Significant Impact of Promoter Hypermethylation and the 540 C>T Polymorphism of *CDKN2A* in Cutaneous Melanoma of the Vertical Growth Phase

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Promoter hypermethylation, mutations, and loss of heterozygosity in the *CDKN2A* gene as well as polymorphisms at the 3'-untranslated region were determined in vertical growth phase melanomas. Methylation-specific polymerase chain reaction *in soluti* and *in situ* showed that 19% of the cases were hypermethylated at the *CDKN2A* promoter region, and some of these cases were heterogeneous with both methylated and unmethylated tumor cells. Methylation was associated with increased tumor cell proliferation by Ki-67 expression ($P = 0.01$) and decreased patient survival ($P = 0.025$). Point mutations in *CDKN2A* were found in 4% of the cases, whereas 90% had loss of heterozygosity at one or more of 4 markers studied. Furthermore, presence of the 540 C>T polymorphism at the 3'-untranslated region of *CDKN2A* (23%) was associated with improved survival in multivariate analysis (hazard ratio, 2.6; $P = 0.02$). Our results suggest that promoter methylation of the *CDKN2A* gene is present in a subgroup of the tumors and associated with increased tumor cell proliferation and reduced survival. Further, the 540 C>T polymorphism might define a distinct subgroup of low-grade vertical growth phase melanomas. These findings support a significant role of the *CDKN2A* gene in melanoma progression. (*Am J Pathol* 2002; 161:229–237)

The tumor suppressor gene *CDKN2A* (*p16*), which is located at chromosome 9p21¹ and encodes two cell cycle inhibitors, p16^{INK4a} and p14^{ARF}, from a partially shared genomic sequence,² is frequently altered in several tumor types.^{3–5} Thus, *p16* is inactivated by germline mutations in about 20% of melanoma-prone families studied,⁶ and has been regarded a melanoma susceptibility gene.^{7,8} In sporadic melanomas, various genetic alterations of *p16* have been reported, such as point muta-

tions (0 to 26%), promoter methylation (0 to 10%), and homozygous deletions (6 to 25%).⁹ Point mutations are rare in most studies (~7%), although they have been reported in up to 26% of the cases.¹⁰

Hypermethylation of the *p16* promoter is frequent in several cancer types,¹¹ being associated with gene silencing.¹² Only a few small studies report on its presence in melanomas,^{13–16} and the frequency seems to be <10%. In contrast, loss of heterozygosity (LOH) at chromosome 9p21 has been reported in 47 to 71%.^{10,15,17–19} The *CDKN2A* gene also carries polymorphisms, and the two most common sites (500 C>G and 540 C>T) are located within the 3'-untranslated region (3'-UTR).²⁰ The functional importance of these polymorphisms is not known.

In a recent study, we found that loss of p16 expression in 45% of vertical growth phase melanomas was associated with significantly increased tumor cell proliferation and reduced survival, indicating a role of p16 protein as an important cell cycle regulator.²¹ These findings are in line with studies on other tumor types, eg, pancreatic carcinoma,²² squamous cell carcinoma of the esophagus,²³ lung carcinoma,²⁴ and leukemia.²⁵ The aim of our present study was to determine the frequency and relative importance of *CDKN2A* promoter hypermethylation, point mutations, LOH, and polymorphisms with respect to p16 protein expression and patient prognosis in this consecutive series of vertical growth phase cutaneous melanoma.

Materials and Methods

Patients and Follow-Up

The patient material of this series is described in detail elsewhere.²¹ Briefly, 202 vertical growth phase melanomas occurring during 1981 to 1997 were initially included. The presence of a vertical growth phase, and the lack of a radial growth phase, ie, adjacent *in situ* or microinvasive component, were used as inclusion criteria

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for the present study.²⁶ In addition, 68 separate biopsies of local (skin, $n = 17$), regional (lymph nodes, $n = 44$), or distant ($n = 7$) metastases from 58 patients with recurrent disease were available for analyses. The hospital records were used to obtain follow-up information with respect to recurrences. Information about cause and date of death was obtained from the Cancer Registry of Norway and Statistics Norway. Complete information on patient survival, and time and cause of death was available in all 202 cases, and 69 patients died during follow-up. Last date of follow-up was December 18th, 1998, and median follow-up time for all survivors (total survival) was 76 months (range, 13 to 210 months).

Clinicopathological Variables

The following variables were recorded: date of histological diagnosis, sex, age at diagnosis, anatomical site of the primary tumor, and presence of local (skin), regional (lymph node), or distant metastases at diagnosis. The hematoxylin and eosin (H&E) slides were re-examined, and the following histological features were also included: tumor thickness according to Breslow,²⁷ level of invasion according to Clark,²⁸ microscopic ulceration, and vascular invasion (each case was closely examined using both H&E- and factor VIII-stained slides).

Immunohistochemistry

As previously described in detail,²¹ staining was performed on formalin-fixed and paraffin-embedded archival tissue. Thin sections (5 μm) from 190 cases with sufficient tumor material left in the tissue blocks, were incubated with the rabbit polyclonal p16 antibody SC-468 (Santa-Cruz Biotechnology, Santa Cruz, CA) at 1:200 overnight in room temperature. Negative controls were incubated with the antibody and blocking peptide (SC-468P). The p53 staining protocol included microwave antigen retrieval (10 minutes at 750 W and 3 $\times$ 5 minutes at 500 W) and incubation for 1 hour (room temperature) with the DO-7 monoclonal antibody (code no. M-700; DAKO, Copenhagen, Denmark) diluted 1:100. After microwave antigen retrieval (10 minutes at 750 W and 4 $\times$ 5 minutes at 500 W), the sections were incubated for 1 hour (room temperature) with the polyclonal Ki-67 antibody (code no. A-047, DAKO) diluted 1:50.

Immunohistochemical staining of the p16 and p53 proteins was recorded as previously described,²¹ considering both the staining intensity and proportion of positive tumor cells. (Intensity: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. Area: 0, no positive tumor cells; 1, < 10% positive tumor cells; 2, 10 to 50% positive tumor cells; 3, > 50% positive tumor cells. Staining index: staining intensity times positive area.) The Ki-67 staining was assessed according to the approach of Weidner and colleagues.²⁹ Briefly, the tumors were scanned at low magnification ($\times 40$ and $\times 100$) to identify the areas of most intense nuclear staining (hot spots). The percentage of immunoreactive tumor cell nuclei (pro-

liferative rate) was then calculated by counting at least 500 cells at $\times 1000$ within the selected areas.

Solution-Phase Methylation-Specific Polymerase Chain Reaction (MSP)

DNA was isolated as previously described,²⁰ and isolated DNA from 81 primary tumors and 12 metastases were modified by the CpGmize DNA Modification Kit and amplified by the CpGWIZ Amplification Kit (Intergen Company, New York, NY) according to the recommendations of the manufacturer. These 81 cases did not differ significantly from the rest of the patients in this series with respect to standard clinicopathological variables and survival.

The protocol used is based on the method described by Herman and colleagues.³⁰ Modified DNA was amplified with primers specific for unmethylated DNA (U-primers) and methylated DNA (M-primers), and the efficiency of the bisulfite modification was determined by primers specific for the wild-type p16 promoter region (W-primers). All cases analyzed were amplified by U-, M-, and W-primers. The products were electrophoresed on a 2% agarose gel containing ethidium bromide and visualized by UV light. Cases not showing amplification with any of the primers ($n = 22$) were regarded to have insufficient quality of DNA. These cases were analyzed separately with respect to clinicopathological variables and p16 expression to exclude any selection bias, and the DNA quality was also checked for by primers for other genes (p53) under optimal conditions. Positive and negative control DNA were included in the kit.

In Situ MSP and Hybridization

Cases showing a mixed pattern with bands after amplification with both U-primers and M-primers ($n = 11$) were analyzed by *in situ* MSP, as described by Nuovo and colleagues³¹ with some modifications. After dewaxing, the sections were subjected to a 15-minute microwave treatment in citric buffer, followed by a mild treatment with Proteinase K (10 $\mu\text{g}/\text{ml}$ for 10 minutes). The tissue morphology was significantly better preserved than after Proteinase K treatment alone. DNA was denatured by 0.4 mol/L of NaOH at 37°C followed by an overnight incubation with 3 mol/L of sodium bisulfite at 55°C in Gene Frames (Advanced Biotechnologies, Surrey, UK), and the modification was completed by a 5-minute incubation with 0.4 mol/L of NaOH. The amplification solution contained 1 $\times$ polymerase chain reaction (PCR) buffer II (Applied Biosystems, Foster City, CA); 3.5 mmol/L MgCl₂; 200 $\mu\text{mol}/\text{L}$ dNTP; 1 $\mu\text{mol}/\text{L}$ U-, M-, or W-primers; 0.1 U/ μl AmpliTac Gold polymerase (Applied Biosystems, Foster City, CA) and 0.1% bovine serum albumin was applied in Gene Frames of 65 μl . After denaturation at 94°C for 7 minutes, 40 cycles of PCR were performed on the Omnislide Thermal Cycler (Hybaid, Ashford, UK): annealing at 55°C for 2 minutes and denaturing at 94°C for 1 minute.

Digoxigenin-labeled PCR products (U, M, and W) from the solution-phase PCR were used as probes for *in situ*

Table 1. Primers Used in PCR Amplification of the Three Exons of the *CDKN2A* Gene

Exon	Primer sequences		Annealing temperature	Product size (bp)
1 α	Forward	5'CGG CTG CGG AGA GGG GGA GAC	66	246
	Reverse	5'CAG CGC CCG CAC CTC CTC TA		
2 (5' end)	Forward	5'GGG CTC TAC ACA AGC TTC CTT	63	277
	Reverse	5'AGC CAG GTC CAC GGG CAG AC		
2 (3' end)	Forward	GGG AGG GCT TCC TGG ACA C	63	243
	Reverse	TTT GGA AGC TCT CAG GGT ACA		
3	Forward	GCC TGT TTT CTT TCT GCC CTC TG	57	144
	Reverse	CGA AAG CGG GGT GGG TTG T		

hybridization. A 100- μ l probe cocktail contained 50 μ l formamide, 30 μ l 25% dextran sulfate, 10 μ l 20 $\times$ standard saline citrate, and 10 μ l probes. Target DNA and probes were co-denatured at 95°C for 9 minutes, incubated overnight at 37°C under coverslips, and washed for 10 minutes in 1 $\times$ standard saline citrate (pH 7.0) at 52°C. The probes were visualized by the DIG Nucleic Acid Detection Kit (Roche, Mannheim, Germany) showing a dark-blue signal in positive cases, and using nuclear fast red as counterstain.

Sections treated with DNase, cases incubated without primers, or cases incubated without probes served as negative controls. All cases were analyzed with the U-, M-, and W-primers and -probes, and the W-probes served as control for the efficiency of the bisulfite treatment.

PCR-SSCP and LOH

Tumor tissue and surrounding normal tissues were dissected from 185 primary tumors with sufficient material left in the tissue blocks. The DNA was isolated separately and screened for polymorphisms at the 3'-UTR of the *CDKN2A* gene as recently described.²⁰

In addition, 50 cases were screened for exon 1 to 3 mutations of the *CDKN2A* gene as reported.¹⁰ The primers used are given in Table 1. All shifts detected by single-stranded conformational polymorphism (SSCP) were confirmed by direct sequencing of both the forward and the reverse strand. The analyses were repeated, starting with PCR amplification of DNA. Direct sequencing was performed using a rhodamine dye terminator cycle sequencing kit (Big Dye, Applied Biosystems), and the sequencing reaction products were electrophoresed on a denaturing polyacrylamide gel in an automated sequencer (ABI 377, Applied Biosystems).

In the same 50 cases, four microsatellite markers on chromosome 9p21, namely *D9S736*, *D9S974*, *D9S171*, and *D9S1870*, were amplified using primer sequences described in the Genome Data Base. One primer in each set was labeled at the 5' end with Cy-5 or FAM fluorescent dyes, and PCR products were electrophoresed on 6% denaturing polyacrylamide gel and detected in an automated sequencer (ABI 377). In case of informative markers LOH was scored when a reduction of at least 50% was seen in one allele in DNA isolated from tumor tissue compared with DNA isolated from the corresponding normal tissue.

Statistics

Analyses were performed using the statistical package SPSS.³² Associations between different categorical variables were assessed by Pearson's chi-square test. Continuous variables not following the normal distribution were compared between two or more groups using the Mann-Whitney U or Kruskal-Wallis H tests. Univariate analyses of time to death because of malignant melanoma were performed using the product-limit procedure (Kaplan-Meier method), with date of histological diagnosis as the starting point. Differences between categories were tested by the log-rank test. The influence of covariates on patient survival was analyzed by the proportional hazards method.³³ Model assumptions were tested by log-minus-log plots.

Results

p16 Protein Expression

The results of the p16 protein expression analysis, as well as the proliferative rate by Ki-67 expression, are previously reported.²¹ Briefly, the immunohistochemical staining pattern for p16 protein was homogeneous throughout the tumor in 75% of the positive cases. Some cases showed a more heterogeneous staining pattern (mosaic type), with groups of positive and negative tumor cells side by side, whereas other cases showed positive and negative areas, such as the case in Figure 1g). Of all 190 primary tumors, 45% showed no or minimal nuclear staining of p16 protein (staining index, 0 to 1), compared with 77% for the metastases (Wilcoxon signed rank test, $P = 0.001$).

Mean proliferative rate, assessed by Ki-67 expression, was 35% in cases with absent or minimal nuclear p16 staining, compared with 24% in the remaining cases with moderate/strong nuclear p16 expression (t -test, $P < 0.0001$).

Solution-Phase MSP

Fifty-nine primary tumors and all 12 metastases showed reproducible amplification with the U- and/or the M-primers. Forty-three primary tumors (73%) were unmethylated, 11 cases (19%) showed bands with both the U- and

Figure 1. a and b: Solution-phase MSP of the *CDKN2A* promoter region. a: Lane 1, 100-bp marker; lane 2, unmethylated control; lane 3, methylated control; lane 4, unmodified DNA wild-type control; lane 5, blank; lanes 6 and 7, case 274 positive with the M-primers; lane 8, blank. b: Lane 1, 100-bp marker; lanes 2 and 3, case 276 positive with the U-primers; lanes 4 and 5, case 393 positive with both the U- and M-primers; lanes 6 and 7, case 396 positive with both the U- and M-primers; lane 8, H₂O. In situ MSP. c: Case 393 shows nuclear blue and granular positivity with the M-primers/probe. d: The same case with the U-primers/probe, note the positive signals in some stromal cells. e: Case 396 showed no positivity with the M-primers/probe, in contrast to the positive reaction for U-primers/probe shown for tumor tissue and overlying epidermis in f. g: Case 393 shows tumor cell heterogeneity with respect to immunohistochemical staining of the p16 protein. h: Positive nuclear staining of the p16 protein in case 396.

Figure 2. Flow chart illustrating the reclassification of cases with bands for both unmethylated and methylated *p16* promoter (mixed cases) after *in situ* MSP. The mixed cases were subjected to *in situ* MSP and subsequently reclassified into predominantly unmethylated (U) and predominantly methylated (M). Ht, heterogeneous cases; Hm, homogeneous cases.

M-primers, whereas 5 cases (8%) had positive M-bands only (Figure 1). Eight metastases (67%) were unmethylated and four (33%) were methylated at the *p16* promoter region (Wilcoxon signed rank test, $P = 0.25$). All five methylated primary tumors (100%) showed complete loss of p16 protein expression, compared with 24 of the unmethylated (55%) and 5 of the mixed cases (45%) (chi square, $P = 0.05$). None of the two mutated cases had promoter hypermethylation.

In Situ MSP and Hybridization

To interpret the 11 cases being positive after both methylation- and unmethylation-specific solution-phase PCR, *in situ* MSP was performed. Apparently, the bisulfite modification was a critical step, and incubations impairing the tissue morphology (NaOH and proteinase K) were necessary to get sufficient DNA modification. However, all mixed cases gave evaluable staining results with blue nuclear grains contrasting the red counterstain (Figure 1). Cases positive with the M-probe/primers in tumor cells and the U-probe/primers only in tumor stroma were recorded as methylated ($n = 4$). Cases with tumor cell positivity for both the U- and M-probe/primers were interpreted as heterogeneous (partial methylation, $n = 7$) and recorded as predominantly methylated if the M-probe/primer positivity was dominant ($n = 2$), and predominantly unmethylated if the U-probe/primer positivity was dominant ($n = 5$). Thus, the cases were regrouped, without knowledge of patient outcome or clinicopathological data, into methylated ($n = 11$, 19%) and unmethylated ($n = 48$, 81%) tumors (Figure 2).

After the *in situ* MSP, there was no significant association between promoter methylation ($n = 11$) and loss of nuclear p16 staining ($P = 0.30$). Four of the seven cases with heterogeneous methylation (partial methylation) showed presence of p16 protein expression, and the protein-staining pattern was heterogeneous in these cases as illustrated in Figure 1g. Methylated cases ($n = 11$) showed significantly increased proliferative rate by Ki-67 expression (Mann-Whitney test, $P = 0.01$), and these cases were significantly thicker (Mann-Whitney test, $P = 0.004$). As illustrated in Figure 1g, histological heterogeneity was observed in some of the cases with

heterogeneous methylation. Still, no particular feature was exclusive to these cases, and four of seven cases did not show histological heterogeneity.

The number of different genetic alterations (promoter methylation, point mutations, LOH, and polymorphisms) was recorded for each case. Two or more alterations, found in 25% of the cases, were significantly associated with loss of p16 protein expression ($P < 0.001$).

Mutations in *CDKN2A*

In total, 50 primary melanomas were screened, and sequence changes were detected in two cases (4%). Case 364: codon 99 (exon 2) CGG>CGA (Arg>Arg) for *p16*, and codon 114 (exon 2) GGC>AGC (Gly>Ser) for *p14*^{ARF}. Case 453: codon 55 (exon 2) GGC>CGC (Gly>Arg) for *p16*, and codon 69 (exon 2) GGG>GCG (Gly>Ala) for *p14*^{ARF}. The mutation for case 453 in *p16* is a novel mutation and is at the residue which is conserved in all four CDK inhibitors.³⁴

LOH

LOH was determined at four different polymorphic markers on chromosome 9p21 in 50 primary melanomas. These markers cover the entire *INK4* locus as well as flanking centromeric and telomeric loci on chromosome 9p21. Our data show that 45 cases (90%) had lost an allele at one or more loci, and 3 cases showed loss at all four loci. LOH at the chromosome 9p21 markers ranged from 34 to 56% of informative cases.

Both mutated cases had LOH at *D9S974*, *D9S736*, and *D9S1870*, whereas one had LOH at *D9S171*. Further, *D9S171* was significantly less frequent in cases with Clark's level 5 of invasion ($P = 0.02$). None of these cases with LOH at this locus were hypermethylated at the promoter region ($P = 0.07$), and the same trend was seen for LOH at *D9S736* ($P = 0.06$). LOH at *D9S1870* were more often found in ulcerated cases ($P = 0.02$) and in cases with strong p53 staining ($P = 0.02$). No significant associations were present between LOH at any of the four markers and p16 protein expression, proliferative rate by Ki-67, tumor thickness, or patient survival.

Polymorphisms in *CDKN2A*

In total, 185 primary tumors had sufficient material left in the paraffin blocks for the study of polymorphisms in the 3'-UTR of the *CDKN2A* gene. The frequency of cases that were heterozygous for the T allele in the 540 C>T polymorphism was 23% ($n = 42$), whereas one case was homozygous. Forty-seven cases (25%) were heterozygous, and three (1.6%) were homozygous for the G allele at the 500 C>G polymorphism.

There was no significant association between either of the polymorphisms and methylation status, LOH, or p16 protein expression. Both mutated cases had the 540 C>T and one had the 500 C>G polymorphism. Further, there was no association with the other clinicopathological variables studied.

Figure 3. Survival curves were estimated according to the Kaplan-Meier method with death as the result of melanoma as end point. **A:** Survival by *CDKN2A* promoter methylation. **B:** Survival by presence of the 540 C>T polymorphism at 3'-UTR of *CDKN2A*.

Survival Analyses

Significantly reduced survival was present for cases showing absent or minimal nuclear p16 protein expression (log rank test, $P = 0.0003$), and recurrence-free survival was also significantly decreased in this subgroup of patients (log rank test, $P = 0.007$).

Estimated 10-year survival for cases regarded as predominantly methylated after *in situ* MSP ($n = 11$) was 31%, compared with 74% for unmethylated cases (log rank test, $P = 0.025$; Figure 3). No significant associa-

tions were present between LOH at any of the four markers and patient survival. However, presence of the 540 C>T polymorphism was significantly associated with improved patient survival (log rank test, $P = 0.03$; Figure 2). No significant survival differences were found with respect to number of genetic alterations (methylation, mutations, LOH, polymorphisms).

In multivariate analysis according to the proportional hazards method, the positive impact on survival of the 540 C>T polymorphism was still significant (Table 2), with a Hazard ratio of 2.6 (L ratio test, $P = 0.02$) for cases without this polymorphism, whereas promoter methylation of *CDKN2A* had no independent prognostic importance, possibly because of the low number of cases included in this subset analysis.

Discussion

Previous studies indicate a major role for *CDKN2A* (*p16*) as a tumor suppressor gene in a variety of human cancers, being inactivated by point mutations, promoter methylation, and homozygous deletions.⁹ Recent publications have also demonstrated that *p16* knockout mice, as well as mice with *p16*-specific mutations or deletions, show an increased incidence of spontaneous and carcinogen-induced cancers including melanoma.^{35,36} We previously showed that loss of p16 protein expression was associated with increased tumor cell proliferation and reduced survival in vertical growth phase cutaneous melanoma,²¹ supporting that *p16* is an important cell cycle regulator in these tumors. However, the mechanisms of inactivation are not completely understood.

In this series of cutaneous melanoma, we found that 19% of the cases were hypermethylated at the promoter region of *CDKN2A*. This frequency is higher than what has been indicated in a few small studies,¹³⁻¹⁶ but lower than recently reported for uveal melanoma (32%).³⁷ The frequency tended to be increased in corresponding metastases (33%), indicating that *p16* hypermethylation might also occur in late tumor progression. Some of the cases were heterogeneous, as also described by others,³⁸ showing both methylated and unmethylated tumor cells by *in situ* PCR, similar to the mixed pattern of p16 protein expression observed by immunohistochemistry.²¹

The importance of *p16* promoter hypermethylation in vertical growth phase melanoma is supported by a significant correlation with increased tumor cell proliferation by Ki-67 expression, indicating loss of cell cycle control.³⁹ Further, there was a significantly reduced patient survival in methylated cases, suggesting a functional importance for disease progression in these tumors. This is in line with studies of *p16* promoter methylation and survival impact in other tumors.⁴⁰⁻⁴³

There was no clear association between promoter methylation and reduced p16 protein expression after *in situ* MSP. This may, in part, be because of the low number of cases analyzed for methylation. Another reason might be partial or monoallelic methylation and heterozygous tumor cells with varying penetrance of the wild-type allele, similar to what has been described for p27 in mela-

Table 2. Multivariate Survival Analysis According to the Proportional Hazards Method, for Patients with Vertical Growth Phase Melanoma, Using Death from Melanoma as End Point

Variable	Categories	n	HR*	P value†
Level of invasion (Clark)	II, III, IV	143	1	
	V	34	5.0	<0.0001
Anatomic site	Trunk	49	3.3	
	Other	128	1	<0.0001
Vascular invasion	Absent	141	1	
	Present	36	1.96	0.034
Proliferative rate by Ki-67 expression	Low‡	46	1	
	High	131	4.69	0.001
Microvessel density	Low§	120	1	
	High	57	2.12	0.015
p53 expression	Absent¶	27	1	
	Present	150	19.5	0.003
p16 expression	Absent/low	80	2.31	
	Present	97	1	0.003
540 C > T polymorphism	Absent	137	2.61	
	Present	40	1	0.019

Only patients with information on all variables were included, $n = 177$.

*Hazard ratio.

†L ratio test.

‡ $\leq 16\%$ (25-percentile).

§Cut points (see text): ≤ 144 vessels/mm² (67th percentile).

¶Staining index = 0.

||Staining index = 0 to 1.

noma.⁴⁴ Also, other mechanisms of inactivation may act in concert with methylation and explain the loss of p16 protein expression. An association between *p16* promoter methylation and reduced protein expression has been reported in other cancer types.^{12,14,37,45–48}

Only two point mutations (4%) were detected in this series, supporting most previous studies that mutational inactivation is not a major mechanism of gene silencing in sporadic primary melanoma. The frequency of mutations varies from 0 to 26% according to recent reports,^{10,13,17,49,50} but differences in case selection exist. Still, our data show that 90% of the cases had LOH at one or more markers within the *INK4* locus, suggesting that monoallelic loss at 9p21 is frequent and might represent one of two or more events causing reduced expression of p16 and also that of p14^{ARF}.¹⁹ Although LOH has been correlated to reduced p16 protein expression,¹⁵ we found no significant association between LOH at any marker and p16 staining, indicating that monoallelic loss alone may not be sufficient for genetic silencing.

Recent evidence strongly suggests that the 3'-untranslated region of mRNA is involved in regulation of gene expression by controlling nuclear export, polyadenylation status, subcellular targeting, translation rates, and mRNA degradation.⁵¹ We recently found a significant overrepresentation of the heterozygote frequency of the 540 C>T polymorphism of *CDKN2A* when compared to controls.²⁰ In our present study, the presence of this polymorphism provided independent prognostic information in multivariate survival analysis, and patients positive for this marker had significantly improved survival. To our knowledge, only Sauroja and colleagues⁵² have reported on possible survival differences with respect to this polymorphism, although the analysis was performed on melanoma metastases and not primary tumors. Our present results strongly suggest a role for the 540 C>T polymorphism of

CDKN2A in the initiation or early progression of a subset of cutaneous melanomas with less aggressive behavior.

In conclusion, point mutations of *CDKN2A* seem to be rare in cutaneous melanoma of the vertical growth phase, whereas promoter hypermethylation was present in a significant subgroup of the cases (19%), being associated with increased tumor cell proliferation and significantly reduced patient survival. Further, the presence of one specific polymorphism (540 C>T) at 3'-UTR, which previously was found to be increased in melanoma cases when compared with the general population, was associated with favorable outcome in multivariate analysis, pointing to the possibility that this might represent a distinct subgroup of low-grade vertical growth phase melanoma. Thus, our findings support a significant role of the *CDKN2A* gene for melanoma progression.

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