OTX1 and *OTX2* Genes in Medulloblastoma

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OBJECTIVE: **To study the prevalence of *OTX1* and *OTX2* gene expression in 60 medulloblastoma specimen samples and to establish correlations between gene expression and clinical and histopathological aspects.**

METHODS: **We performed a retrospective analysis of 60 patients with a diagnosis of medulloblastoma at the Clinicas Hospital of the School of Medicine, University of São Paulo, and the Cancer Hospital of Barretos. We created a database of the 60 patients containing information on the gene expression of *OTX1* and *OTX2* (obtained using real-time polymerase chain reaction) and clinical and epidemiological data. Statistical tests were performed to verify potential correlations of clinicopathological data and follow-up aspects with gene expression.**

RESULTS: **The *OTX1* gene was expressed in 52% of the study population. Expression varied with age (higher in adults), location (predominantly by hemisphere), and histological type (desmoplastic). The *OTX2* gene was expressed in 62% of the study population. Expression varied with age (higher in younger age groups), location (predominantly vermis), and histological type (classic and anaplastic). A statistical correlation between *OTX2* gene expression and the development of leptomeningeal metastases was observed.**

CONCLUSIONS: **The relative expression of *OTX1* and *OTX2* was dependent on patient age, tumor location, and histological variant. In addition, *OTX2* expression might be a predictive factor for leptomeningeal metastases of medulloblastoma. The OTX pathway should be consider as an important venue for medulloblastomas development.**

INTRODUCTION

Medulloblastoma is the most common malignant tumor of the central nervous system (CNS) in children and can also occur in adults.[1-3] It is a highly aggressive and invasive cerebellar tumor classified as a primitive neuroectodermal tumor. It has a high potential for spreading through the cerebrospinal fluid, resulting in a poor prognosis.[4] Although some clinical, radiological, and macroscopic features are relatively similar, the histological and molecular features and prognoses are heterogeneous.[5-7] The World Health Organization has defined 5 histological variants of medulloblastoma: classic, desmoplastic, giant cell, extensive nodularity, and anaplastic.[8] This stratification is currently overlapped by a molecular classification in groups, described by several investigators.[9-14] The classification of medulloblastomas has resulted in the greatest conceptual challenge in devising a merger of histological and molecular classification schemes. Medulloblastoma has long-established histological variants that have clinical utility (e.g., desmoplastic/nodular, medulloblastoma with extensive nodularity, large cell, and anaplastic), and it has been widely accepted that 4 genetic (molecular) groups of medulloblastoma exist: WNT-activated, sonic hedgehog -activated, and the numerically designated group 3 and group 4.[15] Rather than providing a long list of the many possible histological and molecular combinations, the classification lists "genetically defined" and "histologically defined" variants, with the expectation that pathologists with the ability to undertake the molecular

Key words
- Classic
- Desmoplastic
- Medulloblastoma
- *OTX1* gene
- *OTX2* gene

Abbreviations and Acronyms
CNS: Central nervous system
CT: Cycle threshold
PCR: Polymerase chain reaction

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classification will generate an integrated diagnosis that includes both the molecular group and the histological phenotype.[16]

Recent studies have described the involvement of *OTX* genes in the tumorigenic process of medulloblastoma, identifying these genes as oncogenes and as potential biological markers for determining the prognosis.[17-20] *OTX1* and *OTX2* are homeobox genes that control the formation, differentiation, and compartmentalization of the CNS, especially in the cerebellum.[21,22] These genes are extremely active until the ninth week of extrauterine life and are then silenced.[19] The molecular mechanism by which these genes act is not yet completely understood. However, it is known that some of the functions of these genes stem from their ability to control transcription factors in embryonic cells.[23] Thus, *OTX* genes control the cell cycle and the formation of embryonic tissues. Additionally, *OTX2* controls mechanisms involving circadian rhythm, affecting not only sleeping and waking but also the temporal hierarchy of tissue formation.[21] *OTX2* also interacts with the Wnt5 protein of the Wnt pathway and acts on adhesion molecules.[24] Both *OTX1* and *OTX2* genes, in addition to contributing harmonically in embryogenesis, could also be involved in surveying topography and tumor histopathological features. In addition, increased copy numbers and mutations in OTX2 have been reported in patients with medulloblastoma.[25] Previous studies have shown that the expression of *OTX* genes can be inhibited by transretinoic acid,[26,27] offering a promising method for therapeutic approaches to target these genes.[28]

METHODS

We analyzed 60 cases of medulloblastoma that had been surgically resected from 2000 to 2009 at the Hospital das Clinicas, attached to the School of Medicine at the University of São Paulo and the Cancer Hospital of Barretos. The tumor samples were immediately snap-frozen on removal and stored in liquid nitrogen for later RNA extraction. Epidemiological, clinical, surgical, radiological, and follow-up data were retrieved for each case. All the patients or their legal guardian had provided written informed consent, and the respective local research ethics committee had approved the present study. The relative expression of *OTX1* and *OTX2* genes was analyzed using the quantitative real-time polymerase chain reaction (PCR) technique. The results were correlated with the clinical and surgical findings. The 3 nontumor samples used as controls were obtained with consent from the cerebellar tissue of patients aged >6 months. These fragments of cerebellum were obtained from patients who had undergone surgery for removal of cerebellar tissue with non-neoplastic disease (e.g., encephalocele and Chiari malformation) using a standard procedure. Commercially pooled cerebellum was also used (Clontech, Mountain View, California, USA), consisting of a pool of non-neoplastic cerebellum from 24 men and women aged 16–72 years.

RNA Extraction and Complementary DNA Synthesis

Before RNA extraction, a 4-µm-thick section of each sample was obtained for histological assessment to verify the quality of the tissue after hematoxylin and eosin staining. Necrotic and nontumor areas were dissected before the RNA extraction procedure. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Complementary DNA synthesis was performed by reverse transcription using 1 µg of total RNA, previously treated with 1 U of DNase I (FPLC-pure; GE Healthcare,), with Superscript III using oligo dT, random primers, and RNase inhibitor (Invitrogen, Carlsbad, California, USA), following the recommendations of manufacturer.

Quantitative Real-Time PCR

Relative gene expression analyses were performed using quantitative real-time PCR, and the reactions were performed in duplicate. PCR reaction mixtures (12 µL) contained 6 µL of 2× Power Syber Green I Master Mix (Applied Biosystems, Foster City, California, USA), 3 µL of complementary DNA, and 3 µL of forward and reverse primers to a final concentration of 400 nM for *OTX1* and 200 nM for *OTX2*. PCR reactions were run using an ABI Prism 7500 (Applied Biosystems). PCRs were performed as follows: 2 minutes at 50°C, 10 minutes of polymerase activation at 95°C, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Standard curves were established to ensure amplification efficiency, and an analysis of melting curves demonstrated a single peak for the primers. The sequence of primers used was as follows (5′-3′): *OTX1* forward: CAATCACCTAAACAACCAGCA; *OTX1* reverse: GGGCCGTTCACCATCTACCT; *OTX2* forward: AGACCCGGTACCCAGACATCTT; *OTX2* reverse: GCGGCACTTAGCTCTTCGATT. Quantitative data were normalized relative to the internal housekeeping genes: *GUSB*, *BCRP*, and *HPRT*; the primer sequences were as follows (5′-3′): *HPRT* forward: TGAGGATTTGGAAAGGGTGT; *HPRT* reverse: GAGCACACAGAGGGCTACAA; *BCRP* forward: CCTTCG ACGTCAATAACAAGGAT; *BCRP* reverse: CCTGCGATGGCGTTCAC; *GUSB* forward: GAAAATACGTGGTTGGAGAGCTCATT; *GUSB* reverse: CCGAGTGAAGATCCCCTTTTTA. All primers were synthesized using Integrated DNA Technologies (Coralville, Iowa, USA). The geometric mean of housekeeping gene expression was used for relative expression analysis. The equation $2^{-\Delta\Delta CT}$ was applied to calculate the relative expression of the tumor samples versus the median of non-neoplastic samples, where $^{\Delta}CT$ was the cycle threshold (CT) gene—geometric mean CT of the housekeeping genes, and $^{\Delta\Delta}CT$ is the $^{\Delta}CT$ tumor-mean $^{\Delta}CT$ of non-neoplastic tissues.[22]

Statistical Analysis

The statistical analyses of *OTX1* and *OTX2* expression and the relative expression of both genes in vermian and hemispheric locations in medulloblastomas were performed using the Mann-Whitney U test. The statistical analysis of both genes according to histological type was performed using the Kruskal-Wallis test. Overall survival was calculated as the interval from surgery to death, expressed in months. The Kaplan-Meier method and log-rank test were used to investigate the behavior of individual survival time in months for the patients according to age at diagnosis, histological tumor type, tumor mass site, and *OTX1* and *OTX2* gene expression. All conclusions drawn from the analysis were based on an inferential significance α level of <5%. The Statistical Package for Social Sciences, version 15.0, for Windows (IBM Corp., Armonk, New York, USA) and R-Program, version 2.10.1 (R Foundation, Vienna, Austria), were used for the statistical analyses.

RESULTS

Clinical Findings

The data from 60 patients with a pathological diagnosis of medulloblastoma (23 females [38.3%] and 37 males [61.7%]) were

analyzed. Patient age ranged from 2 months to 48 years, 8 months at the diagnosis, with 7 patients (11.7%) <3 years old, 31 (51.7%) aged 3–18 years, and 22 (36.7%) >18 years. Of the 60 patients, 45 had presented with a tumor mass in the cerebellar vermis and 15 in the cerebellar hemisphere. Leptomeningeal dissemination was detected in 9 patients at the diagnosis. The most common histological finding was the classic tumor type in 39 patients (65%), followed by desmoplastic in 11 (18.3%), giant cell in 6 (10%), extensive nodularity in 3 (5.0%), and anaplastic type in 1 patient (1.7%).

The surgical technique used was the posterior fossa craniotomy, in the midline or retromastoid, depending on surgeon preference and tumor topography. Total removal of the tumor was possible for 39 patients, subtotal for 17 patients, and biopsy for 4 patients because of tumor adherence to circumscribed structures. External ventricular shunting was performed in all patients with hydrocephalus, except for those with a previous ventriculoperitoneal shunt.

All patients, except for children aged <3 years, had undergone radiotherapy, and all the patients had also received chemotherapy. The latter was intermittent in 9 patients owing to the development of infections during the adjuvant therapy. The median clinical follow-up period was 35 months (range, 1–144), during which leptomeningeal dissemination was detected in 25 patients (25%) and extraneural dissemination in 1 patient. Disease recurrence, determined from magnetic resonance imaging findings, was detected in 16 patients at an interval of 6–96 months (median, 27) after surgery. Five patients underwent a second surgical resection, two of whom underwent 2 additional surgical interventions for the same purpose. The main cause of death was disease spread, followed by infection. At the study endpoint, the mean death rate was 40% and the 5-year survival rate was 65%.

OTX Gene Expression Analysis

Expression of the *OTX1* and *OTX2* genes should not be present after early childhood. This was also shown by the normal postnatal cerebellum control findings found in the present study. In the present study, the CT levels of *OTX1* in the normal cerebellum samples were 0.00, 0.14, and 0.00, and the CT levels of *OTX2* were 0.39, 1.27, and 1.61. Given that *OTX1* and *OTX2* gene expression is not expected to

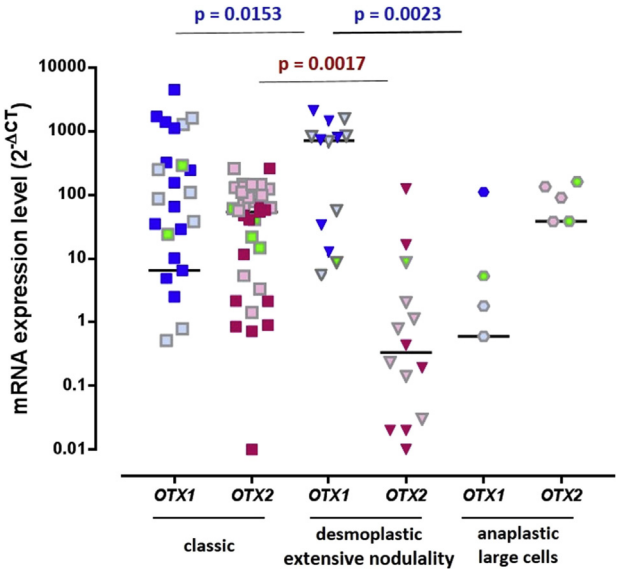

Figure 2. *OTX1* and *OTX2* gene expression stratified by vermian and hemispheric topography and compared with controls. *OTX1* expression was higher in medulloblastomas cases compared with controls and significantly greater in those located in the cerebellar hemisphere ($P = 0.0086$, Mann-Whitney *U* test). In contrast, although *OTX2* expression was also greater in medulloblastoma cases compared with controls, significantly greater expression was observed in tumors in the vermis ($P < 0.0001$, Mann-Whitney *U* test). Patients aged <18 years (*blue and pink shapes*) and all patients aged <3 years (*green shapes*) had presented with vermian tumors.

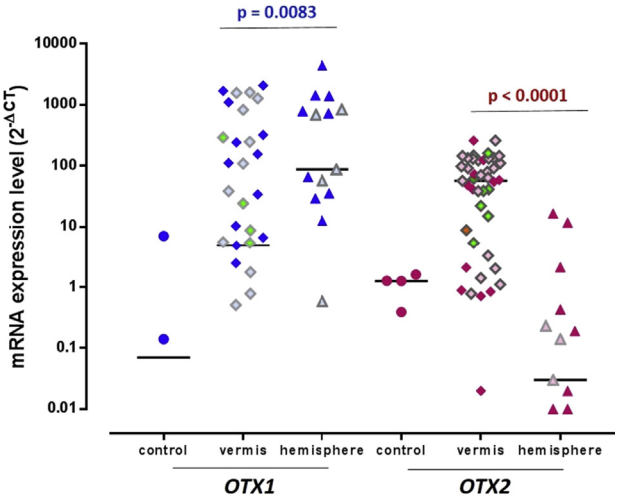

Figure 1. Relative expression of *OTX1* and *OTX2* genes in medulloblastoma.

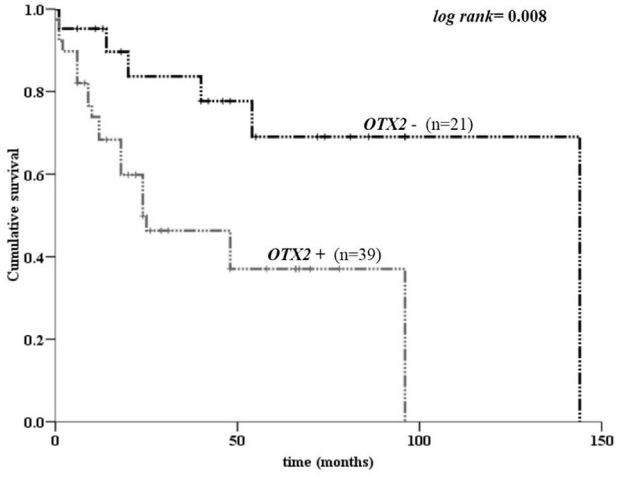

Figure 3. Survival curve showing cumulative survival between patients with *OTX2*-negative and *OTX2*-positive expression.

Table 1. Demographic and Clinical Characteristics

Characteristic	All Patients (n = 60; 100%)	*OTX1* Expression			*OTX2* Expression		
		Negative (n = 28; 46.7%)	Positive (n = 32; 53.3%)	*P* Value	Negative (n = 21; 35%)	Positive (n = 39; 65%)	*P* Value
Age at diagnosis (years)				0.006*			0.002*
<18	36 (60)	22 (78.6)	14 (43.7)		7 (33.3)	29 (74.4)	
≥18	24 (40)	6 (21.4)	18 (56.3)		14 (66.7)	10 (25.6)	
Mean ± SE	15.9 ± 11.9	10.9 ± 9.1	20.1 ± 12.6		23.5 ± 11.7	11.7 ± 9.9	
Gender				0.005*			0.024*
Female	23 (38.3)	16 (57.1)	7 (21.9)		4 (19.0)	19 (48.7)	
Male	37 (61.7)	12 (42.9)	25 (78.1)		17 (81.0)	20 (51.3)	
Histological type				0.004*			0.005*
Classic	39 (65.0)	20 (71.4)	19 (59.4)		9 (42.9)	30 (76.9)	
Desmoplastic/extensive nodularity	14 (23.3)	2 (7.1)	12 (37.5)		10 (47.6)	4 (10.3)	
Large cells/anaplastic	7 (11.7)	6 (21.4)	1 (3.1)		2 (9.5)	5 (12.8)	
Tumor site				0.0083*			<0.0001*
Vermis	45 (75.0)	26 (92.9)	19 (59.4)		9 (42.9)	36 (92.3)	
Hemisphere	15 (25.0)	2 (7.1)	13 (40.6)		12 (57.1)	3 (7.7)	
Spreading CSF				0.264*,†			0.765*,†
No	43 (71.7)	18 (64.3)	25 (78.1)		16 (76.2)	27 (69.2)	
Yes	17 (28.3)	10 (35.7)	7 (21.9)		5 (23.8)	12 (30.8)	
Overall survival (months)	74.2 ± 9.8	58.7 ± 13.2	63.5 ± 7.5	0.092*	108.5 ± 14.8	46.8 ± 7.1	0.008*

Data presented as n (%) or mean ± standard deviation, unless otherwise noted.
SE, standard error; CSF, cerebrospinal fluid.
*Statistically significant.
†Fisher exact test.

occur beyond early childhood, the relative expression levels greater than the highest expression in these normal controls (>1 for *OTX1* and >2 for *OTX2*) were considered to indicate positive expression. Using these criteria, 32 patients (53%) had had positive *OTX1* expression and 39 patients (65%) had had positive *OTX2* expression (Figure 1). *OTX2* expression was observed more frequently in children, especially children aged <3 years. *OTX1* expression was significantly associated statistically with desmoplastic and extensive nodularity type medulloblastomas, with considerably low relative expression in the classic, large cell, and anaplastic histological types. In contrast, the inverse was shown for relative *OTX2* expression, which was greater in classic, large cell, and anaplastic medulloblastomas than in the desmoplastic and extensive nodularity types ($P = 0.009$). Additionally, a statistically significant association was observed between the presence of *OTX1* expression and cerebellar hemispheric topography of the tumor. In contrast, greater *OTX2* expression was detected in patients presenting with a medulloblastoma at the vermian site ($P < 0.0001$; Figure 2).

The 15 patients who had presented with leptomeningeal disease spread during the follow-up period were found to have predominantly *OTX1*-negative, *OTX2*-positive expression profiles. No leptomeningeal dissemination was observed in the patients who had presented with *OTX1*-negative, *OTX2*-negative expression. Furthermore, the presence of *OTX2* expression was significantly related to the risk of leptomeningeal dissemination ($P = 0.033$) and shorter survival compared with *OTX2*-negative expression ($P = 0.020$; Figure 3). Kaplan-Meier analysis of overall survival revealed no statistically significant differences among the different combinations of *OTX1* and *OTX2* expression: *OTX1*-positive, *OTX2*-positive; *OTX1*-negative, *OTX2*-negative; *OTX1*-negative, *OTX2*-positive; *OTX1*-positive, *OTX2*-negative ($P = 0.131$). However, patients with *OTX1*-negative, *OTX2*-positive expression experienced significantly shorter survival ($P = 0.028$; Table 1).

DISCUSSION

Medulloblastoma and Subgroups

In recent years, several groups have attempted to describe the molecular heterogeneity of medulloblastomas,[12,15,29-31] with the aim of a better understanding of their unpredictable behavior and oncogenic pathways. This knowledge could also result in tailored treatments by accessing molecular targets.[13,30] Several investigators

have attempted to classify medulloblastomas, with a consensus reached for 4 main groups.[12,14,15,32,33] Group 1, or WNT medulloblastomas, group 2 or sonic hedgehog medulloblastomas, and groups 3 and 4, with the latter still the least known and the most heterogeneous. Preliminary evidence has also shown the existence of several subgroups. Groups 3 and 4 probably have similarities between themselves and also have relationships or intersections at some point with the hedgehog and WNT pathways.

In 2012, Northcott et al.[29] reported somatic copy number aberrations in 1087 medulloblastoma samples. The most common were duplications, and some had a peculiar relationship with subgroup 4. That study also demonstrated that MYC amplicons were unique in group 3, with a probable correlation with the expression of the OTX2 gene.[29] However, whether the *OTX* gene was present only in group 3 was not reported. Although that study did not establish clinical correlations, the results indicated the importance of *OTX* gene expression in $\geq$1 of the subgroups.[29]

Boulay et al.,[34] using genome-wide maps of chromatin, reported that group 3 medulloblastoma showed OTX2 expression as a major feature of the regulatory landscape of these tumors. In keeping with OTX2-binding data from previous studies, our profiles showed a large number of OTX2-binding sites across the genome.[35]

Several interactions have been described among WNT,[36-38] notch,[39-41] and sonic hedgehog pathways,[42-45] such as the interaction of Gli protein (hedgehog) with the β-catenin complex (WNT) and SuFu protein (hedgehog).[46,47] Similarly, *OTX* genes might interact with the WNT pathway via the Wnt5 factor.[48,49] Thus, an intense interplay between *OTX* gene expression and the medulloblastoma pathways seems likely. These findings also lead to the hypothesis that *OTX* gene expression would be important, not only in group 3, but also in the other subgroups.

Present Series

A high frequency of overexpression of OTX1 (53%) and OTX2 (65%) genes was detected in the present series of 60 patients, stressing the importance of the *OTX* pathway in a relevant proportion of medulloblastoma cases. The relative OTX1 and OTX2 expression levels correlated closely with patient age, with statistically significant findings for OTX2 gene expression in children, especially those aged <3 years.

Considering that the OTX2 gene is a homeobox gene committed to embryogenesis, a delay in silencing this gene after birth might be linked to the tumorigenic process in medulloblastoma. In contrast, the OTX1 gene has been detected among adult patients with medulloblastoma, a finding that suggests reactivation of this gene after its shutdown in early childhood. In the present series, we found a clear relationship between OTX1 expression and desmoplastic and extensive nodularity variants, and OTX2 expression correlated with classic, large cell, and anaplastic variants, as previously described. The large cell and anaplastic variants showed a particular combination of *OTX* gene expression (i.e., low OTX1 and high OTX2 expression) not previously described.

This molecular signature, OTX1-negative, OTX2-positive, might help in determining these variants. The present results have also confirmed OTX1 expression in medulloblastoma located in the cerebellar hemisphere, and OTX2 expression in vermian tumors, as previously reported. These results have corroborated the finding that genotypically distinct cells result in different topographical and histologic variants of medulloblastoma. OTX2-positive cells of the periventricular germinal matrix or granular layer from the midline could lead to vermian tumors. In contrast, OTX1-positive granular layer cells led to hemispheric tumors. However, the relationship between gene expression and hypothetical tumor progenitor cell or topography was not always found, because OTX2 expression was not detected in 20% of the vermian tumors in the present series. In contrast, OTX2 expression was found in 13% of hemispheric tumors, highlighting that these tumor characteristics might be determined by other genes. *NEUROG1* and *MATH1* could also be important in determining the topography of medulloblastomas.[43,50,51] Nevertheless, the present data reinforce the currently held concept that OTX2-positive progenitor cells can induce the tumorigenesis of medulloblastoma in the cerebellar vermis and OTX1-positive progenitor cells can induce the tumorigenesis of medulloblastoma in the cerebellar hemisphere.[25] The relationship of OTX2 and OTX1 genes with the development of metastases has not yet been explored, and our observation that OTX2 expression is related to leptomeningeal spread of the tumor is an original finding. These data were indirectly consistent with previous reports that correlated OTX2 gene expression to the Wnt pathway and adhesion molecules, such as cadherins and integrins, involved in the development of metastasis. Thus, aberrant OTX2 expression might interfere with this protein expression and, thereby, enhance the metastatic potential of the tumor cells.

de Haas et al.[25] reported that protein analysis of classic medulloblastomas showed that OTX2-positive tumors mainly originated from the vermis of children and frequently lacked $p75^{NTR}$ expression. OTX2-negative tumors, which were virtually all OTX1-positive, developed in very young children or adults.[25] They were more frequently located in the lateral hemisphere, were strongly associated with the nodular and desmoplastic pathologic subtypes, and were $p75^{NTR}$-positive. A correlation between younger age and tumor localization in the vermis was also reported by others.[10,52-54] However, the results from the present series found that this was true only for OTX2-expressing tumors. OTX2-negative medulloblastomas in young children were more frequently found in the lateral hemisphere of the cerebellum.

Additionally, OTX2 expression was significantly related to shorter survival. This finding, together with OTX2 expression as a potential predictive factor of leptomeningeal dissemination, and greater expression in more aggressive tumor variants (large cell and anaplastic), points to OTX2 as a potential biological marker of a poor prognosis for patients with medulloblastoma.[13,50,55] Insertion of *OTX* genes in the tumorigenic pathways already known to be involved in medulloblastoma would be the next step to a better understanding of the functional role of these genes. Animal experimental data have shown suppression of OTX2 expression by transretinoic acid,[27,28,56] leading to the absence of CNS formation in murine embryos.[23] Furthermore, primary cultures of anaplastic medulloblastoma presenting with OTX2 amplification subjected to transretinoic acid in vitro have shown a relevant decrease in OTX2 expression and the induction of apoptosis.[26]

The effect of retinoids on medulloblastoma has been appreciated for some time,[27,28,57] and clinical trials implementing retinoid treatment as an adjuvant therapy for medulloblastoma are underway. Such investigations could identify alternative or cooperative

approaches to inducing the downstream antitumor effects of retinoids, such as bone morphogenetic protein 2 secretion and *OTX2* repression.[28,57] Wortham et al.[55] identified the transcriptional regulatory element responsible for *OTX2* repression by retinoids. These results reinforce *OTX2* as a therapeutic target for medulloblastoma. Phase III studies with transretinoic acid used as rescue treatment of medulloblastoma are currently in progress (e.g., ClinicalTrials.gov identifier, NCT00867178; status, active).

CONCLUSIONS

We found that the *OTX1* gene was expressed in a high proportion (53%) of medulloblastomas, with expression increasing with age. *OTX1* expression was associated with hemispheric tumors and desmoplastic and large cell variants but not with leptomeningeal metastases. The *OTX2* gene was also expressed in a large proportion (65%) of medulloblastomas and was associated with pediatric patients, vermian topography, and classic, large cell, and anaplastic variants. The gene was also associated with the development of leptomeningeal metastasis and shorter survival. Our data suggest that the *OTX* gene pathways are involved in the tumorigenic process of most medulloblastomas, acting as an oncogene. The knowledge that their expression can be inhibited by the action of transretinoic acid renders the identification of patients who express these genes essential and offers this patient group new possibilities of treatment and, possibly, longer and improved survival.

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