Meningiomas and schwannomas: Molecular subgroup classification found by expression arrays

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Abstract. Microarray gene expression profiling is a high-throughput system used to identify differentially expressed genes and regulation patterns, and to discover new tumor markers. As the molecular pathogenesis of meningiomas and schwannomas, characterized by *NF2* gene alterations, remains unclear and suitable molecular targets need to be identified, we used low density cDNA microarrays to establish expression patterns of 96 cancer-related genes on 23 schwannomas, 42 meningiomas and 3 normal cerebral meninges. We also performed a mutational analysis of the *NF2* gene (PCR, dHPLC, Sequencing and MLPA), a search for 22q LOH and an analysis of gene silencing by promoter hypermethylation (MS-MLPA). Results showed a high frequency of *NF2* gene mutations (40%), increased 22q LOH as aggressiveness increased, frequent losses and gains by MLPA in benign meningiomas, and gene expression silencing by hypermethylation. Array analysis showed decreased expression of 7 genes in meningiomas. Unsupervised analyses identified 2 molecular subgroups for both meningiomas and schwannomas showing 38 and 20 differentially expressed genes, respectively, and 19 genes differentially expressed between the two tumor types. These findings provide a molecular subgroup classification for meningiomas and schwannomas with possible implications for clinical practice.

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Introduction

Meningiomas represent ~20% of all primary intracranial tumors and arise sporadically or within the neurofibromatosis type 2 syndrome (NF2). Cytogenetically, the characteristic alteration is the partial or total loss of chromosome 22 (up to 70%) (1). Other cytogenetic changes secondary to this anomaly are primarily the deletions of 1p and 14q (2). At the molecular level, half of these tumors have allelic losses including the 22q12 band. The *NF2* gene, located at 22q12.2, is considered the main candidate for the genesis of meningiomas and works as a tumor suppressor gene (TSG). Mutations in this gene have been detected in 60% of meningiomas (3). *NF2* has also been found silenced by promoter hypermethylation (4), although this has not been found in all cases (5). Other TSG epigenetically silenced in meningiomas include *THBS1* (15-30%), *TIMP-3* (24%), *p16(INK4a)* (17%), *MGMT* (6-16%), *p73* (15%), *ER* (15%), *GSTP1* (27%), *RB1* (10%) and *p14(ARF)* (13%) (6,7). In addition, atypical and anaplastic meningiomas show frequent losses in chromosomal arms 1p, 14q, 6q, 9q, 10, 17p, 18p and 18q (1,8-10).

On the other hand, schwannomas are benign encapsulated tumors that grow around the nerves. Schwannomas also arise sporadically or within NF2 syndrome. NF2 is characterized by the presence of bilateral vestibular schwannomas at an early age (11). The main genetic alterations found in this tumor type are loss of heterozygosity (LOH) of the long arm of chromosome 22 (up to 50%) and the mutations in the *NF2* gene (up to 60%) (12,13). Other cytogenetic gains, losses or alterations of specific genes vary (12,14,15). Epigenetic silencing of the *NF2* gene (18-60%) has also been detected in schwannomas (16,17). The relevance of aberrant *NF2* methylation with respect to tumor genesis in both tumor types remains to be clearly determined. As with meningiomas, aberrant promoter hypermethylation of other tumor-related genes has also been described in schwannomas: *THBS1* (30-36%), *TP73* (19-27%), *MGMT* (16-20%), *TIMP-3* (18%), *RB1*/*p16*^{INK4a} (15%) (16,18). For schwannomas and meningiomas it is considered that there might be other TSGs

located at 22q, that could be involved in the development of these tumors, since deletions of 22q outside the *NF2* locus have also been reported (19).

The methods used to classify cancer are currently based on morphological characteristics. The information obtained by this method is limited and omits many tumor characteristics, such as the rate of proliferation, invasion and metastasis ability, and the possible development of cellular mechanisms of resistance to treatment (1,20). Microarray gene expression profiling is a high-throughput system that can be used in combination with conventional diagnostic methods as a useful complement. In addition to the identification of differentially expressed genes and the establishment of gene expression patterns, microarrays can also allow us to discover potential tumoral markers that could improve clinical practice and therapy for cancer (21-23).

Currently there are few studies that have identified patterns of gene expression using microarrays in meningiomas or schwannomas (24-31). In this study we used low density microarray expression profiling in a series of schwannomas and meningiomas to find the differential expression of 96 genes specifically involved in: i) control of the cell cycle and damage to DNA repair; ii) apoptosis and cellular senescence, ii) signal transduction and transcription factors; iv) adhesion; v) angiogenesis; and vi) invasion and metastasis. We also performed correlations with the tumor grade, molecular alterations of the *NF2* gene, LOH in 22q and the methylation status of 25 cancer-related genes, in order to identify molecular markers with diagnostic, prognostic and therapeutic potential.

Materials and methods

Sample and DNA/RNA preparation. Samples and clinical-pathological data were obtained according hospital ethical committee's procedures. DNA was isolated from 65 frozen samples, corresponding to 42 meningiomas and 23 schwannomas, using the Wizard Genomic DNA purification kit (Promega). DNA from the corresponding patients' peripheral blood was also extracted. RNA was isolated using the RNeasy® Mini Kit (Quiagen) and Poly A was obtained using the Oligotex® mRNA Mini Kit (Quiagen). Pathological diagnosis was performed according to the WHO classification (1), and included 32 grade I, 6 grade II, and 4 grade I recurrent meningiomas. As controls 3 commercial human adult normal RNA from cerebral meninges were used (USBiological Catalogs No T5595-7251, T5595-7252A and Biochain Catalog No R1234043-50-BC). No normal Schwann controls were available.

22q LOH. The allelic status of 5 microsatellite markers at the D22S275, D22S264, D22S929, D22S268 and D22S280 loci (22q11-q12.3) was determined by labeling 5' primers with fluorescent markers (6-FAM/HEX and ROX as a size standard, Applied Biosystems, Foster City, CA). Allelic ratios were defined according to previously described criteria: T2xN1/T1xN2 in which LOH <0.6-1.67 >LOH (32).

PCR/dHPLC analysis and direct sequencing of NF2. Genomic DNA amplification was performed by standard PCR methods (total volume of 20 μl). A set of 15 primer pairs were used as described (33). Mutational screening was carried out using dHPLC following manufacturer's protocols (Transgenomic WAVE® dHPLC Systems). Samples with abnormal patterns were sequenced bidirectionally (ABI 3100 Avant, Applied Biosystems), using the Big Dye sequencing kit (Applied Biosystems), to determine the position and nature of the alteration.

MLPA and MS-MLPA. We used a commercial MLPA kit for *NF2* gene analysis (SALSA P044 NF2) and a kit for the methylation status of the promoter region of 25 cancer-related genes (SALSA MS-MLPA Kit ME001; MRC-Holland, Amsterdam, The Netherlands). Information regarding the probe sequences and ligation sites can be found at http://www.mlpa.com. The MLPA protocol was performed as described by the manufacturers, using 100 ng of DNA from control and tumor samples. Data analysis was performed with MRC-Coffalyser software (MRC-Holland).

Expression arrays. For the gene expression analysis by microarray membranes we used the GEArray™ Q Series HS-006 (SuperArray, Bethesda, MD) containing 96 cDNA fragments in quadruplicate, as well as housekeeping genes and negative controls. The manufacturer's protocol was used. The cDNA was obtained from 200 ng of mRNA by reverse transcription. Labeling with [^{33}P]dCTP was performed using linear amplification (AmpoLabeling-LPR.SuperArray). Labeled probes were hybridized in the membranes at 60˚C for 24 h. Membranes were exposed to screen storage (Cyclone Storage Phosphor Screen), and images were obtained with the Cyclone 3000 and analyzed by the Phoretix™ Array software (Nonlinear Dynamics Ltd.).

Q-PCR. To validate the microarray data obtained, an assay was conducted using the ABI PRISM 7900HT Sequence Detection System, which allows for the relative quantification of nucleic acids through the comparative C_T method. *GAPDH* was used as an endogenous control gene. Expression levels of 4 TaqMan® probes (Applied Biosystems) were measured (selected by its various expressions from the 96 genes included in the microarray: *BCL2L1*, *FOS*, *MDM2* and *TIMP1*). The fluorescence data were analyzed with the SDS software to obtain the values of relative expression. To check consistency and correlation of data obtained from the expression by microarray compared with the corresponding data obtained by Q-PCR an intra-class correlation analysis was performed.

Statistical analysis. Analyses were performed using BRB-ArrayTools 3.5.0 Patch_1 by Dr Richard Simon and Amy Peng Lam. Sample data were normalized by using *GAPDH*, *RPL13A* and *ACTB* genes. Genes whose expression differed by at least 1.5-fold from the median in at least 20% of the arrays were retained. Cluster analysis: we used hierarchical clustering to cluster the samples and used R (reproducibility) and D (discrepancy) measures to evaluate the robustness of the clusters. Class comparison: we identified genes differentially expressed among the two classes using a random-variance t-test (F-test for more than 2 classes). Genes were considered statistically significant at $p<0.001$. We also

performed a global test of whether the expression profiles differed between the classes by permuting the labels of which arrays corresponded to which classes. SAM (significative analysis of microarrays): we also identified genes that were differentially expressed among the two classes by using a multivariate permutation test to provide 90% confidence that the false discovery rate (FDR) was less than 10%. The test statistics used are random variance t-statistics for each gene. Class prediction: we developed models for utilizing gene expression profile to predict the class of future samples based on the compound covariate predictor, diagonal linear discriminant analysis, nearest neighbor classification, and support vector machines with linear kernel. The models incorporated genes that were differentially expressed among genes at $p<0.005$ as assessed by the random variance t-test. We estimated the prediction error of each model using leave-one-out cross-validation (LOOCV). 1000 random permutations were used. All array experiment information and data has been loaded into ArrayExpress (www.ebi.ac.uk/arrayexpress).

Results

We analyzed 65 frozen samples, corresponding to 42 meningiomas and 23 schwannomas. Using 5 microsatellites markers, LOH for 22q was detected in 53.6% of GI meningiomas, 83.3% of GII meningiomas, 100.0% of recurrent meningiomas, as well as 30.4% of the schwannomas (Table I). Except for M41 and S23, all samples with LOH showed loss of the 5 analyzed markers. *NF2* gene mutations were identified by PCR/dHPLC and sequencing in 26/65 (40%) of the samples. Meningiomas showed small deletions in 4/42 (9.5%), small insertions in 3/42 (7.1%), nonsense mutations in 4/42 (9.5%), splicing mutations in 4/42 (9.5%) and intronic changes in 3/42 (7.1%), accounting for 42.9% (18/42) of the meningiomas. Schwannomas showed small deletions in 2/23 (8.7%), nonsense mutations in 2/23 (8.7%), splicing mutations in 2/23 (8.7%) and intronic changes in 2/23 (8.7%), accounting for 34.8% (8/23) of the schwannomas. Only the nonsense mutation g.592C>T-arg57stop (S16) has been previously reported (33). Intronic mutation IVS4+47delA was found in 2 meningioma samples (M16 and M17) and 1 schwannoma sample (S8), and splicing mutation IVS8+2T>G was found in 3 meningioma samples (M14, M15 and M24) and 1 schwannoma sample (S11) as well as in their corresponding DNA samples, except for 1 meningioma (Table I).

Multiplex ligation-dependent probe amplification (MLPA) analyses showed complete *NF2* gene loss in 34/65 (52.3%) of the samples, including two samples with partial losses on the other allele. *NF2* gene partial losses also were found in 4/65 (6.15%). MLPA also showed duplications on 1p36 (20%), 8q24 (10.8%), 9q (16.9%), 10q (15.4%), 12q (33.9%) and 17q (4.6%), and deletions on 1p36 (15.4%), 6q25-q27 (21.5%) and 14q (24.6%) (Table I). Methylation-Specific MLPA (MS-MLPA) analysis showed alterations in 10 meningiomas and 2 schwannomas. Hypermethylation was found for genes *CDKN2B* and *RASSF1* in 4 samples each and for *CDH13*, *IGSF4*, *CASP8*, *CDKN1B*, *PTEN* and *CHFR* genes in 1 sample each. *CASP8* and *CDKN1B* genes, studied both in MS-MLPA and microarray, showed low expression in the two samples with promoter hypermethylation.

Microarray expression analysis for 96 cancer-related genes was performed in 23 schwannomas, 42 meningiomas and 3 normal cerebral meninges as controls. No normal Schwann cell controls were available. Microarray data were validated by Q-PCR, and intra-class correlation analysis of the expression levels of *BCL2L1*, *FOS*, *MDM2* and *TIMP1* genes was performed to check consistency and correlation of data obtained. Data acquired in our tumor series clearly identified, by unsupervised hierarchical cluster analysis, two main groups of tumors (I and II) and three groups of genes (A, B and C) (Fig. 1). As a global analysis this statistical method found one group of samples, differentiating between meningiomas (I3) and schwannomas (I2), with expression patterns similar to normal cerebral meninges (I1), and another group of samples, also differentiating between meningiomas (II5) and schwannomas (II4), with expression patterns different from the normal cerebral meninges. Differences in gene expression for groups A and B are responsible for the two main molecular groups of samples, in the same way that genes in group C distinguish each of the two tumor types, schwannomas and meningiomas within each of these two main sample groups.

For class comparison, differentially expressed genes [$p<0.001$ and SAM (significative analysis of microarrays)] among classes were identified. Comparison between normal cerebral meninges and meningiomas showed 7 differentially expressed genes: *FOS*, *ICAM1*, *PLAUR*, *IL8*, *FLT1*, *SERPINE1* and *CDKN1A*. Class comparison including only the 35 meningioma samples in subgroups I3 and II5 found 38 differentially expressed genes (Table II). Analyzing meningiomas by tumor grade and recurrence, the *S100A4* gene was differentially expressed, increasing its expression in samples GII as compared to GI and also in the recurrent meningiomas with respect to GII. SAM analysis also showed 2 genes with decreased expression in samples GII with respect to GI samples: *IFNA1* and *MMP9* (Table II).

Comparative expression patterns between meningiomas and schwannomas showed 16 genes with lower expression and 3 genes with higher expression in meningiomas with respect to schwannomas. As performed with meningiomas, we achieved a class comparison including only the schwannoma samples in subgroups I2 and II4, and we identified 20 differentially expressed genes (Table II).

Class comparison in meningiomas and schwannomas, classifying them by presence or absence of LOH for 22q and for *NF2* gene alteration detected by MLPA, showed 15 genes with lower expression in those samples with LOH and 11 genes with lower expression in those samples with *NF2* gene alteration detected by MLPA (Table II). When the comparison was performed classifying samples by presence or absence of any *NF2* gene alteration (LOH, MLPA or mutational analysis), we found 3 genes differentially expressed (*PLAUR*, *CDKN1A* and *FLT1*), with lower expression in the altered samples (Table II). We did not identify gene ontology (GO) groups of genes whose expressions were differentially regulated among the classes. There were no genes with expression levels significantly related to patient age or gender.

Table I. Data and results for LOH, MLPA and mutational analysis in meningiomas (M) and schwannomas (S).

ID	Grade	Age	Sex	LOH	Loss of *NF2* by MLPA	Gains by MLPA	Losses by MLPA	Methylation by MLPA	NF2 tumor alterations (NM_000268)	NF2 constitutional alterations (NM_000268)
M01	I	70	F	-	-/-	-	-	-	-	-
M02	I	53	F	-	-/-	11p12	-	-	-	-
M03	II	19	M	+	-/+	-	-	-	-	-
M04	II	-	M	+	-/+	05q31.1	04q35	-	-	-
M05	I	-	F	+	-/+	-	-	-	-	-
M06	I	-	F	-	-/-	-	-	-	-	-
M07	I	-	F	+	-/+	-	-	*CDH13*	NC_000022.9:g.57597C>T (IVS8-33C>T)	NC_000022.9:g.57597C>T (IVS8-33C>T)
M08	I	-	F	-	-/-	-	-	-	-	-
M09	I	-	M	-	-/-	-	-	-	-	-
M10	I	-	F	-	-/-	-	-	-	-	-
M11	I	-	M	+	-/+	-	-	*IGSF4*	-	-
M12	I	-	F	+	-/+	-	-	-	-	-
M13	I	-	F	-	-/exon 1-6	08q24	-	-	g.1057C>T-p.Gln212Stop	-
M14	I	38	M	+	-/+	08q24	-	-	NC_000022.9:g.57766T>G (IVS8+2T>G)	NC_000022.9:g.57766T>G (IVS8+2T>G)
M15	I	38	M	+	-/+	-	-	-	NC_000022.9:g.57766T>G (IVS8+2T>G)	NC_000022.9:g.57766T>G (IVS8+2T>G)
M16	R	69	F	+	-/+	08q24	-	*CDKN2B*	NC-000022.9:g.38750delA (IVS4+47delA)	-
M17	I	33	F	+	+/exon 2-8	08q24-15q24.3	06q26	-	NC-000022.9:g.38750delA (IVS4+47delA)	-
M18	I	61	F	+	-/+	-	-	*CDKN2B*	g.1732insTG	-
M19	II	76	M	-	-/-	-	06q26	-	-	-
M20	II	71	F	+	-/+	15q24.3	06q26-14q13	*CDKN2B*	-	-
M21	R	67	M	+	-/+	-	06q26	-	NC-000022.9:g.54688G>A (IVS7+1G>A)	-
M22	I	49	F	+	-/+	08q24	-	-	g.628A>T-p.lys69stop	-
M23	II	63	M	+	-/+	-	06q26-14q13	-	g.1393C>T-p.Gln324Stop	-
M24	II	81	M	+	-/+	-	14q13	*RASSF1*	NC_000022.9:g.57766T>G (IVS8+2T>G)	-
M25	I	37	F	-	-/-	-	-	-	-	-
M26	I	55	F	+	-/+	-	-	-	-	-
M27	I	66	F	+	-/+	-	14q13	*RASSF1*	g.578_589del	-
M28	I	54	M	-	-/-	-	-	-	-	-
M29	I	60	M	+	-/+	-	-	-	-	-
M30	I	65	F	+	-/+	-	-	-	-	-
M31	I	-	F	+	-/+	-	-	*CASP8 RASSF1*	-	-
M32	I	74	F	+	-/+	-	-	-	g.1211delA	-
M33	I	44	F	+	-/+	02q14.2	-	-	g.1438_1442del	-
M34	I	78	F	+	-/+	-	14q13-15q24.3	-	g.1747G>T-p.Glu442Stop	-

Table I. Continued.

ID	Grade	Age	Sex	LOH	Loss of NF2 by MLPA	Gains by MLPA	Losses by MLPA	Methylation by MLPA	NF2 tumor alterations (NM_000268)	NF2 constitutional alterations (NM_000268)
M35	I	46	F	+	-/+	-	-	*CDKN1B*	g.1389_1399del	-
M36	I	61	F	-	-/exon 1-4	-	02q14.2	*CDKN2B*	-	-
M37	R	76	F	+	-/+	05q31.1	-	-	g.1924insC	-
M38	I	60	M	-	-/-	-	-	-	-	-
M39	I	-	-	-	-exon 1-12	-	-	*RASSF1*	-	-
M40	I	50	F	-	-/-	-	-	-	-	-
M41	R	55	F	+	-/+	-	-	-	g.988insA	-
M42	I	70	F	-	-/exon 9-10	04q35	08q24-09q21.3	-	-	-
S01	I	-	M	-	-/-	-	-	*PTEN*	-	-
S02	I	-	M	-	-/-	-	-	-	-	-
S03	I	-	F	-	-/-	-	-	-	-	-
S04	I	-	M	+	-/+	-	14q13	*CHFR*	-	-
S05	I	-	M	-	-/-	08q24	-	-	-	-
S06	I	-	M	-	-/-	-	-	-	-	-
S07	I	-	M	-	-/-	-	-	-	-	-
S08	I	54	M	-	-/-	-	-	-	NC-000022.9:g.38750delA (IVS4+47delA)	-
S09	I	71	F	-	-/-	-	-	-	-	-
S10	I	59	F	+	-/+	-	-	-	g.1143delG	-
S11	I	38	M	-	-/-	08q24	14q13	-	NC_000022.9:g.57766T>G (IVS8+2T>G)	NC_000022.9:g.57766T>G (IVS8+2T>G)
S12	I	-	M	-	-/-	-	-	-	-	-
S13	I	69	M	-	-/-	-	-	-	-	-
S14	I	55	M	-	-/-	-	-	-	g.780_781del	-
S15	I	56	M	+	-+	-	-	-	-	-
S16	I	27	F	+	+/exon 2	-	-	-	g.592C>T-arg57stop	-
S17	I	26	F	-	-/-	-	-	-	-	-
S18	I	49	F	-	-/-	-	-	-	-	-
S19	I	65	M	+	-/+	06q26	-	-	NC_000022.9:g.71259A>C (IVS13-2A>C)	-
S20	I	14	M	-	-/-	-	-	-	-	-
S21	I	37	F	-	-/-	-	-	-	-	-
S22	I	55	F	+	-/+	-	-	-	g.782T>A-leu120stop	-
S23	I	62	F	+	-/+	-	-	-	-	-

R, recurrent meningioma grade I. -/+, negative and positive results.

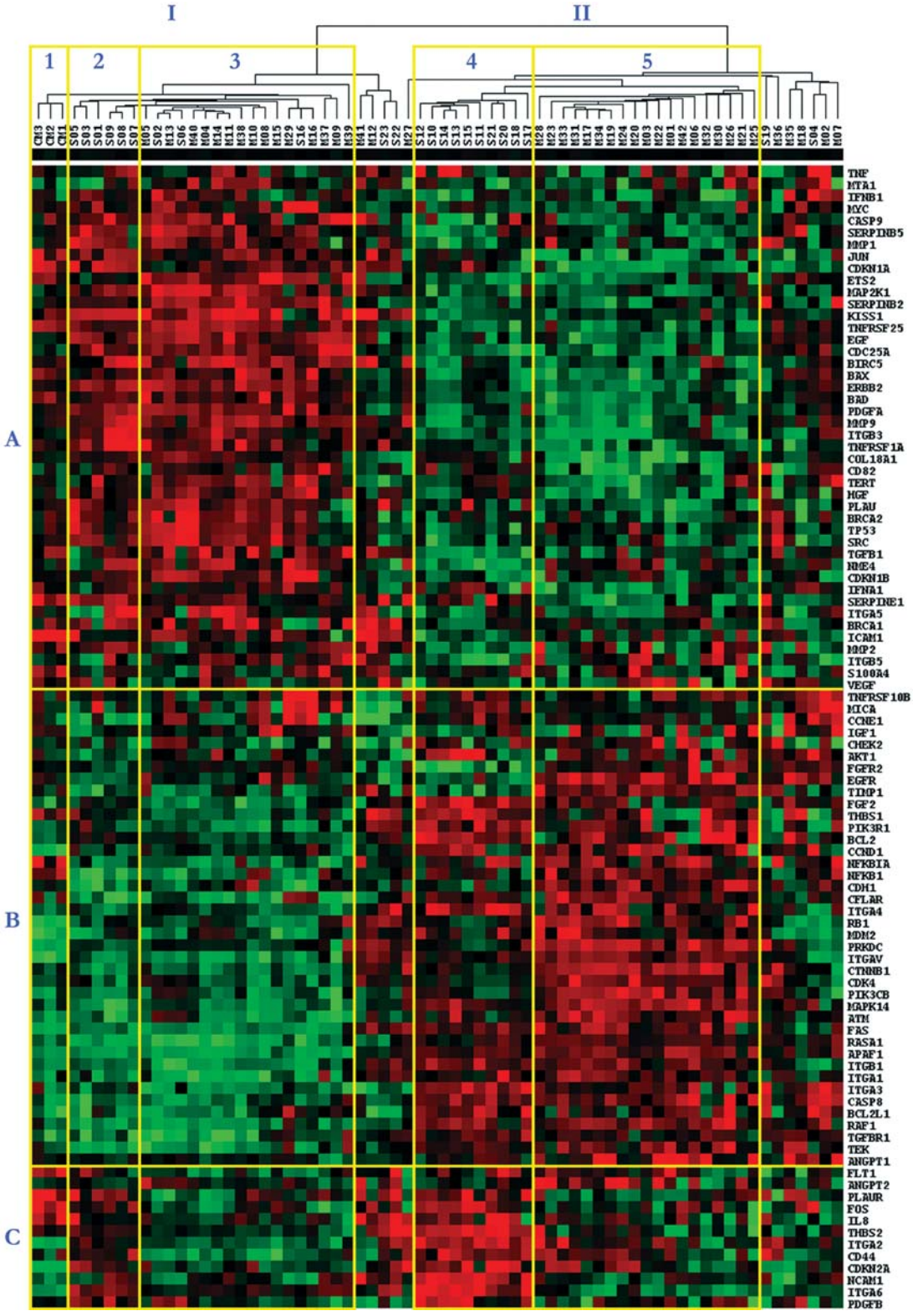

Figure 1. Sorting by unsupervised hierarchical cluster the expressions of 96 cancer-related genes in 3 normal cerebral meninges (CM), 42 meningiomas (M) and 23 schwannomas (S). Analysis identified two main groups of tumors (I and II) and three groups of genes (A, B and C), differentiating between meningiomas (I3 and II5) and schwannomas (I2 and II4) with expression patterns similar to or different to from the normal cerebral meninges (I1). Genes in groups A and B established the two main groups of samples, in which schwannomas and meningiomas can be distinguished by the genes included in group C. Red denotes higher relative expression, and green denotes lower relative expression levels.

Discussion

The mutational study of the *NF2* gene found similar percentages as those already described (Human Gene Mutation Database; www.hgmd.cf.ac.uk), both in total and by type of mutation. The splicing mutation IVS8T+2>G was detected in 3 meningiomas and 1 schwannoma (15.38%), as well as in the peripheral blood DNA from 3 of them, suggesting

Table II. Class comparison and SAM analysis showing the mean of the expression intensities and the ratio for the different classes.

Gene	Ratio M:CM	p-value/SAM
Meningioma/cerebral meninges		
CDKN1A	-5.9	SAM1
FLT1	-7.8	SAM1
FOS	-24.6	p<0.001
ICAM1	-6.9	p<0.001
IL8	-12.2	p<0.001
PLAUR	-10.7	p<0.001
SERPINE1	-6.6	SAM1
Gene	**Ratio I:II**	**SAM**
Grade I and II - meningiomas		
IFNA1	5.8	SAM2
MMP9	5.8	SAM2

Gene	GI:GII	GI:R	GII:R
Grade and R - meningiomas			
S100A4	-2.4	-3.7	-1.5

Gene	CM:I3	CM:II5	I3:II5
Meningioma subgroups I3 and II5			
APAF1	1.7	-11.4	-19.7
ATM	1.1	-2.9	-3.1
BAD	1.2	7.7	6.6
BAX	1.1	7.5	7.0
BIRC5	-1.9	6.5	12.2
CASP9	0.8	2.4	3.1
CDC25A	-2.2	2.3	5.0
CDKN1A	2.1	12.6	6.1
COL18A1	0.9	5.1	5.6
CTNNB1	2.5	-4.9	-12.3
EGF	-2.4	2.5	6.1
ERBB2	-1.7	5.3	9.2
ETS2	0.9	3.7	4.3
HGF	-1.6	2.7	4.3
ICAM1	4.7	8.7	1.9
IFNA1	1.3	8.5	6.5
ITGA1	2.5	-3.3	-8.3
ITGA6	-2.6	1.6	4.2
ITGAV	-2.2	-36.3	-16.3
ITGB1	1.5	-3.5	-5.2
ITGB3	-1.6	3.9	6.3
JUN	2.1	6.7	3.2
KISS1	-2.1	6.1	12.8
MAP2K1	-1.6	1.9	3.1
MAPK14	1.8	-3.3	-6.0
MMP9	-2.1	6.7	14.0

Table II. Continued.

Gene	CM:I3	CM:II5	I3:II5
Meningioma subgroups I3 and II5			
PDGFA	-1.6	3.9	6.2
PIK3CB	1.0	-3.0	-3.0
PLAU	0.9	4.3	4.9
PRKDC	-2.1	-7.8	-3.7
RAF1	2.1	-3.1	-6.5
RASA1	3.2	-5.4	-17.4
SERPINB2	-1.7	2.6	4.4
SERPINE1	2.7	14.0	5.1
TIMP1	1.9	-1.7	-3.2
TNFRSF25	1.5	6.7	4.5
TNFRSF1A	0.9	3.5	3.8
FAS	0.9	-6.3	-5.7

Gene	Ratio M:S	p-value/SAM
Meningioma/schwannoma		
CD44	-2.6	p<0.001
CDKN1A	-2.0	SAM3
CDKN2A	-3.9	p<0.001
COL18A1	-2.0	SAM3
EGFR	4.1	p<0.001
FGFR2	2.3	p<0.001
FOS	-3.9	p<0.001
IL8	-3.5	p<0.001
ITGA2	-3.3	p<0.001
ITGA3	-3.4	p<0.001
ITGA6	-4.3	p<0.001
NCAM1	-2.9	p<0.001
PDGFB	-2.0	SAM3
PLAU	-2.2	SAM3
PLAUR	-2.9	p<0.001
SERPINE1	-3.1	p<0.001
THBS1	-2.6	SAM3
THBS2	-4.7	p<0.001
TIMP1	3.0	p<0.001
Gene	**Ratio +:-**	**p-value/SAM**
LOH meningiomas and schwannomas		
CDKN1A	-2.3	SAM4
CHEK2	-2.0	p<0.001
COL18A1	-2.4	p<0.001
ERBB2	-2.3	SAM4
FLT1	-2.9	p<0.001
FOS	-2.7	SAM4
ITGA2	-2.0	SAM4
ITGA6	-2.4	SAM4
ITGB3	-2.2	SAM4
MMP9	-2.1	SAM4

Table II. Continued.

Gene	Ratio +:-	p-value/SAM
LOH meningiomas and schwannomas		
NCAM1	-2.2	SAM4
PLAUR	-2.1	p<0.001
SERPINE1	-2.2	SAM4
THBS2	-2.0	SAM4
TNF	-2.2	SAM4
MLPA meningiomas and schwannomas		
CDKN1A	-2.4	SAM5
COL18A1	-2.3	SAM5
FLT1	-2.7	p<0.001
FOS	-2.6	SAM5
ITGA3	-2.3	SAM5
ITGA6	-2.6	SAM5
NCAM1	-2.3	SAM5
PLAUR	-2.9	p<0.001
SERPINE1	-2.2	SAM5
THBS2	-2.6	SAM5
TNF	-2.2	SAM5
NF2 meningiomas and schwannomas		
CDKN1A	-2.3	SAM6
FLT1	-2.6	SAM6
PLAUR	-2.8	p<0.001
Gene	Ratio I2:II4	p-value
Schwannoma subgroups I2 and II4		
APAF1	-19.6	p<0.001
CASP8	-6.6	p<0.001
CFLAR	-4.1	p<0.001
ERBB2	3.5	p<0.001
FAS	-7.9	p<0.001
FGF2	-5.2	p<0.001
ITGA2	-7.6	p<0.001
ITGA3	-14.3	p<0.001
ITGA4	-8.2	p<0.001
ITGAV	7.6	p<0.001
ITGB1	-8.1	p<0.001
TGB3	4.7	p<0.001
KISS1	11.4	p<0.001
MAPK14	-7.2	p<0.001
MMP2	3.7	p<0.001
MMP9	6.1	p<0.001
RASA1	-20.8	p<0.001
ITEK	-13.7	p<0.001
TGFB1	2.1	p<0.001
THBS2	-6.3	p<0.001

Table II. Continued.

The p-value/SAM columns show genes found statistically significant at p<0.0001 or by SAM. With 90th percentile confidence, the false discovery rate among significant genes is: SAM1=0.09804, SAM2=0, SAM3=0.08019, SAM4=0.08654, SAM5=0.07059, SAM6=0. CM, normal cerebral meninges; R, recurrent meningiomas grades I; I2, I3, II4, II5, subgroups as detailed in Fig. 1; +/-, negative and positive results as detailed in Table I.

a prevalent mutation in our population. In agreement with previous data (3,4,9,12,34), through the 22q LOH analysis, we detected alterations in 30.4% of the schwannomas and 64.3% of the meningiomas, and a significant increase in this alteration paralleled the increase of aggressiveness in meningioma samples (53.6% in GI, 83.3% in GII and 100.0% of the recurrent meningiomas).

MLPA analysis in meningiomas and schwannomas confirmed the loss of one complete *NF2* allele in all samples with a positive result for 22q LOH. We also found partial losses of the *NF2* gene in 2 samples with 22q LOH and 4 samples without 22q LOH. The MLPA technique contributes to an accurate identification of alterations that cannot be detected by conventional techniques, and has been used previously in two different studies to search for alterations in the *NF2* gene in sporadic and familiar meningiomas, finding 6% and 48% of cases with complete deletions and 18% and 26% of cases, respectively, with partial deletions (35,36).

MLPA analysis showed that gains and losses in regions other than 22q are frequent in GI meningiomas and that their frequency increases with tumoral grade. Atypical and anaplastic meningiomas frequently show loss of 1p (76%), 14q (43%) and 6q (33%), and gains in 9q (33%), 12q (43%) and 17q21 (33%), usually accompanied by 22q LOH (8,9,34). Our results showed frequent losses of 1p36 (15.4%), 14q (24.6%) and 6q (21.5%), and duplications in 9q34 (16.9%), 12q24 (33.9%) and 17q21 (4.6%), suggesting the presence of possible TSGs in these regions. The decreased percentages obtained in our study can be explained by the composition of the series studied, comprised mainly of GI meningiomas.

Additionally, we found duplications in 8q in 5/42 (10.8%) of the meningiomas and 2/23 of the schwannomas. This is the first report of frequent 8q duplications, since the only report available refers to a highly aberrant karyotype in a recurrent malignant meningioma (37). The specific MLPA probe that hybridizes in *PTEN* promoter, a gene virtually excluded in schwannomas and meningiomas (38,39), detected duplications of 10q (15.4%). If this represents a duplication of *PTEN* or a gain of 10q, it would be the first description of this alteration, contrary to its usual loss. We cannot discard the possibility that this region could contain other TSGs.

As previously described, we found aberrant promoter hypermethylation in schwannomas and meningiomas for the *CDKN2B* (6.15%) and *RASSF1* (6.15%) genes (7,40). The silencing of *CDKN2B* (9p21), a cyclin-dependent kinase inhibitor (41), and *RASSF1* (3p21.3), involved in DNA repair and inhibition of the accumulation of cyclin D1 (42), as well

as the presence of another 6 genes (*CDH13*, *IGSF4*, *CASP8*, *CDKN1B*, *PTEN* and *CHFR*) that also showed promoter hypermethylation in one sample each, corroborates previous studies describing that this mechanism could contribute as a viable alternative to the traditional form of deletion plus mutation in the tumorigenesis of these neoplasms (6,16,18).

Taken as a whole, the cluster analysis identified two main groups of tumors (I and II) and three groups of genes (A, B and C), allowing for discrimination between meningiomas and schwannomas within each group (Fig. 1). In the subsequent analysis, the gene expression levels comparison between normal cerebral meninges and meningiomas showed 7 differentially expressed genes: *FOS*, *ICAM1*, *PLAUR*, *IL8*, *FLT1*, *SERPINE1* and *CDKN1A*. In meningioma samples we found low expression of *FOS* (24.6-fold), a known TSG that encodes a nuclear protein involved in signal transduction. Down-regulation of *FOS* might be an important event in the tumorigenesis of meningiomas. *ICAM-1* (19p13) shows variable expression levels in different cancer types (43). Since the over-expression of this gene is important in senescence (44), its negative regulation (6.9-fold in our series) could prevent the inhibition of proliferation in cells with a pro-inflammatory phenotype in meningiomas. Silencing of *PLAUR* (19q13) in meningioma cells inhibits tumor growth and invasion (45). However, the reduced expression of *PLAUR* in our series (10.7-fold) correlates with reported *PLAUR* decreased immunoreactivity in some regions of GII meningiomas (46). *SERPINE1* (7q21.3-q22) enhances or inhibits tumor growth due to an expression-dependent regulation of angiogenesis (47). As described for prostate cell carcinoma (48), in our tumor series we found reduced *SERPINE1* expression (6.6-fold). A high percentage of meningiomas express chemokine receptors, but have low patterns of *IL8* (4q13-q21) expression in the same samples (46,49). These data are consistent with our findings in which meningiomas expressed a lesser amount of *IL8* (12.2-fold) compared to controls. *FLT1* (13q12), a *VEGF* receptor, seems to be a negative regulator of endothelial cell proliferation, and has high affinity for *VEGF* in addition to a low tyrosine kinase activity (50). The low expression of *FLT1* in our series (7.8-fold) could allow the union of other *VEGF* receptors with increased activity, thus promoting vascular proliferation in meningiomas. Finally, decreased expression of the cell cycle regulator *CDKN1A* (6p21.2) has been described in a large number of meningiomas (51). The results obtained in our series also showed a decreased expression of this gene in meningiomas (5.9-fold), preventing its oncosuppressor activity.

The comparison analysis between meningioma subgroups I3 and II5 showed 38 differentially expressed genes between both subgroups, allowing the establishment of a molecular signature for each one. While we cannot say that these groups are related to tumor grade in a statistically significant way because of the small number of GII meningiomas studied, we can suggest that there is a clear trend toward grouping GII samples in group II5, whose global expressions are different from the controls (Fig. 1). This II5 meningioma tumors group includes *IFNA1* and *MMP9*, which were differentially expressed between meningiomas GI and GII when analyzed in the entire pool of meningiomas. *IFNA1* (9p22) induces apoptosis associated with caspases, *PI3K* and *BAX* and induces the expression of *PLAUR* (52,53). Therefore, the lower *IFNA1* expression in GII meningiomas (5.8-fold) promotes the inhibition of apoptosis in these atypical meningiomas and is not favorable to the expression of *PLAUR* which, as noted above, is a constant found in the whole pool of meningiomas when compared to controls. On the other hand, *MMP9* (20q11.2-q13.1) is involved in degradation of the extracellular matrix and its over-expression is associated with the invasive potential of meningiomas (54). Furthermore, the matrix metalloproteinases (MMPs) inhibitor *TIMP1* showed increased expression and *PLAU* showed decreased expression in meningiomas group II5 compared with group I3. The higher expression of *MMP9* in both GI meningiomas and the I3 group, compared with GII meningiomas and the II5 group, respectively, could therefore promote tumor progression (55). Additionally, *S100A4*, involved in the regulation of cell differentiation and progression, is expressed to a lesser extent in GI meningiomas and to a greater extent in GII, and to an even greater extent in GI recurrent meningiomas; accordingly, the increased *S100A4* expression could enhance the invasive phenotype in this neoplasm. Increased expression of *S100A4*, located at 1q, is consistent with the frequent gains in this region in atypical and anaplastic meningiomas (2,34) and therefore could be a candidate in tumor progression for meningiomas.

Using gene expression microarrays in a previous study, all studied GI meningiomas were found to fall into a so-called 'low-proliferative group', all GIII meningiomas fall into a 'high-proliferative group' and GII meningiomas fall into one of these groups (56). Twenty-eight genes were differentially expressed by SAM between GI and GII meningiomas and 1212 genes were differentially expressed between GI and GIII meningiomas ($p<0.05$ and fold >2). Although our series do not include GIII meningiomas, our series showed 38 differentially expressed genes at $p<0.001$ and established a well defined molecular subgroup classification for GI and GII meningiomas. The results reported by Carvalho *et al* support our work and confirm the presence of molecular subgroups in meningiomas (56).

As with meningiomas, schwannomas showed two distinct groups in the cluster analysis (I2 and II4). The comparison between these two subgroups of samples revealed 20 differentially expressed genes, half of which were found differentially expressed between the two subgroups of meningiomas and showing similar variations (Table II). A previous work identified 14 genes including MMPs, growth factors and receptors and tyrosine kinases with increased or decreased mRNA levels in schwannoma compared with normal Schwann cells (25,34). The lack of appropriate Schwann cell controls in our study does not allow the establishment of differences in gene expression between normal and tumoral cells. However, the comparison between meningiomas and schwannomas permits the establishment of differential expression patterns between two types of tumors that share main characteristics: the alterations in the *NF2* gene and the coexistence of neurofibromatosis type II. Our findings showed 16 genes with lower expression and 3 genes with higher expression in meningiomas compared to schwannomas. *CDKN1A*, *FOS*, *IL8*, *PLAUR* and *SERPINE1*

genes expressed 2- to 4-fold less in meningiomas than in schwannomas. However, these genes expressed 6- to 25-fold less in meningioma than in normal controls. *EGFR*, *FGFR2* and *TIMP1* showed higher expression (2- to 4-fold) in meningiomas than schwannomas. These genes showed no differences between controls and meningiomas, but they were expressed more in subgroup II5 of meningiomas. A low immunoreactivity of *EGFR* (7p12) has been associated with poor prognosis in atypical meningiomas (57), and alteration of *EGFR* in NF2-associated tumors has been found in schwannoma rat cell lines (58). Accordingly, assessment of the *EGFR* expression levels in these tumors may be useful for a therapeutic approach. The *FGFR2* gene (10q26) participates in processes such as cell proliferation, migration and differentiation, so this tyrosin kinase receptor is also a potential therapeutic target in many tumor types, especially those of neuronal, epithelial and skeletal origin (59).

We also analyzed expression patterns in search of possible differences between tumors with or without 22q or *NF2* abnormalities. The class comparison analysis showed a group of 15 genes with significantly lower expression in meningioma and schwannoma samples with 22q LOH and a group of 11 genes with significantly lower expression in samples with *NF2* gene alteration identified by MLPA (Table II). In the latter group, only the *ITGA3* gene is not present in both groups (MLPA and LOH). The decreased expression of these genes could be a direct or indirect result of the absence or decreased expression of the *NF2* gene, especially for *CDKN1A*, *FLT1* and *PLAUR*, since they were also found differentially expressed when the comparison was conducted by the presence or absence of any alteration in the *NF2* gene (chromosomal loss, deletion or mutation).

From the 5 genes present in the 22q LOH group but not in the MLPA group, *CHEK2* also located at 22q, displayed half expression; this can be explained by the loss of one of its alleles (LOH). The decreased expression of the other 4 genes (*ERBB2*, *ITGA2*, *ITGB3* and *MMP9*) may be due to the loss of one or more regulatory genes located at 22q. As changes in the *NF2* gene alone do not explain all cases of schwannomas and meningiomas, suggesting the involvement of one or more TSG located on chromosome 22, the study of cellular pathways of *ERBB2*, *ITGA2*, *ITGB3* and *MMP9* could lead to the identification of candidate TSGs on 22q.

With the class comparison results between meningioma and normal cerebral meninges, we decided to search for a predictor to enable us to further classify tumor samples as belonging to one of the two classes, because the classification of tumors based on patterns of gene expression could also be used as a prognostic marker with regard to evolution, disease-free times and survival. Using the expression of 96 cancer-related genes and with an optimized error at $p<0.005$, we propose a set of 7 genes (*FOS*, *ICAM1*, *PLAUR*, *IL8*, *APR*, *FLT1* and *SERPINE1*) that compose the classifier. Once validated, this classifier, which is based on a small number of genes, might permit an accurate diagnosis in cases where the histopathology can not and could have implications for evolution and post-surgical monitoring of patients, especially for those cases with widespread invasion in which is important to check the extent of the resection.

In conclusion, we have established the expression patterns of 96 specific cancer-related genes using low density cDNA microarrays in a series of meningioma and schwannoma tumor samples. In addition to identifying biologically relevant genes in the genesis and progression of theses two tumor types characterized by the *NF2* gene alterations and that coexist in the *N*F2 syndrome, expression analyses provided a molecular tumor sub-classification as a basis for future studies to identify potential phenotypes and to improve current methods of classification, which may include invasive capacity, metastatic potential, tumor recurrence or resistance and sensitivity to specific therapies.

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