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CDKN2A/p16 Is Inactivated in Most Melanoma Cell Lines[1]

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ABSTRACT

The *CDKN2A* gene maps to chromosome 9p21–22 and is responsible for melanoma susceptibility in some families. Its product, p16, binds specifically to CDK4 and CDK6 *in vitro* and *in vivo*, inhibiting their kinase activity. *CDKN2A* is homozygously deleted or mutated in a large proportion of tumor cell lines and some primary tumors, including melanomas. The aim of this study was to investigate the involvement of *CDKN2A* and elucidate the mechanisms of p16 inactivation in a panel of 60 cell lines derived from sporadic melanomas. Twenty-six (43%) of the melanoma lines were homozygously deleted for *CDKN2A*, and an additional 15 (25%) lines carried missense, nonsense, or frameshift mutations. All but one of the latter group were shown by microsatellite analysis to be hemizygous for the region of 9p surrounding *CDKN2A*. p16 was detected by Western blotting in only five of the cell lines carrying mutations. Immunoprecipitation of p16 in these lines, followed by Western blotting to detect the coprecipitation of CDK4 and CDK6, revealed that p16 was functionally compromised in all cell lines but the one that carried a heterozygous *CDKN2A* mutation. In the remaining 19 lines that carried wild-type *CDKN2A* alleles, Western blot analysis and immunoprecipitation indicated that 11 cell lines expressed a wild-type protein. Northern blotting was performed on the remaining eight cell lines and revealed that one cell line carried an aberrantly sized RNA transcript, and two other cell lines failed to express RNA. The promoter was found to be methylated in five cell lines that expressed *CDKN2A* transcript but not p16. Presumably, the message seen by Northern blotting in these cell lines is the result of cross-hybridization of the total cDNA probe with the exon 1β transcript. Microsatellite analysis revealed that the majority of these cell lines were hemi/homozygous for the region surrounding *CDKN2A*, indicating that the wild-type allele had been lost. In the 11 cell lines that expressed functional p16, microsatellite analysis revealed loss of heterozygosity at the markers immediately surrounding *CDKN2A* in five cases, and the previously characterized R24C mutation of *CDK4* was identified in one of the remaining 6 lines. These data indicate that 55 of 60 (92%) melanoma cell lines demonstrated some aberration of *CDKN2A* or *CDK4*, thus suggesting that this pathway is a primary genetic target in melanoma development.

INTRODUCTION

A predisposition gene for familial melanoma has been localized to chromosome bands 9p21–p22 by linkage analysis in several cohorts of pedigrees (1–4). Positional cloning of a candidate tumor suppressor gene in this region centromeric of the *IFNA* gene cluster was accomplished simultaneously by two groups (5, 6). The gene (*CDKN2A*) thus isolated was found to be identical to a previously characterized cell cycle regulatory gene encoding the CDK[4] inhibitor, p16 (7). Initially, the *CDKN2A* gene was found to be homozygously deleted in a total of 161 of 336 (48%) cell lines derived from various tumor types (5, 6). More recently, mutations and deletions have been found *in vivo* in a high proportion of a limited range of cancer subtypes, such as pancreatic, esophageal, bladder, and non-small cell lung carcinomas, and gliomas (reviewed in Refs. 8 and 9). These data clearly highlight the role this gene plays as a suppressor of carcinogenesis in a wide variety of histologically different cell types.

More specifically, melanoma cell lines (5, 9–14) and some fresh melanomas (14–19) show deletions or interstitial mutations of *CDKN2A*, indicating the importance of *CDKN2A* in the development of this particular cancer. Furthermore, the identification of germ-line mutations of *CDKN2A* in affected members of familial melanoma kindreds has given unequivocal proof of its role in melanoma predisposition (reviewed in Refs. 8 and 20). The differences in the percentages of melanoma cell lines and primary tumors with inactivation of *CDKN2A* reported in the literature have been interpreted in at least two ways: either the detection of deletions and mutations in fresh tumors is hindered by the presence of contaminating normal tissue in the sample, and/or aberrations of *CDKN2A* are more common in cell lines as the culturing procedure selects for those either already carrying an inactive *CDKN2A* allele or acquiring inactivation of *CDKN2A in vitro*.

Overexpression of wild-type p16 inhibits progression of cells through the G_1 phase of the cell cycle by binding to CDK4/cyclin D (or CDK6/cyclin D) complexes and blocking the kinase activity of the enzyme (Ref. 7 and reviewed in Refs. 21–23). Loss of functional p16 results in abnormal proliferation by removing a key cell cycle checkpoint and allowing cells to progress, unrestrained, into S phase. Missense and nonsense mutations in *CDKN2A* generate p16 variants that are greatly impaired in their ability to inhibit CDK4/cyclin D or CDK6/cyclin D catalytic activity, and this is due primarily to the mutant proteins being unable to form stable complexes with the kinase (reviewed in Ref. 20; Ref. 24). It is interesting to note, however, that not all mutant forms of p16 appear to be functionally compromised (25, 26).

In this study, we have investigated the involvement of *CDKN2A* in a panel of 60 melanoma cell lines in an effort to elucidate the principal mechanisms of p16 inactivation and the degree to which aberrations of this locus contribute to melanoma development. Cell lines were initially screened for homozygous deletions, and those that retained *CDKN2A* were further analyzed for intragenic mutations, heterozygosity of 9p microsatellite markers, RNA expression, and p16 expression and function.

MATERIALS AND METHODS

Cell Lines. Melanoma cell lines A2058 and NK14 were provided by George Todaro and Rose Ann Padua, respectively. SK-MEL-13 and SK-MEL-28 were from Lloyd Old; BA, WW, and BL were from Craig Beattie; Colo239F, HT144, C-32, and RPMI7932 were from the American Type Culture Collection; ME1402, ME10538, Newton, and WSB were from Peter Hersey; and IPC298 was from Christian Aubert. Cell lines MM96 to 649 were established by P. G. Parsons, and cell lines AF-6, A04-GEH, A06-MLC, CJM, JA-M, JLO, MW, and RM were established by Michelle Down. Cell lines were grown in RPMI 1640 plus 10% FCS.

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[4] The abbreviations used are: CDK, cyclin-dependent kinase; RT-PCR, reverse transcription-PCR; LOH, loss of heterozygosity; MOPS, 4-morpholinepropanesulfonic acid.

RNA and DNA Extraction. DNA was extracted as described previously (27). Total RNA was isolated from cell pellets with Qiagen RNeasy Total RNA extraction kits, according to the manufacturer's instructions. Purified RNA was quantified by spectrophotometric measurement.

PCR Analysis. PCR amplification of *CDKN2A* was performed under similar conditions to those described previously by Pollock *et al.* (13). The exon 1β primers used were 5′-CCGCGAGTGAGGGTTTTCGT-3′ and 5′-CAAAACAAGTGCCGAATGCG-3′. The latter primer is located flanking E1β (28), and the forward primer is within the exon. Exon 1α of *CDKN2A* was amplified using primers 2F and 1108R (5). For Exon 1β and 1α of *CDKN2A*, a "touchdown" thermal cycling routine of two cycles at each degree between 65°C and 56°C, followed by 15 cycles at 55°C, was used. Exon 2 was amplified using primers 42F and 551R (5), and exon 3 was amplified using primers X3.90F and 530R (29). For exons 2 and 3, a touchdown thermal cycling routine of two cycles at each degree between 60°C and 56°C, followed by 25 cycles at 55°C, was used. Each cycle consisted of 45 s at 94°C, 90 s at the annealing temperature, and 90 s at 72°C. As a positive control, amplification of a portion of the 3′ untranslated region of the human *MXI1* gene, which maps to chromosome 10, was carried out using the primers described by Wechsler *et al.* (30). Amplification of a 270-bp fragment corresponding to exon 2 of *CDK4* was carried out using the primers described by Wölfel *et al.* (31) with a standard 30-cycle routine consisting of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s. PCR reactions consisted of 100 ng of template DNA, 10–20 pmol of each primer, 200 nM deoxynucleotide triphosphates, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 6% DMSO, and 0.5 unit DynaZyme (Finnzymes Oy). Products were resolved on 1.5% agarose gels and then purified using Qiagen QIAquick gel purification columns according to the manufacturer's instructions.

RT-PCR. RNA was reverse transcribed with Superscript II reverse transcriptase (Life Technologies, Inc.) according to the manufacturer's instructions. Two μl of the cDNA were amplified using primers 305F and 530R (29), which generated a 226-bp product containing the P114L mutation in MM628. A touchdown thermal cycling routine of two cycles at each degree between 65°C and 56°C, followed by 15 cycles at 55°C, was used. Each cycle consisted of 45 s at 94°C, 90 s at the annealing temperature, and 90 s at 72°C. Duplicate reactions were then run out on 1.5% agarose gels, and the bands were excised and purified using Qiagen QIAquick gel purification columns.

DNA Sequencing. To minimize possible sequencing artifacts induced by PCR, products from at least two different PCR reactions were combined before purification and then sequenced using an ABI dye-terminator sequencing kit (Applied Biosystems, Inc.) according to the manufacturer's directions. Extension products were then purified by ethanol precipitation as described by the manufacturer. Reactions were then resolved on a model 383A ABI automated DNA sequencer.

Genotyping. Simple tandem repeat polymorphisms were typed for the *D9S162, IFNA, D9S1749, D9S942, D9S171,* and *D9S162* loci using PCR. Primer sequences were obtained from the Genome Database, and reactions consisted of 100 ng of template DNA; 5–10 pmol of each primer; 200 nM of dATP, dGTP, and dTTP; 2 nM dCTP; 10 mM Tris-HCl; 1.5 mM $MgCl_2$; 50 mM KCl; 0.1% Triton X-100; 0.5 unit DynaZyme; and 1 μCi of [α-^{32}P]dCTP. PCR conditions consisted of an initial denaturation step at 94°C for 180 s, followed by 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s. For the *D9S974* locus, the reaction conditions included 5% DMSO and a 58°C annealing temperature; otherwise, the PCR was performed similarly. For the *D9S1748* locus, which is situated 236 bp 3′ to E1β (Fig. 1; Ref. 28), the forward primer was 5′ end-labeled with [γ^{32}P]dATP according to the protocol supplied with T4 polynucleotide kinase from New England Biolabs. PCR reactions contained 10 pmol of the forward primer (1 pmol labeled and 9 pmol unlabeled), 10 pmol of the reverse primer, and 200 μM of each deoxynucleotide triphosphate; otherwise, PCR conditions and cycling were the same. Products were denatured at 95°C and electrophoresed through 7 M urea-6% polyacrylamide gels, which were typically run for 3–6 h at 1000–1500 V, dried, and exposed to X-ray film for 1–18 h. The marker order was compiled from data contained within the reports of Cairns *et al.* (32) and Ohta *et al.* (18). Cell lines were deemed to have LOH of this region if they contained a single allele at three or more contiguous markers flanking *CDKN2A*, and including *D9S942*, as the probability that three of these highly polymorphic markers were homozygous by chance was very low ($P < 0.003$; calculated by multiplication of the average homozygosity of each marker).

Western Blotting. Cells (10^6) were harvested and lysed in NETN [20 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl, 5 μg/ml each leupeptin, pepstatin, aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5% NP40] supplemented with 300 mM NaCl. Fifty μg of protein lysate were separated by 15% SDS-PAGE and electrotransferred to polyvinylidene difluoride membrane (Bio-Rad). Membranes were blocked in 5% BLOTTO for $\geq$1 h; probed with either anti-p16 antibody (33), anti-CDK4 antibody (Santa Cruz Biotechnology), or anti-CDK6 antibody (Santa Cruz Biotechnology); washed in PBS-Tween 20; reprobed with the secondary antibody; and after extensive washing, developed using ECL detection according to the manufacturer's instructions (DuPont NEN).

Immunoprecipitation. One μg of protein lysate was used for each cell line. This was mixed with 30 μl of a 50% suspension of anti-p16 antibody conjugated to protein A-Sepharose and incubated for at least 1 h at 4°C on a rocker. The immunoprecipitate was then pelleted, washed three times in NETN, and finally resuspended in 100 μl of 1× protein loading buffer [5×: 4.2 g of Tris base, 10 ml of glycerol, 15 g of SDS, in 75 ml H_2O (pH 6.8), 0.01% bromphenol blue, and 25% β-mercaptoethanol], and denatured by boiling. Thirty μl were loaded into lanes of a mini-gel and subjected to 15% SDS-PAGE electrophoresis and immunoblotting as specified in "Western Blotting."

Northern Blotting. Thirty μg of each sample were loaded onto 1.2% agarose/MOPS/formaldehyde gels. Electrophoresis was performed for 3 h at 70 V in 1× MOPS [200 mM MOPS (pH 7), 5 mM sodium acetate, and 1 mM EDTA (pH 8)]. Gels was then capillary-blotted overnight onto Hybond-N filters (Amersham Corp.). Photos were taken of both the ethidium bromide-stained gels and filters to check for even loading and good transfer, respectively. The full-length p16 cDNA cloned in pBluescript was a gift from Dr. C. Hussussian. The plasmid was labeled with [α-^{32}P]dCTP using a Megaprime DNA labeling kit (Amersham), and the probe was purified on Qiagen QIAquick gel purification columns to remove unincorporated nucleotides. Prehybridization and hybridization were performed at 65°C in a modified Church and Gilbert buffer (7% SDS freshly weighed, 0.263 M Na_2HPO_4, 1 mM EDTA, and 1% BSA). Washes were performed at 65°C to a stringency of 0.1× SSC, 0.1% SDS. Filters were exposed to X-ray films overnight at −70°C.

RESULTS AND DISCUSSION

Cell Lines with Homozygous Deletion of *CDKN2A*

To determine the extent of the *CDKN2A* homozygous deletions and the possible involvement of the recently described exon 1β (E1β), all four exons of *CDKN2A* were coamplified with a fragment of the *MXI1* gene by PCR and run on 1.5% agarose gels. The exon 1 and exon 2 deletion status for 30 of 60 of these melanoma cell lines has been published previously (13). Complete or partial deletions of *CDKN2A* were seen in 26 (43%) of the melanoma cell lines studied, a proportion similar to that reported by others: 27%, Ohta *et al.* (10); 42%, Luca *et al.* (12); and 40%, Maelandsmo *et al.* (17). Table 1 summarizes the extent of these deletions and provides evidence that the region of homozygous deletion on 9p can be limited to a single exon of *CDKN2A*. In a study defining the breakpoints in a variety of T-cell lines, Cayuela *et al.* (34) found that 10 of 17 breakpoints on chromosome 9 were localized between E1β and exon 3, possibly suggesting the presence of sequences in this region that promote recombination. Twelve of the 26 melanoma cell lines in the present study also had breakpoints within this region, supporting this hypothesis. Two melanoma cell lines have been reported that carry deletions that remove E1β but not E1α (35), raising the possibility that E1β may play a role in melanoma development. However, in our study, there were no cell lines in which only E1β was deleted, indicating that E1β plays a limited role, if any, in melanoma tumorigenesis.

Cell Lines Carrying at Least One *CDKN2A* Allele

Sequencing of *CDKN2A*. For the 34 cell lines that retained at least one *CDKN2A* allele, the entire coding sequence of p16 was amplified

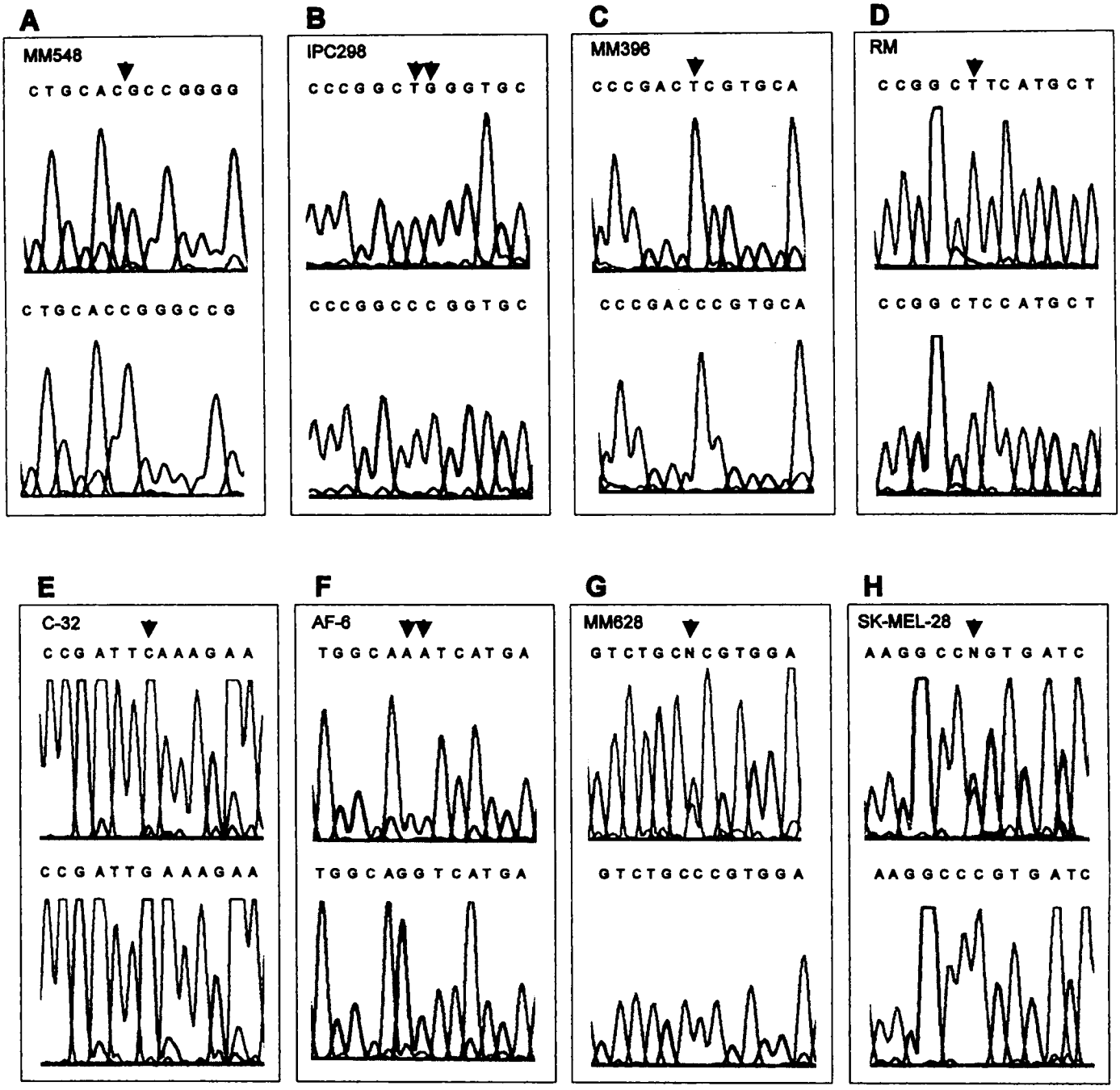

Fig. 1. Mutations of *CDKN2A* (*A–G*) and *CDK4* (*H*) in melanoma cell lines. The *upper panel* in each case represents the mutated cell line, whereas the *lower panel* contains the wild-type sequence for the corresponding region. The positions of mutated bases are indicated by *arrows* above the automated DNA sequence traces. In *B* and *D*, the sequence is shown 5′→3′ for the noncoding strand; in all other cases, the sequence is shown 5′→3′ for the coding strand.

and directly sequenced. In summary, 15 of 34 cell lines carried mutations, of which eight have been previously published (13). Sequence data for the unpublished mutations is presented in Fig. 1, A–G, and a summary of all mutations that have been further studied is presented in Table 2. As expected, the high proportion of C→T transitions at dipyrimidines and tandem CC to TT mutations reported in these new melanoma cell lines further supports the notion that UV radiation is involved in the genesis of a large proportion of melanomas (13). Two of these mutations are particularly worthy of comment; C-32 carries a transversion that results in the substitution of the stop codon with a serine and the addition of 13 amino acids before the p16 protein is terminated at a downstream stop codon, and AF-6 carries a tandem CC→TT mutation involving the nucleotides on either side of the exon 2 splice acceptor site, thereby destroying the consensus sequence. All 34 lines, regardless of the presence or absence of a mutation, were subsequently analyzed for heterozygosity of this region of 9p, and 32 of these were analyzed for p16 expression and function.

Zygosity. LOH was determined by analyzing a panel of microsatellite markers spanning the *CDKN2A* region on chromosome 9p. A summary of these results is shown in Table 3. As expected with Knudson's two-hit model for inactivation of tumor suppressor genes, 14 of 15 cell lines carrying an intragenic mutation were hemizygous for the region surrounding *CDKN2A* (*i.e.*, had no wild-type allele).

Table 1 *Summary of homozygous deletion status determined by PCR in 26 melanoma cell lines*[a]

Cell line	Exon 1β	Exon 1α	Exon 2	Exon 3
MM470	+	−	+	+
ME1402	+	−	−	+
MM229	+	+	−	−
MM409	+	+	−	−
MM488	+	+	−	−
MM648	+	+	−	−
Newton	+	−	−	−
WSB	+	−	−	−
MM127	+	−	−	−
MM253	+	−	−	−
MM418	+	−	−	−
MM595	+	−	−	−
Colo 239F	−	−	−	−
ME10538	−	−	−	−
SK-MEL-13	−	−	−	−
MM170	−	−	−	−
MM455	−	−	−	−
MM466	−	−	−	−
MM472	−	−	−	−
MM608	−	−	−	−
HT144	−	−	−	−
A04-GEH	−	−	−	−
A06-MLC	−	−	−	−
CJM	−	−	−	−
MW	−	−	−	−
WW	ND	−	−	ND

[a] The remaining 34 cell lines in this study did not have homozygous deletions of these exons (Table 3). +, exon was present; −, homozygous deletion; ND, not determined.

These zygosity studies also confirmed that the tandem mutations identified in Table 2 are carried on the one allele. One mutated cell line, MM628, was found to be heterozygous for the region surrounding *CDKN2A*. To exclude the presence of a small undetectable region of LOH, RT-PCR and subsequent sequencing were then performed on this cell line. Fig. 1*G* shows the expression of both the wild-type and mutant alleles. In addition, of the 19 cell lines that showed a wild-type genomic sequence for *CDKN2A*, 11 demonstrated LOH in the region surrounding *CDKN2A* (discussed later).

p16 Expression and Analysis of *in Vitro* Function. The 34 cell lines that carried at least one *CDKN2A* allele were analyzed for the presence of p16 by Western blotting. If present, p16 function at the cellular level was assayed by immunoprecipitation of the p16 and subsequent Western blotting for the coprecipitation of endogenous CDK4 or CDK6. In the absence of protein, expression of *CDKN2A* RNA was assayed by Northern blotting to further elucidate the mechanism of p16 inactivation. A summary of these data are presented in Table 4, and a comprehensive discussion follows in the subsequent sections.

Table 2 *Summary of CDKN2A mutations detected in melanoma cell lines*

Cell line	Base[a]	Codon	Sequence[b]	Base change	Amino acid change
AF6	Int-1/151		tgaCCtgc	CC→TT	Splice
BA	238	80	accCgac	C→T	Arg→stop
BL	262	88	cctCccg	C→T	Glu→Lys
C-32	470	Stop	tttCaat	C→G	stop→Ser (+ 13 αα)
NK14	171/172	57/58	cgcCCga	CC→TT	Arg→stop
IPC298	296/297	99	ggcCCgg	CC→TG	Arg→Pro
RM	4	2	gctCcat	C→T	Glu→Lys
MM384	142/143	48	gggCCga	CC→TT	Pro→Leu
MM396	242	81	gacCcgt	C→T	Pro→Leu
MM426	262	88	cctCccg	C→A	Glu→stop
MM473	247	83	gtgCacg	C→T	His→Tyr
MM485	330	110	gccCcag	C→T	Trp→stop
MM548	295	99	ggcCCGg	Del CCG	Delete Arg
MM622	198	67	gccTgtg	Ins T	frameshift
MM628	341	114	tgcCcgt	C→T	Pro→Leu

[a] Nucleotide numbering starts at the A of the initiation codon.
[b] The sequence is written 5′→3′ for the strand containing the pyrimidine. Mutated bases are shown in capitals.

Table 3 *Summary of 34 melanoma cell line genotypes for eight markers mapping to chromosome 9p*[a]

Cell line	*D9S162*[b]	IFNA	*D9S1749*	*D9S974*	*D9S942*	*D9S1748*	*D9S171*	*D9S126*
BA	+[c]	+	+	+	+	+	+	+
BL	+	+	+	+	+	+	+	+
C-32	+	+	+		+	+	+	+
NK14	+	+	+	+	+	+	+	+
MM396	+		+	+	+	+	+	+
MM485	+	+	+	+	+	+	+	+
MM548	+	+	+	+	+	+	+	+
MM622	+	+	+	+	+	+	+	+
IPC298	+	+	+	+	+	+	+	+
AF-6		+	+	+	+	+	+	+
RM		+	+	+	+	+	+	+
MM383	+	+	+	+	+	+	+	+
MM540	+	+	+	+	+	+	+	+
MM386	+	+	+	+	+	+	+	+
MM576	+	+	+	+	+	+	+	+
JA-M		+	+	+	+	+	+	+
JLO		+	+	+	+	+	+	+
MM426	+	+	+	+	+	+	+	+/+
MM384	+	+	+	+	+	+/+	+	+
MM649	+	+	+	+	+	+	+	+
MM473	+	+	+/+	+	+	+	+/+	+/+
RPMI 7932	+	+/+	+/+	+	+	+	+	+
MM329	+/+	+/+	+/+		+	+	+/+	+/+
MM369	+	+/+	+	+	+	+	+/+	+/+
MM370	+/+	+	+/+	+	+	+	+	+/+
SK-MEL-28	+/+	+		+/+	+/+	+	+	+
MM96	+/+	+	+	+/+	+/+	+/+	+	+
A2058	+	+/+	+/+	+	+/+	+	+	+/+
MM415	+	+/+	+/+	+/+	+/+	+	+/+	+/+
MM598	+/+	+/+	+	+/+	+/+	+	+/+	+/+
MM200	+/+	+		+/+	+/+	+/+	+/+	+/+
MM603	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+
MM647	+	+/+	+/+	+	+/+	+/+	+/+	+/+
MM628	+/+	+/+	+/+	+	+/+	+/+	+	+/+

[a] The remaining 26 cell lines in this study had partial or complete homozygous deletions of *CDKN2A* (Table 1).
[b] Marker order is from telomere (*D9S162*) to centromere (*D9S126*). Blanks indicate samples not done.
[c] +, hemizygosity or homozygosity; +/+, heterozygosity.

Table 4 *Summary of CDKN2A/p16 status in 34 melanoma cell lines without homozygous deletions*

Cell line	Zygosity[a]	*CDKN2A* mutation	*CDKN2A* RNA[b]	p16 protein	p16 binding to CDK4[c]	p16 binding to CDK6[c]
BL	+	E(88)K		+	−	+
IPC 298	NA	R(99)P	NA	NA	NA	NA
MM384	+	P(48)L		+	−	+
MM396	+	P(81)L		+	−	−
MM473	+	H(83)Y		+	−	−
MM628	+/+	P(114)L[d]		+	+	+
BA	NA	R(80)STOP	NA	NA	NA	NA
MM485	+	W(110)STOP	+	?[e]		
MM622	+	Ins 198 + 1	+	?[e]		
NK14	+	R(58)STOP	+	?[e]		
RM	+	E(2)K	+	−		
MM548	+	Del R(99)	+	−		
AF-6	+	splice site mtn	−	−		
C-32	+	stop codon mtn	−	−		
MM426	+	E(88)STOP	−[f]	?[e]		
MM647	+/+	WT	Aberrant	−		
MM383	+	WT	−[f]	−		
MM540	+	WT	−[g]	−		
MM96L	+	WT	+[h]	−		
MM386	+	WT	+[h]	−		
MM598	+/+	WT	+[h]	−		
MM649	+	WT	+[h]	−		
RPMI7932	+	WT	+[h]	−		
A2058	+/+	WT		+	+	+
JA-M	+	WT		+	+	+
JLO	+	WT		+	+	+
MM200	+/+	WT		+	+	+
MM329	+	WT		+	+	+
MM369	+	WT		+	+	+
MM370	+	WT		+	+	+
MM415	+/+	WT		+	+	+
MM576	+/+	WT		+	+	+
MM603	+/+	WT		+	+	+
SKMEL28	+/+	WT[i]		+	+	+

[a] Zygosity was determined from Table 3, where samples were defined to be hemi/homozygous when three or more contiguous markers flanking *CDKN2A* and including *D9S942* showed a single allele. In the case of MM384, sequencing revealed only the mutant allele; therefore, it has been scored as hemizygous for the region. NA, cells were not available for further studies; WT, wild-type.

[b] Northern analysis was not done (blanks) in those cell lines that expressed p16 protein.

[c] CDK4/CDK6 binding analyses were not done (blanks) in those cell lines that did not express p16 protein.

[d] The mutation in this cell line is heterozygous. Refer to "Results and Discussion" for details.

[e] The putative protein resulting from these mutations could not be detected with our COOH-terminal peptide-directed antibody.

[f] This cell line is not methylated within the promoter of the *CDKN2A* gene.

[g] This cell line is heavily pigmented, and Southern blot analysis for methylation of the *CDKN2A* promoter was not possible.

[h] Southern blot analysis revealed that the DNA from these cell lines was methylated; presumably the signal on the Northern blot is due to the alternative transcript containing exon 1β. Refer to "Results and Discussion" section for details.

[i] This cell line has an R24C CDK4 mutation.

Cell Lines Carrying Missense Mutations

Western blotting demonstrated that five of seven of the cell lines carrying missense mutations expressed p16, *i.e.*, BL, MM384, MM396, MM473, and MM628 (Fig. 2*A* and Table 4). These cell lines were also shown to express CDK4 and CDK6 proteins, although the levels varied widely (Fig. 2*A*). p16 function was then analyzed in these cell lines by immunoprecipitating p16 and performing Western blotting to detect bound CDK4 and CDK6 proteins (Fig. 2*B*). MM396 and MM473 carried mutations that completely abrogated the binding of p16 both to CDK4 and to CDK6, whereas BL and MM384 carried mutations that inhibited binding to CDK4 while still allowing binding to CDK6 (Fig. 2*B* and Table 4). Reymond and Brent (36) reported that the G101W mutation in p16 also inhibits binding to CDK4 but not CDK6. Together, these data suggest that the p16 binding sites for CDK4 and CDK6 are distinct, or that there is a second region of p16 that is specifically needed for binding to one of these two kinases but not for binding to the other. We have not been able to identify mutations that affect CDK6 binding but not CDK4 binding, suggesting that, at least in melanocytic cells, CDK4 is the primary functional substrate of p16. The remaining cell line found to express p16 was MM628, which carried a heterozygous P114L substitution. p16 was consistently expressed at very low levels in this cell line, and immunoprecipitation demonstrated that this protein was capable of binding CDK4 and CDK6 (Fig. 2*B*). As Parry and Peters (37) have demonstrated previously, the P114L mutant protein does not bind either CDK4 or CDK6; the binding activity in MM628 was presumably due to the expression of the wild-type allele. The expression of both wild-type and mutant alleles was unexpected but suggests that haploinsufficiency for p16 may be enough for melanoma initiation.

Of the remaining two of seven lines that carried a missense mutation, IPC298 was not analyzed further due to the lack of available cells, and RM, which carried an E2K substitution, was found not to express p16 protein (Fig. 3 and Table 4), although Northern analysis of the latter cell line indicated the presence of *CDKN2A* RNA (data not shown). The mutation in this line occurs at nucleotide +4 relative to the initiation codon. This position comprises part of the consensus

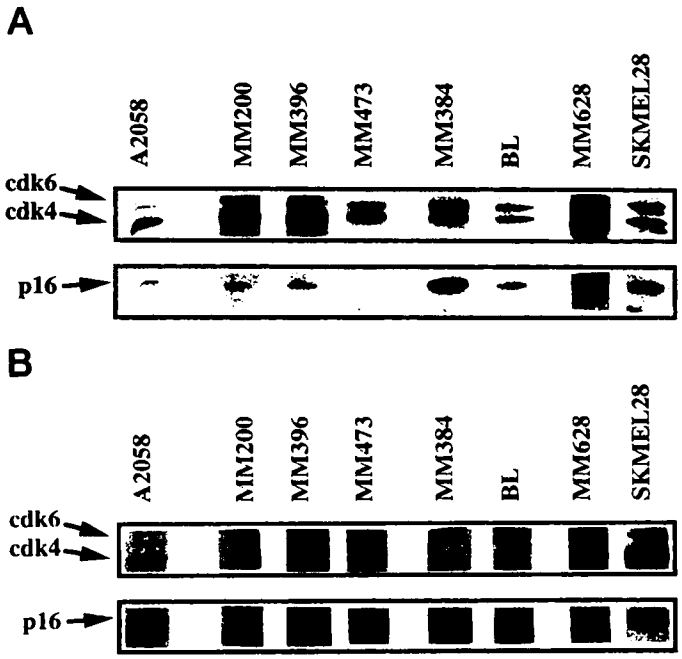

Fig. 2. Western blot analysis of cell lines carrying a *CDKN2A* missense mutation and expressing p16 protein. *A*, equal quantity of cell lysates were loaded onto minigel lanes and probed with anti-p16, anti-CDK4, and anti-CDK6 antibodies. *B*, equal quantities of cell lysates were immunoprecipitated with anti-p16 antibody and immunoblotted for p16, CDK4, and CDK6.

sequence for translation initiation by eukaryotic ribosomes (38, 39). Disruption of this consensus sequence by nucleotide substitution at position +4 has been shown previously to completely inhibit proinsulin synthesis from a construct transfected into COS cells (38). The single base substitution in RM cells is, therefore, most likely responsible for the absence of p16 expression through inability of the cellular translational machinery to recognize the initiating methionine.

Cell Lines Carrying More Severe Mutations

p16 was not detectable by Western blotting in any of the remaining seven of eight cell lines tested, carrying more severe mutations (BA was not analyzed further due to the lack of available cells). Of these seven lines, four carried either nonsense or frameshift mutations that would have resulted in a truncated protein not detectable by the COOH-terminally-directed antibody (33) used in this study (Table 4). Although the presence or absence of p16 cannot be proven in these cell lines, various reports by others indicate that these truncation mutations are likely to impair or abolish the inhibitory function of p16. Parry and Peters (37) reported that nonsense mutations identical to those carried by MM485 and NK14, when expressed *in vitro*, cannot bind to either CDK4 or CDK6. Moreover, Lilischkis *et al.* (40) concluded that the introduction of a stop codon upstream of codon 131 is likely to impair or abolish the inhibitory function of p16. Based on this evidence, MM622 would also be expected to encode a nonfunctional protein because the one nucleotide insertion at nucleotide 198 results in the introduction of a premature stop codon at codon 119. Northern analysis was performed on these four cell lines and demonstrated *CDKN2A* RNA expression for all but MM426 (Fig. 4, Table 4, and data not shown). The reason for this lack of *CDKN2A* mRNA expression is unclear because this cell line is not methylated (results not shown). It has been suggested that loss of *CDKN2A* exon 3 sequences could lead to a higher instability both of the protein and the mRNA (41). Moreover, Nakagawa *et al.* (42) showed that four non-small cell lung carcinoma cell lines had detectable *CDKN2A* mRNA by RT-PCR but not by Northern blot, and three of four of these cell lines carried nonsense mutations, suggesting that *CDKN2A* mRNA containing some nonsense mutations may be more rapidly degraded.

MM548, which carries an in-frame 1 amino acid deletion at codon 99, expressed mRNA but did not express p16 protein (Fig. 3 and Table 4). It is not clear why this mutation would result in loss of p16 expression; however, the deletion of a single amino acid may lead to a conformational change resulting in a highly unstable protein. AF-6 carries a tandem CC→TT mutation, and neither p16 nor *CDKN2A* mRNA expression was detected, presumably due to the disruption of the consensus splice site sequence. C-32 does not express detectable p16 or *CDKN2A* mRNA; however, further experimentation is needed to determine whether this lack of expression is due to possible RNA instability from the addition of an extra 13 amino acids at the COOH-terminal end of the protein or to unrelated transcriptional silencing of the promoter via methylation, mutation, or rearrangement.

Cell Lines Carrying Wild-Type *CDKN2A* but not Expressing p16 Protein

Of the 19 cell lines carrying at least one wild-type *CDKN2A* allele by sequencing, 8 did not express p16 by Western blot analysis (Fig. 3 and Table 4). Northern blotting revealed that one of eight cell lines (MM647) expressed an aberrant mRNA (~3.5 kb) for *CDKN2A* (Fig. 4). Splice donor and acceptor sites have been sequenced for all *CDKN2A* exons in this cell line and appear to be wild-type (data not shown). One possible hypothesis for this aberrant message is partial intron retention due to the generation of an intronic splice acceptor site. The analysis herein could not determine whether this was indeed the case. Microsatellite analysis in MM647 indicated heterozygosity for all but one of the markers surrounding the *CDKN2A* locus; therefore, the absence of a wild-type transcript indicates that more than one mechanism of *CDKN2A* inactivation is potentially operating in this cell line (Table 3).

Another two of eight cell lines (MM383 and MM540) did not express mRNA for *CDKN2A* (Fig. 4 and Table 4). The *CDKN2A* promoter was analyzed for methylation in these cell lines, because this has been reported as a possible mechanism of *CDKN2A* inactivation in some human cancers (43–45). The presence of a methylated 5′ CpG island encompassing exon 1α of *CDKN2A* was assessed according to Merlo *et al.* (45) by double digestion with *Eco*RI and the methylation-sensitive restriction enzyme *Sac*II. The loss of a 4.3-kb *Eco*RI restriction fragment after digestion with *Sac*II indicated that MM383 was not methylated at this site. Other mechanisms of transcriptional silencing potentially operating in MM383 are promoter sequence mutations, mRNA instability, or aberrations of regulatory factors. Other investigators have reported absence of *CDKN2A* mRNA in otherwise wild-type cell lines that have not undergone

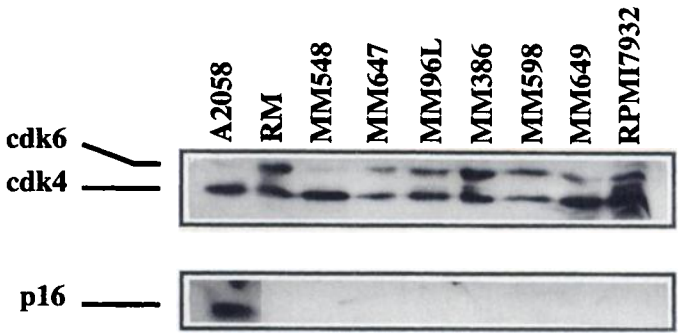

Fig. 3. Western blot analysis of cell lines carrying either a *CDKN2A* mutation or a wild-type gene and not expressing p16 protein. Equal quantity of cell lysates were loaded onto minigel lanes and probed with anti-p16, anti-CDK4, and anti-CDK6 antibodies.

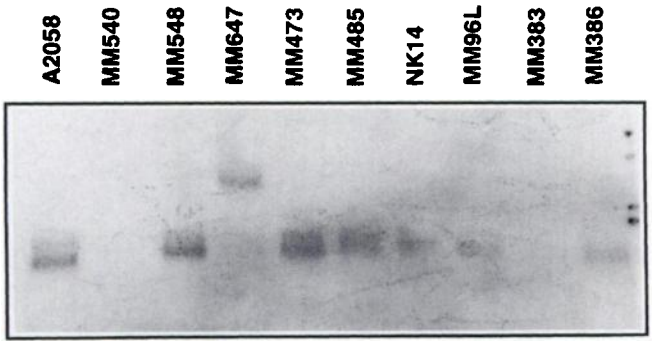

Fig. 4. A representative Northern blot of melanoma cell lines. All cell lines expressed normal *CDKN2A* mRNA, except for MM647, which expressed an aberrantly sized variant, and MM540 and MM383, which expressed no transcripts.

methylation of 5′ CpG islands (34, 46), and Goldstein *et al.* (47) have reported an uncharacterized mutation that prevents transcription of one *CDKN2A* allele in a melanoma kindred. MM540 could not be tested for potential CpG island methylation because repeated attempts at DNA digestion failed due to the very high melanin content in this cell line.

Of the remaining five of eight cell lines studied that did not express p16 (MM96L, MM386, MM598, MM649, and RPMI7932), all expressed *CDKN2A* mRNA (Fig. 4, Table 4, and data not shown) as assessed by probing with a total cDNA probe, suggesting the possibility of a posttranscriptional mechanism of inactivation. Because the potential existed that the transcript observed with the total cDNA probe was due to the alternative transcript containing exon 1β, the *CDKN2A* promoter was analyzed for methylation in these five lines as described above (45), and all were found to be methylated (data not shown). These results suggest that in these cell lines, the *CDKN2A* message detected by Northern analysis is the alternative transcript of *CDKN2A* containing exon 1β, although exon-specific hybridization to the Northern blots is needed to formally prove this hypothesis. Because several other investigators have indicated potential posttranscriptional silencing of *CDKN2A* by reporting the existence of cell lines that express *CDKN2A* mRNA but which are negative for p16 (48–52), our findings raise the possibility that the true frequency of promoter methylation has been underestimated due to the comigration of the two *CDKN2A* transcripts and their cross-hybridization, resulting from the common exon 2 sequences.

Cell Lines Carrying Wild-Type *CDKN2A* and Expressing Functional p16 Protein

The remaining 11 cell lines expressed a functional p16 protein, as assayed by p16 immunoprecipitation and subsequent Western blotting to detect the coprecipitation of CDK4 or CDK6 (Fig. 2, Table 4, and data not shown). It is interesting to note that 5 of 11 of these functionally wild-type cell lines demonstrated LOH of the region surrounding *CDKN2A*. Reed *et al.* (53) have suggested that haploinsufficiency of *CDKN2A* is enough to initiate melanoma tumorigenesis; however, the relevance of this LOH to the *in vivo* formation of the primary melanomas from which these cell lines were derived is unknown, because matched tumor material was not available for comparison. Another possibility is that the 9p LOH in these five cell lines in the absence of *CDKN2A* inactivation indicates the existence of another tumor suppressor gene in the region.

In summary, this study has demonstrated some form of *CDKN2A* aberration in 54 of 60 (90%) cell lines, suggesting that inactivation of p16 plays a primary role in the development of melanoma. The p16 protein affects the pRb-regulated cell cycle pathway by influencing its phosphorylation by CDK4/cyclin D complexes; therefore, an inactive or absent p16 fails to inhibit CDK4 and allows unrestrained pRb phosphorylation and release of transcription factors involved in cell cycle progression. An exhaustive search for alterations in other members of this growth regulatory pathway may reveal that aberration of this pathway by any mechanism is an essential step for melanoma tumorigenesis to occur (or at least the *in vitro* propagation of a melanoma cell line), a hypothesis for which there is already mounting evidence (54, 55). To this end, exon 2 of the *CDK4* gene was sequenced in all cell lines to detect the previously characterized R24C mutation (31). SK-MEL-28, a cell line carrying a functional p16 and exhibiting no LOH on 9p21, was the only line found to carry the R24C mutation (Fig. 1*H*). As overexpression of CDK4 would also lead to unrestrained pRb phosphorylation, amplification of *CDK4* was investigated in 9 of 11 of these cell lines by Southern blotting. Although none exhibited an increase in the copy number of this gene compared to the control probe (data not shown), *CDK4* overexpression at the RNA or protein level was not examined and represents an additional mechanism by which this pathway could be compromised (17). Overexpression of the *cyclin D1* gene or inactivation of pRb both represent further mechanisms by which this cell cycle control pathway could be abrogated (17, 54). It is also possible that some of these cell lines have acquired mutations in genes that are involved in other growth-regulatory pathways, *e.g.*, p53 (15, 56) or $p21^{ras}$ (57).

In conclusion, this study has demonstrated *CDKN2A* or *CDK4* aberrations in up to 55 of 60 (92%) of the melanoma cell lines examined. Specifically, 48 of 60 (80%) of cell lines were found to have an absent or nonfunctional p16; 1 of 60 carried a heterozygous *CDKN2A* mutation and expressed a functional p16; 5 of 60 (8%) were shown to have LOH of the region surrounding wild-type *CDKN2A*; and 1 of 60 carried an R24C CDK4 mutation. These data support the hypothesis that aberration of this p16/CDK4/pRb pathway is an essential step for melanoma tumorigenesis to occur, with the caveat that concordance of this data with that derived directly from uncultured melanoma tissue is necessary to evaluate any selective factors arising from tissue culture. We have also shown the benefits of examining *CDKN2A*/p16 status at the DNA, RNA, and protein levels to both identify the maximum number of *CDKN2A*/p16 aberrations and determine the mechanisms of inactivation. Some reports have interpreted the absence of protein to represent homozygous deletions and the presence of RNA as evidence for p16 expression; however, this study provides evidence that this is clearly not always the case.

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