with classic histology, an origin in the vermis, and young age. OTX1 expression correlated with nodular/desmoplastic histology and localization in the hemispheres of the cerebellum. Analysis of OTX2 expression in human embryonal brain suggested that at least part of the classic medulloblastomas may also originate from the EGL, but from another region than the nodular/desmoplastic medulloblastomas.

MATERIALS AND METHODS

Patients

Patient material, including tumors and cell lines, for the panel analyzed by reverse transcriptase–polymerase chain reaction (RT-PCR) was obtained from 37 children and 8 adults diagnosed with medulloblastoma at the Department of Neuropathology, University of Bonn, Bonn, Germany. The cell lines we used were the 4 classic medulloblastoma cell lines D341, MHH-MED3, WU¨-1580, and D283, and the nodular/desmoplastic medulloblastoma cell line DAOY. Clinical data available for all these patients, including the patients from whom we had only tumor-derived cell lines, were age and histology. The median age at diagnosis was 5 years (range, 2–51 years). Five (11%) patients were younger than 3 years of age at diagnosis. Thirty-eight (84%) patients were younger than 19 years of age at diagnosis. Tumors and cell lines from this panel were used to obtain RNA for RT-PCR analysis.

Patient material for the tumor panel analyzed by immunohistochemistry was embedded in paraffin. From 10 medulloblastoma samples from this panel, sufficient fresh-frozen tumor material was available for RNA isolation and Northern blot analysis. Also, from these same 10 samples together with 14 other medulloblastoma samples and the 5 medulloblastoma cell lines D425, UW228-2, D341, MED8A, and DAOY, we isolated genomic DNA, which we used for Southern blot analysis. RNA and protein from the cell lines D425, D341, D283, D556, Med8A, DAOY, and UW228-2 were used for additional Northern and Western blot analysis. The diagnosis of medulloblastoma was confirmed by histologic assessment of the tumor specimen by at least 2 neuropathologists. Samples were collected from 82 children and 25 adults diagnosed with medulloblastoma between 1985 and 2003 at the Academic Medical Center, Amsterdam, The Netherlands. Time of formaldehyde fixation of tissue obtained at autopsy is not known. Samples were collected from the cerebellum of 20 fetuses (9–42 weeks of gestation), 11 children (3 days postpartum to 12 years of age), and 15 adults (24–94 years of age). Informed consent was obtained for the use of brain tissue.

Southern Blot Analysis

Genomic DNA was isolated from 16 medulloblastoma tumors and 5 medulloblastoma cell lines. After isolation, 10 μg Acquisition of tissue specimens and access to medical records were approved by the ethics committee of the Academic Medical Center, Amsterdam. All data were collected retrospectively. Survival was expressed as crude survival. The median age at diagnosis was 9 years (range, 1–53 years). Fourteen patients (11%) were younger than 3 years of age at diagnosis. Ninety-four patients (88%) were younger than 19 years of age at diagnosis. Seventy-five patients (70%) were male, 37 patients (35%) were female, and gender was unknown in 5 (5%) patients. Metastatic stage was assessed by magnetic resonance imaging and lumbar cerebrospinal fluid sampling in 90 patients (84%). Seventy-one patients (78%) had no metastases at diagnosis, and 19 (21%) were stage ≥M1. Data about radiation therapy were available for 92 patients. Eighty-five of these patients (92%) received standard neuraxis radiation. In 91 cases, we had information about chemotherapy. Fifty-seven patients (63%) received chemotherapy. Nine of 10 patients under the age of 3 received chemotherapy.

Brain Tissue

Brain tissue samples were obtained at diagnostic biopsies or autopsy from the cerebellar region of 45 patients of the Academic Medical Center, Amsterdam, The Netherlands. Time of formaldehyde fixation of tissue obtained at autopsy is not known. Samples were collected from the cerebellum of 20 fetuses (9–42 weeks of gestation), 11 children (3 days postpartum to 12 years of age), and 15 adults (24–94 years of age). Informed consent was obtained for the use of brain tissue.

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Brain Tissue

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). Twenty micrograms total RNA was separated on a glyoxal gel followed by blotting on Hybond-N (Amersham Biosciences, Freiburg, Germany) according to standard procedures. Hybridization of the probes to the RNA blots was performed according to Church and Gilbert (15). A GAPDH probe was used as control for RNA loading of the lanes. Hybridized probe was visualized on a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA). Probes for the Northern blot were obtained by standard PCR on cDNA. For ZIC1 (GenBank NM_003412), a 300-bp fragment was generated using the primers 5′-ACGATTACAGTCTCCAGGTCG-3′ and 5′-GCCCT- TTACATGCGGATATAA-3′ (reverse). An OTX2 (GenBank NM_021728) probe of 292 bp was generated using the primers 5′-ACGATTACAGTCTCCAGGTCG-3′ (forward) and 5′-ATCTGCAAATCCAGAAGAA-3′ (reverse). A GAPDH (GenBank NM_002046) probe of 292 bp was generated using the primers 5′-CTGAGAGCAGGAGGAGAGGT-3′ (forward) and 5′-GGTGCTAAGCAGTGTGTTTCT-3′ (reverse). Fragments were cloned into pGEM-T Easy Vector (Promega). The OTX1 probe was obtained from the IMAGE consortium (clone no. 1872843). The identity of the clones was confirmed by sequence analysis.

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of DNA was digested using TaqI. The digested DNA was precipitated and electrophoresed on a 0.8% agarose gel and transferred to a Hybond N+ filter (Amersham) by Southern blotting according to standard procedures. For hybridization, an OTX2 (GenBank AF298117) and OTX1 probe (GenBank NM_014562) were obtained by standard PCR on genomic DNA. A 992 bp OTX2 fragment from intron 2 was generated using the primers 5’-GGTTGCTGGGAGAGAC-3’ (forward) and 5’-ATGCATCTGTTGAGCTC-3’ (reverse) and detected an 1160-bp fragment on Taq1-digested genomic DNA. The 898-bp OTX1 fragment was generated using the primers 5’-GCAACACCTCGTATGCA-3’ (forward) and 5’-GGCAGAACAGCCAGTAG-3’ (reverse) and detected a 2222-bp fragment on Taq1-digested genomic DNA. A DLK1 (Delta-like-1; GenBank NM_003836) probe of 2428 bp was generated using the primers 5’-GGTTGCTGGTGGAGAGAC-3’ (forward) and 5’-AGGCTTGTGATGAGC-3’ (reverse). The DLK1 probe detected a 2428-bp fragment on Taq1-digested genomic DNA. The DLK1 probe was used as a DNA loading control of the samples. The fragments were cloned into pGEM-T Easy Vector (Promega, Madison, WI) and sequenced. The identity of the OTX2/DLK1 and OTX2/DLK1 probes was confirmed.

Western Blot Analysis

Protein was isolated from 7 medulloblastoma cell lines. Protein concentration was measured using the DC Protein Assay (Bio-Rad Laboratories, Life Science Group, Hercules, CA). Aliquots of 10 μg cell-extracted protein prepared in SDS sample buffer were incubated for 5 minutes at 95°C. Denatured proteins were separated by SDS PAGE and then transferred to a Hybond N+ filter (Amersham) by Southern blotting. The membrane was incubated with the OTX2-specific monoclonal antibody, which was made in the laboratory of Dr. G. Corte, Genoa, Italy (16). The antibody was used in a 1:2000 dilution. The membrane was incubated for one hour followed by incubation for one hour with a 1:2000 dilution of horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (Amersham Biosciences). Immune complexes were detected by ECL Plus Western blotting detections reagents using peroxidase-conjugated secondary antibodies (Amersham Biosciences). Coomassie staining was used as control for protein loading of the lanes.

Semiquantitative Reverse Transcriptase–Polymerase Chain Reaction

The following primer sets were used for the RT-PCR for OTX1 (forward) 5’-TCAAACACACCCCCACAGGCCG-3’ and OTX1 (reverse) 5’-GAATTTGCAGACTTCTCAG-3’; OTX2 (forward) 5’-CAGATTGGCTGGGACATGCG-3’ and OTX2 (reverse) 5’-TTCCGACATCAGTCTGCG-3’; β2-microglobulin (β2M) 5’-TCTCTTCTTCTGCTGTC-3’ (forward) and 5’-AGTCTGCTCATTCTAGTCG-3’ (reverse). These primer combinations gave products of 368 bp, 200 bp, and 148 bp, respectively, and all cross intron sequences. Duplex RT-PCRs were carried out in a final volume of 10 μL containing 100 ng of cDNA, 10 pmol of each B2M primer and 2.5 pmol of the OTX1 or OTX2 primers, 10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, and 200 mM of each deoxynucleotide. The forward primers of OTX1, OTX2, and B2M were labeled with the immunofluorescent dye IRD-800 (MWG Biotech, Ebersberg, Germany). The PCR program consisted of initial denaturation at 94°C for 5 minutes, followed by 32 cycles of 94°C for 30 seconds, 57°C for 40 seconds, and a final extension step at 72°C for 10 minutes. The number of cycles was determined to be in the exponential phase by kinetic experiments using 20 to 38 cycles. All reactions were performed several times, at least twice, and always with positive and negative controls and were highly reproducible. The products were analyzed on 4.5% denaturing acrylamide gels using a LI-COR 4200 semiautomatic DNA sequencer. Expression of OTX1 and OTX2 was not quantified but scored as “+” or “−.”

Immunohistochemistry

All tissue sections were embedded in paraffin. The Manual Tissue Arrayer 1 from Beecher Instruments (Sun Prairie, WI) was used to compose 2 medulloblastoma tissue arrays. The recipient paraffin blocks contained, respectively, 34 and 63 0.6-mm tumor samples in triplet. The other 10 medulloblastoma specimens were not suitable for the array because of the tumor size or the heterogeneity of the tumor and were analyzed separately. Sections were made of the 2 arrays (4 μm) and the other tumor samples (5 μm). All sections were deparaffinized according to standard procedures and blocked in 0.3% H₂O₂ in methanol at room temperature for 20 minutes. All sections were incubated in TrisEDTA for 10 minutes at 100°C, blocked with 10% normal goat serum for 15 minutes, and incubated with the OTX2-specific monoclonal antibody (16) (dilution 1:500) or a P75NTR-specific monoclonal antibody (DakoCytomation, Glostrup, Denmark; dilution 1:50) for one hour at room temperature. Detection of antibody binding was performed using Powervision poly-HRP (Powervision; Immunologic DPV-999HPR, Brisbane, CA) according to the protocol. Immunohistochemistry of primary tumor sections was scored blind on separate occasions by 2 neuropathologists. Tumors were scored positive for OTX2 or P75NTR when in at least one of the sections of the triplet more than 10% of the cells staining was detected. However, in only 3% of the cases OTX2 staining was not reproducible in every section, and in only 11% of the cases the final score was based on only one section as a result of loss of the other sections on the tissue array. For P75NTR, these numbers were 3% and 5%, respectively.

Mutation Analysis

PCR and sequencing were used to screen for mutations in the OTX2 gene in 18 tumors from the tumor panel analyzed by immunohistochemistry, Southern and Northern blot analysis, and the 5 medulloblastoma cell lines, DAOY, D341, D425, UW228-2, and MED8A. OTX2 consists of 3 exons in the coding region. The primer pairs for amplification of each exon and flanking intronic sequences were designed on the basis of
the genomic sequence NT_026437. The following primer sets were used: exon 1 (forward) 5’-TAAACCAGCCCTCTGGTTG-3’, (reverse) 5’-GTGGGGAAGTTGTTGTGTTT-3’; exon 2 (forward) 5’-GCTGAGGCTGAGGAAC-3’, (reverse) 5’-TGCCCTAAGTTGGAAG-3’; exon 3 primer set A (forward) 5’-GCAGGAAAATTTGTGTTCTT-3’, (reverse) 5’-ATGCCCCAAATGGGAGTTTT-3’; exon 3 primer set B (forward) 5’-CATCTCCCCTGTGAACTGATC-3’, (reverse) 5’-GCCTGGCTAAAACGGAATG-3’. DNA (50 ng) was amplified using a Biometra Thermocycler using the following PCR conditions: a denaturation step at 94°C for 10 minutes, then 40 cycles of denaturation at 94°C for one minute, one minute annealing at 58°C, 60°C, 60°C, and 58°C, for exon 1, exon 2, exon 3 part A, and exon 3 part B, respectively, followed by extension at 72°C for one minute and finishing with 72°C for 10 minutes. PCR products were sequenced using the ABI dye terminator cycle sequencing kit version 2.0 and an ABI 3100 Prism sequencer. Sequences were analyzed with Phred, Phrap, and PolyPhred analysis.

Statistical Analysis

Relationships among tumor characteristics, clinical variables, and OTX2 expression were tested using the Pearson χ² test. The crude survival time (CS) was measured from the date of diagnosis to the date of death or last control date. Distribution of CS was estimated by the Kaplan-Meier method; the log rank test was used to compare the equality of each survival distribution. The significance level for all tests was set at 0.05 using a 95% confidence interval. All statistical calculations were performed in SPSS 12.5 for Windows.

RESULTS

OTX1 and OTX2 Expression in SAGE Libraries

We have previously observed a high OTX2 expression in 2 medulloblastoma SAGE libraries ([3] and unpublished data). Since then, numerous medulloblastoma as well as other tumor and normal tissue SAGE libraries have been constructed (see Cancer Genome Anatomy Project; http://cgap.nci.nih.gov/sage) (17, 18). Analysis of all these SAGE libraries for the presence of the SAGE tags for OTX1 (GCGGTTCCAG) and OTX2 (ACCAACTGGT) confirmed that OTX2 is highly expressed in a subset of medulloblastoma tumors and cell lines (Fig. 1). In contrast, OTX2 expression is hardly found in other tumors and tissues, except for moderate expression in several human embryonal stem cell lines, and weak expression in a few other tumors and tissues. OTX1 expression was only weakly detected in one medulloblastoma cell line and one tumor, as well as in some brain, breast, prostate, and colon tumors (data not shown). OTX1 and OTX2 expression was undetectable in SAGE libraries of normal cerebellum.

Southern Blot Analysis

The SAGE data suggested that a subset of medulloblastomas is marked by high OTX2 expression. Recently, OTX2 gene amplifications were found in a subset of medulloblastoma tumors and/or cell lines (5, 6). We therefore performed Southern blot analysis for 24 medulloblastoma tumors and 5 medulloblastoma cell lines. OTX2 was amplified in the medulloblastoma cell line D425, confirming the results of both Di et al and Boon et al, but not in any of the 24 tumors and other cell lines (Fig. 2). We also analyzed the same medulloblastoma series for OTX1 copy number changes, but no amplifications were found (data not shown). In addition, the
coding sequence of the OTX2 gene was sequenced in 18 medulloblastoma tumors and the 5 cell lines. No mutations were found. The observed amplification shows that OTX2 probably functions as an oncogene in medulloblastoma. Although the frequency of amplification is low and may suggest a bias toward cell lines (see “Discussion”), the data lends further support for a role of OTX2 in medulloblastoma pathogenesis.

OTX1 and OTX2 mRNA Expression Correlates With Nodular/Desmoplastic or Classic Histology

We studied OTX1 and OTX2 mRNA expression by Northern blot analysis of 10 medulloblastoma tumors and 2 human fetal brain samples of 16 and 24 weeks of gestation. All tumors expressed OTX1, OTX2, or both (Fig. 3A). Five medulloblastomas showed very high OTX2 expression, whereas 2 other tumors had substantial OTX1 signals. The remaining 3 tumors only weakly expressed OTX1 or OTX2. The fetal brain samples showed expression of OTX1, but not of OTX2. The filter was also hybridized with a probe for the medulloblastoma marker ZIC1, which was expressed in all samples (11).

To extend the analysis of OTX1 and OTX2 gene expression, a series of 40 medulloblastoma tumors and 5 cell lines were analyzed by semiquantitative RT-PCR. A representative example of the RT-PCR results for OTX2 is shown in Figure 3B. Again, virtually all tumors (i.e. 44 of 45) and cell lines expressed OTX1, OTX2, or both, but normal adult cerebellum was negative for both genes. Thirteen samples expressed OTX1 only, 16 samples expressed OTX1 and OTX2, whereas 15 samples expressed only OTX2 (Table 1). Expression of OTX2, regardless of OTX1 expression, strongly correlated with a classic histology (29 of 34 cases), whereas expression of OTX1 only, without OTX2 expression, was strongly associated with nodular/desmoplastic medulloblastoma (9 of 11 cases) (χ²-test, p ≤ 0.001). In samples with both OTX1 and OTX2 expression, OTX2 determines the correlation with histology, because 14 of 16 samples with expression of both genes were of the classic phenotype.

OTX2 Protein Analysis

Seven medulloblastoma cell lines were analyzed for OTX2 protein expression by Western blot analysis using a monoclonal antibody specific for OTX2 (16) (Fig. 4). OTX2 protein was found to be strongly expressed not only in the D425 cell line with the OTX2 amplification, but also in the D283, D341, and D556 cell lines. Very low levels of OTX2 were detected in the MED8A cells, but no OTX2 was found in the DAOY or UW228-2 cells (Fig. 4). The presence or absence of OTX2 protein expression correlated well with the presence

<table>
<thead>
<tr>
<th>Histology</th>
<th>OTX1 Only (n = 13)</th>
<th>OTX1 and OTX2 (n = 16)</th>
<th>OTX2 Only (n = 15)</th>
<th>None (n = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classic</td>
<td>4</td>
<td>14</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>Desmoplastic</td>
<td>9</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* OTX2-positive and OTX2-negative versus histology, χ² test, p = 0.001.
or absence of OTX2 mRNA expression as shown by Northern blot analysis (Fig. 4). Only in the MED8A cells with very low levels of OTX2 protein, no detectable signal was found for OTX2 mRNA. Interestingly, the D425 cell line, which has the OTX2 amplification, showed the highest level of OTX2 mRNA expression, but this cell line did not have the highest level of OTX2 protein, indicating that there are other mechanisms that may regulate the OTX2 protein expression as well.

**OTX2 Protein Expression in Tumors**

Because OTX2 expression strongly correlated with a classic histology, we analyzed a series of 107 classic medulloblastomas at the protein level. A tissue array, containing 107 classic medulloblastomas, was stained with the same OTX2 antibody as used for the Western blot analysis (16). All immunostainings were easily interpretable as positive (strong nuclear staining) or negative (completely absent). Normal cerebellum tissue surrounding the tumor was always negative (Fig. 5A, B). A clear positive OTX2 signal was found in 83 of 107 classic tumors (78%). This is in agreement with the PCR data that showed OTX2 expression in 29 of 34 (85%) of the classic medulloblastomas. We also analyzed nodular/desmoplastic medulloblastomas and found OTX2 expression in some of the tumors. However, as a result of the low number of nodular/desmoplastic cases available, we could not draw any conclusions about the frequency of OTX2 staining in this pathologic subtype. An interesting observation on the OTX2 staining in the nodular/desmoplastic medulloblastomas was that, in contrast to the classic medulloblastomas in which the OTX2 staining was in most cases present in all tumor cells, in the nodular/desmoplastic medulloblastomas with OTX2 expression, the protein was mainly detected in the less differentiated cells surrounding the nodules, but there was none or much less in the more differentiated cells within the nodules (Fig. 5C).

**OTX2 Protein Expression Correlates With Localization of the Tumor**

Because there is an association between classic medulloblastomas and an origin in the vermis of the cerebellum (7, 13), we analyzed the site of tumor at diagnosis in our array series. For 97 classic medulloblastomas, we had information on the localization of the tumor at diagnosis. Indeed, 76 of 97 (78%) classic medulloblastomas originated from the vermis. There was a significant correlation between OTX2 expression and an origin in the vermis ($\chi^2$ test, $p < 0.01$) (Table 2). We conclude that only classic medulloblastomas with OTX2 expression are preferentially found in the vermis of the cerebellum. In contrast, classic medulloblastomas without OTX2 expression are more frequently found in the hemispheres, just like nodular/desmoplastic medulloblastomas (7, 13). These data suggest that expression of OTX1 is strongly correlated with localization in the hemispheres.

Interestingly, it was previously reported that the nodular/desmoplastic histology and localization in the hemispheres is associated with expression of the low-affinity nerve growth factor receptor (P75NTR or NGFR) (13). Combined with our data, this would suggest an inverse correlation between P75NTR and OTX2. We therefore tested P75NTR expression on our
tissue array of classic medulloblastomas. Eighty-seven samples were stained for $P75^{NTR}$ expression, of which 20 (23%) were positive. $P75^{NTR}$ expression in medulloblastomas was indeed inversely correlated with OTX2 expression ($\chi^2$ test, $p \leq 0.001$) (Table 3).

For 90 samples (70 OTX2-positive and 20 OTX2-negative) analyzed by immunohistochemistry, we had access to clinical data, including follow up. Thirty-two patients (36%) had died from disease. The median follow-up time was 27 months, ranging from one to 233 months. OTX2 expression did not correlate with outcome, metastatic disease, or tumor recurrence, or with chosen treatment modalities like chemotherapy, radiotherapy, or extent of surgery (data not shown). However, age at diagnosis was correlated with OTX1 or OTX2 expression.

**Correlation of OTX2 Expression and Age of Patients With Medulloblastoma**

The age at diagnosis of the 107 patients with classic medulloblastomas analyzed on the tissue array showed that OTX2-positive tumors mainly arise in children (Fig. 6). OTX2 was expressed in 89% of tumors in children (≤18 years), whereas in adults (>18 years), this was only 38% (Table 4). OTX2-negative tumors in contrast are found either in very young children or in adults (Table 4). In children, patients with OTX2-negative tumors are significantly younger than patients with OTX2-positive tumors (average age, 4.0 years vs 7.7 years). Similar results were found for the tumors analyzed by RT-PCR (data not shown). The 2 age groups of OTX2-negative tumors did not significantly differ regarding localization of the tumor, although numbers are small for a reliable analysis. The minor difference in $P75^{NTR}$ expression between both groups was not significant.

**TABLE 2. Correlation Between Localization of the Tumor at Diagnosis and OTX2 Protein Expression in Classic Medulloblastomas (n = 97) as Determined by Immunohistochemistry**

<table>
<thead>
<tr>
<th>Localization (n = 97)</th>
<th>OTX2-Negative (n = 19)</th>
<th>OTX2-Positive (n = 78)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vermis (n = 76)</td>
<td>10</td>
<td>66</td>
</tr>
<tr>
<td>Hemispheres (n = 21)</td>
<td>9</td>
<td>12</td>
</tr>
</tbody>
</table>

$^*$, $\chi^2$ test, $p \leq 0.001$.  

**TABLE 3. Negative Correlation Between OTX2 and $P75^{NTR}$ Protein Expression in Classic Medulloblastomas as Determined by Immunohistochemistry (n = 85)$^*$

<table>
<thead>
<tr>
<th>$P75^{NTR}$-Positive (n = 19)</th>
<th>$P75^{NTR}$-Negative (n = 66)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTX2-positive (n = 66)</td>
<td>9</td>
</tr>
<tr>
<td>OTX2-negative (n = 19)</td>
<td>10</td>
</tr>
</tbody>
</table>

$^*$, $\chi^2$ test, $p \leq 0.001$.  

**OTX2 Expression in Normal Cerebellum During Human Development**

It is currently assumed that nodular/desmoplastic medulloblastomas originate from the EGL in the cerebellum. The origin of classic medulloblastomas is less clear. Both an origin from the EGL and the ventricular matrix has been proposed. In our study, we found a strong correlation between the expression of OTX2 and classic medulloblastomas. Studies in rodents have shown that OTX2 is expressed in cells of the EGL during development, but not in cells of the ventricular matrix (19). We therefore have examined the expression of OTX2 during normal human cerebellum development. Different ages of human fetal, postnatal, and adult cerebella were immunohistochemically analyzed for the presence of the OTX2 protein. OTX2 protein was only detected in cerebella of the fetal stage (Fig. 7A, B), whereas other cerebella (11 children and 15 adults; age range, 0–94 years old) were negative for OTX2. The earliest embryonal stage analyzed was in 2 fetuses that were 9 weeks old. No cerebellum has been developed yet at this stage, but the fourth ventricle with the upper and lower rhombic lip is clearly visible. The neuro-epithelial cells of the rhombic lip were negative for OTX2 expression. In contrast, cells of the choroid plexuses and some matrix cells of the spinal cord were positive for OTX2, as well as the retina and the epithelial cells of the skin (data not shown). Also, early stages of the EGL appeared to express OTX2. This was evident in the embryonal stages, 23 to 42 weeks, for which we only analyzed the cerebellum. OTX2 protein was detected from week 23 on in a small number of presumptive neuronal precursor cells of the EGL. Figure 7A shows positive OTX2 staining in the mitotic neuroblasts in the subpial stratum of the EGL, but not in the postmitotic fusiform neurons in the substratum of the EGL. At later stages (26 weeks and later), OTX2 expression was also detected in cells of the internal granular layer (IGL) (Fig. 7B) and in Purkinje cells and neurons of the dentate nuclei. However, in the Purkinje cells and the neurons of the dentate nuclei, the staining was restricted to the cytoplasm (Fig. 8). These data
show that, similar to previous findings in rodents, OTX2 is also expressed in the EGL in humans, but not in precursor cells of the ventricular matrix. This suggests that not only nodular/desmoplastic, but also part of the classic medulloblastomas may originate from the EGL. However, the OTX2 staining in Purkinje cells, the progeny of the ventricular neuroepithelium of the velum medullare, does not exclude an origin of some classic medulloblastomas in the ventricular matrix.

DISCUSSION

OTX1 and OTX2 in Medulloblastoma

The transcription factors OTX1 and OTX2 are essential for normal brain development and are switched off after birth. Here, we show that almost all medulloblastomas and cell lines express either OTX1 or OTX2 or both and that the OTX2 gene is highly amplified in the medulloblastoma cell line D425. No expression of either OTX1 or OTX2 was detected in postnatal normal cerebellum. These data suggest that OTX2 is an oncogene important in the tumorigenesis of medulloblastomas. Recently, Boon et al (5) and Di et al (6) also reported OTX2 amplifications in medulloblastoma tumors and cell lines. Although Boon et al (5) detected amplified OTX2 in 8 of 42 medulloblastoma tumors and 2 of 11 medulloblastoma cell lines, Di et al (6) found no OTX2 amplifications in 44 analyzed tumors, but only in 3 of 13 medulloblastoma cell lines. Our data and data from Di et al (6) strongly suggest that amplification of OTX2 is not a frequent event in medulloblastoma tumors, but rather is found in medulloblastoma cell lines. Although this might suggest that OTX2 amplification is an artifact caused by cell culturing, Di et al reported that OTX2 amplification was observed in 2 cell lines obtained from the same patient, one from the primary tumor (D425) and one from a metastasis (D458) (6). This indicates that OTX2 amplification may indeed be present in medulloblastoma tumors, but probably in a small subgroup that can be more easily taken in culture than tumors without amplification. The more frequent observation of OTX2 amplification in tumors by Boon et al (5) could therefore result from analysis of a different clinical subset by them.

Although the amplification rate of OTX2 might be low, our OTX2 expression data, analyzed at the mRNA and protein level in 2 independent medulloblastoma panels, showed that more than two thirds of the tumors were positive for OTX2. These data are in line with recent data from Yokota et al (20) who also detected OTX2 mRNA expression in 18 of 25 medulloblastomas (72%). Although our analyses showed that almost all medulloblastomas express either OTX1 and/or OTX2, the analyses of SAGE libraries indicate that OTX expression is highly specific for medulloblastomas. Other brain tumors like ependymomas, astrocytomas, oligodendrogliomas, meningiomas, and glioblastomas did not express OTX1 or OTX2 (Fig. 1).

Expression of OTX1 or OTX2 Correlates With Histology, Tumor Localization, and Age

The expression of OTX genes in medulloblastomas appears to correlate with some of the major biologic parameters of this tumor. Most strikingly, there is a strong correlation with the histopathologic classification of medulloblastomas. Presence of OTX2 mRNA expression, regardless of OTX1 expression, strongly correlated with classic histology. However, sole expression of OTX1 strongly correlated with nodular/desmoplastic histology. Protein analysis of a series of classic medulloblastomas indeed showed that the majority (78%) of these tumors expressed OTX2. OTX2 staining was also observed in some nodular/desmoplastic medulloblastomas (Fig. 5C), but as a result of the very low numbers of nodular/desmoplastic tumors we could use for immunohistochemistry, we did not include them in our statistical analyses. However, the lesser degree of expression of OTX2 in the majority of nodular/desmoplastic medulloblastomas as seen by RT-PCR and immunohistochemistry may be differentiation-dependent. The strong OTX2 staining in the classic medulloblastomas may be the result of the higher degree of poorly differentiated cells in this pathologic subtype. In the nodular/desmoplastic tumors in which we detected OTX2 protein, the protein was indeed also located in the proliferating and poorly differentiated cells around the nodules, whereas the more differentiated cells in the nodules show no or much less OTX2 staining. The OTX2 staining in the proliferating mitotic neuroblasts, but not in the postmitotic neurons in the EGL of the developing cerebellum (Fig. 7A), is also an indication that OTX2 staining may be restricted to less differentiated cells. Further studies and more nodular/desmoplastic medulloblastomas are necessary to examine this in more detail.

The protein analysis of the classical medulloblastomas showed that the OTX2-positive tumors mainly originated from the vermis of children and frequently lacked p75NTR expression. OTX2-negative tumors, which are virtually all OTX1-positive, either arise in very young children or in adults. They are more frequently located in the lateral hemispheres, are strongly associated with the nodular/desmoplastic pathologic subtype, and are p75NTR-negative. A correlation between younger age and localization of the tumor in the vermis has been

TABLE 4. Average Ages (Years ± Standard Deviation) of Patients With Medulloblastoma With and Without OTX2 Protein Expression as Determined by Immunohistochemistry

<table>
<thead>
<tr>
<th>Category</th>
<th>Average Age</th>
<th>OTX2-Negative Tumors</th>
<th>OTX2-Positive Tumors</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td>21.7 ± 15.3</td>
<td>9.9 ± 8.0 (n = 83; 1–46 years)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Children (&lt;18)</td>
<td>4.0 ± 2.3</td>
<td>7.7 ± 4.2 (n = 74; 1–16 years)</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>Adults (&gt;18)</td>
<td>30.5 ± 10.2</td>
<td>28.0 ± 8.8 (n = 9; 19–46 years)</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

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reported by others as well (13, 14, 21–24), but our results show that this is true only for the OTX2-expressing tumors. OTX2-negative medulloblastomas arising in young children are more frequently found in the lateral hemispheres of the cerebellum. Recent data from Salsano et al (14) for MATH1 suggest that this subset will also express MATH1, just like the OTX2-negative medulloblastomas from adults.

OTX2 protein expression appeared to have no prognostic value. Our RT-PCR analysis showed that almost all OTX2-negative tumors do express OTX1. It has been demonstrated in transgenic mice models that OTX1 and OTX2 are functionally equivalent proteins (25, 26). This may also be the case in tumors, which would explain why there is no prognostic effect of OTX2. Whether the presence of OTX2 amplification in tumors has any prognostic value is currently unknown, because larger series have to be analyzed.

Expression of OTX Genes in Normal Cerebellum and the Origin of Medulloblastomas

The expression pattern of OTX2 protein in human embryonal cerebellum appeared to be very similar to what has been reported for rodents (19, 27, 28). Both in rats and mice, Otx2 expression is also detected in granule cells and their
precursors, but not in cells of the ventricular matrix. In embryonal and early postnatal stages, Otx2 is first only expressed in cells of the EGL and later also in cells of the IGL. At later postnatal stages, the Otx2 expression in EGL cells disappears, but is still detectable in IGL cells until P15 in mice. Frantz et al (19) also observed that in rats, both Otx1 and Otx2 are expressed in the EGL, but in 3 spatially distinct domains of the cerebellum. Both genes are expressed in opposing gradients. Otx1 expression was strongest in anterior regions and declined toward more posterior regions, whereas the highest levels of Otx2 expression were found in the most posterior regions of the cerebellum and declined anteriorly. A region of overlap in midcerebellum defines a domain in which both genes are expressed. Whether these gradients of Otx1 and Otx2 expression are also present during human fetal cerebellum development remains to be analyzed. Our results offer only preliminary observations and do not represent a definitive mapping of Otx2 protein in the developing human cerebellum. Further studies in this regard are necessary to elucidate the precise expression pattern of Otx2 during the development of the human cerebellum.

The expression patterns of Otx1 and Otx2 during normal cerebellum development in rodents and in humans and our results for the expression of these genes in medulloblastomas suggest that most medulloblastomas originate from progenitor cells in the EGL, but from different regions. Nodular/desmoplastic medulloblastomas are indeed thought to arise from the EGL, but it has been postulated that classic medulloblastomas have their origin in cells derived from the ventricular matrix. This hypothesis is mainly based on the lack of expression in classical medulloblastomas of genes shown to be expressed in the EGL such as P75NTR and MATH1 (13, 14). A positive marker reported for classic medulloblastomas is calbindin-D28K (12). In normal cerebellum, calbindin-D28K is expressed in Purkinje cells and their precursors in the ventricular neuroepithelium of the velum medullare (29), suggesting that a subset of classic medulloblastomas may be derived from the ventricular matrix as opposed to EGL (7, 8, 12). Our results, however, clearly demonstrate that Otx2 is preferentially expressed in the majority of classic medulloblastomas, and also in a small number of presumptive neuronal precursor cells of the EGL, but not in precursor cells of the ventricular matrix. These data suggest that not only nodular/desmoplastic medulloblastomas, but also part of the classic medulloblastomas, which will then be Otx2-positive and calbindin-D28K-negative, may originate from the EGL. If the human embryonal EGL shows a similar tripartite expression pattern for Otx1 and Otx2 as the rat, it can be speculated that the nodular/desmoplastic medulloblastomas with only Otx1 expression are derived from cells from the anterior EGL, whereas tumors expressing only Otx2 (but no calbindin-D28K) are derived from cells from the posterior EGL, and tumors expressing both Otx1 and Otx2 have an origin in the cerebellar vermis. It is noteworthy that in addition to Otx2 mRNA expression confirmed in the present study, it has been previously shown that calbindin-D28K is also highly expressed in the human medulloblastoma cell line D283 Med, which was derived from a metastatic classic medulloblastoma (30). Thus, the expression of Otx2 and calbindin-D28K in medulloblastomas and in Purkinje cells, suggest that a subset of the classic medulloblastomas may still have their origin in the ventricular matrix.

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REFERENCES

features, proliferation index and apoptotic index. J Neurooncol 2002;59:49–61


