Alterations of the Tumor Suppressor Genes *CDKN2A* (*p16^{INK4a}*), *p14^{ARF}*, *CDKN2B* (*p15^{INK4b}*), and *CDKN2C* (*p18^{INK4c}*) in Atypical and Anaplastic Meningiomas

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We investigated 67 meningothelial tumors (20 benign meningiomas, 34 atypical meningiomas, and 13 anaplastic meningiomas) for losses of genetic information from chromosome arms 1p and 9p, as well as for deletion, mutation, and expression of the tumor suppressor genes *CDKN2A* (*p16^{INK4a}/MTS1*), *p14^{ARF}*, *CDKN2B* (*p15^{INK4b}/MTS2*) (all located at 9p21) and *CDKN2C* (1p32). Comparative genomic hybridization and microsatellite analysis showed losses on 1p in 11 anaplastic meningiomas (85%), 23 atypical meningiomas (68%), and 5 benign meningiomas (25%). One atypical meningioma with loss of heterozygosity on 1p carried a somatic *CDKN2C* mutation (c.202C>T: R68X). Losses on 9p were found in five anaplastic meningiomas (38%), six atypical meningiomas (18%), and one benign meningioma (5%). Six anaplastic meningiomas (46%) and one atypical meningioma (3%) showed homozygous deletions of the *CDKN2A*, *p14^{ARF}*, and *CDKN2B* genes. Two anaplastic meningiomas carried somatic point mutations in *CDKN2A* (c.262G>T: E88X and c.262G>A: E88K) and *p14^{ARF}* (c.305G>T: G102V and c.305G>A: G102E). One anaplastic meningioma, three atypical meningiomas, and one benign meningioma without a demonstrated homozygous deletion or mutation of *CDKN2A*, *p14^{ARF}*, or *CDKN2B* lacked detectable transcripts from at least one of these genes. Hypermethylation of *CDKN2A*, *p14^{ARF}*, and *CDKN2B* could be demonstrated in one of these cases. Taken together, our results indicate that *CDKN2C* is rarely altered in meningiomas. However, the majority of anaplastic meningiomas either show homozygous dele-

tions of *CDKN2A*, *p14^{ARF}*, and *CDKN2B*, mutations in *CDKN2A* and *p14^{ARF}*, or lack of expression of one or more of these genes. Thus, inactivation of the G₁/S-phase cell-cycle checkpoint is an important aberration in anaplastic meningiomas. (*Am J Pathol* 2001; 159:661–669)

Meningiomas are common primary tumors of the central nervous system. The annual incidence has been estimated to be ~6 per 100,000 population.¹ Most meningiomas are slowly growing benign tumors that histologically correspond to grade I of the World Health Organization's classification of tumors of the nervous system.¹ Atypical meningiomas (World Health Organization grade II) are a histologically defined subgroup of meningiomas that are associated with a significantly higher risk of local tumor recurrence than benign meningiomas, even after gross total resection.¹ Approximately 2 to 3% of all meningiomas show histological features of malignancy and are therefore classified as anaplastic meningiomas (World Health Organization grade III).¹ Anaplastic meningiomas are associated with a poor prognosis, as indicated by a median survival time of <2 years after diagnosis.²

The most common genetic alteration in meningiomas is loss of heterozygosity (LOH) at polymorphic markers on 22q, which has been detected in 40 to 70% of all meningiomas.^{3,4} LOH on 22q is typically associated with mutation, deletion, and/or loss of expression of the neurofibromatosis type 2 gene (*NF2*) at 22q12.2.^{5–7} *NF2* alterations have been detected in >50% of sporadic meningiomas of all malignancy grades, indicating that inactivation of the *NF2* gene product merlin/schwannomin represents an important early event in meningioma development.^{5–7}

We have previously shown by comparative genomic hybridization (CGH) and molecular genetic analyses that atypical and anaplastic meningiomas demonstrate an

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accumulation of multiple genomic abnormalities in addition to *NF2* alterations and LOH on 22q.⁸ The most common additional aberration, which was detected in more than two-thirds of atypical and anaplastic meningiomas, was the loss of genetic material from the short arm of chromosome 1. In addition, losses on 9p were frequently found in anaplastic meningiomas but only rarely in atypical and benign meningiomas. Preliminary analysis of the *CDKN2A* gene at 9p21 indicated that >20% of anaplastic meningiomas had homozygously lost this gene.⁸ *CDKN2A* encodes the p16^{INK4a} protein, which functions as regulator of G₁/S-phase transition by inhibiting the activity of cyclin-dependent kinases Cdk4 and Cdk6.⁹ The p14^{ARF} protein is transcribed from a unique exon 1 β and exons 2 and 3 of *CDKN2A* using an alternative reading frame.⁹ Transcription of the *p14ARF* gene is regulated independently from *CDKN2A* by a distinct promoter.^{9,10} The product of *p14ARF* regulates the activity of the p53 tumor suppressor protein by binding to Mdm2 and inhibiting Mdm2-mediated degradation of p53.¹⁰ *CDKN2A* belongs to a family of genes coding for structurally related inhibitors of cyclin-dependent kinases that are important in cell-cycle regulation at the G₁/S-phase transition.⁹ Other members of this family are *CDKN2B* at 9p21 (encoding p15^{INK4b}), *CDKN2C* at 1p32 (encoding p18^{INK4c}), and *CDKN2D* at 19q13 (encoding p19^{INK4d}).⁹ *CDKN2A* and *CDKN2B* are well-known tumor suppressor genes that are frequently aberrant in various types of human tumors. Mutation of the *CDKN2C* gene has also been reported in human tumors, albeit at much lower frequency.^{11–13}

We have now investigated 67 meningiomas of all World Health Organization grades for deletion, mutation, and expression of the *CDKN2A*, *p14ARF*, *CDKN2B*, and *CDKN2C* genes. All tumors were additionally investigated for amplification of the *MDM2* and *CDK4* genes and for mutation of the *TP53* gene. Our data indicate that *CDKN2C* is rarely altered in meningiomas and, therefore, does not represent the major target of the frequent losses on 1p in atypical and anaplastic meningiomas. However, the majority of anaplastic meningiomas shows aberrations of the *CDKN2A*, *p14ARF*, and *CDKN2B* genes.

Materials and Methods

Tumor Samples and DNA and RNA Extraction

We investigated meningiomas from 67 patients (26 male, 41 female; mean age at operation, 61 years; range, 6 to 90 years). All tumors were histologically reclassified according to the third edition of the World Health Organization classification of tumors of the nervous system.¹ Seven tumors from our previous series,⁸ which were originally classified as anaplastic meningioma, had to be reclassified as atypical meningioma. The main reasons for the reclassification were that the mitotic count was <20 mitoses per 10 high-power fields and that brain invasion is no longer regarded as a histological marker of malignancy. The present tumor series thus included 20 benign meningiomas (World Health Organization grade

I), 34 atypical meningiomas (World Health Organization grade II), and 13 anaplastic meningiomas (World Health Organization grade III) (Table 1). Forty-three tumors were primary tumors and 24 tumors were recurrences. One case was a spinal metastasis of an intracranial anaplastic meningioma. None of the primary and only one of the recurrent tumors had been treated by irradiation before operation. Parts of each tumor were snap-frozen immediately after operation and stored at -80°C . Extraction of high-molecular weight DNA and RNA from frozen tumor tissue was performed by ultracentrifugation as described elsewhere.¹⁴ Peripheral blood samples for the extraction of constitutive (leukocyte) DNA were available from 53 patients.

CGH and Microsatellite Analysis

Fifty-five of the meningiomas had been previously investigated for chromosomal imbalances by CGH.⁸ Five tumors were additionally analyzed by CGH using the same protocol. All 53 tumors for which corresponding leukocyte DNA was available were evaluated for LOH at the following microsatellite markers: *D1S224* (1p22), *D1S496* (1p34), *D1S468* (1p36), *D9S171* (9p21, proximal to *CDKN2A*, *p14ARF*, and *CDKN2B*), *D9S157* (9p21, distal to *CDKN2A*, *p14ARF*, and *CDKN2B*), and *D9S168* (9p22–23). Polymerase chain reaction (PCR) amplification and assessment for allelic loss was performed as described.¹⁵ LOH results of 40 tumors had been reported on previously.⁸

Single-Strand Conformation Polymorphism (SSCP)/Heteroduplex Analysis

The mutation analyses of *CDKN2A* and *p14ARF* (exon 1 α , exon 1 β , exons 2 and 3), *CDKN2B* (exons 1 and 2), and *CDKN2C* (exons 2 and 3) were performed by a combined SSCP/heteroduplex analysis.¹⁶ The oligonucleotide primer sequences used for amplification of the individual exons of these genes are summarized in Table 2. All tumors were additionally analyzed for mutations in exons 5 to 8 of the *TP53* tumor suppressor gene.¹⁶ PCR products spanning *CDKN2C* exon 3 and *CDKN2A* exon 2 were cut with restriction enzymes (*AluI* and *SmaI*, respectively) before SSCP/heteroduplex analysis. PCR products showing aberrant SSCP/heteroduplex band patterns were sequenced in both directions using the BigDye cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 377 semiautomated DNA sequencer (Applied Biosystems).

Determination of Gene Dosage

All tumors were screened by duplex-PCR analysis for homozygous deletions of *CDKN2A*, *p14ARF*, *CDKN2B*, and *CDKN2C*, as well as for amplification of *CDK4* and *MDM2* as described.^{13,17} The individual PCR products were separated on 3% agarose gels and ethidium bromide-stained fragments were recorded by the Gel-Doc 1000 system (Bio-Rad, Hercules, CA). Quantitative anal-

Table 1. Summary of Selected Patient Data, Tumor Diagnoses, and Molecular Genetic Findings

Case no.	Sex	Age (y)	Subtype	WHO grade	Losses on 1p*	Losses on 9p*	p14 ^{ARF} gene alt.	p14 ^{ARF} expr.	CDKN2A gene alt.	CDKN2A expr.	CDKN2B gene alt.	CDKN2B expr.	CDKN2C gene alt.	CDKN2C expr.
MN34	m	55	anapl	III	+	+	c.305G>T (G102V)	+	c.262G>T (E88X)	+		++		++
MN49A	f	55	anapl	III	+			(+)		+		n.d.		(+)
MN63B	f	78	anapl	III	+		c.305G>A (G102E)	+	c.262G>A (E88K)	+		+		+
MN64	m	6	anapl	III			HD	(+)	HD	(+)	HD	+		++
MN67	f	70	anapl	III	+		HD	(+)	HD	—	HD	—		+
MN70	f	41	anapl	III	+	+	HD	n.d.	HD	n.d.	HD	n.d.		n.d.
MN74	f	67	anapl	III	+	+		n.d.		n.d.		n.d.		n.d.
MN77	m	66	anapl	III	+			n.d.		n.d.		n.d.		n.d.
MN85	f	42	anapl	III			n.d.	—		—		—		(+)
MN88B	m	51	anapl	III	+			(+)		(+)		+		+
MN90	m	58	anapl	III	+	+	HD	(+)	HD	(+)	HD	(+)		+
MN91A	m	66	anapl	III	+	+	HD	—	HD	—	HD	—		(+)
MN100	f	45	anapl	III	+		HD	—	HD	(+)	HD	—		+
MN4	m	70	atyp	II	+			+		++		+		++
MN14	f	66	atyp	II	+			+		++		+		++
MN16	f	78	atyp	II	+			++		++		+		++
MN20	m	44	atyp	II	+			+		+		+		++
MN22	f	71	atyp	II				+		+		(+)		+
MN40	f	71	atyp	II	+			++		++		+		++
MN42	f	58	atyp	II				+		(+)		+		++
MN47	f	81	atyp	II				(+)		+		+		+
MN56B	m	47	atyp	II	+	+		+		+		+	c.202C>T (R68X)	++
MN59A	m	68	atyp	II	+			(+)		++		+		+
MN60	f	72	atyp	II				(+)		+		+		+
MN61	f	74	atyp	II	+		HM	—	HM	—	HM	—		(+)
MN62	f	74	atyp	II	+			+		++		++		++
MN65	f	76	atyp	II	+	+		+		++		++		++
MN66	f	90	atyp	II	+			(+)		++		+		+
MN68	f	43	atyp	II	+			+		+		+		++
MN69	m	38	atyp	II	+			++		++		++		++
MN71	f	76	atyp	II	+		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.
MN72	f	76	atyp	II	+			+		—		(+)		+
MN73	f	66	atyp	II				n.d.		n.d.		n.d.		n.d.
MN83	m	79	atyp	II	+	+	HD	n.d.	HD	n.d.	HD	n.d.		n.d.
MN84	m	63	atyp	II				+		(+)		+		+
MN86	f	79	atyp	II	+			+		+		(+)		+
MN89	m	78	atyp	II				+		—		—		+
MN101	f	54	atyp	II	+	+		+		+		+		+
MN102	f	74	atyp	II	+			++		++		++		(+)
MN103	f	70	atyp	II	+			(+)		+		(+)		+
MN104	m	56	atyp	II	+	+		+		+		++		++
MN105	f	42	atyp	II	+			++		+		+		++
MN106	f	38	atyp	II				+		+		++		+
MN107	m	71	atyp	II				+		(+)		+		+
MN108	f	39	atyp	II	+			(+)		n.d.		n.d.		n.d.
MN109	f	74	atyp	II				+		+		+		+
MN110	m	62	atyp	II		+		(+)		+		+		++
MN1	m	65	men	I				n.d.		n.d.		n.d.		n.d.
MN2	m	42	fib	I				(+)		—		(+)		+
MN7	f	50	fib	I		+		+		+		+		++
MN10	f	38	trans	I				+		+		+		++
MN11	m	49	trans	I				+		++		+		++
MN12	m	74	men	I	+			(+)		++		+		++
MN15	f	69	men	I				+		++		+		+
MN17	f	55	fib	I	+			(+)		++		+		++
MN19	f	52	fib	I	+	+		+		(+)		+		++
MN27	f	73	trans	I	+			+		+		+		++
MN31	m	47	ang	I				+		(+)		+		++
MN36	f	53	fib	I				(+)		(+)		+		++
MN37	f	71	fib	I				+		+		+		++
MN38	m	53	trans	I				+		+		+		+
MN41	f	55	fib	I				+		(+)		+		+
MN45	f	65	trans	I				++		++		++		++
MN51	m	63	psam	I				+		+		+		(+)
MN53	m	73	secr	I				+		+		+		+
MN58	f	59	men	I				+		+		+		+
MN111	m	49	trans	I				+		+		++		+

Abbreviations used: f, female; m, male; y, years; anapl, anaplastic; ang, angiomatous; atyp, atypical; fib, fibroblastic; men, meningotheliomatous; psam, psammomatous; trans, transitional; secr, secretory; gene alt., gene alterations detected; expr., expression; HD, homozygous deletion; HM, CpG island hypermethylation; n.d., not determined.

*, Determined by CGH and/or microsatellite analysis.

†, LOH restricted to markers distal to *D1S496*. The expression data obtained by duplex reverse transcription-PCR were semiquantitatively graded as follows: —, no detectable expression; (+), weak expression; +, moderate expression; ++, strong expression.

Table 2. Summary of Oligonucleotide Primers Used for SSCP/Heteroduplex Analysis (Application 1), Expression Analysis (Application 2), and Bisulfite Sequencing (Application 3) of *CDKN2A*, *p14^{ARF}*, and *CDKN2B*

Primer name	Sequence	Fragment length	Application
CDKN2A-exon1 α /F	5'-gaa gaa aga gga ggg gct g-3'	340 bp	1
CDKN2A-exon1 α /R	5'-gcg cta cct gat tcc aat tc-3'		
CDKN2A-exon2/F	5'-gaa aat tgg aaa ctg gaa gc-3'	508 bp	1
CDKN2A-exon2/R	5'-tct gag ctt tgg aag ctg t-3'		
CDKN2A-exon3/F	5'-ccg gta ggg acg gca aga ga-3'	169 bp	1
CDKN2A-exon3/R	5'-ctg tag gac cct cgg tga ctg-3'		
p14 ^{ARF} -exon1 β /F1	5'-gtc cca gtc tgc agt taa gg-3'	192 bp	1
p14 ^{ARF} -exon1 β /R1	5'-tga gcc gcg gga tgt gaa c-3'		
p14 ^{ARF} -exon1 β /F2	5'-ccg cga gtg agg gtt ttc g-3'	233 bp	1
p14 ^{ARF} -exon1 β /R2	5'-cct aga atg ggc tag aga cg-3'		
CDKN2B-exon1/F	5'-aag agt gtc gtt aag ttt acg-3'	315 bp	1
CDKN2B-exon1/R	5'-aca tcg gcg atc tag gtt cca-3'		
CDKN2B-exon2/F	5'-tga gta taa cct gaa ggt gg-3'	521 bp	1
CDKN2B-exon2/R	5'-ggg tgg gaa att ggg taa g-3'		
CDKN2A-RT/F	5'-caa cgc acc gaa tag tta cgg tc-3'	320 bp	2
CDKN2A-RT/R	5'-tct atg cgg gca tgg tta ctg-3'		
p14 ^{ARF} -RT/F	5'-tgc gca ggt tct tgg tga cc-3'	199 bp	2
p14 ^{ARF} -RT/R	5'-cat cat gac ctg gtc ttc ta-3'		
CDKN2B-RT/F	5'-gga cga acg gga ggg taa tg-3'	641 bp	2
CDKN2B-RT/R	5'-gcc ttc atc gaa tta ggt ggg tg-3'		
B2M-RT/F	5'-acc ccc act gaa aaa gat ga-3'	114 bp	2
B2M-RT/R	5'-atc ttc aaa cct cca tga tg-3'		
CDKN2A-Seq/F	5'-ggg ttt ttt aga gga ttt gag gga tag g-3'	392 bp	3
CDKN2A-Seq/R	5'-cta cct aat tcc aat tcc cct aca aac ttc-3'		
p14 ^{ARF} -Seq/F1	5'-gtt gag ggt ggg aag atg gtg-3'	550 bp	3
p14 ^{ARF} -Seq/F2	5'-gtt tgt agt taa ggg ggt agg ag-3'	254 bp	
p14 ^{ARF} -Seq/R	5'-cta act cct caa taa cat caa cac-3'		
CDKN2B-Seq/F1	5'-gat tag gag ttg agg gta gtg-3'	720 bp	3
CDKN2B-Seq/F2	5'-gag ttt aag ggg gtg ggg ag-3'	512 bp	
CDKN2B-Seq/R1	5'-cac ctt ctc cac taa tcc-3'		

ysis of the signal intensities obtained for the target gene and the reference gene was performed with the Molecular Analyst software (version 2.1, Bio-Rad). Tumors showing evidence for homozygous deletion of *CDKN2A*, *p14^{ARF}*, and *CDKN2B* by duplex-PCR were additionally analyzed by Southern blot analysis. For Southern blot analysis, 2.5 μ g of DNA were digested with the restriction enzyme *TaqI*, electrophoretically separated on 0.8% agarose gels, and alkali-blotted to Hybond-N⁺ membranes (Amersham-Pharmacia Biotech, Freiburg, Germany). The membranes were sequentially hybridized with α -[³²P]-dCTP-labeled, PCR-generated probes for *CDKN2A* (exon 1 α), *p14^{ARF}* (exon 1 β), *CDKN2B* (exon 1), and the reference locus *D2S44* (pYNH24, obtained from American Type Culture Collection, Rockville, MD). Hybridized membranes were exposed to imaging plates (Fuji, Kanagawa, Japan) and analyzed using the FLA-2000 Phosphor and Fluorescent image analyzer (Fuji). Quantitative densitometric analysis of the gene copy number was performed with the Mac BAS version 2.5 software (Image Reader, version 1.4E, and Image Gauge, version 3.0) as described.¹⁸

Expression Analyses

For expression analyses at the mRNA level, 3 μ g of total RNA from each tumor were reverse-transcribed into cDNA in a total volume of 50 μ l using random hexanucleotide primers and Superscript reverse transcriptase (Gibco BRL, Eggenstein, Germany). Expression of

CDKN2A, *p14^{ARF}*, and *CDKN2B* was determined by duplex reverse transcription-PCR, using β -2-microglobulin (B2M) mRNA for reference (primer sequences are listed in Table 2). Duplex reverse transcription-PCR for *CDKN2C* mRNA expression was performed according to Husemann and colleagues.¹³ As reference tissue, we used leptomeningeal tissue samples obtained at autopsy and nonneoplastic brain tissue (cortex and white matter from the temporal lobe) from a patient operated on for chronic epilepsy. PCR products were separated on 2% agarose gels, and ethidium bromide-stained bands were recorded by the Gel-Doc 1000 system.

Bisulfite Sequencing

Those tumors that lacked detectable transcripts from either *CDKN2A*, *p14^{ARF}*, or *CDKN2B* but showed neither mutations nor homozygous deletions of these genes were analyzed for hypermethylation of the CpG islands within the first exons of these genes. Genomic DNA (1 μ g) was treated with sodium bisulfite as described by Herman and colleagues.¹⁹ PCR analyses were performed with 70 to 80 ng of the bisulfite-modified DNA as template using 0.625 U of HotStar *Taq* DNA polymerase (Qiagen, Hilden, Germany) in 1 $\times$ Qiagen PCR-buffer with 0.2 mmol/L of each dNTP and 0.5 μ mol/L of each primer (Table 2) under the following PCR conditions: 15 minutes initial denaturation at 95°C were followed by 40 cycles of 30 seconds at 94°C, 30 seconds at the appropriate T_m value for each primer pair, 90 seconds at 72°C, and a final

Figure 1. **a** and **b**: Demonstration of LOH at microsatellite markers on 1p (**a**) and mutation of the *CDKN2C* gene in the atypical meningioma MN56B (**b**). Microsatellite analysis of tumor DNA (lane **T**) from MN56B and corresponding blood DNA (lane **B**) showed LOH at markers *D1S468* and *D1S224* in the tumor DNA (**a**, arrowheads). MN56B carried a somatic nonsense *CDKN2C* mutation (c.202C>T: R68X). **c** and **d**: *CDKN2A* and *p14^{ARF}* point mutations in two anaplastic meningiomas (MN34, MN63B). SSCP/heteroduplex analysis was performed after restriction digestion of the PCR products with *Sma*I. Both tumors showed an aberrant band corresponding to an undigested double-stranded PCR product (**c**, arrowhead). On sequencing, point mutations altering the *Sma*I restriction site were found in both tumors but not in the corresponding blood DNAs (**d**). MN34 showed a nonsense mutation in *CDKN2A* (c.262G>T: E88X) and a missense mutation in *p14^{ARF}* (c.305G>T: G102V). The mutation identified in MN63B also affected both genes (c.262G>A: E88K in *CDKN2A* and c.305G>A: G102E in *p14^{ARF}*). The sequences shown are derived from reverse sequencing of the coding strand. **Arrows** point to the mutation sites.

extension of 5 minutes. In the case of *p14^{ARF}* and *CDKN2B*, a second round of PCR was performed using 1 μ l of the previous PCR reaction as template and nested forward primers in combination with original reverse primers (Table 2). The re-amplification was done for 35 cycles under the same PCR conditions as described above. The PCR products were purified with the Jetquick gel extraction spin kit (Genomed, Bad Oeynhausen, Germany), subjected to cycle sequencing with the BigDye cycle sequencing kit and analyzed on an ABI PRISM 377 semi-automated DNA sequencer.

Results

Table 1 provides an overview of the 67 meningiomas investigated, including selected clinical data of the patients, histopathological diagnoses, and molecular genetic findings. Losses of genetic material from 1p were found in 11 of 13 anaplastic meningiomas (85%), 23 of 34 atypical meningiomas (68%), and 5 of 20 benign meningiomas (25%). The region of common loss on 1p mapped to 1p34-pter, distal to the marker *D1S468*, a finding in line with our previous study on a smaller series of cases.¹⁵

Duplex-PCR analysis of *CDKN2C* (exon 2) showed no homozygous deletion. Mutation analysis identified one

Figure 2. Demonstration of a complex LOH pattern on 9p in the anaplastic meningioma MN34. This particular tumor showed retention of heterozygosity at *D9S157* and *D9S171* (9p21), as well as LOH at markers located between *D9S157* and *D9S171* (shown is *D9S974*) and distal to *D9S157* (*D9S168* at 9p22-p23). **Arrows** point to lost alleles in tumor DNA.

atypical meningioma (MN56B) that carried a somatic *CDKN2C* nonsense mutation (c.202C>T: R68X) (Figure 1b). CGH analysis of tumor MN56B revealed loss of 1p and microsatellite analysis showed LOH at all informative loci on 1p, indicating complete loss of wild-type *CDKN2C* in MN56B (Figure 1a). None of 59 meningiomas investigated by duplex reverse transcription-PCR lacked detectable *CDKN2C* transcripts (Table 1).

Losses of alleles from 9p were found in 5 of 13 anaplastic meningiomas (38%), 6 of 34 atypical meningiomas (18%), and 1 of 20 benign meningiomas (5%) (Table 1). Eleven tumors demonstrated losses that included chromosome band 9p21 where *CDKN2A*, *p14^{ARF}*, and *CDKN2B* are located. One anaplastic meningioma (MN34) showed LOH at the *D9S168* locus (9p22-p23) but retention of heterozygosity at *D9S157* and *D9S171*, both located at 9p21 (Figure 2). CGH analysis indicated loss restricted to 9p24 in this tumor.⁸ Nevertheless, MN34 showed loss of one copy of *CDKN2A*, *p14^{ARF}*, and *CDKN2B* by duplex-PCR and Southern blot analysis. We therefore investigated MN34 for LOH at three additional microsatellite markers located at 9p21 between *D9S157* and *D1S171* (*D9S974*, *D9S942*, *D9S1748*). These loci demonstrated loss of one allele in MN34 (Figure 2). Taken together, these findings indicate a complex rearrangement of 9p in MN34, which resulted in interstitial and terminal losses of 9p sequences. Tumors MN31 and MN73 were previously reported to demonstrate LOH at *D9S168*.⁸ However, re-analysis of these two cases did not confirm this finding and CGH analysis also showed no losses on 9p in these cases.⁸

Determination of *CDKN2A*, *p14^{ARF}*, and *CDKN2B* gene dosage by duplex-PCR and Southern blot analysis identified homozygous deletions including all three genes in 6 of 13 anaplastic meningiomas (46%) and 1 of 34 atypical

Figure 3. Demonstration of homozygous *p14^{ARF}*, *CDKN2A* and *CDKN2B* deletions by duplex-PCR (a) and Southern blot analysis (b). Shown are results for seven meningiomas. MN63B retained two copies of each gene whereas MN19 and MN65 carried hemizygous deletions. MN64, MN67, MN90, and MN91A all show homozygous deletions (arrows). *INFG* and *9qSTS* were used as reference for the duplex-PCR analyses. *D2S44* served as reference to adjust for differences in DNA loading of the individual lanes of the Southern blot. The sizes of the respective PCR fragments (a) and hybridized restriction fragments (b) are given on the right.

meningiomas (3%), but none of 20 benign meningiomas (Figure 3; Table 1). Mutation analysis of *CDKN2A*, *p14^{ARF}*, and *CDKN2B* identified somatic point mutations within exon 2 of *CDKN2A* and *p14^{ARF}* in the anaplastic meningiomas MN34 and MN63B (Figure 1, c and d). The mutation detected in MN34 results in a premature stop of translation at position 88 of p16^{INK4a} (c.262G>T: E88X). The same mutation causes a single amino-acid exchange at codon 102 of p14^{ARF} (c.305G>T: G102V). MN63B carried a point mutation predicting single amino-acid exchanges in p16^{INK4a} (c.262G>A; E88K) and p14^{ARF} (c.305G>A; G102E) (Figure 1, c and d).

On reverse transcription-PCR analysis, five tumors without homozygous loss or mutation of either *CDKN2A*, *p14^{ARF}*, or *CDKN2B*, including one anaplastic meningioma (MN85), three atypical meningiomas (MN61, MN72, MN89) and one benign meningioma (MN2), lacked detectable transcripts for at least one of the p16^{INK4a}, p14^{ARF}, or p15^{INK4b} proteins (Figure 4, Table 1). These

Figure 4. Expression of transcripts from *CDKN2A*, *p14^{ARF}*, and *CDKN2B* in meningiomas. Shown are results obtained by duplex reverse transcription-PCR using β -2-microglobulin (B2M) mRNA as reference. The individual lanes correspond to: 1, MN10; 2, MN27; 3, MN37; 4, MN38; 5, MN41; 6, normal leptomeningeal tissue; 7, MN34; 8, MN63B; 9, MN85; 10, MN91A; 11, MN16; 12, MN40; 13, MN61; 14, MN89; 15, MN2; 16, MN45; 17 and 18, two different samples of normal leptomeningeal tissue; 19 and 20, two different samples of nonneoplastic brain tissue; 21, water control. Lanes 1 to 5 and 15 to 16 contain samples from benign meningiomas (World Health Organization grade I), lanes 11 to 14 from atypical meningiomas (World Health Organization grade II), and lanes 7 to 10 from anaplastic meningiomas (World Health Organization grade III). Transcripts from all three genes are present in the normal brain samples (lanes 19 to 20). The leptomeningeal tissue samples expressed *p14^{ARF}* mRNA and low levels of *CDKN2B* mRNA but lacked detectable *CDKN2A* transcripts. Most benign meningiomas expressed all three genes whereas transcripts from one or more of the genes were not detectable in tumors MN85, MN91A, MN61, MN89, and MN2 (arrows). Among these tumors, MN91A had a homozygous deletion involving *CDKN2A*, *p14^{ARF}*, and *CDKN2B*, whereas tumor MN61 showed hypermethylation of these genes (see Figure 5).

five tumors were investigated by bisulfite sequencing of the CpG islands within the first exons of *CDKN2A*, *p14^{ARF}*, and *CDKN2B*. The atypical meningioma MN61 demonstrated CpG island hypermethylation of all three genes (Figure 5). The other four tumors showed no 5'-CpG island hypermethylation of these genes.

Mutation analysis of exons 5 to 8 of the *TP53* gene revealed no mutations in any of the meningiomas of our series. One atypical meningioma (MN16) demonstrated a ninefold amplification of the *CDK4* and *MDM2* genes, as previously reported.⁸ This particular tumor neither

Figure 5. Hypermethylation of the CpG islands in the first exons of *CDKN2A*, *p14^{ARF}*, and *CDKN2B* in the atypical meningioma MN61. Shown are results of sequencing of sodium bisulfite-modified tumor and corresponding blood DNA. All sequences are derived from reverse sequencing of the coding strand. Arrows point to methylated CpG residues in tumor DNA that were not modified by the bisulfite treatment.

showed homozygous losses nor point mutations of *CDKN2A*, *p14^{ARF}*, *CDKN2B*, or *CDKN2C*. Transcripts from all four genes were expressed in MN16 (Table 1). No amplification of *CDK4* and/or *MDM2* was detected in the remaining 66 meningiomas investigated.

Discussion

The molecular genetic alterations underlying the progression of meningiomas are still poorly understood. Cytogenetic studies indicated that atypical and anaplastic meningiomas frequently show complex numerical and structural aberrations involving several chromosomes in addition to 22q.^{20,21} LOH and CGH analyses revealed that chromosome arms 1p, 6q, 10p, 10q, 14q, and 18q are rarely altered in benign meningiomas but are frequently affected by losses in atypical and anaplastic meningiomas.^{8,22–24} These chromosome arms are, therefore, expected to carry yet unknown meningioma progression-associated genes.

Several studies indicated that losses of 1p are of particular importance in the pathogenesis of atypical and anaplastic meningiomas.^{8,22,24,25} We found 1p losses in 85% of the anaplastic meningiomas and 68% of the atypical meningiomas, but only 25% of the benign meningiomas of our series. We previously mapped a candidate tumor suppressor region to 1p34-pter, distally to the anonymous locus *D1S496*.¹⁵ This region includes the chromosomal segment 1p36 that is commonly deleted in diverse human tumor types, including neuroblastoma, malignant melanoma, oligodendroglioma, and different carcinomas.^{13,26} The present tumor series included 35 cases from our previous study. Microsatellite analysis of the 22 newly analyzed tumors did not further narrow down the candidate gene region at 1p34-pter. However, because only three microsatellite loci from 1p were analyzed, we may have missed cases with small deletions. Another group reported on a meningioma-associated tumor suppressor gene region that is located closer to the centromere, ie, that maps to 1p32 between *D1S2713* and *D1S2134*.²⁷

To date, only a few candidate genes from 1p have been evaluated for tumor-associated alterations in meningiomas. One study suggested that the alkaline phosphatase gene (*ALPL*) at 1p34-p36.1 is a meningioma suppressor gene because its expression was found to be down-regulated in the majority of meningiomas with loss of 1p.²⁵ However, structural *ALPL* alterations have not been documented in meningiomas so far and functional evidence for tumor suppressor properties of its gene product is lacking. Analysis of the *hRAD54* gene (1p32) revealed no mutations in a series of 29 meningiomas with 1p deletions.²⁸ We investigated the *CDKN2C* gene at 1p32 for mutation and expression in meningiomas. This gene maps outside of the regions of common deletion identified by LOH analyses.^{15,27} Nevertheless, the 1p losses detected in 37 of 39 meningiomas from our present tumor series included the *CDKN2C* locus at 1p32. *CDKN2C* codes for an 18-kd protein (p18^{INK4c}) that binds to Cdk6 and Cdk4 and inhibits their kinase activi-

ty.²⁹ Overexpression of p18^{INK4c} *in vivo* inhibits cell proliferation and growth in a pRb-dependent manner.²⁹ Point mutations or homozygous deletions of *CDKN2C* have been detected in small fractions of different tumor types, including breast carcinomas,¹¹ acute lymphoblastic leukemias,³⁰ multiple myelomas,³¹ and anaplastic oligodendrogliomas.¹³ We found a novel *CDKN2C* nonsense mutation (c.202C>T: R68X) in an atypical meningioma. This mutation results in a truncated protein lacking three of the five ankyrin repeats that are important for the correct tertiary structure and stability of the protein.³² In addition, the truncated protein has lost its binding sites for Cdk4 and Cdk6 and thus can no longer inhibit the activity of these proteins.³³ The absence of detectable *CDKN2C* alterations in 66 of 67 meningiomas studied and the presence of *CDKN2C* transcripts in all tumors investigated by reverse transcription-PCR, however, indicate that this gene is not the major target of the common 1p losses in meningiomas. In line with our results, a recent study on 40 meningiomas identified only a single tumor with a homozygous deletion of the *CDKN2C* locus.³⁴ The remaining 39 meningiomas neither showed *CDKN2C* point mutations nor loss of expression.

The *CDKN2A*, *p14^{ARF}*, and *CDKN2B* genes at 9p21 are well-known tumor suppressor genes that are inactivated at high frequency in a large variety of human tumors.⁹ We found homozygous deletions including all three genes in 46% of anaplastic meningiomas, 3% of atypical meningiomas, but none of 20 benign meningiomas. Two additional anaplastic meningiomas carried *CDKN2A* and *p14^{ARF}* point mutations. Our data seem to be at variance with other studies reporting that *CDKN2A* alterations are either rare³⁵ or absent in meningiomas.^{36,37} However, these studies were based on small tumor series (generally <30 meningiomas), which mostly consisted of benign meningiomas of World Health Organization grade I. According to our data, alterations of *CDKN2A*, *p14^{ARF}*, and *CDKN2B* are predominantly found in anaplastic meningiomas. Thus, the discrepant results between our study and former reports are likely because of the higher fraction of anaplastic meningiomas in our series.

We identified two anaplastic meningiomas with point mutations in exon 2 of *CDKN2A*. One of these mutations (c.262G>T: E88X) predicts a truncated p16^{INK4a} protein, which is functionally impaired because it lacks parts of ankyrin repeat 3 and 4 as well as important residues for the interaction with Cdk4 and Cdk6.^{38,39} The second mutation detected (c.262G>A; E88K) maps to a site implicated in the binding of p16^{INK4a} to Cdk4 and Cdk6³⁹ and thus likely results in a functional impairment of the protein. The significance of these mutations is supported by the fact that both have been repeatedly found as somatic alterations in different types of human tumors.⁹ In addition to an effect on p16^{INK4a}, both mutations also result in missense mutations of p14^{ARF}. Recent studies indicated that the C-terminal domain encoded by exon 2 of *p14^{ARF}* contains an important nucleolar localization signal.^{40, 41} Mutations in exon 2 may therefore disrupt the nucleolar localization of p14^{ARF} and thereby interfere with p14^{ARF}-regulated Mdm2-dependent stabilization of p53.^{40,41}

Transcriptional silencing by CpG methylation of 5'-regulatory sequences represents an epigenetic mechanism for the inactivation of various tumor suppressor genes, including the *CDKN2A*, *p14^{ARF}*, and *CDKN2B* genes.⁴² Five tumors of our meningioma series without demonstrated homozygous deletion or mutation of *CDKN2A*, *p14^{ARF}*, and/or *CDKN2B* lacked detectable transcripts for at least one of the p16^{INK4a}, p14^{ARF}, or p15^{INK4b} proteins. However, only one of these tumors showed 5'-CpG hypermethylation of *CDKN2A*, *p14^{ARF}*, and *CDKN2B*. Tse and colleagues³⁷ investigated 23 meningiomas for methylation of *CDKN2A* and found partial rather than complete methylation in five tumors. The methylation status was not consistently associated with the expression of p16^{INK4a}. Thus, it seems that the regulation of *CDKN2A* and *CDKN2B* transcription in meningioma cells is complex and may be influenced by yet unknown mechanisms in addition to hypermethylation.

In addition to the *CDKN2* genes, other cell cycle-regulatory genes, such as *CDK4*, *CDK6*, *RB1*, and the cyclin D genes (*CCND1*, *CCND2*, and *CCND3*), are frequently aberrant in malignant tumors. We identified only one atypical meningioma with co-amplification of *CDK4* and *MDM2* among a total of 74 meningiomas investigated (present study and Weber and colleagues⁸). Two other studies did not detect any *CDK4* amplification in meningiomas.^{36,37} Thus, amplification of *CDK4* and/or *MDM2* is rare in meningiomas. Amplification of *CDK6* or any of the cyclin D genes has not been systematically investigated in meningiomas. Nevertheless, CGH studies did not reveal any amplification involving sequences from 7p13 (*CDK6*), 11q13 (*CCND1*), 12p13 (*CCND2*), or 6p21 (*CCND3*),^{8,43} suggesting that amplification of these genes is infrequent in meningiomas. The possibility of *RB1* gene aberrations in those anaplastic meningiomas that have no detectable *CDKN2A* or *CDKN2B* alterations remains to be investigated. An immunohistochemical analysis of 23 meningiomas showed no aberrant pRB1 expression.³⁷ The significance of this finding, however, is limited because no molecular genetic analyses were performed and most tumors were benign meningiomas.

In line with previous studies,^{44,45} we found no mutations in exons 5 to 8 of the *TP53* gene in our meningioma series. A single anaplastic meningioma with a *TP53* missense mutation was reported by Wang and colleagues.⁴⁶ Although *TP53* mutations are apparently rare in meningiomas, nuclear immunoreactivity for p53 protein has been detected in variable fractions of meningiomas.^{45,46}

In summary, we performed a detailed analysis of human meningiomas for alterations of the tumor suppressor genes *CDKN2A*, *p14^{ARF}*, *CDKN2B*, and *CDKN2C*. We found *CDKN2C* on 1p32 rarely altered in meningiomas and can, therefore, exclude this gene as the major target of the frequent losses on 1p in atypical and anaplastic meningiomas. Most anaplastic meningiomas either show homozygous *CDKN2A*, *p14^{ARF}*, and *CDKN2B* deletions, *CDKN2A* and *p14^{ARF}* mutations, or lack detectable expression of transcripts from one or more of these genes. Thus, inactivation of the G₁/S-phase cell-cycle checkpoint is an important feature of anaplastic meningiomas

that likely contributes to the rapid growth and malignant behavior of these tumors.

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