Chromosome Band 9p21 Is Frequently Altered in Malignant Peripheral Nerve Sheath Tumors: Studies of *CDKN2A* and Other Genes of the pRB Pathway

Jeanne-Marie Berner,[1] Therese Sørlie,[2] Fredrik Mertens,[3] Jørn Henriksen,[1] Gunnar Sæter,[4] Nils Mandahl,[3] Anton Brøgger,[2] Ola Myklebost,[1] and Ragnhild A. Lothe[2]*

[1]Department of Tumor Biology, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, Oslo, Norway
[2]Department of Genetics, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, Oslo, Norway
[3]Department of Clinical Genetics, University Hospital, Lund, Sweden
[4]Department of Oncology, The Norwegian Radium Hospital, Montebello, Oslo, Norway

Malignant peripheral nerve sheath tumors (MPNSTs) are frequently associated with the disease neurofibromatosis type 1. Only few recurrent cytogenetic changes have been reported, including rearrangements of the short arm of chromosome 9. By fluorescence in situ hybridization with a centromere 9 probe, and by allelic imbalance studies with seven 9p21–23 markers in nine familial and three sporadic MPNSTs, we found interstitial deletions that supported *CDKN2A* as a possible target gene. Nine MPNSTs showed aberrations of *CDKN2A* by Southern blot analyses, and in four of these, expression of *CDKN2A* could not be detected by Northern blot analysis. No mutations of *CDKN2A* were identified by sequencing of the coding region, and gene inactivation by promoter methylation was not found. In the 9p allelic imbalance studies, a novel allele was detected at one locus in one tumor. Analyses of additional markers (n = 8) excluded mismatch repair deficiency as an important mechanism in the genesis of these tumors. The tumors were analyzed further for alterations in other candidate cell cycle-associated genes. In total, 11/12 MPNSTs showed DNA changes in one or more of the genes *CDKN2A*, *CDKN2B*, *RB1*, *CDK4*, *MDM2*, and *CCND2*. The present study suggests that disruption of the pRB pathway is common in MPNST, and that dose reduction of *CDKN2A* is particularly frequent and contributes to MPNST development. *Genes Chromosomes Cancer 26:151–160, 1999.*
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INTRODUCTION

The severity of von Recklinghausen disease, or neurofibromatosis type 1 (NF1), including the tendency to develop malignant disease, varies considerably (Riccardi, 1992). Approximately half of all malignant peripheral nerve sheath tumors (MPNSTs) develop in patients with NF1.

Although the MPNSTs characterized cytogenetically until now had complex karyotypes with no consistent aberration pattern (Mertens et al., 1995), previous analyses indicate several genetic alterations of possible importance in the genesis of MPNST. Individuals with NF1 carry a germline mutation of *NF1*, and the deletions of 17q loci found in MPNST from such patients suggest somatic inactivation of the second allele (Skuse et al., 1989; Menon et al., 1990; Glover et al., 1991). On the other hand, actual mutation of both alleles has been demonstrated in one tumor only (Legius et al., 1993). Loss-of-function mutations of the *TP53* gene have been suggested to contribute to MPNST development. However, a limited number of tumors have been analyzed, and few mutants have been described (Nigro et al., 1989; Menon et al., 1990; Legius et al., 1994). Seven of the MPNSTs included in this study have been analyzed for mutations in *TP53*, but no mutations were found (Lothe et al., 1995).

By comparative genomic hybridization (CGH), a molecular cytogenetic technique that is used to identify net gains and losses in the entire genome and thereby provides a copy number karyotype of each tumor (Kallioniemi et al., 1992), we have previously found 9p deletions in 4 of 10 MPNSTs (Lothe et al., 1996). By cytogenetic banding analysis, chromosome arm 9p is also found to be rearranged in sarcomas, including MPNST (Mertens et al., 1995). *CDKN2A* maps to 9p21 and is a possible target for the observed deletions. This gene encodes the cyclin-dependent kinase inhibitor $p16^{INK4A}$, and the inactivation of *CDKN2A* may lead to increased phosphorylation of the retinoblastoma protein (pRB).

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*Correspondence to: Ragnhild A. Lothe, Department of Genetics, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, N-0310 Oslo, Norway. E-mail: rlothe@radium.uio.no

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TABLE 1. Patient Characteristics

Patients	Sex	Age	Diagnosis[a]	Tumor site	Tumor size (diameter)	Tumor status[b]/ grade
1	M	33	NF1 and MPNST	Thoracic wall	8 cm	R/4
3	M	33	NF1 and MPNST	Lower trunk	12 cm	P/3
			NF1 and INF	Gluteal	13 cm	P
6	M	17	NF1 and MPNST	Thigh	12 cm	P/3
7	F	17	NF1 and MPNST	Retroperitoneal	7 cm	P/4
8	F	71	NF1 and MPNST	Upper trunk	10 cm	P/3
9	F	19	NF1 and MPNST	Retroperitoneal	10 cm	P/2
32	M	41	NF1 and MPNST	Shoulder	6 cm	R/2
753	F	15	NF1 and MPNST	Retroperitoneal	6–10 cm	P/2
2367	M	24	NF1 and MPNST	Thigh	16 cm	P/2
2362	F	65	NF1 and DFS	NA[c]	NA	NA
5	F	77	MPNST	Gluteal	18 cm	P/4
650	F	45	MPNST	Gluteal	6 cm	P/2
1347	M	71	MPNST	Back	8 cm	P/3

[a]NF1 = neurofibromatosis; MPNST = malignant peripheral nerve sheath tumor; DFS = dermatofibrosarcoma protuberans; INF = intramuscular neurofibroma.
[b]P = primary; R = local recurrence.
[c]NA = information not available.

In the present study, we investigated 9p changes in MPNST, by microsatellite analysis, and the involvement of *CDKN2A* as a possible target. We also searched for gene alterations in other components of the pRB pathway, including *CDK4*, *CCND1*, *CCND2*, *CCND3*, and *RB1*.

MATERIALS AND METHODS

Patients and Tumors

Tissue specimens were taken from 12 MPNSTs from 9 patients with NF1 and from 3 sporadic cases (Table 1). Additional information on the patients and tumors has been provided elsewhere (Lothe et al., 1995; Maelandsmo et al., 1995; Mertens et al., 1995). Tumors 753, 2367, 32, 650, and 1347 in this article are samples from patients admitted to the University Hospital, Lund, Sweden, and correspond to numbers 2, 3, 5, 6, and 8 in Mertens et al. (1995). Numbers 1, 3, and 5–9 are tumor samples from patients admitted to the Norwegian Radium Hospital, Oslo, Norway, and the numbers are the same as in Lothe et al. (1995). Patients numbers 1, 5, and 8 correspond to MS9, MS8, and MS1 in Maelandsmo et al. (1996), respectively. From patient 3, we also analyzed an intramuscular neurofibroma. Two biopsy specimens from a mucinous and a nonmucinous part of tumor 9 were analyzed, and no differences were observed. In addition, one dermatofibrosarcoma protuberans from an NF1 patient (case 2362 from University Hospital, Lund), was included.

Fluorescence In Situ Hybridization (FISH) Analysis

In order to evaluate the copy number of chromosomes 9 and 12, we analyzed interphase nuclei from 10 MPNSTs by FISH using centromere-specific probes. Five tumors were analyzed according to a previously described protocol (Lothe et al., 1995). Briefly, each probe (D9Z1 and D12Z3, Oncor, Gaithersburg, MD) was hybridized separately in a formamide-based solution with dissociated and fixed nuclei from frozen tumors. After posthybridization washing, the probes were detected by a two-step immunoreaction and visualized by rhodamine. Fluorescent signals were counted in approximately 100 nuclei from each tumor for each probe. Normal lymphocyte preparations (interphase and metaphase) were used as controls for the hybridizations. FISH analysis of the remaining five MPNSTs was performed using probes directly labeled with SpectrumOrange fluorophore (CEP9, 9p11–q11 and CEP12, 12p11–q11). These analyses were done according to the protocol supplied by the manufacturer (Vysis, Downers Grove, IL).

DNA Isolation

Normal DNA from all individuals were isolated from peripheral blood cells. Both normal and tumor DNA were extracted according to standard procedures (manual procedure or automated by use of a DNA extractor from Applied Biosystems, Foster City, CA) using phenol and chloroform extraction followed by ethanol precipitation.

Analysis of Microsatellite Loci

Microsatellite loci containing dinucleotide repeats were analyzed according to previously described procedures (Giercksky et al., 1997). Briefly, primer sets specific for each locus were used to

amplify the repeats and short flanking sequences in template DNA by multiplex polymerase chain reaction (PCR). The products were labeled radioactively during the amplification reaction, followed by separation in denaturing polyacrylamide gels.

The loci included in the study were (chromosomal location in parentheses): D9S199 (9p23), D9S157 (9p22–23), D9S162 (9p22–23), D9S171 (9p21), D9S126 (9p21), D9S265 (9p21), D9S169 (9p21), D9S152 (9q21–22), D1S201 (1p), D1S104 (1q), D6S289 (6p), D6S292 (6q), D11S915 (11p), D11S917 (11q), D12S77 (12p), D12S80 (12q), and RB1.20 [a variable number of tandem repeats (VNTR) locus, 13q14]. Allelic imbalance (AI) at these loci was scored by visually comparing the tumor DNA pattern with the constitutional heterozygosity pattern. By AI, we mean in this context loss of heterozygosity (LOH; the complete absence of one allele) or a skewed intensity ratio between the two alleles in tumor compared to constitutional DNA. All positive findings at microsatellite loci were analyzed twice by independent PCR and electrophoretic runs.

Southern Blot Analysis

DNA from tumor and peripheral blood cells from each patient was digested to completion by the restriction enzymes *Hin*dIII or *Bam*HI. The DNA fragments were separated in an 0.8% agarose gel and transferred by alkaline blotting onto Hybond N+ membranes (Amersham Life Science, Little Chalfont, U.K.). The probes were radioactively labeled by random priming (Feinberg and Vogelstein, 1983), and the filters were hybridized in formamide-SSC solution as described (Maelandsmo et al., 1995). The net signals from specific bands were corrected for unequal sample loading by calibration relative to the signal obtained from the *APOB* (chromosome 2) control probe. A signal of at least three times higher intensity than signals from the corresponding normal sample (DNA from peripheral blood cells) was scored as an amplification. Signals weaker than 25% or 75% were scored as a homozygous or heterozygous deletion, respectively. Quantitation of signal intensity was done either by two-dimensional densitometry on a Molecular Dynamics Laser Densitometer or by use of a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

The following probes were used: *MDM2* [location 12q15; kindly provided by Drs. D. L. George and B. Vogelstein (Oliner et al., 1992)]; *CDK4* [12q13; Dr. P. Meltzer (Su et al., 1994)]; *CCND1* (11q13), *CCND2* (12p13), and *CCND3* (6p21) [Dr. D. Beach (Xiong et al., 1992)]; *CDKN2A* and *CDKN2B* [9p21; Dr. D. Beach (Serrano et al., 1993; Hannon and Beach, 1994)]; *APOB* (clone pB27) [2p24–23; Dr. J. Breslow (Huang et al., 1985)].

Mutation Analysis of *CDKN2A*

The coding sequences of the *CDKN2A* gene, exons 1–3 with short flanking sequences, were amplified by PCR as previously described (Esteve et al., 1996) and submitted to direct sequencing by two different approaches. Briefly, single-stranded templates were obtained by asymmetric PCR. Templates were purified on Chromaspin columns (Clontech Life Technologies, Palo Alto, CA) and sequenced in both directions using the USB/Amersham PCR Product Sequencing kit with 7-deaza dGTP as described elsewhere (Esteve et al., 1996). Alternatively, double-stranded templates were purified on Microspin columns (Amersham Pharmacia Biotech) and sequenced using the ABI Prism Dye Terminator Cycle Sequencing Reaction Kit on an ABI 373 DNA Sequencer following the manufacturer's protocol (Perkin Elmer, Applied Biosystem Division, Foster City, CA).

Methylation Analysis

Hypermethylation of *CDKN2A* was analyzed by a methylation-specific PCR method as described previously (Herman et al., 1996). Briefly, 1 μg of genomic DNA was denaturated by NaOH and further modified by sodium bisulfite, converting all unmethylated cytosines to uracils. Modified DNA was purified by Wizard DNA purification resin according to the manufacturer (Promega, Madison, WI). PCR was performed with specific primer sets for methylated *CDKN2A* (p16-M : 5′-TTA-TTAGAGGGTGGGGCGGATCGC-3′ and 5′-GACCCCGAACCGCGACCGTAA-3′) and unmethylated *CDKN2A* (p16-U: 5′-TTATTAG-AGGGTGGGGTGGATTGT-3′ and 5′-CAAC-CCCAAACCACAACCATAA-3′; positions of bisulfite modified DNA are underlined, potential methylation sites in are given in bold) (Herman et al., 1996). The PCR products were evaluated on polyacrylamide gels.

Northern Blot Analysis

Transcription of *CDKN2A* was analyzed by Northern blot hybridization in eight MPNSTs from which frozen tissue was available. Total RNA was isolated from tissues by TRIzol Reagent (Total RNA Isolation Reagent, Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. Five micrograms of total RNA were electrophoresed through 1% agarose-formaldehyde gels, transferred to Hybond N+ membranes (Amersham, Amer-

sham, U.K.), and the filters were hybridized as previously described (Maelandsmo et al., 1995). To adjust for unequal amounts of RNA loaded in each lane, the filters were rehybridized with a kinase-labeled oligonucleotide probe specific for human 18S rRNA. No frozen tissue was available from primary tumor 1, but transcripts were analyzed from xenograft material passage from this tumor. The sarcoma cell lines "tax" and "ohs" and the MPNST MS5 were used as positive controls (Maelandsmo et al., 1995). The MPNSTs MS2x and MS7 have homozygous deletions of *CDKN2A* and were used as negative controls (Maelandsmo et al., 1995).

RESULTS

Chromosome 9 Analysis

FISH analysis using a centromere 9-specific probe revealed a dominating disomic pattern in all tested tumors (n = 10). An average of 85% (range, 70%–95%) of the nuclei of each tumor had two centromeres (Fig. 1).

AI was found by microsatellite analysis at one or more 9p loci in 6 of 12 MPNSTs, and only one tumor (1347) showed AI at 9q (D9S152; Table 2, Figs. 1 and 2). Based on the deletion patterns, a smallest region of overlap could be delineated between D9S162 and D9S169 (Fig. 2).

The gross integrity of the *CDKN2A* and *CDKN2B* genes was analyzed by Southern blot hybridization. This revealed aberrations of *CDKN2A* in 9 of 12 tumors and of *CDKN2B* in 7 of the same tumors (Table 2). Two of 3 sporadic cases and 7 of 9 familial cases had *CDKN2A* aberrations. Cases 5 and 650 showed reduction of *CDKN2A* signal intensity, 35% and 50%, respectively, indicating heterozygous deletions. In six other MPNSTs, an aberrant restriction fragment was found in tumor DNA, but not in the corresponding normal blood DNA (Figs. 1 and 3A). In three of these (6, 32, and 2367), the *CDKN2A* probe detected an extra *Bam*HI fragment in addition to the normal bands. In the other 3 cases (1, 3, and 753), the largest *Bam*HI fragment was lost and one additional *Bam*HI fragment of smaller size was detected. Homozygous deletions of *CDKN2A* and *CDKN2B* were found in case 7. We detected a single copy of the *CDKN2B* gene in six MPNSTs. No changes in *CDKN2A* or *CDKN2B* were detected in the benign tumor from patient 3, nor in the dermatofibrosarcoma protuberans.

Sequencing of the three *CDKN2A* exons revealed no mutations in any of the 12 MPNSTs, although in tumor 3 a polymorphism was detected in codon 148 (GCG→ACG/Ala→Thr). Furthermore, *CDKN2A* was not found to be hypermethylated in any of the MPNSTs analyzed (n = 12), as PCR products were obtained only for reactions with primers specific for unmethylated *CDKN2A*.

Transcription of *CDKN2A* was analyzed by Northern blot analysis in 8/12 MPNSTs from which frozen tissue samples were available (Table 2, Fig. 3B). No mRNA was detected in four of the six tumors with *CDKN2A* gene alterations. Tumor 5 had a heterozygous deletion of *CDKN2A*, and a low mRNA level was seen (Fig. 3B). In tumor 753, *CDKN2A* was rearranged, and we detected a transcript of smaller size expressed at a high level (Fig. 3B). One of the two tumors without detectable *CDKN2A* aberrations showed a low-level transcription of the gene (tumor 8), whereas tumor 9 did not express detectable *CDKN2A* mRNA.

Alteration of Genes Encoding Proteins Involved in the pRB Pathway

By Southern blot hybridization, we determined the copy number of *CDK4* and *MDM2* (both in 12q13–15) in all tumors. An increased copy number was detected in only one tumor (no. 5), in which both genes were amplified more than 10-fold (Fig. 4). FISH analysis of centromere 12 revealed a normal copy number in the majority (74%) of the nuclei analyzed in this sample (Fig. 4). In eight other tumors, between 79% and 93% of the nuclei were disomic, whereas tumor 6 had three or four 12-centromeres in 43% of the nuclei. *CCND2* (12p13) also showed amplification in tumor 5, and the probe detected a rearranged and amplified restriction fragment (Fig. 4). *CCND1* and *CCND3* were not amplified in any of the tumors. An intragenic *RB1* VNTR locus (RB1.20) showed AI in five samples (3, 5, 6, 8, and 1347).

Microsatellite Instability

One of 12 MPNSTs (8.3%) showed microsatellite instability. A novel allele was seen at locus D9S171 in tumor 1, which is shown in Lothe (1997). Analysis of eight additional microsatellite loci mapping to four different chromosomes revealed no novel alleles in any of the tumors, thus microsatellite instability was found in 1 of 16 (6.3%) genotypes evaluated.

DISCUSSION

Previous CGH and cytogenetic banding analyses suggested loss of 9p to be a common aberration in MPNST (Mertens et al., 1995; Lothe et al., 1996). Recurrent abnormalities of chromosomal band 9p21 are found in a variety of human cancers, including malignant melanomas (Fountain et al., 1992), gliomas (Olopade et al., 1992), lung cancers (Olopade

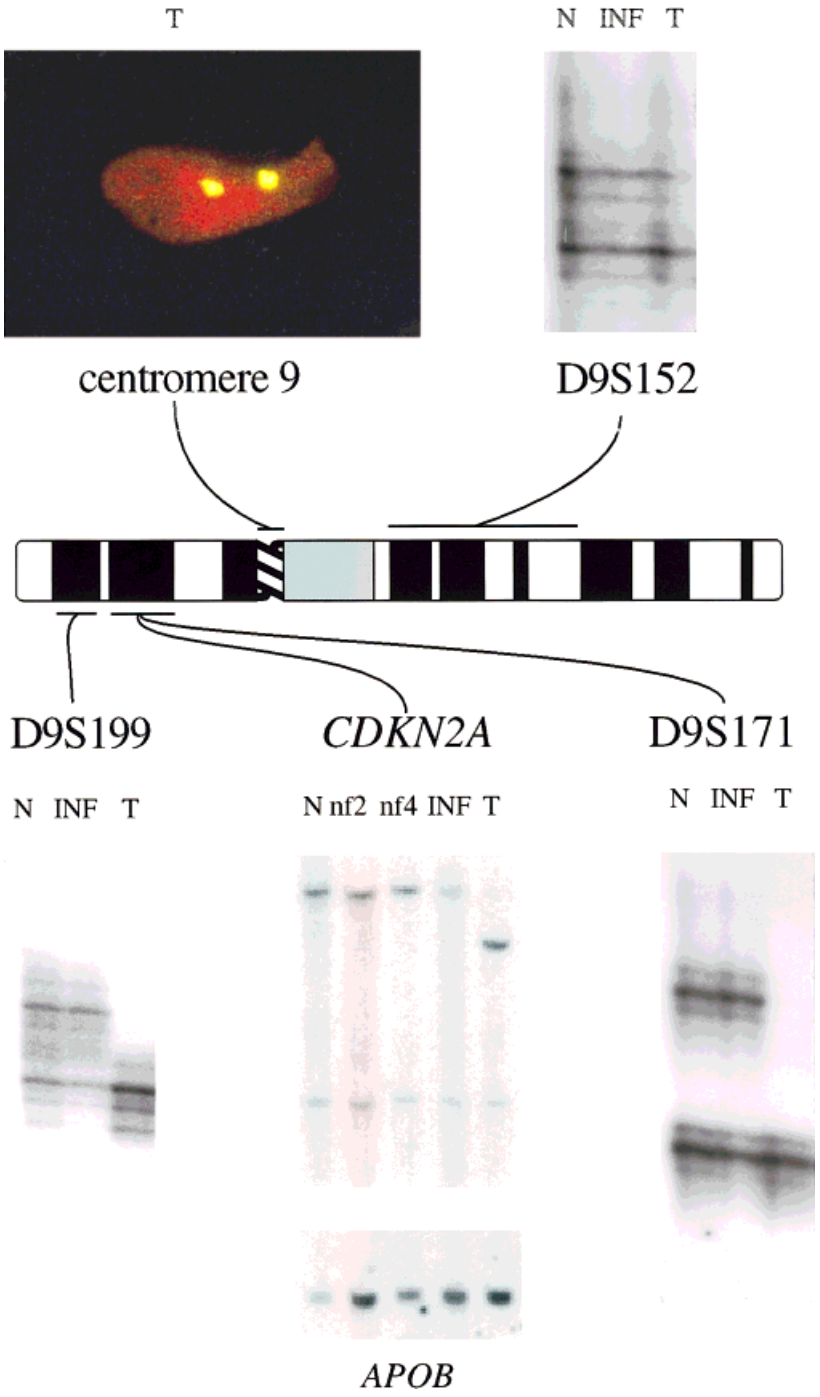

Figure 1. Analysis of chromosome 9 loci in tumors from case 3. The map positions of the loci are indicated on the chromosome 9 ideogram. FISH with the centromere 9 probe showed a disomic pattern in the MPNST (here denoted T). AI was detected by PCR-based analyses of microsatellites at D9S199 and D9S171, but not at D9S152 (9q) in the MPNST. No changes were seen in the intramuscular neurofibroma (INF). Southern blot analysis (*Bam*HI) demonstrated rearrangement of *CDKN2A*, i.e., detection of a restriction fragment with abnormal electrophoretic mobility, in the MPNST, but not in the INF nor in the benign neurofibromas of the skin (nf2, nf4) from this patient. The blot was also hybridized with *APOB* as a loading control. N = DNA from peripheral blood. Note that the nf2 and nf4 samples are not included in the present study, but only in this illustration.

et al., 1993), and leukemias (Diaz et al., 1988). *CDKN2A*, mapping to 9p21, has been proposed as a candidate tumor suppressor gene. The gene encodes $p16^{INK4A}$, a protein that binds to cyclin-dependent kinases 4 and 6 and inhibits the ability of these kinases to interact with D-type cyclins and to phosphorylate the retinoblastoma protein, thus abrogating the cell cycle checkpoint in G1 (Serrano et al., 1993).

TABLE 2. Alterations at Chromosome Arm 9p in MPNST

Patients[a]	Tumor[b]	Gene structure[c]		Expression[d]	Allelic imbalance[e]	Loss of 9p
		CDKN2A	*CDKN2B*	*CDKN2A*	9p21–23	CGH[f]
1 NF1	MPNST	R	N	−	AI	nd
3 NF1	INF	N	N	nd	N	nd
3 NF1	MPNST	R	d	−	AI	Loss
6 NF1	MPNST	r	N	−	N	N
7 NF1	MPNST	D	D	nd	N	Loss
8 NF1	MPNST	N	N	+	N	N
9 NF1	MPNST	N	N	−	N	nd
32 NF1	MPNST	r	d	−	AI	N
753 NF1	MPNST	R	d	+[g]	N	N
2367 NF1	MPNST	r	d	nd	AI	Loss
2362 NF1	DFS	N	N	nd	N	N
5	MPNST	d	d	+	AI	Loss
650	MPNST	d	d	nd	N	N
1347	MPNST	N	N	nd	AI	N

[a]NF1 = neurofibromatosis type 1.
[b]MPNST = malignant peripheral nerve sheath tumor; INF = intramuscular neurofibroma; DFS = dermatofibrosarcoma protuberans.
[c]R = detection of a restriction fragment of abnormal mobility and only residual amounts of the normal restriction fragments; r = detection of a restriction fragment of abnormal mobility as well as the normal fragments; D = homozygous deletion; d = heterozygous deletion; N = normal/wild-type.
[d]nd = not done/not determined; + = transcript detected; − = no transcript detected.
[e]AI = allelic imbalance (see Fig. 2); N = retained heterozygosity.
[f]Data from Lothe et al. (1996); N = no 9p deletion detected.
[g]A transcript of aberrant size.

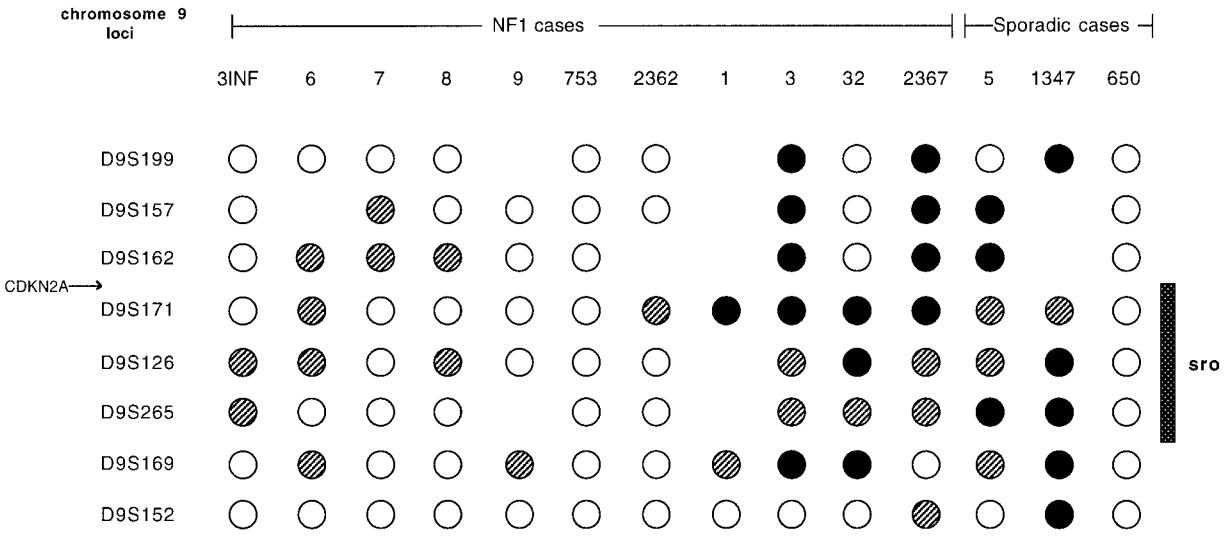

Figure 2. AI at 9p loci in MPNST. Solid circle indicates AI in tumor DNA compared to normal DNA; empty circle, retained heterozygosity in tumor DNA; cross-hatched circle, homozygosity (not informative); no circle, not done/not determined. NF1 cases = patients with neurofibromatosis type 1 and MPNST or dermatofibrosarcoma protuberans (case 2362); sporadic cases = patients with MPNST without NF1. AI was found in the MPNST, but not in the benign tumor (INF = intramuscular neurofibroma) from case 3. The smallest region of overlap (sro) is indicated.

Homozygous deletions of *CDKN2A* were initially reported in a high percentage of human cancer cell lines derived from various histologic types (Kamb et al., 1994a; Nobori et al., 1994). Although complete inactivation of this gene is not as frequent in primary tumors (Cairns et al., 1994), deletion of one or both gene copies has been observed in several cancer types, including sarcomas (Maelandsmo et al., 1995). A melanoma susceptibility locus has been mapped to this region, and several studies suggest *CDKN2A* to be the target, because mutations were frequent in melanoma cell lines and were demonstrated in the germline of members of familial melanoma kindreds showing link-

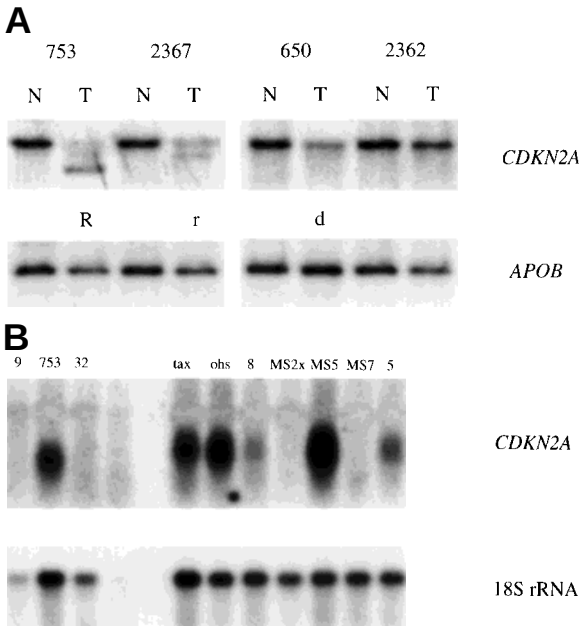

Figure 3. **A:** Southern blot demonstrating alterations of *CDKN2A* in MPNST. DNA from four MPNSTs (here denoted by T) and corresponding normal blood (N) was digested with *Bam*HI, electrophoresed, transferred to a nylon membrane, and hybridized with a *CDKN2A* probe and subsequently with *APOB* (chromosome 2) for loading control. R = detection of a restriction fragment of abnormal mobility and only residual amounts of the normal restriction fragment; r = detection of a restriction fragment of abnormal mobility as well as the normal fragment; d = heterozygous deletion of *CDKN2A*. **B:** *CDKN2A* expression in MPNST. The Northern blot was hybridized to *CDKN2A* and to 18S rRNA as a loading control. Tumor 753 with rearranged *CDKN2A* has a transcript of aberrant size. The sarcoma cell lines tax and ohs, and the MPNST MS5 known to express *CDKN2A*, were used as positive controls (Maelandsmo et al., 1995). MS2x and MS7 have homozygous deletions of *CDKN2A* and were used as negative controls (Maelandsmo et al., 1995).

age to 9p21 markers (Hussussian et al., 1994; Kamb et al., 1994b).

In this study, the AI pattern of 9p21–23 loci revealed the smallest region of overlap between D9S162 and D9S169, thus including *CDKN2A* as a possible target. Southern blot analysis revealed alterations of *CDKN2A* in 75% of the MPNSTs (Table 2). The single case with a homozygous deletion suggests a frequency comparable to that found in sarcomas in general (Maelandsmo et al., 1995). The six cases showing restriction fragments of reduced size compared to the wild-type fragment could be caused by a mutation creating an additional restriction site, but is more likely to be a result of interstitial deletion. Rearrangements of *CDKN2A* have been reported previously in a low frequency among various other tumor types (Orlow et al., 1995; Stranks et al., 1995; Yao et al., 1998). The frequent finding of *CDKN2A* rearrangement in MPNST could suggest a slightly different mechanism for inactivation of *CDKN2A* in this group of sarcomas. In three of these cases with rearrangements (1, 3, and 753), we found the remaining allele to be deleted, suggesting that the *CDKN2A* gene was completely inactivated in these samples. By Northern blot analysis, we could not detect any *CDKN2A* transcripts in tumor 1 or 3, whereas a transcript of smaller size was observed in tumor 753 (Fig. 3B). This is probably a truncated transcript due to a partial deletion of the gene, but we do not know how this affects the protein coding region. It should be noted that transcription of *CDKN2A* is generally not detected by Northern blot in normal tissue, but that a high transcription level is frequently found in tumors, including sarcomas (Maelandsmo et al., 1995). Elevated $p16^{INK4A}$ expression in tumor cells is correlated with nonfunctional pRB protein (Serrano et al., 1993).

Five cases had heterozygous changes of *CDKN2A*, including three cases (6, 32, and 2367) with rearrangement of one allele and two cases with heterozygous deletion of the gene (5 and 650). If *CDKN2A* is a tumor suppressor gene that functions by complete inactivation, we should expect the other allele to be inactivated by mutation. Sequencing of the coding region of the gene did not reveal any mutations in any samples, similar to the findings in studies of large panels of human sarcomas, where no mutations were found (Meye et al., 1998; Yao et al., 1998). However, it cannot be excluded that mutations exist in other parts of the gene. Northern blot analysis showed a low-level transcription of *CDKN2A* in a sample with a heterozygous deletion of the gene (case 5), indicating that the remaining allele was intact (Fig. 3B). In the other samples with heterozygous changes of *CDKN2A* where RNA was available (6 and 32), no transcription of the gene was seen, suggesting that the gene was inactivated. It has been reported that de novo methylation of CpG islands spanning exon 1 and part of exon 2 may inactivate *CDKN2A* (Merlo et al., 1995), but we could not detect methylation of *CDKN2A* in any of the MPNSTs.

On the basis of these results, it cannot be ruled out that a second tumor suppressor located in the vicinity of *CDKN2A* might be the target for 9p rearrangements in MPNST. The *CDKN2B* gene, another member of the $p16^{INK4A}$ family, is located adjacent to *CDKN2A* on 9p21 and has been found to be deleted together with *CDKN2A* in cancer cell lines (Hannon and Beach, 1994; Kamb et al., 1994a). However, we found *CDKN2B* to be altered in fewer cases than was *CDKN2A*, and only in samples where *CDKN2A* also was rearranged. On the other hand, *CDKN2A* yields two transcripts derived from alternative first exons (Mao et al.,

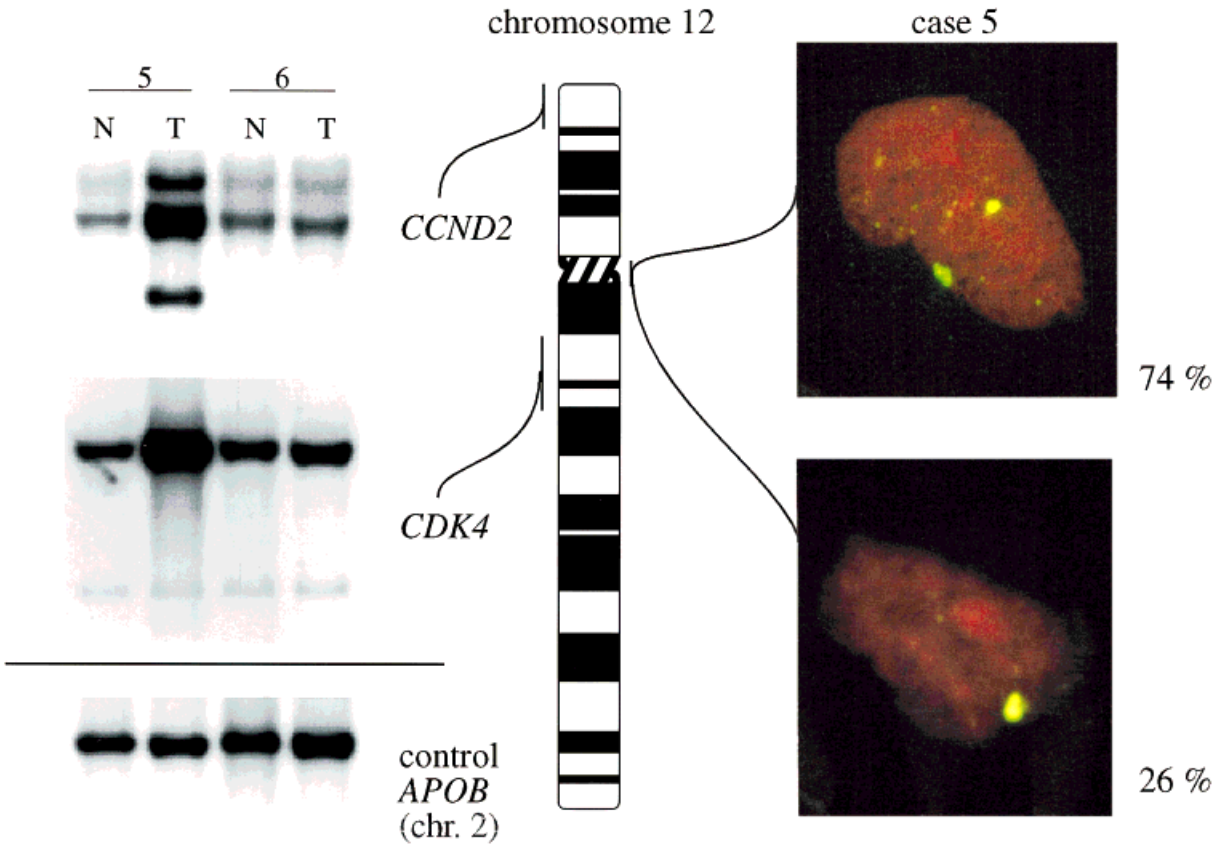

Figure 4. The left part of the figure is a Southern blot showing amplification of *CDK4* and *CCND2* in tumor 5. DNA from two MPNSTs (here denoted by T) and corresponding normal blood (N) was digested with *Hin*dIII, electrophoresed, transferred, and hybridized with *CDK4* and subsequently *CCND2*. The blot was also hybridized with *APOB* as a loading control. The *CCND2* probe detected an amplified and rearranged restriction fragment in tumor 5. FISH (the right part of the figure) with the centromere 12 probe showed disomy in sample 5 in 74% of the nuclei analyzed (the remaining 26% showed one centromeric signal), confirming region specific amplification and not polysomy of the whole chromosome. The map positions of the loci are indicated on the chromosome 12 ideogram.

1995; Stone et al., 1995), coding for two distinct proteins, $p16^{INK4A}$ and $p14^{ARF}$ (mouse homologue = $p19^{ARF}$), which participate in different cell cycle-inhibitory functions (Quelle et al., 1995). Recently, $p14^{ARF}$ was found to block MDM2-induced degradation of p53 (Pomerantz et al., 1998; Zhang et al., 1998). Consequently, deletion of the *CDKN2A* locus impairs both the pRB and the p53 tumor suppressor pathways.

If the frequent alterations of the *CDKN2A* gene are selected for by deranging of the pRB-mediated G1 checkpoint, it would be likely that there were other aberrations of this pathway in the tumors where the gene seemed to be intact. Inactivation of the pRB pathway by amplification of the *CDK4* and *cyclin D* genes has been reported previously in sarcomas (Maelandsmo et al., 1995). Amplification of *CDK4*, *CCND2*, and *MDM2* was observed in only one of the present tumors (case 5), confirming the results of a previous CGH analysis in which chromosome 12 sequences were found to be amplified (Lothe et al., 1996). Interestingly, in this sample we found heterozygous deletion of the *CDKN2A* gene, suggesting that the presence of the remaining allele is counteracted by *CDK4* amplification. Detection of separate amplicons involving components of the same pathway may reflect tumor heterogeneity or perhaps a synergistic growth advantage. The observation is in accordance with previous findings in other sarcomas (Maelandsmo et al., 1995).

A novel allele was seen at locus D9S171 in tumor 1, and we evaluated the possibility of genome instability by analyzing eight additional microsatellites. As in previous studies of human sarcomas, we found infrequent microsatellite instability, suggesting that mismatch repair deficiency is not a major contributor to the genesis of MPNST (Wooster et al., 1994; Tarkkanen et al., 1996).

Among three tumors without alterations of *CDKN2A* or *CDK4*, AI of the *RB1* locus was detected in tumors 8 and 1347, leaving only tumor 9 without any detected alterations in the analyzed

genes involved in the pRB pathway. Thus, our results clearly demonstrate the importance of inactivation of the pRB pathway for MPNST development or progression, both in familial and sporadic cases. Although we cannot draw any conclusion from the single intramuscular neurofibroma or from the dermatofibrosarcoma protuberans, it is noteworthy that pRB aberrations were not seen in either of these. Because MPNSTs are of neuroectodermal origin, it might be relevant that loss of function of *CDKN2A* and of pRB-mediated growth control have been a frequent observation in other cancers of such origin, including melanomas (Bartkova et al., 1996), glioblastomas (Schmidt et al., 1994; Ishimura et al., 1996; Ueki et al., 1996), and Ewing tumors (Kovar et al., 1997).

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