

# Gene Expression Profiling of the Hedgehog Signaling Pathway in Human Meningiomas

Ingrid Laurendeau,<sup>1</sup> Marcela Ferrer,<sup>2</sup> Delia Garrido,<sup>2</sup> Nicky D'Haene,<sup>3</sup> Patricia Ciavarelli,<sup>2</sup> Armando Basso,<sup>2</sup> Michel Vidaud,<sup>1</sup> Ivan Bieche,<sup>1</sup> Isabelle Salmon,<sup>3</sup> and Irene Szijan<sup>2\*</sup>

<sup>1</sup>UMR745, INSERM, Université Paris-Descartes, Faculté des Sciences Pharmaceutiques et Biologiques, Paris, France; <sup>2</sup>Cátedras de Genética y Biología Molecular y de Matemáticas, Facultad de Farmacia y Bioquímica; División de Neurocirugía del Hospital de Clínicas "José de San Martín," Universidad de Buenos Aires, Buenos Aires, Argentina; <sup>3</sup>Department of Pathology, Erasme University Hospital, Université Libre de Bruxelles, Bruxelles, Belgium

The Hedgehog (Hh) signaling pathway has an important role during embryogenesis and in adult life, regulating proliferation, angiogenesis, matrix remodeling and stem-cell renewal. Deregulation of the Hh pathway is involved in tumor development, since mutations in several components of this pathway were found in patients with basal cell carcinoma, medulloblastoma and other tumors; however, the role of Hh in meningiomas has not been studied yet. Meningiomas represent 30% of primary cranial tumors, are mostly benign and prevail in the second half of life. Novel therapies for meningiomas such as targeted molecular agents could use Hh pathway components. To provide information concerning molecular alterations, by use of real-time RT-PCR, we studied expression at the mRNA level of 32 Hh pathway and target genes in 36 meningioma specimens of different grades. mRNA levels of 16 genes, involved mainly in Hh pathway activation and cell proliferation, increased in meningiomas in comparison with normal tissue, whereas those of 7 genes, mainly related to Hh pathway repression, decreased. The most significant changes occurred in signal transduction (*SMO*) and *GLI*-transcription factor genes, and the target *FOXM1* mRNA attained the highest values; their overexpression was found in aggressive and in benign tumors. Some proliferation-related genes (*SPPT1*, *IGF2*) were overexpressed in higher meningioma grades. A correlation in expression between genes with a similar function was also found. Our results show a marked activation of the Hh pathway in meningiomas, which may be important for their biological and clinical characterization and would be useful for gene therapy.

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Online address: <http://www.molmed.org>

doi: 10.2119/molmed.2010.00005

## INTRODUCTION

The study of genetic alterations involved in tumorigenesis is important for molecular characterization of tumors and offers useful targets for therapy. The Hedgehog (Hh) signaling pathway is closely related to processes such as cell proliferation, differentiation, angiogenesis, cellular matrix remodeling and stem-cell homeostasis, all of which are involved in tumorigenesis and metastasis (1). Deregulation of this pathway was found in different tumors such as basal cell carcinoma, medulloblastoma, rhabdomyoma, rhabdomyosarcoma and

other tumors (2–4), but there have been no reports on meningiomas so far.

The Hedgehog pathway plays a fundamental role in multiple developmental processes as well as in adult tissues and is conserved across the animal kingdom from insects to vertebrates (1,5,6). Hh ligands have evolved from one in *Drosophila melanogaster* to three in vertebrates: Sonic hedgehog (SHH), the most widely expressed in mammalian tissues; Indian hedgehog (IHH), specific for bone development; and Desert hedgehog (DHH), restricted to gonads (1). Hh proteins are synthesized, palmitoylated by a palmitoyltransferase

specific for SHH (HHAT), cholesterol-modified and released by means of the transmembrane protein Dispatched (DISP) (5–7). They act at short or long distance, and their gradient determines the development and differentiation of cells (7,8).

The Hh signaling function is accomplished through the process of ligand binding to the receptor Patched (PTCH), transduction of the signal mediated by the transmembrane protein Smoothed (SMO) and activation of specific transcription factors regulating the Hh target gene expression. The PTCH receptor is suppressed in response to ligand binding, something unusual for a signal receptor, whereas SMO is activated (1,5–7). Other proteins that modulate the Hh function are Hh interacting protein (HHIP), involved in a negative feedback;

**Address correspondence and reprint requests to Irene Szijan, Genética y Biología Molecular, Facultad de Farmacia y Bioquímica, UBA, Junín 956, 1113 Buenos Aires, Argentina.**

Phone/Fax: 5411 4964 8296; E-mail: [iszijan@ffybu.uba.ar](mailto:iszijan@ffybu.uba.ar), [iszijan@fibertel.com.ar](mailto:iszijan@fibertel.com.ar).

Submitted January 27, 2010; accepted for publication March 25, 2010; Epub

([www.molmed.org](http://www.molmed.org)) ahead of print March 26, 2010.

the growth arrest specific 1 (GAS1), regulating the range and level of signaling; and Rab23, a small G-protein having a major role in the subcellular localization of Hh pathway components (1,9,10).

Activation of SMO initiates a signal transduction cascade involving several intermediate proteins such as the Kinesin family (Kif7, Kif27) that interact with microtubules and other proteins; a Fused homolog, the serine-threonine kinase 36 (STK36) not essential for mammals; the Suppressor of fused (SUFU) a strong inhibitor of the transcription factors GLI in mammals (related to *Drosophila*'s Krüppel-like zinc finger proteins named GLI after glioblastoma); and a  $\beta$ -transducin-repeat-containing E3 ligase (BTRC), which binds the phosphorylated GLI2 and GLI3 for their processing into a repressor (GLI3) or to ubiquitine-proteasome-mediated degradation (GLI2) (1,5,6,11). The relative levels of GLI transcription factors GLI1/GLI2 (activators) and GLI3 (repressor) determine which target genes are expressed; another member, GLI4, and GLI-similar 1 and 2 (GLIS1/GLIS2) are additional transcription factors (7,12–15).

Several genes are regulated by the Hh signal: (a) MTSS1 (metastasis suppressor 1)—a regulator of actin filaments assembly—which is expressed in embryonic tissues including the developing central nervous system and downregulated or missing in metastatic cancer cell lines (16); (b) the Forkhead family of transcription factors, which are associated with cell growth and proliferation, such as FOXE1, FOXA2 and FOXM1 (a regulator of cell-cycle gene-expression), and all have been found in human cancers (17–19); (c) SPP1 (osteopontin) and OSF (periostin)—secreted extracellular matrix-associated proteins—which have a crucial role in cell adhesion, prevention of apoptosis, invasion and migration (20,21); (d) IGF2 (insulin-like growth factor 2) and CCND2, a cyclin required for cell cycle  $G_1/S$  transition, both of which are overexpressed in tumors (22,23); (e) PTHR1, a parathyroid hormone receptor, which is over-

expressed in osteosarcoma and bone metastases of other tumors (24).

Activation of the Hh pathway, therefore, stimulates several processes related to cancer such as cell-cycle machinery, expression of angiogenic and antiapoptotic factors and stem-cell renewal, which may involve transformation into cancer stem cells (6,20,21,23).

The most common mutations that activate the Hh pathway occur in (a) *PTCH* or *SUFU*, causing the loss of their function; (b) *SMO*, activating it; or (c) *GLI1*, amplified in 15% of glioblastomas. The nevoid basal cell carcinoma syndrome (NBCC) caused by *PTCH* mutations is associated with development of tumors such as basal cell carcinoma, medulloblastoma and meningioma (1,2).

Specific inhibitors of Hh signaling will provide an effective therapy for a wide range of malignancies. The most common ways to target this pathway are (a) modulating SMO by its antagonists, the plant alkaloid cyclopamine or the derived synthetic compounds, which have been used in a phase 1 clinical trial (25–29); (b) using SHH-neutralizing antibodies (30); and (c) inhibiting GLI1 with a small interfering RNA (siRNA) (31).

Meningiomas represent 30% of primary cranial tumors (Central Brain Tumor Registry of the United States [CBTRUS]) prevailing in the second half of life. They are mostly sporadic and often considered benign; however, three malignancy grades are recognized by WHO: grade I or benign (MGI); grade II or atypical (MGII), associated with a high rate of recurrence; and grade III or anaplastic (MGIII), highly aggressive with a short survival time.

Emerging therapies for meningiomas are also being developed, and the components of the Hh pathway are candidates for targeting. Herein we investigated the expression of a large panel of components and related molecules involved in the Hh pathway. Using the real-time quantitative RT-PCR, we quantified the expression of 32 genes at the mRNA level in 36 meningioma specimens of different grades and attempted

to determine a link between gene expression and pathological parameters.

## MATERIALS AND METHODS

### Patients and Samples

Samples of 36 meningiomas were obtained by surgical excision from patients with sporadic tumors at the Neurosurgery departments of Brussels (26 patients) and Buenos Aires (10 patients) from 2003 to 2005. They included 23 MGI tumor samples with a similar number of meningothelial and transitional meningiomas, nine MGII and four MGIII. Specimens of adjacent normal meningeal tissue from five patients and of normal cerebellum from one patient were used as sources of normal RNA. Immediately after surgery, tumors and normal tissue samples were stored in liquid nitrogen. The patients were 21 women and 15 men with a mean age of 63 (range 41–80) years. This study received ethical approval from the Ethical Committee of "Hospital de Clinicas Jose de San Martin." The patients had given a written consent to use their biopsy samples for this study.

### RNA Extraction and cDNA Synthesis

Total RNA was extracted from meningioma specimens using the acid-phenol guanidinium method. Frozen tissues were triturated in liquid nitrogen in a mortar, and the powder was homogenized in the extraction solution, vortexed and treated with chloroform on ice. Iso-propanol was added to the upper phase, and the precipitated total RNA was washed, dissolved in sterile water, quantified by UV absorbance at 260 and 280 nm and assessed qualitatively by gel electrophoresis to check the presence of 18S and 28S RNA bands. RNA was reverse transcribed to cDNA as previously reported (32).

### Real-Time Quantitative PCR

The theoretical bases and PCR reaction conditions have been described (32). Briefly, quantitative values were obtained from the threshold cycle number

**Table 1.** Oligonucleotide primer sequences used

Gene	Primer forward	Primer reverse
IHH	5' AGG CCG GCT TTG ACT GGG TGT ATT 3'	5' GCG GCC GAG TGC TCG GAC TT 3'
SHH	5' CCG GCT TCG ACT GGG TGT ACT A 3'	5' CGC CAC CGA GTT CTC TGC TTT 3'
DHH	5' CCG GCT TCG ACT GGG TCT ACT AC 3'	5' GAC CGC CAG TGA GTT ATC AGC TTT 3'
HHAT	5' CCT GGA TGC TGG CCT ATG TCT T 3'	5' GCT CCT GCT GCT GCA TCT GTT 3'
DISP1	5' ACT TCT CTG ATC CAT TGC TGG GTT 3'	5' GAC CAA TCT CTG GCC TAT TGC TGT 3'
DISP2	5' TGT GCA GCA CCA TGT GGT CA 3'	5' AGC AAT CAG CTG GGA ATA GCT CTT 3'
PTCH1	5' CCC CTG TAC GAA GTG GAC ACT CTC 3'	5' AAG GAA GAT CAC CAC TAC CTT GGC T 3'
PTCH2	5' GAT GGG GCC ATC TCC ACA TT 3'	5' CGC CGC AAA GAA GTA CCT TAC A 3'
SMO	5' GCT ACT TCC TCA TCC GAG GAG TCA 3'	5' GGC GCA GCA TGG TCT CGT T 3'
HHIP	5' GGG CGC CTG GAG AAT AAG ATA TTT 3'	5'GTGGAGAGCAAAGTGCACATTTGA 3'
GAS1	5' CGT CAT TGA GGA CAT GCT GGC TAT 3'	5' TTC TCC TTG ACC GAC TCG CAG AT 3'
RAB23	5' GAA AGT AGT AGC CGA AGT GGG AGA T 3'	5' AGT GCC TCA GCT TCC TCA TTC TT 3'
KIF7	5' GTC CCA GTG CGA GAT GAA CCT 3'	5' CGG AGC GTC ACC ACC TTG T 3'
KIF27	5' AGC TTG CCT GAG TCC TGT TGA GAT TA 3'	5' GCT TCT CGC AAA TTC ACC ACC TTA 3'
STK36	5' ACC CCA GAT TGT GAA CGA GCA T 3'	5' CAT TGT CAC TGT CTG GCT CCT CAT 3'
SUFU	5' GCT GCT GAC AGA GGA CCC ACA 3'	5' GTG CAG ACA CCA ACG ATC TGG A 3'
BTRC	5' GTC TAC GGA CCC TTG TGG AGC AT 3'	5' GGG CAG CTG GAT CAT TTA GGA AGT 3'
GLI1	5' CCA ACT CCA CAG GCA TAC AGG AT 3'	5' CAC AGA TTC AGG CTC ACG CTT C 3'
GLI2	5' AAG TCA CTC AAG GAT TCC TGC TCA 3'	5' GTT TTC CAG GAT GGA GCC ACT T 3'
GLI3	5' CGC GAC TGA ACC CCA TTC TAC 3'	5' GTG TTG TTG GAC TGT GTG CCA TT 3'
GLI4	5' CCA TGG GCA TCA ACA TGG CT 3'	5' TCC TCT ACG TCT TGG AGA TCC AGG T 3'
GLIS2	5' GTG TCG CTG GGC CAA GTG TAA 3'	5' CGG GCT TGA CAT GGT AAT CGT T 3'
GLIS1	5' CCC AGC CCA CAA GGT TAC CA 3'	5' CAT CCG GTA GCA GTC GCC ATA 3'
MTSS1	5' CTG CGG CCA GTG ATT GAA GAA 3'	5' GGG CAG TTT GTG AGG GTC CAT 3'
FOXE1	5' GCG CTG GGA GGC TGC TAC AA 3'	5' TGG CGG ACA CGA ACC GAT CTA T 3'
FOXA2	5' TGG GAG CGG TGA AGA TGG AA 3'	5' GAG GAG TAG CCC TCG GGC TCT 3'
FOXM1	5' GGG AGA CCT GTG CAG ATG GTG A 3'	5' TCG AAG CCA CTG GAT GTT GGA T 3'
SPP1	5' TCG CAG ACC TGA CAT CCA GTA CC 3'	5' CCA TTC AAC TCC TCG CTT TCC AT 3'
OSF	5' GTC CTA ATT CCT GAT TCT GCC AAA 3'	5' GGG CCA CAA GAT CCG TGA A 3'
IGF2	5' CGA CCG TGC TTC CGG ACA AC 3'	5' AGG CGC TGG GTG GAC TGC TT 3'
CCND2	5' GCT GTC TCT CTG ATC CGC AAG CAT 3'	5' GGC AAA CTT AAA GTC GGT GGC A 3'
PTHR1	5' ACA ACA GGA CGT GGG CCA ACT AC 3'	5' CGG TCA AAC ACC TCC CGT TCA 3'
MKI67	5' ATT GAA CCT GCG GAA GAG CTG A 3'	5' GGA GCG CAG GGA TAT TCC CTT A 3'

at which the increase in the signal associated with the exponential growth of PCR product begins to be detected. The amount of RNA added to reaction mix was determined by normalization of each sample to an endogenous RNA control (TBP [TATA box-binding protein]). The results, presented as N-fold differences in target gene expression relative to the *TBP* gene and termed N target, were determined as  $N_{target} = 2^{\Delta Ct_{tumor}}$ , where the  $\Delta Ct$  value of the sample was determined by subtracting the average Ct value of the target gene from the average Ct value of the *TBP* gene. The  $N_{target}$  values of the tumor samples were subsequently normalized such that the mean of the normal tissue sample  $N_{target}$  value was 1.

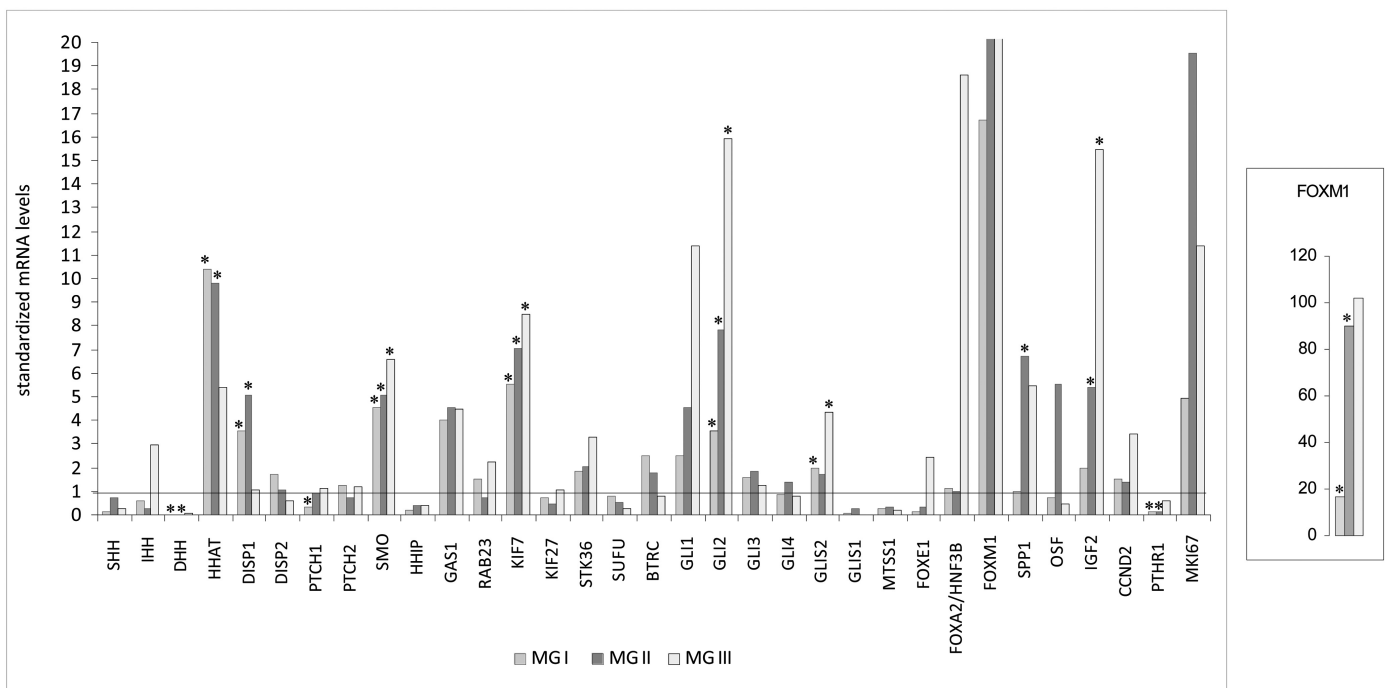
Primers for *TBP* and target genes were chosen with the assistance of the oligo 4.0 program. Searches in dbEST, htgs and nr databases confirmed the total gene specificity of the nucleotide sequences chosen as primers and the absence of single nucleotide polymorphisms. The primer pairs were selected to be unique relative to the sequences of closely related family member genes or the corresponding retropseudogenes. To avoid amplification of contaminating genomic DNA, one of the two primers was placed at the junction between two exons. For each primer pair, we performed no-template control and no-RT control (RT-negative) assays, which produced negligible signals that were usually greater than 40 in Ct values, sug-

gesting that primer-dimer formation and genomic DNA contamination effects were negligible. Table 1 lists the nucleotide sequences of the primers.

Experiments were performed with duplication for each data point. Positive controls for all genes were obtained by performing an RNA pool control, which was prepared by mixing identical amounts of RNA from various human normal and tumor tissues. PCR amplification was performed in an ABI Prism 7700 Sequence detection system using the SYBR® Green PCR Core Reagent kit (Perkin-Elmer Applied Biosystems, Oak Brook, IL).

**Statistical Analyses**

Because the mRNA levels did not fit a Gaussian distribution, they were charac-



**Figure 1.** mRNA levels of 32 Hedgehog pathway genes, target genes and the proliferation marker *MKI67* in different meningioma grades (MGI, MGII and MGIII). Median standardized mRNA levels in meningiomas were determined relative to the median mRNA level in normal meninges, which has an assigned value of 1 and is indicated by a horizontal line. Statistically significant differences in mRNA levels between meningiomas and normal tissue are denoted by asterisk. The tests used and *P* values are as in Table 2.

terized in each group by their median values and ranges. Comparisons between the medians of target gene mRNA levels and histopathological parameters were checked by the nonparametric Kruskal–Wallis test. To evaluate the significance of multiple analyses, pair confrontations between the means of group ranges were performed by Conover’s method (33).

The relationships between mRNA levels of the various target genes were performed by the nonparametric Spearman rank correlation test. Differences between two populations were judged significant at confidence level greater than 95% ( $P < 0.05$ ).

## RESULTS

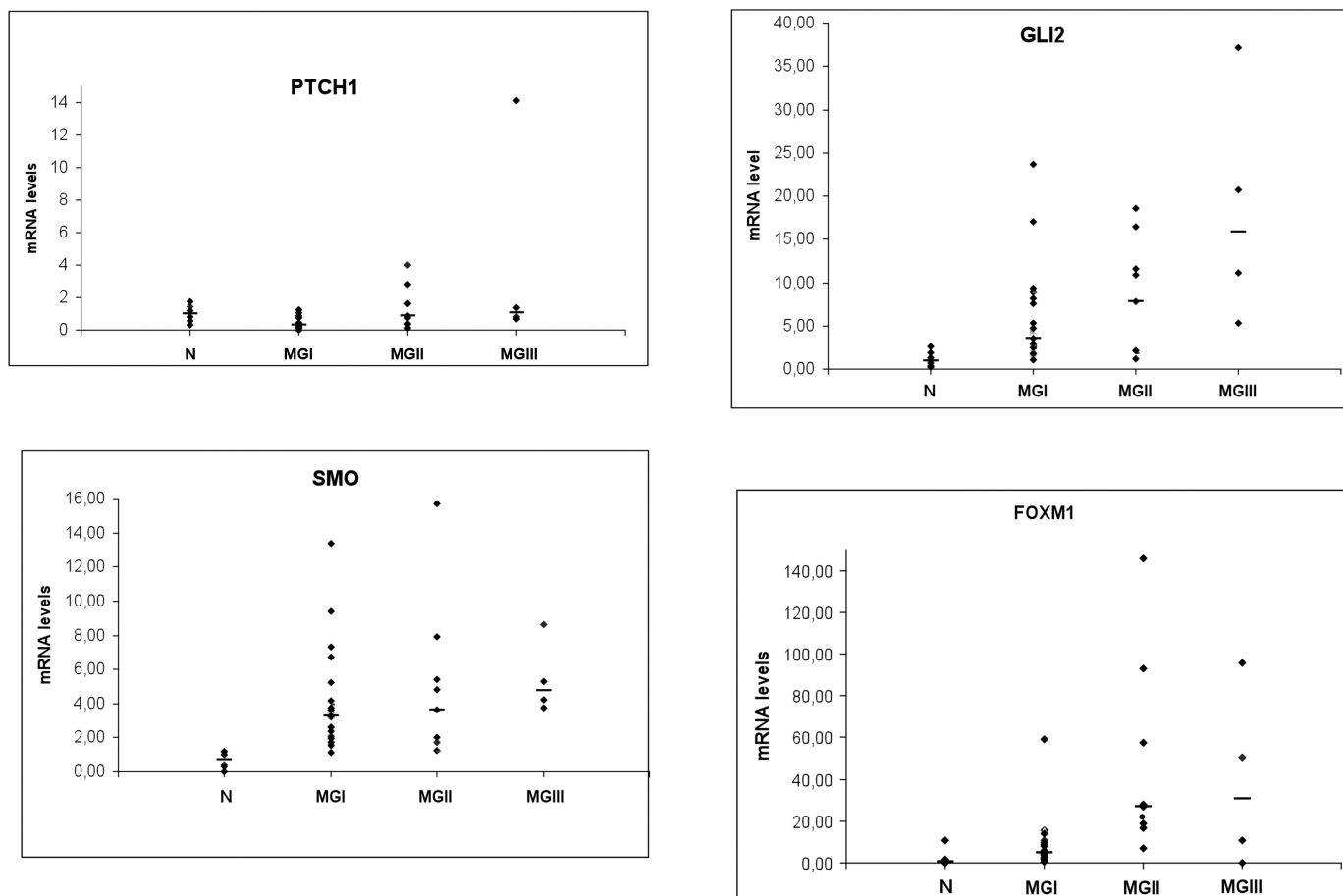
### Hedgehog Pathway mRNA Levels in Meningiomas

Figure 1 shows the median mRNA levels of 32 Hedgehog pathway and target genes as well as of the proliferation

marker *MKI67* in various grades of meningiomas compared with normal tissue. mRNA levels of 16 genes, mainly involved in Hh pathway activation and cell proliferation, were increased (overexpressed) in meningiomas, whereas mRNA levels of seven genes, mainly related to Hh pathway repression, were decreased (underexpressed). The 9 remaining genes (*SHH*, *IHH*, *DISP2*, *PTCH2*, *KIF27*, *RAB23*, *GLI3*, *GLI4* and *FOXE1*) did not show quantifiable changes in mRNA levels between normal and tumor tissues.

**Increased mRNA levels (overexpressed genes).** The following 11 genes showed a significant increase in mRNA levels in all or some grades of meningiomas compared with normal tissue: (a) *HHAT* and *DISP1*, involved in Hh ligand formation and release from producing cells, were overexpressed in 50% to 89% and 25% to 78% of meningiomas regardless their grade, respectively; (b) *SMO*, which initiates a signal transduction cas-

cade, showed an mRNA level increase in 56% to 100% of meningiomas; (c) *KIF7*, a signaling intermediate proposed as *cos2* protein homolog in mammals, was overexpressed in 56% to 100% of all the grades of meningiomas, gradually increasing from MGI to MGIII; (d) *GLI1*, *GLI2* and *GLIS2* transcription factors were overexpressed in 39% to 75%, 52% to 100% and 26% to 100% of all the grades of meningiomas, respectively, with the highest mRNA values in MGIII and the lowest in MGI; (e) *FOXM1* mRNA, a GLI target, proliferation-specific transcription factor, increased in 22% to 89% of tumors, showing the highest levels in comparison with those of other genes; (f) *SPP1*, *IGF2* and *CCND2*, the other GLI-target genes encoding an extracellular matrix-associated protein, a growth factor and a cyclin, respectively, were overexpressed in 13% to 75%, 26% to 89% and 35% to 75% of meningiomas regardless of the grade, respectively; *SPP1* and *IGF2* mRNA levels increased



**Figure 2.** mRNA levels of *PTCH1*, *SMO*, *GLI2* and *FOXM1*. The figure shows standardized mRNA levels of each gene in six individual normal meninges (N), 23 grade I meningiomas (MGI), 9 grade II meningiomas (MGII) and 4 grade III meningiomas (MGIII). Median values are indicated for normal meninges and each tumor group by horizontal bars. *P* values for the three tumor grades versus normal tissue are as in Table 2.

mostly in MGII and MGIII, the more aggressive tumors.

A nonsignificant increase in median mRNA level of five additional genes was found; however, they showed values higher than normal in a considerable amount of meningiomas: 22%–75% (*GAS1*, *STK36*, *BTRC*, *FOXA2* and *OSF*).

**Decreased mRNA levels (underexpressed genes).** A significant decrease in the mRNA level of the following genes was found in meningiomas in comparison with normal tissue: (a) *DHH*, a gonad-specific Hh ligand, was underexpressed in 50% to 71% of meningiomas regardless of grade; (b) *PTCH1*, a Hh ligand receptor that represses the Hh pathway, was underexpressed in 11% to 43% of tumors; (c)

*PTHR1*, a parathyroid receptor participating, coupled with Hh ligand IHH, in the regulation of chondrocyte differentiation, showed reduced mRNA levels in 22% to 35% of meningiomas regardless of grade.

A nonsignificant decrease in the median mRNA level of four additional genes (*HHIP*, *SUFU*, *MTSS1* and *GLIS1*) was found in meningiomas; some of them, such as the negative Hh pathway regulators *HHIP* and *SUFU*, showed values lower than normal in 13%–50% of tumors.

Figure 2 shows an example of the mRNA levels of *PTCH1*, *SMO*, *GLI2* and *FOXM1* genes in each of the six normal tissues (N), 23 MGI, 9 MGII and 4 MGIII.

### Comparison of the Hedgehog Pathway and Related mRNA Levels with the Grades of Meningiomas

mRNA levels of 9 of 23 Hh pathway genes and 5 of 9 Hh-inducible genes, related to cell proliferation/invasion, differed significantly as regards total or each grade of meningiomas and normal tissue (Table 2), although the changes were not always statistically significant for the MGIII group, probably owing to the small sample size. It should be noted that the *PTCH1* mRNA level decreased only in MGI compared with normal tissue, whereas *SPP1* and *IGF2* mRNA levels increased in the intermediate grade (MGII) or in MGII and MGIII, respectively, but not in the benign MGI grade.

**Table 2.** Hedgehog pathway mRNA levels and tumor grades, changes in expression and *P* values (Kruskal-Wallis test).

Gene	MGI-MGIII versus normal	MGI versus normal	MGII versus normal	MGIII versus normal	MGI versus MGII	MGI versus MGIII	MGI versus MGII-MGIII
<i>DHH</i>	D (0.0018)	D (0.0005)	D (0.007)	NS	NS	NS	NS
<i>HHAT</i>	I (0.0160)	I (0.015)	I (0.028)	NS	NS	NS	NS
<i>DISP1</i>	I (0.0006)	I (0.0006)	I (0.004)	NS	NS	NS	NS
<i>PTCH1</i>	NS	D (0.0089)	NS	NS	D (0.018)	D (0.011)	D (0.0011)
<i>SMO</i>	I (0.0001)	I (0.0003)	I (0.0004)	I (0.0095)	NS	NS	NS
<i>KIF7</i>	I (0.0008)	I (0.002)	I (0.012)	I (0.0095)	NS	NS	NS
					MGII versus MGI	MGIII versus MGI	MGII-MGIII versus MGI
<i>GLI1</i>	I (0.019)	NS (0.06)	NS	NS	NS	NS	I (0.0508)
<i>GLI2</i>	I (0.0003)	I (0.026)	I (0.018)	I (0.0095)	NS	I (0.014)	NS (0.068)
<i>GLIS2</i>	I (0.0024)	I (0.0083)	NS	I (0.0095)	NS	NS	NS
<i>FOXM1</i>	I (0.0007)	I (0.0098)	I (0.0008)	NS	I (0.0002)	NS	I (0.0073)
<i>SPP1</i>	I (0.047)	NS	I (0.012)	NS	I (0.0057)	NS	I (0.0027)
<i>IGF2</i>	I (0.0014)	NS	I (0.0004)	I (0.0095)	I (0.0013)	I (0.029)	I (0.0037)
<i>CCND2</i>	I (0.022)	NS	NS	NS	NS	NS	NS
<i>PTHR1</i>	D (0.011)	D (0.0026)	D (0.025)	NS	NS	NS	NS

I and D indicate expression levels that are increased and decreased in the first group versus other groups, respectively. *P* values in parentheses; NS, not significant. Comparison of mRNA levels with the grades of meningiomas were as follows: MGI versus MGII, MGI versus MGIII and MGI versus MGII-MGIII for the first six genes and MGII versus MGI, MGIII versus MGI and MGII-MGIII versus MGI for the next eight genes.

A significant difference in mRNA levels between various grades of meningiomas was found for (a) *GLI2*, *GLIS2* and *IGF2*, higher in malign tumors (MGIII) than in MGI or MGII grades; (b) *FOXM1*, *SPP1* and *IGF2*, increased in the intermediate grade (MGII) compared with MGI; (c) *PTCH1*, decreased in MGI in comparison with MGII and MGIII (Table 2).

### Relationship between mRNA Values of Hh Pathway and Related Genes in Meningiomas

Table 3 lists the more relevant correlations ( $P < 0.01$ ) between mRNA levels of Hh pathway genes as well as between them and those of their target genes in meningiomas. The most significant values were found for the following genes: (a) *DISP1* mRNA levels, encoding the Hh ligand-secretion receptors, correlated with those of a membrane protein *GAS1* ( $r = 0.65$ ), both increased in meningiomas thus supporting the actual increase of *GAS1* mRNA despite its nonsignificance; (b) Hh ligand receptors *PTCH1/2* mRNA levels, reduced in meningiomas, corre-

lated with those of *RAB23* ( $r = 0.54$ ) a regulator of *GLI2/GLI3* activities, as well as with those of *GLI1/GLIS2* ( $r = 0.68, 0.53$ ) which increased in meningiomas, data that have no logical explanation and might reflect the importance of relative activators and repressors levels; (c) *SMO* expression correlated with that of a high number of Hh pathway and target genes such as *GAS1* ( $r = 0.57$ ), *KIF7* ( $r = 0.73$ ); the transcription factors *GLI2/GLIS2*, which showed the highest correlations,  $r = 0.85$  and  $0.75$ , respectively; and a target gene, the growth factor *IGF2* ( $r = 0.52$ ) in full agreement with the role of *SMO* as a first transducer of the Hh cascade; (d) *KIF7* mRNA levels correlated with those of other positive Hh pathway regulators such as *SMO* and *STK36* ( $r = 0.66$ ) and more closely with those of transcription factors *GLI2/GLIS2* ( $r = 0.76$  and  $0.78$ , respectively); (e) *SUFU* mRNA levels correlated with those of *BTRC* ( $r = 0.54$ ), both Hh pathway repressors in mammals; (f) the expression of transcription factors *GLI2* and *GLIS2* showed a correlation between them ( $r = 0.70$ ) and with other positive Hh pathway regulators in menin-

giomas such as *GAS1* and *STK36* ( $r = 0.56, 0.64$ ), more strongly with *KIF7* ( $r = 0.76, 0.78$ ) and *SMO* ( $r = 0.85, 0.75$ ) as stated before and with the *GLI2* target *IGF2* ( $r = 0.52$ ); (g) *MTSS1* mRNA levels correlated with those of *PTHR1* ( $r = 0.57$ ), in full agreement with the underexpression of both genes in meningiomas but not with the expression of *PTHR1* in other tumors (sarcomas and bone metastases); (h) *IGF2* mRNA levels correlated mainly with those of *SMO* and *GLI2* as stated before, consistent with the association of the Hh pathway activation and cell proliferation, and with those of *FOXM1* ( $r = 0.58$ ) and *CCND2* ( $r = 0.66$ ), as expected from the close association between progression to DNA replication and cell proliferation.

### DISCUSSION

The altered expression of 16 Hh pathway genes and their targets in meningiomas is in full agreement with their function as activators or inhibitors. *HHAT*, *DISP1*, *SMO*, *STK36* and *GLI1/2* involved in Hh pathway activation were overexpressed, five of them with a

**Table 3.** Correlations between mRNA levels of Hedgehog pathway and target genes; P values and correlation coefficients (Spearman rank correlation test).<sup>a</sup>

	DISP1/Disp2	PTCH1/PTCH2	SMO	KIF7	STK36	SUFU	GLI2	GLI4	GLIS2	MTSS1	FOXM1	IGF2
HHAT	0.01/ <b>0.44</b>			1.90E03/ <b>0.48</b>			2.40E03/ <b>0.47</b>					
PTCH1/PTCH2		3.50E03/ <b>0.46</b> 2.40E03/ <b>0.47</b>										
HHIP				2.60E03/ <b>0.47</b>	1.30E03/ <b>0.5</b>	2.80E03/ <b>0.47</b>	3.50E04/ <b>0.56</b>		2.10E04/ <b>0.58</b>			
GAS1	3.20E05/ <b>0.65</b>	5.90E04/ <b>0.54</b>	2.70E04/ <b>0.57</b>	1.80E03/ <b>0.49</b>			1.20E06/ <b>0.76</b>		8.10E04/ <b>0.52</b>			
RAB23			3.10E06/ <b>0.73</b>						6.00E07/ <b>0.78</b>			
KIF7	1.10E05/ <b>0.51</b> 2.80E03/ <b>0.47</b>											
STK36	1.10E03/ <b>0.51</b>		1.70E03/ <b>0.49</b>	2.60E05/ <b>0.66</b>		5.80E04/ <b>0.54</b>	3.20E04/ <b>0.56</b>	7.30E05/ <b>0.62</b>	4.00E05/ <b>0.64</b>			
BTRC				2.00E03/ <b>0.48</b>				4.30E03/ <b>0.45</b>		0.01/ <b>0.39</b>		
GLI1		1.20E05/ <b>0.68</b>	3.30E03/ <b>0.46</b>	0.01/ <b>0.41</b>			0.01/ <b>0.42</b>		3.10E04/ <b>0.56</b>		0.01/ <b>0.42</b>	
GLI2	0.01/ <b>0.41</b>		4.70E08/ <b>0.85</b>	1.20E06/ <b>0.76</b>								
GLI3			1.70E03/ <b>0.49</b>									
GLI4	2.40E04/ <b>0.57</b>						4.10E03/ <b>0.45</b>		0.01/ <b>0.39</b>	2.10E05/ <b>0.66</b>		
GLIS2	0.01/ <b>0.44</b>	7.20E04/ <b>0.53</b>	1.60E06/ <b>0.75</b>	6.00E07/ <b>0.78</b>			8.10E06/ <b>0.7</b>					
FOXM1			2.80E03/ <b>0.47</b>				0.01/ <b>0.42</b>					
SPP1											0.01/ <b>0.43</b>	1.70E03/ <b>0.49</b>
IGF2			9.70E04/ <b>0.52</b>	2.80E03/ <b>0.47</b>			9.50E04/ <b>0.52</b>		3.40E03/ <b>0.46</b>		2.10E04/ <b>0.58</b>	2.20E03/ <b>0.66</b>
CCND2												
PTHR1										2.30E04/ <b>0.57</b>		

<sup>a</sup>P values of 0.01 or lower and respective correlation coefficients (in **bold**) are separated by a slash. The values of DISP1 and DISP2 as well as PTCH1 and PTCH2 are enclosed in the same cell, denoting DISP2 and PTCH2 by *italics*. Correlation coefficient values higher than 0.7 are underlined.

significant difference, whereas *PTCH1*, *HHIP* and *SUFU*, which repress the Hh pathway, were underexpressed. In addition, the well-known inducible genes *FOXA2/M1*, *SPP1*, *OSF*, *IGF2* and *CCND2*, dealing with cell proliferation, invasion/migration and DNA replication were also overexpressed. Activation of these genes was reported to occur in several types of cancers (2,3,21,22). Conversely, the metastasis suppressor *MTSS1* is underexpressed in meningiomas.

On the other hand, the altered expression of seven genes in meningiomas does not fully agree with the function assigned to them in the Hh pathway. The following genes, presumably negative regulators, showed an increase in mRNA levels: (a) the growth arrest-specific 1 gene (*GAS1*) which, despite its name, participates in cerebella cell proliferation (34), thus suggesting multiple functions for this gene; (b) kinesin family member *KIF7*, a presumed homolog of *Drosophila's* Hh-suppressor *cos2* (6); (c) *BTRC*, the E3 ligase for GLI3 processing to repressor form—however, it may also be associated with tumorigenesis through a dysregulation of the proteolysis of its substrates (11,35); (d) *GLIS2* factor, a well-known Wnt/ $\beta$ -catenin pathway repressor, may also have another role relative to tumorigenesis in this pathway (15). Changes in the expression of these genes may reflect a complex regulation of the Hh pathway to maintain correct cell functioning. Conversely, the positive regulators *DHH*, a Hh ligand for gonads, and *PTHR*, a parathyroid hormone receptor involved in bone metabolism and metastasis, showed a significant decrease in mRNA levels in meningiomas. This underexpression of both genes may be explained by their lack of specificity for neural tissues. Hh-ligand mRNA levels did not increase in meningiomas compared with normal tissue; moreover, one of them, *DHH*, decreased. Thus, their role in meningioma development may not be relevant.

A significant change in mRNA levels of several Hh pathway and target genes

was found in low-grade tumors (MGI) in comparison with normal tissue: *HHAT*, *DISP1*, *SMO*, *KIF7*, *GLI2*, *GLIS2* and *FOXM1* mRNA levels increased in MGI, and those of *PTCH1*, the tumor suppressor, decreased (Table 2). Thus, these genes could have a relevant role in the initial steps of tumorigenesis. Moreover, *PTCH1* mRNA levels decreased only in low-grade meningiomas, suggesting that this change is not significant for progression to a more malignant state, which would be attained by other genes overexpressed in MGII and MGIII.

A significant increase in mRNA levels of *GLI1*, *FOXM1*, *SPP1* and *IGF2* was found in the more invasive tumors (MGII + MGIII or MGIII) compared with lower meningiomas grades. Therefore, these genes could be the markers for invasiveness and aggressiveness. The significant changes in expression of these genes, as well as that of *PTCH1*, between MGI and MGII/MGIII suggest different biological functions involved in the development of the three types of meningiomas. Moreover, the benign MGI tumors (95% of total meningiomas) showed a lower number of genes with an altered expression than the atypical (4.7%–7.2%) and anaplastic (1%–2.8%) tumors, both of them presenting similar alterations: an increased expression of genes involved in cell proliferation and invasiveness (*FOXM1*, *SPP1* and *IGF2*).

A wide variation in the increased mRNA levels of several genes (*HHAT*, *SMO*, *KIF7*, *GLI1*, *GLI2*, *FOXM1*, *SPP1* and *IGF2*) occurred between meningiomas and normal tissue, suggesting a strong upregulation of these genes in meningiomas. The number of MGIII tumors was low and the differences among them resulted in no statistical significance in the expression of many genes. However, several relevant Hh pathway genes—such as *SMO*, *GLI2*, *GLIS2* and the target growth factor *IGF2*—showed a significant increase in MGIII versus normal and versus MGI (only *GLI2* and *IGF2*).

### Hh Pathway Members with Similar Function Show a Correlation in Their Expression

A positive correlation between the *SMO* and *GLI* family expression is in full agreement with the activating action of *SMO* on the transcription of these factors. The closest association of *SMO* with *GLI2*/*GLIS2* suggests that they have the most relevant function in the Hh pathway of meningiomas. This is supported by the fact that *GLIS2* was found mainly in neural tissues (36). mRNA levels of *GLI* family members correlated between them and with those of other regulating factors, showing the closest associations for *GLI2*. These results support a relevant role of *GLI2* in meningiomas.

The *KIF7* expression correlates with that of many Hh pathway-activating genes; moreover, its strong correlation with *GLI2*/*GLIS2* transcription factors suggests an important role for *KIF7* in the Hh pathway of meningiomas. Similarly, the expression of *GAS1* correlates with Hh pathway-activating genes, although not as strongly as *KIF7*, supporting its positive role in this pathway in meningiomas. Hh pathway members with an activating role such as *SMO*, *GLI* family and *KIF7* also correlate in their expression with that of the growth factor *IGF2*, in full agreement with the association between Hh pathway activation and cell proliferation.

In conclusion, our results show, to our knowledge for the first time, an activation of the Hh pathway in meningiomas. The positive Hh pathway regulators such as *HHAT*, *DISP*, *SMO*, *KIF7*, *GLI1*/*GLI2* and *GLIS2* are overexpressed in all meningioma grades, including benign tumors, whereas Hh target genes, involved in cell proliferation and invasiveness—such as *IGF2* and *SPP1*—are overexpressed in meningioma grades II and III but not in grade I. *FOXM1*, the *GLI* target gene, shows the most marked rise in mRNA levels, 90- to 100-fold above normal values in meningioma grades II and III and a 17-fold increase in benign tumors. Our results should be a significant step toward an advance in the bio-

logical and clinical characterization of meningiomas. They may also be useful to consider the Hh pathway and related genes as targets for gene therapy.

### ACKNOWLEDGMENTS

The authors are grateful to patients and their families for authorizing the molecular assays of their tumor biopsies. We thank Rex Davis for language supervision. This work was supported by the grant from CONICET PIP 5196, Buenos Aires, Argentina.

### DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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