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Original Paper

Lack of genetic and epigenetic changes in meningiomas without NF2 loss

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Abstract

Approximately 60% of sporadic meningiomas are caused by inactivation of the *NF2* tumour suppressor gene. The causative gene for the remaining meningiomas is unknown. Previous studies have shown that these tumours have no recurrent karyotypic abnormalities. They differ from their *NF2*-related counterparts in that they are more often of the meningothelial subtype and are located preferentially in the anterior skull base. To gain more insight into the aetiology of these tumours, we studied genetic and epigenetic alterations in 25 meningiomas without *NF2* involvement. We first established a genome-wide allelotype using 3 microsatellite markers per chromosome arm. Loss of heterozygosity (LOH) was detected at a low frequency and no indication for the location of putative tumour suppressor genes could be established. We next screened the subtelomeric regions by using 2–3 polymorphic markers close to each telomere. Again no evidence for LOH of a particular chromosome arm was obtained, and no LOH was found in the genomic regions containing the *NF2*-related ERM family members ezrin and radixin, DAL-1, protein 4.1R, and TSLC1. Mutations in the X-chromosome based family member, moesin, were analysed by SSCP and were not detected. Microsatellite instability was studied using 6 commonly used markers but none of these was altered in any meningioma. Methylation was detected in 5 of 16 genes (*NF2*, p14^{ARF}, *CDH1, BRCA1, RB1*) previously shown to be silenced in a variety of tumour types. However, methylation percentages for these genes were generally higher in a group of *NF2*-related meningiomas, with the exception of the *BRCA1* gene. The *NF2* gene was methylated in only 1 of 21 tumours. In conclusion, meningiomas with an intact *NF2* gene have a normal karyotype and no obvious genetic or epigenetic aberrations, suggesting that the gene(s) involved in the pathogenesis of these tumours are altered by smaller events than can be detected with the techniques used in our study.

Keywords: **meningioma; allelotyping; methylation**

Introduction

Meningiomas originate from the meninges covering the central nervous system. Meningiomas comprise about 20% of all primary intracranial tumours. Although generally a benign tumour, meningioma often recurs and is malignant in 5–10% of all cases. Meningiomas are amongst the best karyotyped human tumours. Consistent cytogenetic changes have been described, with loss of chromosome 22 being the most prominent [1,2]. The *NF2* gene is located on this chromosome. *NF2* is a tumour suppressor gene involved in the hereditary condition neurofibromatosis (NF) type 2, which is also characterized by the growth of meningiomas. In approximately 60% of the sporadic meningiomas, the *NF2* gene is inactivated by a small mutation, and this is most frequently accompanied by loss of the second allele, usually reflected by loss of the entire chromosome 22 [3,4]. This correlation was previously shown to be highly significant, indicating that loss of parts of chromosome 22 may be interpreted as inactivation of the *NF2* gene [5]. Cytogenetic and molecular investigations have failed to detect either aberrations of chromosome 22 or mutations in the *NF2* gene in approximately 40% of sporadic meningiomas, thus suggesting that an alternative pathogenetic mechanism is responsible for the development of these tumours.

We previously showed that these *NF2*-intact meningiomas have, besides retention of chromosome 22, some other common clinical and histopathological characteristics: they appear to have a normal karyotype, they are more often of the syncytial (meningothelial) type and they occur more often at the anterior skull base [6,7]. The histological distinction was also made by Wellenreuther *et al*, and Evans

et al, who showed the absence of *NF2* mutations in meningothelial meningiomas [5,8]. To date, not much is known about the genetic alterations in these tumours. Using CGH analysis, the group of Dumanski suggested that candidate tumour suppressor regions might be located on chromosome arms 1p and 3p. However, the frequency of these alterations was low [9]. The elucidation of the molecular pathway(s) that may operate in the tumourigenesis of these tumours is pivotal in order to be able to develop strategies for adjuvant treatment, for instance the use of small molecule inhibitors, when complete resection of the tumour cannot be achieved due to a difficult anatomical location. Therefore, we performed an intensive genetic screen to try and find evidence for the position of candidate tumour suppressor genes for these tumours, including allelotyping, a search for possible mitotic recombinations, methylation of promoter-associated CpG islands, analysis of *NF2*-related genes of the ERM family and putative microsatellite instability.

Materials & Methods

Patients and samples

A set of 25 meningiomas was selected based on the retention of chromosome 22 as determined with a set of 6 microsatellite markers as described previously [6]. All markers were located near or in the *NF2* gene on chromosome 22 (see below for names of the markers used). Patient and tumour data are given in Table 1. The mean age of the patients was 60 years. Eight patients were male, the others female. Twenty cases were graded I according to the World Health Organization grading criteria [10]. Retention of the *NF2* region on chromosome 22 was confirmed for all tumours (not shown). CT scans and/or MRIs were retrieved for review of the tumour location from the files of the Department of Neuroradiology of the Erasmus MC. For methylation profiling, a control group of 13 meningiomas that showed loss of heterozygosity in the *NF2* region was used.

Table 1. Patient data, tumour location, and grade

ID	Age (years)	m/f	Diagnosis	Location	Grade
MN385	39	M	Syncytial	R and L olfactory	I
MN388	74	M	Syncytial anaplastic	Sellar	III
MN392	66	M	Transitional	L sphenoid	II
MN395	54	F	Transitional	Sellar	I
MN399	69	M	Transitional	L frontal-parietal	I
MN402	69	F	Transitional	olfactory	I
MN408	53	F	Transitional	L sphenoid	I
MN412	75	M	Transitional	L parietal	I
MN418	69	F	Microcystic	R frontal	II
MN419	73	M	Syncytial	R orbita (intra-ossal)	I
MN420	44	F	Syncytial	R sphenoid	I
MN430	63	F	Secretory	L frontal	I
MN431	69	F	Syncytial	L sphenoid	I
MN432	62	M	Transitional	L sphenoid	I
MN441	56	F	Transitional	R frontal-parietal	I
MN442	59	F	Transitional	R olfactory	I
MN443	54	M	Syncytial	L temporal	II
MN445	55	F	Syncytial	R and L olfactory	I
MN446	59	F	Transitional	Olfactory	I
MN448	55	F	Anaplastic	L parietal-temporal	III
MN452	57	F	Syncytial	Sellar	I
MN498	33	F	Syncytial	R sellar	I
MN505	55	F	Syncytial	L temporal	I
MN519	65	F	Syncytial	L sphenoid	I
MN540	61	F	Syncytial	R frontal	I

M/F = male/female; R = right; L = left.

Sample handling and characterization

Use of tissues for scientific purposes was approved by the institutional review board. The samples were used according to the Code for proper secondary use of human tissue in The Netherlands, as developed by the Dutch Federation of Medical Scientific Societies (FMWV) (version 2002).

DNA isolation

DNA was isolated from fresh or fresh frozen tumour material by proteinase K digestion (20 mg/ml) in tissue lysis buffer (10 mM Tris.HCl, 10 mM EDTA, 150 mM NaCl, 0.4% SDS). Control DNA from the same patient was isolated from peripheral blood lymphocytes by the salting out procedure described by Miller *et al* [11].

Allelotyping

196 markers were used for allelotyping. For each chromosome arm, 3 markers were selected such that they were spread out over the entire arm. In addition, we used 2–3 polymorphic markers for each telomere. Markers were analysed with a radioisotope or with a fluorescent label (HEX or FAM): template DNA was amplified in a total volume of 15 µl reaction mixture containing 2.5 µM dNTPs, 10 pmol of the appropriate primer combination, (2.5 pmol when a fluorochrome was attached) and 0.25 units of Taq polymerase (Promega, Madison, WI). Non-endlabeled primer products were labeled with α-32P-dATP during cycling. Thermal cycling consisted of initial denaturation at 95 °C for 5 min, followed by 30 cycles of each 55 °C for 45 sec, 72 °C for 45 sec, and 94 °C for 45 sec. The final elongation step was 72 °C for 10 min. PCR-products were separated on 6% denaturing polyacrylamide slab gels or on a ABI PRISM 3100 (Applied biosystems, Palo Alto, CA). Detection was performed by autoradiography followed by quantification using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) or Genotyper software (licensed by Applied Biosystems, Palo Alto, CA). An allele was considered to be lost when the ratio of the tumour alleles was lower than 0.6 or higher than 1.67 compared to the ratio in the matching control DNA of the same patient [12,13].

The following markers were used: D1S2660, D1S468, D1S1609, D1S2836, D1S1612, D1S552, D1S1665, D1S534, D1S518, D1S549, D2S405, D2S0323, D2S1780, D2S140, D2S125, D2S423, D2S498, D2S435, D2S1788, D2S1326, D2S1397, D3S4545, D3S1270, D3S2387, D3S2748, D3S1311, D3S2432, D3S1766, D3S2460, D3S1607, D3S2427, FGA, D4S230, D4S412, D4S243, D4S1627, D4S1614, D4S3034, D4S2936, D4S2366, D4S408, D4S1652, D4S2930, D4S2427, D5S644, D5S498, D5S407, D5S820, ACTBP2, D5S2505, D5S417, D5S1981, D5S1492, D5S2073, D5S408, D6S281, D6S426, D6S477, D6S1617, D6S263, D6S446, D6S1027, D6S503, D6S429, D6S1003, D7S531, D7S2201, D7S517, D7S559, D7S2423, D7S493, D7S555, D7S803, D7S636, D8S307, D8S569, D8S373, D8S264, D8S277, D8S373, D8S1836, D8S298, LPL, D8S273, D8S267, D9S153, D9S252, D9S171, D9S1858, D9S288, D9S1838, D9S158, D9S180, D9S242, D10S169, D10S217, D10S1435, D10S249, D10S212, D10S1651, D10S217, AFMa086wg9, D10S451, D10S192, D10S677, D11S1776, D11S4200, D11S971, D11S488, D11S2362, D11S4046, D11S912, D11S968, THO, D12S341, D12S327, D12S396, D12S1064, D12S372, D12S352, D12S392, D12S1723, D13S802, D13S260, D13S285, D13S293, D14S267, D14S292, D14S118, D14S288, D14S306, D14S1426, D15S205, D15S974, D15S120, D15S642, D15S212, D16S476, D16S310, D16S521, D16S2622, D16S3027, D16S520, D16S539, D17S695, D17S786, D17S849, D17S1308, D17S928, D17S784, D17S960, D17S654, D18S476, D18S40, D18S70, D18S59, D18S476, D18S844, D18S51, D19S394, D19S247, D19S1034, D19S886, D19S254, D19S589, D19S210, D19S434, D19S200, D20S96, D20S473, D20S906, D20S173, D20S171, D20S94, D20S454, D21S1437, D21S1446, D21S266, D21S1912, D21S1435, D21S2055, UT762, D22S686, D22S685, NF2CAV, NF2CAIV, NF2CT3.1, D22S929, D22S684, D22S683, D22S444, D22S445, D22S1169, DXS1003, DXS991, DXS15.

Microsatellite instability

The presence or absence of microsatellite instability was tested according to the international criteria for the determination of microsatellite instability with the markers BAT25, BAT40, D2S123, D5S346, MSH6 and BAT26, as described in Boland *et al*, [14]. PCR, gel electrophoresis and detection were performed as described for allelotyping.

Mutation analysis

Mutations in the coding region of the moesin gene were determined by single strand conformation polymorphism (SSCP) analysis with the primers listed in Table 2. During PCR, alpha ^{32}P-dATP was incorporated and the radioactive products were separated on 8% acryl : bisacryl (49 : 1) gels with 10% glycerol in 1× TBE buffer (0.89M Tris, 0.89M Boric acid, 0.02M EDTA, pH 8.0). The gels were run at room temperature overnight at 7 Watts. Detection of bands was by autoradiography.

Table 2. Primer sequences used for SSCP analysis of the coding sequence of moesin

Exon	Forward reverse	Size	Moesin primers
1	F		CAA AGG GCT ATG AGG CTC AG
1	R	303	GGA AGC CGG GCC ACA TAA AG
2	F		GAG AAG GAG AGG AGG CTA AG
2	R	147	GTA ACC TGC TTC CTT TGA TC
3	F		CCA GGA ATG TTC TGG AAA AG
3	R	189	CTC ACA CAG AGG AAG TAT TC
4	F		CCT TAT GGC CAA GGC AAA GG
4	R	319	GTC CAT AAC CCT TAC TCT TC
5	F		CAT TCA CCC TGT AAG GGA AG
5	R	156	CTC ATC ACC CAT TGT CTT TC
6	F		GCC CTA GAT GTT AAC GTG AC
6	R	238	CAC AAG CCC CAC TTT GTG AC
7	F		CTT GGT CAG AGG GAG GAA TC
7	R	151	CTT CTT GTC TTG CCC TTG TC
8	F		CCT TTG AGA AGC TTC CTT GC
8	R	225	CTG ATT GCT GAT TTC CCA CC
9	F		CCA ATC TCC AAG GTG ACT CC
9	R	214	CAG GCT TTT GTG GAG CGT TG
10	F		CAG TTC CCA TAA TCC CAG CC
10	R	234	GTG CTC TTC ACT GCC TTC TC
11	F		GCT TTG CTT TAG GCC CTG TG
11	R	142	CAC AGG CTT CCA ATT TAT CC
12	F		GCA TAT AGT TTC CTT GCC CC
12	R	284	CTC TCC TTC TGT CAC TGG AC
13	F		GAG TTA GGT GTA GGA GTG TG
13	R	257	CCT CTG TGT TCC CAT ACA TC

Methylation specific PCR

Aberrant DNA methylation in the CpG Island of the genes *NF2, RARβ*, *MGMT, DAPK, CDH1, p14ARF, GSTP1, p15, VHL, p16INK4a, THBS1, CACNA1G, BRCA1, APC, p73* and *RB1* was determined by chemical modification of genomic DNA with sodium bisulfite and followed by methylation-specific PCR. The bisulfite modification procedure was carried out using the Intergen CpGenome DNA modification kit (Intergen, Purchase, NY). In brief, 1 μg of genomic DNA was denatured by sodium hydroxide and then chemically modified by sodium bisulfite for 20 hours. The unmethylated cytosine was converted to uracil, whereas methylated cytosine remains unchanged. The modified DNA was recovered by ethanol precipitation and resuspended in PCR-grade water. Primer sequences were based on previous reports and are listed in Table 3. One μl of bisulfite-modified DNA was amplified in a total volume of 15 μl containing 1× PCR buffer (16.6 mM ammonium sulphate, 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol), 0.25 mM deoxynucleotide triphosphate, 10 pmol of each primer, and 1 unit of AmpliTaq Gold polymerase (Roche) at 95 °C for 10 min. It was then followed by 35 cycles of amplification at 95 °C for 45 s, the specific annealing temperature for 45 s, and 72 °C for 45 s. In vitro methylated DNA (Intergen) was used as a positive control for methylation, and water was used as a negative control. Ten

Table 3. Primer sequences for methylation-specific PCR

Gene	Forward primer (5′3′)	Reverse primer (5′3′)	Temperature (°C)	bp (°C)	Ref
NF2	M: GTGGAGTTATTTTAAAGGAGGC	M: TAACACAACCAAACCAAAAACTAA	62	107	38
	U: GTGGAGTTATTTTAAAGGAGGT	U: TAACACAACCAAACCAAAAACTAA	62	107	
NF2	M: GAGTTATTTTAAAGGAGGCGGGAC	M: TAACACAACCAAACCAAAAACTAA	60	104	38
	U: GAGTTATTTTAAAGGAGGTGGGAT	U: TAACACAACCAAACCAAAAACTAA	60	104	
NF2	M: TAAAGGAGGCGGGACGGAGC	M: TAACACAACCAAACCAAAAACTAA	60	95	38
	U: TAAAGGAGGTGGGATGGAGT	U: TAACACAACCAAACCAAAAACTAA	60	95	
RARß	M: TCGAGAACGCGAGCGATTCG	M: GACCAATCCAACCGAAACGA	59	146	39
	U: TTGAGAATGTGAGTGATTTGA	U: AACCAATCCAACCAAAACAA	59	146	
MGMT	M: TTTCGACGTTCGTAGGTTTTCGC	M: GCACTCTTCCGAAAACGAAACG	66	81	39
	U: TTTGTGTTTTGATGTTTGTAGGTTTTTGT	U: ACTCCACACTCTTCCAAAAACAAAACA	66	93	
DAPK	M: GGATAGTCGGATCGAGTTAACGTC	M: CCCTCCCAAACGCCGA	64	98	39
	U: GGAGGATAGTTGGATTGAGTTAATGTT	U: CAAATCCCTCCCAAACACCAA	64	106	
CDH1	M: TTAGGTTAGAGGGTTATCGCGT	M: TAACTAAAAATTCACCTACCGAC	57	115	39
	U: TAATTTTAGGTTAGAGGGTTATTGT	U: CACAACCAATCAACAACACA	57	97	
p14^{ARF}	M: GTGTTAAAGGGCGGCGTAGC	M: AAAACCCTCACTCGCGACGA	64	122	39
	U: TTTTTGGTGTTAAAGGGTGGTGTAGT	U: CACAAAAACCCTCACTCACAACAA	64	132	
GSTP1	M: TTCGGGGTGTAGCGGTCGTC	M: GCCCCAATACTAAATCACGACG	55	91	39
	U: GATGTTTGGGGTGTAGTGGTTGTT	U: CCACCCCAATACTAAATCACAACA	55	97	
p15	M: GCGTTCGTATTTTGCGGTT	M: CGTACAATAACCGAACGACCGA	60	148	40
	U: TGTGATGTGTTTGTATTTTGTGGTT	U: CCATACAATAACCAAACAACCAA	60	154	
VHL	M: TGGAGGATTTTTTTGCGTACGC	M: GAACCGAACGCCGCGAA	60	158	41
	U: GTTGGAGGATTTTTTTGTGTATGT	U: CCCAAACCAAACACCACAAA	60	165	
p16^{INK4a}	M: TTATTAGAGGGTGGGGCGGATCGC	M: GACCCCGAACCGCGACCGTAA	65	150	41
	U: TTATTAGAGGGTGGGGTGGATTGT	U: CAACCCCAAACCACAACCATAA	65	151	
THBS1	M: TGCGAGCGTTTTTTTAAATGC	M: TAAACTCGCAAACCAACTCG	56	74	
	U: GTTTGGTTGTTGTTTATTGGTTG	U: CCTAAACTCACAAACCAACTCA	54	115	
CACNA1G	M: GTTTTTTCGGGGCGGTTTC	M: TTCCGACTTCTTCGCTTCG	62		
	U: GTTTTTTTTTGGATTTTTGTTTTTTG	U: TTTATTCCAACTTCTTCACTTCA	60		
BRCA1	M: GGTTAATTTAGAGTTTCGAGAGACG	M: TCAACGAACTCACGCCGCGCAATCG	65	182	42
	U: GGTTAATTTAGAGTTTTGAGAGATG	U: TCAACAAACTCACACCACACAATCA	61	182	
APC	M: TATTGCGGAGTGCGGGTC	M: TCGACGAACTCCCGACGA	64	100	43
	U: GTGTTTTATTGTGGAGTGTGGGTT	U: CCAATCAACAAACTCCCAACAA	62	110	
p73	M: GGACGTAGCGAAATCGGGGTTC	M: CGTCGCACCCCGAACATCG	62	67	44
	U: AGGGGATGTAGTGAAATTGGGGTTT	U: CCATCACAACCCCAAACATCA	62	71	
RB1	M: GGGAGTTTCGCGGACGTGAC	M: ACGTCGAAACACGCCCCG	55	172	45
	U: GGGAGTTTTGTGGATGTGAT	U: ACATCAAAACACACCCCA	55	172	

µl of PCR product was loaded on 2% agarose gels stained with ethidium bromide and visualized under UV illumination. Samples were scored as methylated when there was a clearly visible band on the gel with the corresponding primers. Electrophoresis results were interpreted by two independent investigators. All experiments were repeated to ensure the reproducibility of the results.

Results

Loss of heterozygosity is rare in non-NF2 meningiomas

To search for a common region of loss as an indication for the location of a causative tumour suppressor gene, a genome-wide allelotyping study with markers covering all chromosome arms was performed on 25 meningiomas without evidence for loss of the *NF2* region. Very few LOH events were detected with this approach. To make sure that no LOH events were missed because an important gene is located close to a telomere, we also screened the tumour DNAs for LOH using polymorphic markers located at sub-telomeric regions. Such an approach should reveal all LOH events originating from the two mechanisms most frequently observed in model systems (mitotic recombination and loss followed by reduplication). Again only very few LOH events were found. In general, 30 LOH events appeared to have arisen through terminal deletions/recombinations (68%), while 14 events appeared to be interstitial, with retention of chromosomal parts on both sides of the LOH region. In four cases, the LOH was detected only with the sub-telomeric markers. In one case, testing two different sub-telomeric markers for one chromosome arm gave conflicting results: loss for the first and retention for the second marker. The combined allelotyping results are presented in Figure 1. In Figure 1a, the chromosome arms showing loss are indicated per individual tumour, and in Figure 1b, the losses are quantified per chromosome arm. The percentage loss found varied between zero for chromosome arms 2q, 5p, 6, 8q, 9q, 10p, 11q, 16q, 17q, 20p and chromosomes 13, 18, 19 and 21, and 19% for chromosome arms 2p and 4q. In Figure 1c, the percentage LOH for all markers per tumour is presented. In Figure 1d, a schematic representation is given of the regions of LOH on affected chromosomes.

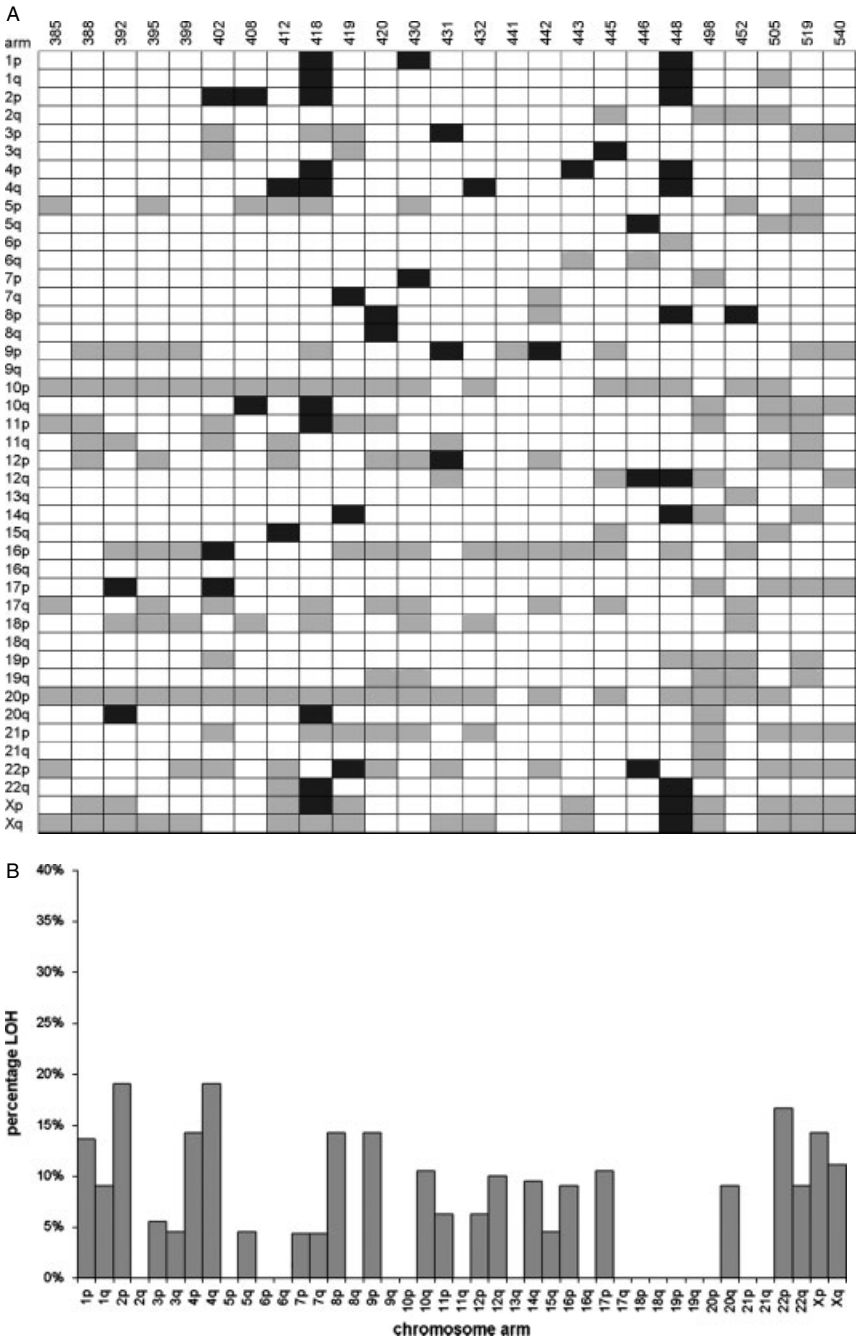

Figure 1. Results of allelotype analysis of non-NF2 meningiomas with 196 microsatellite markers. (a) Overview of the losses per tumour. White, retention, black, LOH, grey, not informative. (b) Summary of the LOH analyses for all tumours. The highest percentage of loss is 19%. Several chromosomes have no LOH for the tested markers in any of the tumours. (c) Summary of the LOH analysis per tumour. Note that some tumours have no LOH for any of the markers tested. Tumours 418 and 448 have more alterations than the other tumours (on 10 and 11 chromosomes, respectively). (d) Schematic representation of the areas of LOH on the affected chromosomes. White, retention, black, LOH, grey, not informative

Screening of the ERM family and other meningioma-related genes suggests that these genes are not involved

The NF2 protein is related to the ERM (ezrin, radixin, moesin) family of membrane-cytoskeleton linking proteins [15]. The ezrin and radixin genes are located on chromosome arms 6q25-26 and 11q23, respectively. The *DAL-1* gene (EPB41) is on chromosome arm 18p, the *protein 4.1R* gene on chromosome arm 1p33-32 and the protein 4.1R interacting protein *TSCL1* on chromosome arm 11q23.2. No evidence for LOH of these genes was obtained. None of the tumours showed loss on chromosome arms 6q, 11q or 18p. Three tumours (418, 430 and 448) showed loss on chromosome 1p, which could encompass the EPB41 gene. The moesin gene is located on the X-chromosome. Because both males and females have only one active X chromosome, inactivation of a putative tumour suppressor gene on this chromosome might occur preferentially by a small genetic event that is difficult to

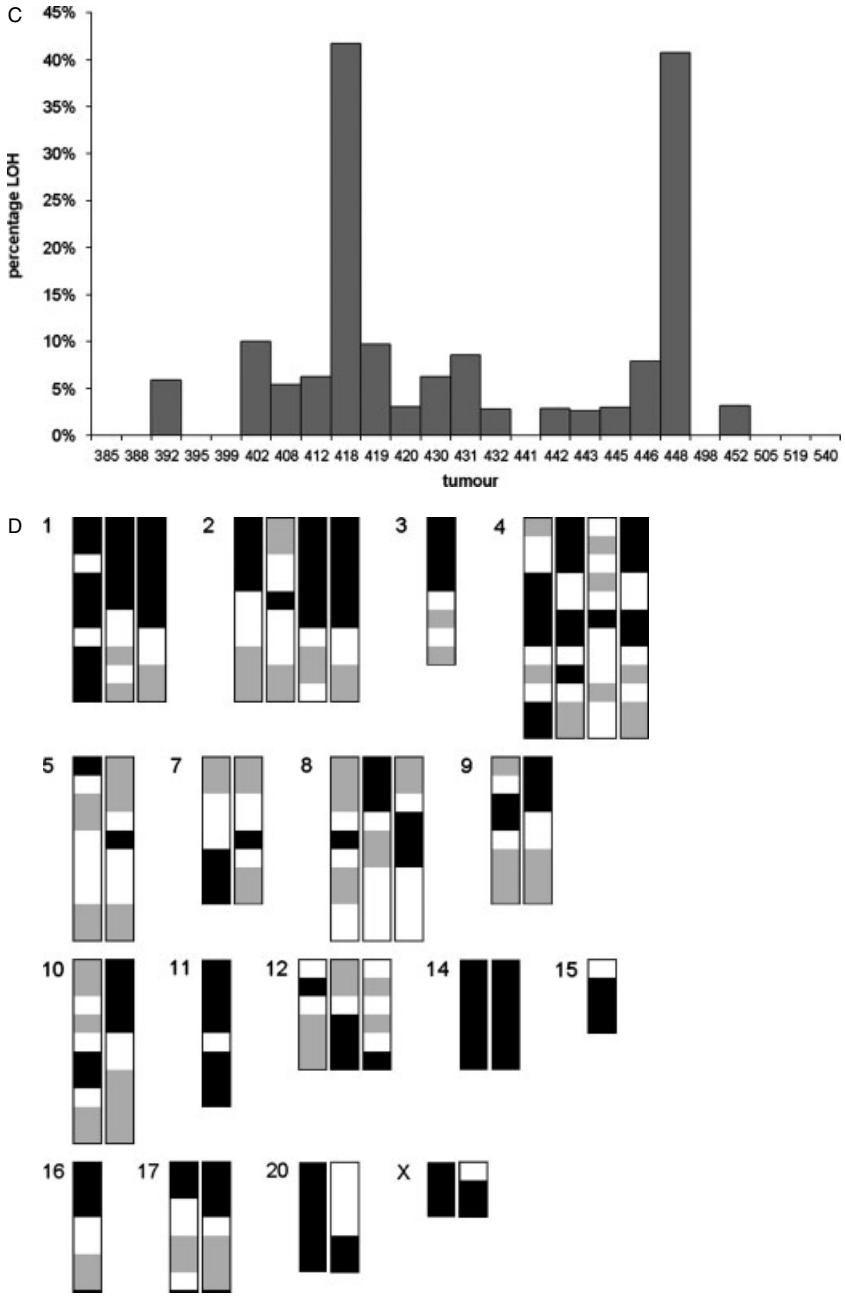

Figure 1. Continued

detect by allelotyping. We therefore tested for possible mutations in the moesin gene by screening all thirteen exons for sequence variations in 22 NF2 intact meningiomas by SSCP. No tumour specific mutations were found. No polymorphisms were detected. We therefore conclude that it is unlikely that the ERM proteins and other meningioma related genes are involved in the pathogenesis of NF2 intact meningiomas.

NF2 intact meningiomas have a microsatellite stable profile

About 15% of all colorectal cancers have a normal karyotype and LOH is also an uncommon finding in these tumours. However, these tumour genomes are highly unstable because of mutations in genes involved in mismatch DNA repair. Mutations in mismatch repair genes lead to a phenomenon called microsatellite instability (MSI), which results in alterations in the size of multiple microsatellite repeats in the tumour genome. These DNA alterations are thought to enhance the mutation frequency of relevant cancer genes [16]. The BAT25, BAT40, MSH6, D2S123, D5S346 and BAT26 microsatellites are commonly used to establish MSI in tumour samples. We used these markers to analyse our set of 25 meningiomas for signs of MSI. The PCR products generated by these markers gave similar allele sizes for tumour and blood DNA of the same patient (results not shown). It therefore appears that meningioma tumours without loss of the *NF2* tumour suppressor gene do not show any microsatellite instability.

Frequency of methylation in NF2 intact meningiomas

Besides genetic alterations such as deletions, mitotic recombinations and mutations, tumour suppressor genes can also be inactivated by epigenetic events [17]. We therefore examined, in addition to the promotor region of the *NF2* gene, 15 commonly used genes for signs of CpG island hypermethylation in 21 specimens of NF2-intact meningiomas and 13 NF2-related tumours. DNA isolated from tumours and peripheral blood from the same patients was subjected to methylation-specific PCR. In general, gene fragments tested were unmethylated, in blood as well as in the tumours. Methylation of tumour suppressor genes was found in 13 of 21 tumours (62%). No methylation was found for the genes p73, $p16^{INK4A}$, *p15, MGM-T, DAPK*, *VHL, THBS1, CACNA1G, APC, GSTP1* and *RAR*b. The methylation frequencies for the remaining genes were as follows: *NF2*, 5%, $p14^{ARF}$, 19%; *CDH1*, 33%; *BRCA1*, 29%; *RB1*, 29% (Figure 2). Three patients had methylation of three genes, five had methylation of two genes, and four patients had one methylated gene.

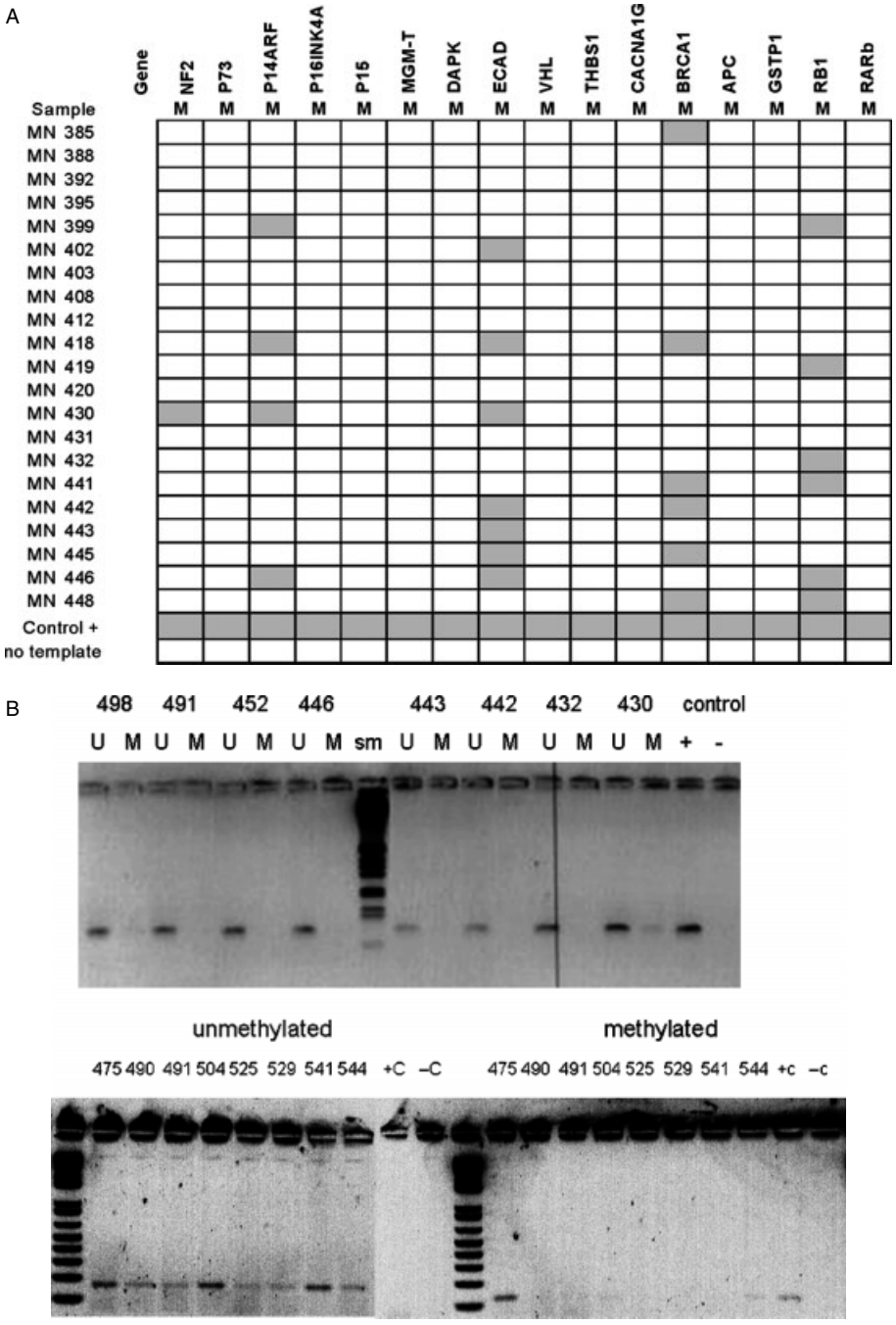

Figure 2. Methylation profiling of 16 differentially expressed genes in non-NF2 meningiomas. A; Summary of methylation in tumours. Grey boxes represent samples that are methylated. B; Representative examples of methylation profiling of the *NF2* gene (upper panel) and the *CDH1* gene (lower panel). U; unmethylated, M; methylated, sm; size marker, +; positive control, −; negative control

In the matching blood samples, methylation was also present at *CDH1* (15%), *GSTP1* (5%) and *RB1* (42%). We included a set of 13 NF2-related meningiomas. Methylation of the $p14^{ARF}$ (60%), *CDH1* (100%), and *RB1* (46%) genes was also seen in the NF2-related tumours. Furthermore, methylation of the $p16^{INK4A}$ (60%), *DAPK* (15%) and *GSTP1* (20%) genes was exclusively seen in NF2-meningiomas. All other genes were negative for methylation. In general, methylation was more frequent in *NF2*-related tumours. Only methylation of the *BRCA1* gene differed significantly between the two meningioma subtypes and control blood.

Discussion

Meningiomas can be divided into at least two subgroups based on their genetic alterations. In about 60% of the tumours, the *NF2* tumour suppressor gene plays a causative role. The current study was carried out in order to provide information about the location of possible candidate tumour suppressor genes that might be involved in the *NF2* intact meningiomas. Previously, we showed that these tumours mostly have a normal karyotype and that they also differ from the NF2-related tumours based on their intracranial location and their histology [6]. We here show that these *NF2* intact meningiomas show few signs of genetic or epigenetic aberrations.

We used genome-wide allelotyping to search for common regions of loss. We also tested sub-telomeric markers to visualize telomeric LOH events resulting from reduplication or mitotic recombination. This approach was used before to identify the *RUNX1/AML1* gene as a tumour suppressor gene in diploid cases of acute myeloid leukemia [18]. A low frequency of loss was revealed with both allelotyping approaches, with LOH on a specific chromosome arm in 19% or less of tumours. In 68% of LOH events, the loss pattern included the telomere. Therefore, LOH analysis at telomeric regions appears to be an effective approach to identify chromosomes harboring critical tumour suppressor genes. LOH has been used extensively as a tool to gather information about the location of tumour suppressor genes involved in specific tumour types. However, it is pivotal to realize that the frequency of LOH as the method of inactivation for the second copy of a tumour suppressor gene can vary between 70–95% for *RB1* [19] and *NF2* [20], and 12.5–25% for *APC* [21]. For most genes, this frequency is not known. In those cases where the preferred method of inactivation is not LOH, the location of the gene will most likely not be detected with this method.

The highest percentage of loss found in our series of tumours was 19 for chromosome arms 2p and 4q. For chromosome arm 2p, however, 3 of the 4 tumours showing LOH did not share loss of the same marker, which makes it unlikely that the same gene was targeted. Although LOH of the chromosome 1p36 region is the second most common alteration in meningiomas, limited data exist on the role of this region in NF2 intact tumours. In fact, it appears more frequently altered in higher grade tumours [22], supporting a role in progression. In a previous study on *NF2* intact meningiomas, Dumanski found that 2 out of 25 tumours had concurrent loss of 1p and 3q [9]. In our series 1p was also targeted in 3 of 25 cases. Concurrent loss at chromosome arm 3q was not seen. The region at chromosome arm 1p overlaps the location of the protein 4.1R gene. In these three tumours, protein 4.1R could be the target of deletion. Loss of protein expression has been shown in 6 of 15 sporadic meningiomas [23].

Other frequently deleted genes in meningiomas are EPB41L3 (*DAL-1*) at chromosome 18p11.32 and *TSLC1* at chromosome 11q23.2. However, in our series of NF2-intact meningiomas, no LOH was seen in these regions, suggesting that these genes are not major targets in the pathogenesis of this group.

Two tumours are remarkable because of their relatively high frequency of LOH: cases 418 and 448 (Figure 1c). Both tumours were classified as belonging to the *NF2* intact group, however, they had LOH on chromosome 22, but this was outside the *NF2* gene region. We excluded the possibility that in these tumours the gene is homozygously deleted on the basis of the signal intensity of the labeled PCR products for these markers, which was not significantly lower than other, adjacent, markers (not shown). It could be that the LOH targets another gene adjacent to the NF2 gene, as has been suggested for schwannomas [24]. It is furthermore remarkable that both tumours show rather similar LOH patterns: loss on chromosome 1p and q, 2q, 4p and q, and chromosome Xp. Both are in fact more aggressive tumours (grade II and III) and this suggests that these LOHs represent secondary, progression steps. Progression has been studied in *NF2*-related meningiomas, where LOH on 1p, 10q, 14q, 18q, and 9p was observed to be associated with higher grade and atypia [25,26]. Progression in *NF2* intact meningiomas may therefore proceed through a different pattern. We conclude that karyotyping, comparative genomic hybridization and LOH analysis show that loss of genomic information is rare in *NF2* intact meningiomas.

Moesin is a member of the ERM-family of proteins from which merlin, the product of the *NF2* gene, derives its name. Other family members include ezrin, radixin and protein 4.1. Physical interaction between ezrin and merlin has been shown [27]. It is not unthinkable that the structural homology between these proteins also indicates a functional homology. Although ezrin has been shown to have a role in metastasis suppression [28,29], so far no evidence exists to implicate ezrin dysfunction in meningiomas. The role of radixin in cancer is unknown. The gene encoding moesin, MSN, is located on the X chromosome. Since men have only one X chromosome, and women have

only one active X chromosome, moesin could function as a tumour suppressor gene by inactivation without the need to lose the second copy of the gene. Therefore, we wanted to exclude this possibility by mutation analysis. No mutations or polymorphisms were found. Thus, our data seem to exclude a role of the ERM members in meningioma pathogenesis.

Microsatellite length instability (MSI), probably resulting from defective DNA mismatch repair mechanisms, has been described in a variety of cancers. Such genetic instability may play a significant role in tumour formation. In meningioma, several groups studied MSI with conflicting results [30,31]. Here we show that meningiomas not caused by inactivation of *NF2* have a microsatellite stable profile. Therefore, we conclude that this specific form of instability plays no role in these tumours.

Besides inactivation of the tumour suppressor gene by LOH, a specific gene can also be inactivated by hypermethylation. Determining the methylation status of this subset of meningiomas could therefore provide clues whether this mechanism of gene inactivation might play a role. Methylation as the method of inactivation of NF2 in our series of tumours seems unlikely: only 1 of the 21 studied tumours showed a weak signal with methylation specifc primers. This seems to differ from the findings of Lomas *et al*, [32], who found a methylation frequency of 17% for meningiomas without apparent NF2 involvement. They also found more methylation in transitional meningiomas, and more NF2 mutations in the meningothelial tumours. This is in contrast with our previous findings and those of Wellenreuther *et al* and Evans *et al*, showing that *NF2* intact meningiomas are more often of the syncytial (meningothelial) type [6,7] and are less likely to carry *NF2* mutations [5,8]. Methylation of *CDH1*, $p14^{ARF}$ and $p16^{INK4A}$, and, to a lesser degree, *RB1*, was much lower in *NF2* intact tumours when compared to *NF2*-related tumours (*CDH1*: 33 vs. 100%, p14ARF: 19 vs. 60%, $p16^{INK4A}$: 0 vs. 60%, *RB1*: 29 vs 46%). On the contrary, methylation of *BRCA1* was higher in *NF2* intact meningiomas (29 vs 0%). Kirsch *et al*, studied expression and LOH for the *BRCA1* and *BRCA2* genes in 60 meningiomas, but found no evidence of a role for these genes in meningioma [33]. However, these authors did not distinguish between *NF2*-related and *NF2* intact meningiomas. Thus, the possibility remains that methylation of *BRCA1* contributes towards development of a subgroup of *NF2* intact meningiomas. An interesting finding was the high degree of methylation of the cell cycle genes $p14^{ARF}$, $p16^{INK4A}$ and *RB1* in *NF2*-related meningiomas. Since the proteins encoded by these genes are involved in the regulation of cell cycle checkpoints, their absence might explain why in these tumours, many more karyotypic abnormalities are found. Loss of E-cadherin expression is in many tumour types linked to tumour cell invasiveness and metastatic potential [34,35]. In meningiomas, E-cadherin expression has been studied with immunohistochemistry only. Previously, Figarella-Branger *et al* suggested that E-cadherin expression is higher in meningothelial tumours [36], which correlates well with our findings that *NF2* intact meningiomas, which are more often of the meningothelial type, have a lower methylation level of the E-cadherin gene (33%) than *NF2*-related meningiomas (100%). The significance of this finding is, however, not clear at the moment since meningiomas do not become invasive and do not metastasize.

We conclude that *NF2* intact meningiomas differ genetically from *NF2*-related meningiomas. Karyotyping, comparative genomic hybridization and LOH analyses have shown that the genome of these tumours is largely intact, that they display no MSI and that methylation of tumour suppressor gene promoters is not extensive. Calpain-dependent degradation could be an option for these tumours, where activation of this pathway does not have to coincide with LOH [37]. But when we accept that all neoplasms develop through an accumulation of genetic and/or epigenetic changes in oncogenes and tumour suppressor genes, at least three possible explanations may explain our lack of success. First, it is possible that these tumours develop through inactivating mutations in different causative genes with each gene contributing to a modest percentage of tumours, a second possibility is that the inactivating mutations are too small to have been identified with the techniques used here or alternatively, that *NF2* intact meningiomas are caused by an oncogene, activated by a point mutation.

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