ORIGINAL ARTICLES

Mutation and homozygous deletion analyses of genes that control the G1/S transition of the cell cycle in skin melanoma: p53, p21, p16 and p15

José Luis Soto Martínez[a], Carmen M. Cabrera Morales[a], Sabio Serrano Ortega[b] and Miguel Ángel López-Nevot[a]

[a]Servicio de Análisis Clínicos e Inmunología. Hospital Universitario Virgen de las Nieves. Universidad de Granada. Granada. Spain. [b]Servicio de Dermatología. Hospital Universitario San Cecilio. Granada. Spain.

Introduction. **The role of genes involved in the control of progression from the G1 to the S phase of the cell cycle in melanoma tumors is not fully known.**

Material and methods. **The aims of our study were to analyse alterations in p53, p21, p16 and p15 genes in melanoma tumors and melanoma cell lines by single strand conformational polymorphism (SSCP), and to detect homozygous deletions. We analysed the DNA from 39 patients with primary and metastatic melanomas, and from 9 melanoma cell lines.**

Results. **The SSCP technique showed heterozygous defects in the p53 gene in 8 of 39 (20.5%) melanoma tumors: three point mutations in intron sequences (introns 1 and 2) and exon 10, and three new polymorphisms located in introns 1 and 2 (C to T transition at position 11701 in intron 1; C insertion at position 11818 in intron 2; and C insertion at position 11875 in intron 2). One melanoma tumor exhibited two heterozygous alterations in the p16 exon 1 (stop codon and missense mutation). No defects were found in the remaining genes. Homozygous deletions were more frequent in melanoma cell lines than in melanoma tumors in p21, p16 and p15 (22.2%, 44.4%, and 44.4% versus 7.7%, 2.5%, and 5.1% respectively). TP53 did not show homozygous deletions.**

Conclusions. **Our results suggest that these genes are involved in melanoma tumorigenesis; but perhaps not in the major targets. Other suppressor genes that may be informative of the mechanism of tumorigenesis in skin melanomas need to be studied.**

Key words: skin melanoma, single strand conformational polymorphism (SSCP), p53, p21, p16, p15.

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INTRODUCTION

The transition from phase G1 to S of the cell cycle is controlled by sequential activation of cyclin/Cdk complexes (cyclin-dependent kinases)[1]. Active cyclin/Cdk complexes phosphorylate and inactivate members of the retinoblastoma protein (Rb) family, which are negative regulators of G1 and S-phase progression, leading to the induction of E2F-regulated gene expression and cell proliferation. Inhibitors of cyclin/Cdk complexes, by binding to these complexes, negatively regulate cell cycle progression[2].

Two families of Cdk-inhibitors (CKI) control the actions mediated by cyclin/Cdk complexes. p21 (also called WAF1)[3,4] is the founding member of the Cip/Kip family of CKI, which also includes p27[5] and p57[6]. Another class of Cdk inhibitors, the so-called INK4 proteins (named for their ability to inhibit cdk4), specifically target the cyclin D-dependent kinases[7]. To date, four INK4 proteins have been identified: the founding member p16 (INK4a)[8], and three other closely related genes designated p15 (INK4b)[9], p18 (INK4c)[10], and p19(INK4d)[10].

In response to irradiation and chemotherapy, p53 protein is stabilised and mediates apoptosis and cell cycle arrest. Whereas the mechanisms of p53-dependent apoptosis are not well understood, p53-dependent cell cycle arrest is known to be primarily

Correspondence: Dra. Carmen M. Cabrera.
Servicio de Análisis Clínicos e Inmunología.
Hospital Universitario Virgen de las Nieves.
Avenida Fuerzas Armadas N°2.
18014 Granada. Spain.
E-mail: mcabrm@fundacionhvn.org

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mediated by p21, a potent inhibitor of cyclin-dependent kinases that is transactivated by p53 and p73[11]. In addition to p21, several other cell cycle regulators are induced by p53, such as GADD45 and members of the 14-3-3 family[12].

The TP53 suppressor gene and Cdk-inhibitors such as p16, p15 and p21 are targets of tumoral process in different types of tumors[13-17]. Mutations in the p53 gene occur frequently in skin tumors such as basal cell carcinoma (BCC) and squamous cell carcinoma (SCC)[18,19]. In human melanoma, p53 mutations are apparently not commonly detected[20-22], and consist mainly of C to T transitions located on dipyrimidine sites originated by UV radiation[23]. In contrast, p16 is deleted or mutated in human sporadic melanomas and derived cell lines[24], and it appears to be the predisposing mutation in some familial melanoma kindreds[25]. A low incidence of mutations has been described for the p15 gene in sporadic melanoma tumors[26]; however, no structural defects have been detected in the p21 gene in human melanoma.

In order to investigate the role of the genes involved in the control of G1/S phase cell cycle progression in human melanomas, the aim of our study was to determine the presence of mutations and homozygous deletions in p53, p21, p15 and p16 genes in primary and metastatic melanomas and melanoma cell lines.

MATERIAL AND METHODS

Tumor samples

Thirty-nine specimens of skin melanoma were obtained from the Department of Surgery at the Hospital Universitario San Cecilio of Granada, Spain (table 1). Melanoma tumors were dissected from normal tissues in fresh samples under sterile conditions, and tumor tissues were frozen in liquid nitrogen and stored at -80°C until DNA isolation. DNA was obtained from peripheral blood from each patient. The following 9 melanoma cell lines were included in this study: MZ2-MEL, MEL-3.0, MEL-2.2, and Mi-13443 were provided by Dr. T. Boon (Ludwig Institute of Cancer Research, Brussels, Belgium); and M31-L, M42-L, M52-L, M34-L, and M59-L were established in our laboratory as described previously[27]. The clinical and pathological characteristics of primary melanoma tumors and derived metastases are described in table 1. Of the 39 tumors studied, 14 were primary (36%) and the rest were metastatic (18 lymph node metastases and 4 subcutaneous metastases).

DNA isolation

DNA was isolated from tumor samples and peripheral blood lymphocytes with the MicroTurboGen Genomic DNA Isolation Kit (Invitrogen, San Diego, California) and the "Quiagen" kit (Wetsburg, Leusden, The Netherlands) respectively.

Mutation analysis of p53, p21, p16 and p15 genes

Point mutations were detected by changes in single-stranded conformational polymorphism (SSCP) of DNA amplified by polymerase chain reaction (PCR), as described by Orita et al[28] with slight modifications[29]. TP53 exons 2-11, p21 exon 2, p15 exon 2, and p16 exons 1-2 were amplified. The sequences of the primers used and fragments (bp) amplified are shown in table 2. All p53 primers used were provided by Clontech (Human p53 Amplier Panels, Palo Alto, CA). A portion of p53 intron 1, exon 2, intron 2 and exon 3 was amplified using the primers PU2 (sense) and PD3 (antisense). p21 exon 2 was amplified in two overlapping fragments with the following primer pairs: p21-L1/p21-R1 and p21-L2/p21-R2 (table 2).

Amplifications were performed with 100 ng genomic DNA and α^{32}P-dCTP (300 Ci/mmol) in a final volume of 25 µl. The PCR conditions for p53 exons 2-11 were as follows: 35 cycles at 95°C/30 s, 66°C/45 s and 72°C/1.5 min, with a 10-min extension after the last cycle. p21 exon 2, p15 exon 2, and p16 exons 1-2 were amplified under the same PCR conditions: in a touchdown PCR procedure the temperature of the reaction was lowered by 1°C every second cycle from 68°C to 60°C, at which temperature 30 cycles were carried out.

Amplified samples (2.5 µl) were mixed with 9 µl of sequencing stop solution (USB, Cleveland, OH, USA), 1.5 µl of 0.08N NaOH, and 15 µl of 0.1% SDS, denatured for 10 min at 95°C, and the samples were quickly cooled in dry ice. Samples of 3 µl were loaded onto a 6% non-denaturing acrylamide gel containing 10% glycerol, and run at room temperature for 4 h at 22 W. Gels were dried at 80°C under a vacuum and exposed to x-ray films for 4-16 h.

DNA sequencing

Asymmetric PCR reactions were purified from agarose gels and reamplified. PCR products were cloned in the PCR 4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Groningen, The Netherlands). After transformation several clones were picked and sequenced. Sequence analysis was carried out with the Sequenase DNA Sequencing kit (USB), using α^{35}S-dATP (DuPont-NEN, Boston, MA) incorporation. Aliquots of the reaction mixture were run on a 6% denaturing acrylamide gel.

Homozygous deletion analysis

Homozygous deletions in the p53, p21, p15 and p16 genes were detected by PCR analysis of genomic DNA according to the conditions and primers reported above.

TABLE 1. **Melanoma tumor samples**

Tumor	Histopathology	Breslow (mm)*	Clark	Tumor sample
M3	SSM	5.5	IV	P
M4a	NM	-	-	Sm
M4b	NM	-	-	Sm
M5	-	-	-	P
M6	-	-	-	Nm
M7a	SSM	4	IV	Nm
M7b	SSM	4	IV	Sm
M8	SSM	0.5	II	P
M13	SSM	3.9	III	P
M18	NM	5	V	Nm
M19	-	-	-	Nm
M21	SSM	3.5	III	P
M22	-	-	-	Nm
M23	SSM	9	IV	Nm
M24	ALM	-	-	Nm
M31	SSM	3	IV	Nm
M32	-	-	-	Nm
M34	SSM	16	V	P
M37	SSM	1.8	III	Nm
M38	NM	9	V	P
M40	NM	3.4	IV	Nm
M42	NM	1.5	IV	Nm
M43	SSM	2.5	IV	P
M44a	NM	10	IV	P
M44b	NM	10	IV	Sm
M45	-	-	-	Nm
M46	-	-	-	P
M49	-	-	-	P
M50	-	-	-	Nm
M52	LMM	-	-	Nm
M53	-	-	-	Nm
M55	ALM	-	V	Nm
M56	LMM	1	III	P
M59	NM	10.1	III	P
M60	SSM	3	III	P
M71	NM	-	-	P
M72	SSM	0.6	III	P
M73	SSM	2.5	III	P
M74	-	-	-	Nm

SSM: superficial spreading melanoma; NM: nodular melanoma; LMM: lentigo maligna melanoma; ALM: acral lentigo melanoma; P: primary melanoma; Nm: lymph node metastases; Sm: subcutaneous metastases. *Breslow vertical tumor thickness.

To confirm the homozygous deletions, multiple PCRs from tumoral DNA were compared with DNA from the patient's peripheral blood lymphocytes (PBLs).

RESULTS

Intronic polymorphisms and heterozygous point mutations in the p53 gene

Of a total of 39 melanoma tumors and 9 melanoma cell lines studied by PCR-SSCP, we detected defects in the p53 gene in 8 of 39 (20.5%) melanoma tumors, and did not find any alterations in melanoma cell lines (table 3). Mutation analysis showed three heterozygous single point mutations in the p53 sequence and four different polymorphisms, three of which have not been described to date.

The G to C transversion found at position 11827 in p53 intron 2 in M4, M7, M38 and M53 melanoma tumors was previously described by Oliva et al[30] (fig. 1). In this study we found 3 new polymorphisms located at intron 1 and 2 of the p53 gene when we compared genomic DNA from melanoma tumors and DNA from autologous PBLs with control PBLs (fig. 1). The C to T transition was found at position 11701 of p53 intron 1 in melanoma tumors M53 and M71 (fig. 2); a C insertion was found at position 11818 of p53 intron 2 in melanoma tumors M7, M42, M53 and M71; and a C insertion was found at position 11875 of p53 intron 2 in melanoma tumors M7, M38, M42, M53 and M71 (table 3).

A heterozygous C deletion in p53 exon 10 at position 172628 produced a stop codon and truncated the p53 protein in melanoma tumor M34 (fig. 3). Melanoma

TABLE 2. **Oligonucleotide primer sequences**

Exon	Primer	Sequence 5'→3'	Fragment (bp)
TP53, exons 2 to 11			
2-3	PU2 (sense)	TCCTCTTGCAGCAGCCAGACTGC	265
	PD3 (antisense)	AACCCTTGTCCTTACCAGAACGTTG	
4	PU4 (sense)	CACCCATCTACAGTCCCCCTTGC	307
	PD4 (antisense)	CTCAGGGCAACTGACCGTGCAAG	
5	PU5 (sense)	CTCTTCCTACAGTACTCCCCTGC	211
	PD5 (antisense)	GCCCCAGCTGCTCACCATCGCTA	
6	PU6 (sense)	GATTGCTCTTAGGTCTGGCCCCTC	182
	PD6 (antisense)	GGCCACTGACAACCACCCTTAACC	
7	PU7 (sense)	GTGTTATCTCCTAGGTTGGCTCTG	139
	PD7 (antisense)	CAAGTGGCTCCTGACCTGGAGTC	
8	PU8 (sense)	ACCTGATTTCCTTACTGCCTCTTGC	200
	PD8 (antisense)	GTCCTGCTTGCTTACCTCGCTTAC	
9	PU9 (sense)	GCCTCTTTCCTAGCACTGCCCAAC	102
	PD9 (antisense)	CCCAAGACTTAGTACCTGAAGGGTG	
10	PU10 (sense)	TGTTGCTGCAGATCCGTGGGCGT	130
	PD10 (antisense)	GAGGTCACTCACCTGGAGTGAGC	
11	PU11 (sense)	TGTGATGTCATCTCTCCTCCCTGC	153
	PD11 (antisense)	GGCTGTCAGTGGGGAACAAGAAGT	
p21, exon 2			
	p21-L1 (sense)	GATGTCCGTCAGAACCCATG	258
	p21-R1 (antisense)	TGCCTCCTCCCAACTCAT	
	p21-L2 (sense)	ATGAGTTGGGAGGAGGCA	181
	p21-R2 (antisense)	ATGCTGGTCTGCCGCCGTT	
p16, exons 1 and 2			
1	MTS1-L1 (sense)	GAAGAAAGAGGAGGGGCTG	340
	MTS1R1 (antisense)	GCGCTACCTGATTCCAATTC	
2	p16-L2 (sense)	GTCATGATGATGGGCAGC	307
	p16-R2 (antisense)	CTGAGGGACCTTCCGCG	
p15, exon 2			
	MTS2-L2 (sense)	TAAGTTTAACCTGAAGGTGG	500
	MTS2-R (antisense)	GGGTGGGAAATTGGGTAAG	

tumors M42 and M43 showed heterozygous point mutations at p53 introns 1 and 2. The C to T transition at position 11701 in intron l, observed in melanoma tumor M42, contrasted with the absence of this transition in autologous PBLs. In contrast, a C insertion was found at position 11818 in intron 2 of p53 in autologous PBLs compared with control PBLs, but this mutation was not seen in melanoma tumor M43.

Mutation analysis of p16, p21 and p15 cyclin-Cdk inhibitors

Only two heterozygous alterations in p16 exon 1 were observed in melanoma tumor M13, whereas no defects were seen in the p21 and p15 genes. The G to A transition produced a stop codon at position 149 (fig. 4) and T to C transition at position 298 resulted in substitution of proline for leucine (table 3).

Homozygous deletions in the genes that govern G1 to S progression

PCR analysis of the p53 gene did not show homozygous deletions in melanoma tumors or melanoma cell lines. Studies of the Cdk inhibitor genes (p21, p16 and p15) revealed a higher incidence of homozygous deletions in melanoma cell lines than in melanoma tumors (table 4). In tumors we found 7.7%, 2.5% and 5.1% homozygous deletion in p21, p16 and p15 respectively, in comparison to 22.2%, 44.4% and 44.4% deletion in melanoma cell lines.

DISCUSSION

Polymorphisms versus mutations in the p53 gene in human melanoma

The major carcinogenic agent in most skin cancers is well established as solar ultraviolet light[31]. This is absorbed in DNA, with the formation of UV-specific dipyrimidine photoproducts. About 50% of all skin cancers exhibit p53 mutations[23], and these mutations are characterised by a specific signature attributed to the UVB part of the solar spectrum. The impact of UVB radiation can be clearly inferred from the characteristic point mutations in p53 found in human SCC and BCC, consisting of C to T or CC to TT transitions at dipyrimidine sites[32].

These findings contrast with the situation in human melanomas, in which p53 mutations are not com-

TABLE 3. **Polymorphisms (P) and heterozygous point mutations (M) in p53, p21, p16 and p15 genes**

Tumor	
TP53	
M4	G→C transversion at 11827 (intron 2) (P)
	C insertion at 11875 (intron 2) (P)
M7	G→C transversion at 11827 (intron 2) (P)
	C insertion at 11818 (intron 2) (P)
	C insertion at 11875 (intron 2) (P)
M38	G→C transversion at 11827 (intron 2) (P)
	C insertion at 11875 (intron 2) (P)
M42	C→T transition at 11701 (intron 1) (M)
	C insertion at 11818 (intron 2) (P)
	C insertion at 11875 (intron 2) (P)
M43	C insertion at 11875 (intron 2) (P)
	C deletion at 11818 (intron 2) (M)
M53	C→T transition at 11701 (intron 1) (P)
	G→C transversion at 11827 (intron 2) (P)
	C insertion at 11818 (intron 2) (P)
	C insertion at 11875 (intron 2) (P)
M71	C→T transition at 11701 (intron 1) (P)
	C insertion at 11818 (intron 2) (P)
	C insertion at 11875 (intron 2) (P)
M34	C deletion at 17628 (codon 350: CTC→CTA, exon 10), stop codon at the beginning of exon 11 (M)
p16, exon 1	
M13	G→A transition at 149, TGG (Trp)→TGA (stop codon) (M)
	T→C transition at 298, CTC (Leu)→CCC (Pro) (M)
p21, and p15	
	No alterations found

PU2 11701 Exon 2
TCC TCT TGC AGC AGC CAG ACT GCC* ttc cgg gtc act gcc atg gag gag ccg cag tca gat cct
T

agc gtc gag ccc cct ctg agt cag gaa aca ttt tca gac cta tgg aaa ct g tga gtg gat cca ttg gaa ggg

11818 11827 11875
cag gcC* acc acc ccG* acc cca acc cca gcc ccc tag cag aga cct gtg gga agc gaa aat tcA* tgg
insC C insC

Exon 3 PD3
gac tga ctt tct gct ctt gtc ttt cag act tcc tga aaa CAA CGT TCT G GT AAG GAC AAG GGT T

Fig. 1. Polymorphisms detected in intron 1 and intron 2 sequences of the p53 gene in melanoma tumors. Each polymorphism and its positions are indicated by an asterisk (*), to determinate the presence of the polymorphisms the DNA sequence from melanoma tumors and autologous peripheral blood lymphocytes (PBLs) were compared with control PBLs.

monly detected. The results of the present study support earlier findings that such mutations are indeed infrequent in this type of tumor. The influence of UVB radiation in these mutations is not clear. p53 mutations in primary melanoma tumors induced by UVB radiation have been described previously by Zerp et al[22]. However, in the tumors examined in our study we did not find p53 alterations originated by UVB radiation (table 3). In contrast, we detected two mutations located in intronic sequences (C deletion at position 11818 in intron 2, and C to T transition at position 11701 in intron 1) (table 3) and C deletion at codon 350 of p53 exon 10, which produced a stop codon and truncated protein. More than 90% of the mutations reported in non-melanoma skin cancers and different types of tumor are clustered between exons 4 and 8[33]. This region is highly conserved throughout evolution and contains the DNA-binding domain of p53, which is essential for its activity[34]. This contrasts with the trans-activation domain (encoded by exons 2 and 3) and the regulatory region (encoded by exons 9 to 11), where few mutations have been described. Unlike other tumor suppressor genes, such as Rb an adenomatous polyposis coli (APC) which are frequently inactivated either by frameshift mutations or by gene deletion, 90% of p53 alterations are missense mutations, which lead to the accumulation of mutant p53 protein in tumor cells. This selection for point

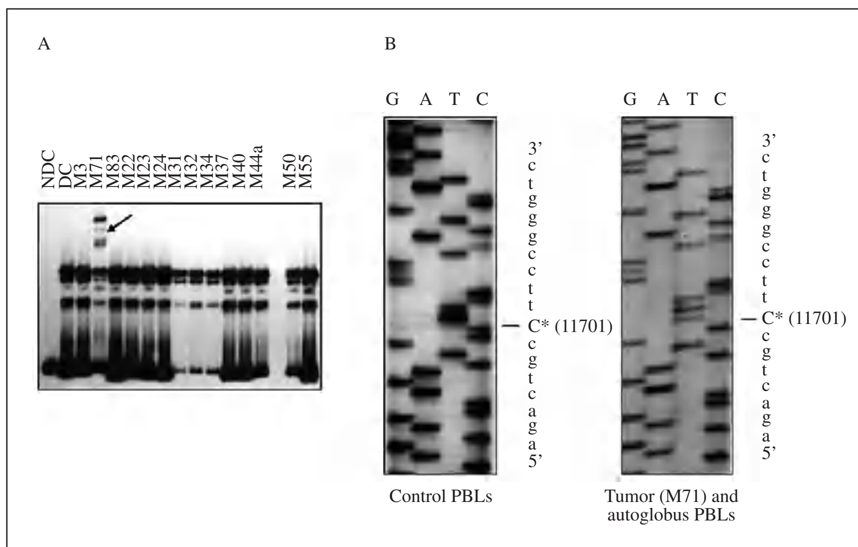

Fig. 2. New polymorphism found in intron 1 of the p53 gene (C to T transition) at position 11701. (A) Polymerase chain reaction-single stranded conformational polymorphism (PCR-SSCP) analysis of melanoma tumors. The arrow indicates the shifted band in melanoma tumor M71. DC: denaturing control; NDC: non-denaturing control. (B) DNA sequence of melanoma tumor M71 and autologous peripheral blood lymphocytes (PBLs) showing the C to T base change (shown by an asterisk *) at position 11701 compared with control PBLs.

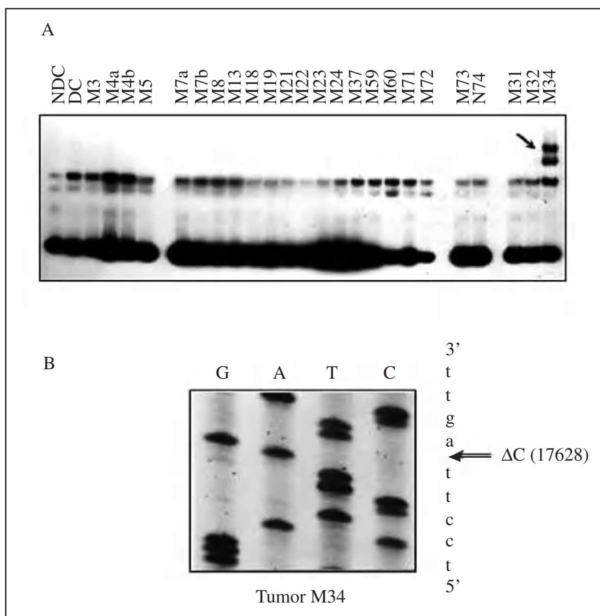

Fig. 3. (A) Autoradiograph of single stranded conformational polymorphism (SSCP) analysis of p53 exon 10 in melanoma tumors. Tumor M34 presented a shifted band (indicated by an arrow). DC: denaturing control; NDC: non-denaturing control. (B) Nucleotide sequence analysis of melanoma tumor M34 showed a heterozygous C deletion at position 17628 (indicated by an arrow).

mutations suggests that mutant p53 is not completely inactive and may have a dominant oncogenic function in the development of the tumor. Therefore, the p53 polymorphisms detected in these melanoma tumors appear to be their most frequent characteristic.

At least 12 intronic p53 polymorphisms have been described in the human p53 gene. These include between others a variable number tandem repeat (VNTR) region[35] and *Hae*III restriction fragment length polymorphism (RFLP)[36] in intron 1, a G to C transversion in intron 2[30], a 16-bp duplication in intron 3 (5'-gacctggagggctggg-3')[37], a *Msp*I RFLP in intron 6 (G to A transition 61 bp downstream from exon 6)[38,39], a G to C transversion 37 bp upstream to exon 7[40], an *Apa*I RFLP in intron 7[41], and A to T transversion in intron 10 (http://www.iarc.fr/p53/polymorphisms)[42]. The melanoma tumors and melanoma cell lines studied here showed the G to C transversion at position 11827 of p53 intron 2, previously described by Oliva et al[30] in four melanoma tumors (M4, M7, M38, and M53), and three new polymorphisms: C to T transition at position 11701 of p53 intron 1; C insertion at position 11818 of p53 intron 2; and C insertion at position 11875 of p53 intron 2 (fig. 1). Moreover, we found three mutations in the p53 gene (table 3), the incidence of mutations detected in the p53 gene accounted for only 7.7% (3 of 39 melanoma tumors) in contrast to 18% (7 of 39 melanoma tumors) of polymorphisms found in these tumors.

Associations between cancer phenotypes and inherited p53 intronic polymorphisms have been observed in studies of epithelial cancers including ovarian, breast, colon, thyroid, nasopharyngeal, and lung cancer[39,40,43-46]. The frequency of G to C transition at position 11827 in intron 2 of the p53 gene (3 of 39 melanoma tumors, 7.7%) is low compared to the frequency of the A1 allele (G to C transition at position 11827) described previously in Caucasian individuals[47]. The polymorphisms that we detected in these melanoma tumors may play a role in the risk of developing skin melanoma in these patients.

Alterations in cyclin-Cdk inhibitors: p16, p15 and p21

Our results revealed the low incidence of mutations in cyclin-Cdk inhibitors in both melanoma tumors and melanoma cell lines. We detected only two mu-

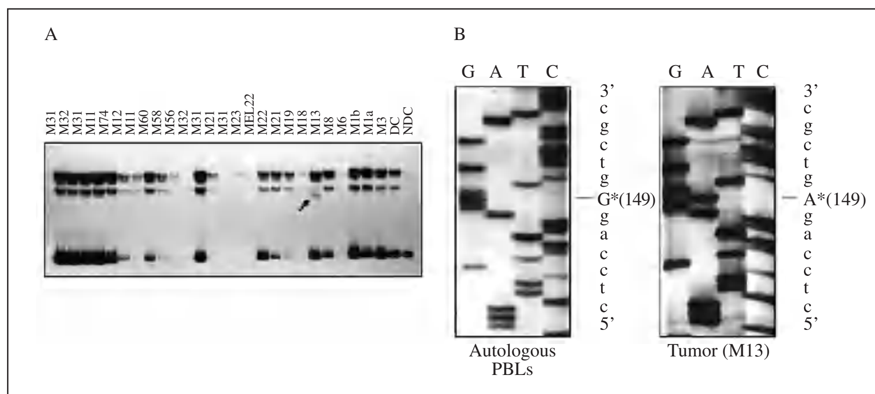

Fig. 4. Analysis of G to A transition at exon 1 of p16 in melanoma tumor M13. (A) Polymerase chain reaction-single stranded conformational polymorphism (PCR-SSCP) of melanoma tumors and melanoma cell line showing the shifted band (indicated by an arrow) in melanoma tumor M13. DC: denaturing control; NDC: non-denaturing control. (B) Sequence analysis of tumor M13 compared with its autologous peripheral blood lymphocytes (PBLs). The heterozygous G to A transition at position 149 is indicated by an asterisk (*).

TABLE 4. **Homozygous deletions in p53, p21, p16 and p15 genes**

	p53	p21	p16	p15
Tumors	0/39 (0%)	3/39 (7.7%)	1/39 (2.5%)	2/39 (5.1%)
M21		exon 2		
M32		exon 2	exons 1-2	exon 2
M34		exon 2		exon 2
Cell lines	0/9 (0%)	2/9 (22.2%)	4/9 (44.4%)	4/9 (44.4%)
MZ2-MEL			exon 1	
MEL-3.0			exon 1	exon 2
MEL-2.2			exon 1	exon 2
M34-L		exon 2	exon 1	exon 2
M59-L		exon 2		exon 2

tations in exon 1 of the p16 gene, both in melanoma tumor M13: G to A transition at position 149 producing a stop codon, and a missense mutation Leu^{298} (leucine → proline). The incidence of homozygous deletions of the p21, p16 and p15 genes was higher in melanoma cell lines (22.2%, 44.4% and 44.4% respectively) than in melanoma tumors (7.7%, 2.5% and 5.1% respectively).

The low incidence of p16 mutations found in primary melanomas is concordant with previous reports[48,49]. In contrast, melanoma cell lines show a high incidence of mutations in p16, with homozygous deletion being the main mechanism of alteration[48]. The presence of germline mutations in approximately 40% of melanoma-prone families confirms p16 as a major gene involved in melanoma predisposition. Homozygous deletions and mutations in p16 have been reported in cell lines derived from a wide variety of tumor types[7,50]; however, the frequency of aberrations detected in unculturated tumors is much lower, even in those tumors displaying loss of heterozygosity (LOH) on chromosome 9p21-22[51]. Because p16 has been shown to play a role in replicative senescence[52], it is likely that a proportion of aberrations are late-stage events that are selected by cell culture. These results suggest that in sporadic melanoma tumors, p16 might not be a target of mutation in contrast to familial melanoma. Only 0.2%-2% of sporadic-melanoma patients harbour inactivating germline p16 mutations, most of these germline and sporadic mutations have been shown to affect p16 protein function as CDK4 binding or Rb phosphorylation[53].

p16 and p15 genes are localised in the same chromosome region: 9p21. Previous studies have reported a low frequency of homozygous codeletion of both genes[26,54]. The melanoma tumors and melanoma cell lines analysed in this study showed homozygous codeletion of p15 and p16 in only one melanoma tumor (M32) and three melanoma cell lines (MEL-3.0, MEL-2.2 and M34-L), with frequencies similar to those described previously in melanoma tumors[26,54]. The 9p21 locus has a complex genomic organization encoding two different tumor suppressor proteins, p16 and p14 (also known as ARF). The two products of the CDKN2A locus negatively regulate the Rb and p53 pathways, and their loss predisposes to the development of melanoma. ARF inhibits MDM2-mediated ubiquitylation and subsequent degradation of p53. p16 controls the G1/S transition by inhibition of Rb hyperphosphorylation mediated by CDK4/6-cyclin-D complex. CDK4 mutations abrogate the interaction with p16, decreasing the selective pressure for p16 loss and p53 mutations[24].

The p21 Cdk-inhibitor gene is located at 6q21.2, and its expression has been shown to be regulated largely at the transcriptional level by both p53-dependent and -independent mechanisms by a variety of trans-

cription factors that are induced by a number of different signaling pathways[55]. p21 gene mutations appear to be an uncommon event not detected to date. However, p21 may be involved in melanoma tumorigenesis, as suggested by the frequency of homozygous deletion that we found in melanoma tumors (7.7%) and melanoma cell lines (22.2%).

We conclude that although none of the cell cycle regulators analysed here can be singled out as a main target for melanoma tumorigenesis, G1/S checkpoint defects are one of the significant factors in the development of melanoma tumors. However, this influence appear to be low in tumors, unlike the situation in melanoma cell lines. Other suppressor genes will be investigated to identify the main targets in the pathogenesis of melanoma.

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