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Alterations in CDKN2A Locus as Potential Indicator of Melanoma Predisposition in Relatives of Non-Familial Melanoma Cases

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Aim. To examine constitutional alterations of CDKN2A/p16INK4A locus as a potential indicator of melanoma predisposition among the first-degree relatives of patients with malignant melanoma.

Method. The study included eight families with a single member affected with melanoma. Members of the families were screened for allelic cosegregation with 9p21 region polymorphic markers IFNA, D9S126, and D9S104. The patient's tumors were screened for loss of heterozygosity (LOH) with the same markers, as well as for single strand conformational polymorphism (SSCP) variability of CDKN2A. In suspect cases, constitutional DNA was examined by SSCP and direct sequencing.

Results. LOH was detected in four cases, and SSCP indicated variability in at least one CDKN2A exon in these tumor samples. In three of four LOH cases, the remaining allele cosegregated within the family, which was interpreted as a preliminary indicator of potential genetic predisposition. In one of these three families, we found constitutional CDKN2A mutations in the patient and one of the relatives. In the second family, only the patient had the constitutionally altered gene, whereas no constitutional CDKN2A alterations were detected in the third family. All significant mutations were different and had not been reported before.

Conclusion. We detected one case of melanoma predisposition among unaffected family members, which corresponded to statistical expectations for such a small number of screened families. Since constitutional mutations of CDKN2A exons have limited incidence, our stepwise approach seemed to be more informative and more affordable than straightforward CDKN2A sequencing of all subjects.

Key word: *alleles; genes, p16; genetic predisposition to disease; genetic screening; melanoma*

Melanoma is one of the most common cancers in young adults. For North American whites, the lifetime risk of melanoma has been estimated at approximately 1 in 80 (1,2). Based on data from 1960s, the annual incidence of melanoma in Croatia has been estimated at 1.5/100,000 (3). However, a recent regional study (4) has suggested about 4 times greater value, which falls within the lower range of incidence in other European countries (5).

Approximately 10% of melanoma cases occur in individuals with a familial predisposition (6,7). Linkage analysis of families with multiple melanoma cases provided evidence of a locus for familial melanoma susceptibility in the 9p21 chromosomal region. The region was defined with IFNA and CA-repeat marker D9S126 (8), and a more centromeric marker, D9S104, was soon included in melanoma studies (9). Approximately one half of melanoma kindred show evidence of genetic linkage to 9p21 locus (10-13), but mutations and deletions of its coding region are also found in sporadic melanoma cases (14).

The CDKN2A gene (also known as p16, p16I NK4a, or MTS1), which encodes p16INK4A protein, was localized to chromosome 9p21 (15,16), and recognized as a candidate melanoma tumor suppressor gene (17). The p16INK4A protein inhibits the activity of CDK4 or CDK6, two protein kinases, which would otherwise phosphorylate the retinoblastoma protein and thus allow the cell to pass through the G1 cell-cycle checkpoint (18). Therefore, lack or impaired function of p16INK4A may result in dysregulated cellular proliferation.

Constitutional CDKN2A mutations can apparently be found in about one-fourth of familial melanoma kindred, most frequently as a cosegregating mutant allele (19). However, actual reports for different populations give a wide range of estimates, from several percent to 50% (20). Although these CDKN 2A mutations are less frequent than 9p21 linkage, their incidence is greater in those familial melanoma kindred that show linkage to the locus (13,21). However, loss of heterozygosity (LOH) and microsatellite instability in the 9p21 region are found at considerably greater incidence than mutations of CDKN2A exons, ie, in about 50% of primary tumors as well as metastases (22).

In the pilot study conducted in Croatia, we examined the 9p21 locus for potential indicators of an increased melanoma risk among the first-degree blood relatives of melanoma patients. The eight families included in the study did not have a family history of melanoma. We screened the tumor samples for LOH, using IFNA, D9S126, and D9S104. Peripheral blood samples of the patients and their relatives for allelic cosegregation were also screened with the same markers. Cosegregation of the LOH-retained allele was considered the first potential predisposition indicator. In these samples, CDKN2A was analyzed for single strand conformational polymorphism (SSCP) variability, and finally sequenced in the case of positive finding. The aim of the study was to develop and test an affordable procedure for early detection of familial melanoma predisposition.

Material and Methods

The samples for analysis – peripheral blood and paraffin blocks of melanoma tissue – were collected from eight patients with the diagnosis of cutaneous malignant melanoma and their first-degree relatives (blood samples only) who wanted to know if they had any detectable indication of genetic predisposition for developing this disease (Table 1). The patients generally came from Zagreb and Split area. Their age ranged between 35 and 80 years. The maximum tumor thickness according to Breslow varied between 2 and 5, and to Clark between 2 and 5 (23). All subjects involved in the study were fully informed and signed the written consent to participation, according to ethical principles.

Since no data were available for Croatian population, at the beginning of the study we selected 20 healthy donors matched by age and sex with the members of the affected families to get an overview of the allelic size and frequency for the markers used in the study. The resulting allele distribution was in good agreement with data from other population studies (Table 2, ref. 8).

Table 2. Allele frequency of 9p21 polymorphic markers in 20 healthy donors*

	9p21 polymorphic markers					
	D9S104		D9S126		IFNA	
Allele	bp	f	bp	f	bp	f
1	199	5	248	4	150	1
2	197		246		148	8
3	195	4	244		146	28
4	193	1	242	2	144	1
5	191	2	240		140	1
6	189	6	238	32	138	1
7	187	6				
8	185	11				
9	183	2				
10	181	1				
MH (%)	81.0	68.0	68.0	26.0	72.0	40.0

*Abbreviations: bp – base pairs, f – frequency, MH – maximum heterozygosity.

Constitutional DNA was isolated from peripheral blood leukocytes according to standard procedures (24). After erythrocyte lysis, leukocyte pellet was digested in buffer composed of sodium dodecyl sulfate (SDS), Tris, NaCl, and ethylenediaminetetraacetic acid (EDTA), with proteinase K. After phenol-chloroform extraction, DNA was precipitated in ethanol. Tumor tissue was embedded in paraffin blocks. Paraffin slices, 4-8 μm thick, were stained with hematoxilin and eosin and evaluated by a pathologist. Thereafter, DNA was extracted from unstained paraffin slices. Tumor tissue was digested in lysis buffer (Tween 20, Triton X100, EDTA, dithiotreitol, and TrisCl with proteinase K) overnight and then boiled for 10 minutes to degrade proteolitic enzymes (25).

Table 1. An overview of 9p21 polymorphic marker screening and single strand conformational polymorphism (SSCP) analysis in all study subjects

		Polymorphic markers							
		D9S104†		D9S126†		IFNA†		SSCP variability‡	
No. of subject	Family members*	blood	tumor	blood	tumor	blood	tumor	blood	tumor
13	**father** metastasis	2, 3	2, LOH	3, 3	3, 3	1, 3	1, 3	e2?	e2
14	daughter	1, 2		1, 3		3, 5		e2?	
15	son	2, 3		3, 3		1, 5		e2?	
16	**father** C4 B2	7, 9	7, 9	1, 6	1, 6	2, 3	2, 3		none
17	daughter	7, 9		3, 6		–			
18	son	7, 9		1, 6					
19	**father** C5 B5	4, 7	4, LOH	–	–	4, 5	4, 5		e2
20	daughter	6, 7							
21	son	7, 7							
22	**mother** C3 B2	1, 1	1, 1	5, 3	5, 3	–	–		none
23	son	1, 6		5, 3					
24	son	1, 9		5, 4					
28	**mother** C3 B3	4, 5	4, 5	3, 2	3, LOH	5, 5	5, 5	e2?	e2
29	daughter	4, 4		2, 3		3, 5		none	
31	**mother** C4 B5	5, 6	5, 6	2, 3	2, 3	–	2, 4		none
32	son	4, 7		2, 3					
34	**father** C4 B5	6, 2	6, 2	3, 3	3, 3	2, 5	2, 5		none
35	daughter	6, 8		3, 3		2, 5			
36	daughter	2, 8		3, 3		5, 5			
40	**mother** C4 B4	5, 2	–			3, 4	3, LOH	none	e2
41	daughter	–				3, 4		none	
42	daughter	3, 4				5, 4		none	

*The affected member is listed first, in bold (C-Clark, B-Breslow).
†Under the polymorphic marker headings (D9S104, D9S126, and IFNA) constitutional (blood sample) alleles are listed on the left side of the column, and on the right side tumor alleles are shown. LOH (loss of heterozigosity) indicates the missing allele. Empty field denotes no analysis, and minus (–) means inconclusive reading.
‡CDKN2A exons showing variability; questionmark denotes possible variations.

DNA samples were typed for three short tandem repeat polymorphisms, IFNA, D9S126, and D9S104, spanning chromosome 9p21. Single strand conformational polymorphism polymerase chain reaction (SSCP-PCR) was developed for 3 exons of CDKN2A/p16 (Table 3).

Table 3. Primer pairs used in this study

Polymorphic marker	Amplimer size (bp)	Primer sequence
D9S104	181-199	5'-ACTGGGACTCTAACTAATGT
		3'-GATCTGGGTATGTCTTTCTG
IFNA	138-150	5'-GTAGGTGGAAACCCCCACT
		3'-TGCGCGTTAAGTTAATTGGTT
D9S126	238-248	5'-ATTGAAACTCTGCTGAATTTTCTG
		3'-CAACTCCTCTTGGGAACTGC
p16 exon 1	340	5'-GAAGAAAGAGGAGGGGCT
		3'-GCGCTACCTGATTCCAATTC
p16 exon 2	367	5'-ACACAAGCTTCCTTTCCGTC
		3'-TCTGAGCTTTGGAAGCTCTC
p16 exon 3	169	5'-CCGGTAGGGACGGCAAGAGA
		3'-CTGTAGGACCCTCGGTGACTGA

PCR reaction was performed in 25 mL reaction mixture containing 100 ng of template DNA or 1-5 mL of crude extract from paraffin-embedded tissue prepared after microdissection, 200 mmol/L deoxynucleoside triphosphate (dNTP), 1.5 mmol/L MgCl2, 10 pmol/L each primer, 1.25 U Taq polymerase (Ampli Tag Gold® DNA polymerase, Roche Molecular Systems, Inc., Mannheim, Germany) in 10 mmol/L Tris-HCl buffer. PCR system (Gene Amp PCR system 2400, Applied Biosystems, Foster City, CA, U.S.A.) was set for 25 to 35 cycles, with the following parameters:

D9S104 – 94 °C for 5 min + 30 x [94 °C for 30 s + 55 °C for 30 s + 74 °C 30 s] + 74 °C for 10 min;

D9S126 – 94 °C for 5 min + 30 x [94 °C for 30 s + 55 °C for 30 s + 74 °C for 30 s] + 74 °C for 10 min;

IFNA – 94 °C for 8 min + 30 x [94 °C for 30 s + 56 °C for 30 s + 74 °C for 30 s] + 74 °C for 10 min; and

p16 exons – 95 °C for 5 min + 35 x [95 °C for 30 s + 50 °C for 30 s + 72 °C for 45 s] + 72 °C for 7 min.

For LOH analysis, 5-10 μL of PCR product was loaded on 0.1×40×32 cm native polyacrylamide gels, with 10% D9S126, 12% D9S104, or 15% IFNA, and 3.3% cross-linking, and run for 8-12 h at 10-15 V/cm in 1xTris/borate/EDTA (TBE) buffer.

SSCP screening was performed on either 6%-native 0.1×40×32 cm polyacrylamide gels with 1.6% cross-linking or 9%-native 0.1×40×32 cm polyacrylamide gels with 3.3% cross-linking, and run for 3-12 h at 10–15 V/cm in 1xTBE buffer.

DNA was visualized by the silver staining method (26). Gels were fixed by submersion in ethanol, oxygenated with HNO_3, followed by treatment with $AgNO_3$, and visualized with Na_2CO_3/formalin. The reaction was stopped with acetic acid.

Before CDKN2A sequencing, amplified PCR products were purified with QIAquick purification kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Each PCR product was sequenced in both directions by using the same primers and the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Weiterstadt, Germany). Sequencing analysis was performed on an automatic sequencer (ABI PRISM 377 DNA, version 2.1.1, PE, Applied Biosystem Division) (27).

Results

Of eight melanoma cases, LOH in the 9p21 region was found in four tumor samples (two for D9S104 marker – subjects No. 13 and 19 from the Table 1; one for D9S126 – subject No. 28; and one for IFNA – subject No. 40) (Fig. 1). We proceeded with SSCP analysis of CDKN2A exons in the tumor tissue and found variability in exon 2 in the same four tumors with LOH.

Allelic screening of constitutional DNA with the same markers revealed that both unaffected children in the family 13-15 (Table 1) had the D9S104 allele 2, which was retained in the patient's tumor LOH (Fig. 1a). In the family 28-29, where the allele 3 of the LOH marker D9S126 remained in the patient's tumor DNA, the unaffected member also had this allele (Fig. 1b). In the family 40-42, only a single relative (subject No. 41) had IFNA allele 3, which was retained in the tumor tissue of the affected member (Fig. 1c).

These four relatives with LOH-retained alleles were potential candidates for genetic predisposition to melanoma. Exclusion of the families where LOH

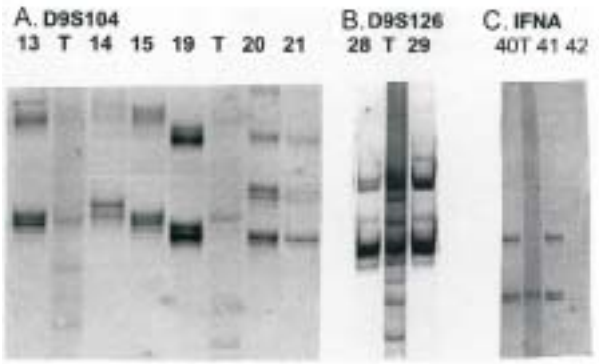

Figure 1. Typing for polymorphic markers **A.** D9S104, **B.** D9S126, and **C.** IFNA. For each marker, only the families with LOH in the affected member's tumor are shown. The lane numbers for constitutional DNA reflect the sample numeration in the Table 1, and tumor DNA ("T") belongs to the patient on the left.

Table 4. Constitutional CDKN2A sequence alterations in affected members and their relatives in families 13-15 and 28-29*

No.	Status	Alteration	Exon	Base change	AA change	Shown in figure No.
13	affected	insertion G	2	TAC 387 TAG	Y 129 stop	3A
		C → T	3	CGC 249 CGT	postcoding	not shown
14	relative	C → G	3	TCT 268 TGT	postcoding	3B
15	relative	deletion G	2	GCG 178 CGG	A 60 R, f → 145stop	3C
		C → T	2	CTG 388 TTG	L 130 L	3D
		G → A	2	CGC 432 CAC	R 144 H	3E
		C → T	3	CGC 249 CGT	postcoding	3F
		G → A	3	CTG 252 CTA	postcoding	3F
28	affected	insertion T	2	GCT 352 TGC	A 118 C, f → 119stop	3G
		G → T	2	GAA 445 TAA	E 149 stop	not shown
		G → C	2	GAG 262 CAG	E 88 Q	3H
		C → T	3	CGC 249 CGT	postcoding	3I
		G → A	3	CTG 252 CTA	postcoding	3I
29	relative	C → G	3	TCT 268 TGT	postcoding	3J

*Only single nucleotide hemizygous alterations were detected, as compared to Gene Data Bank ascension no U 17075 and other references (33). Frameshift is indicated by "f →", arrow pointing at the ensuing stop codon. The last column lists sequencing illustrations from the Figure 3.

was not found in the tumor tissue of affected member was further supported by SSCP analysis, which revealed no variable CDKN2A exons in these tumors (Table 1). The odds to find constitutional CDKN2A mutations in their unaffected relatives were estimated as too low to proceed with sequencing. As for the family 19-21, the LOH for D9S104 in the patient's tumor was accompanied by a few less visible bands of different sizes, suggesting a microsatellite instability. However, the remaining clearly visible allele 4 was not found in his relatives, and they were thus not included in further analysis.

For the three suspect families, SSCP analysis of CDKN2A exons was performed on blood samples of all members (Fig. 2). Possible variations appeared in exon 2 for the members of 13-15 family and for the affected subject No. 28, although not as clearly as in the tumors. As these SSCP results did not seem very informative, constitutional DNA sequencing was performed in all three suspect families. In the family 40-42, no convincing CDKN2A alterations that could affect the gene transcription were found in subjects No. 40 and 41 (few suspect polymorphisms found in the postcoding region are not presented here), and the subject No. 42 was not analyzed (no cosegregation of the LOH-retained allele). The sequence analysis of

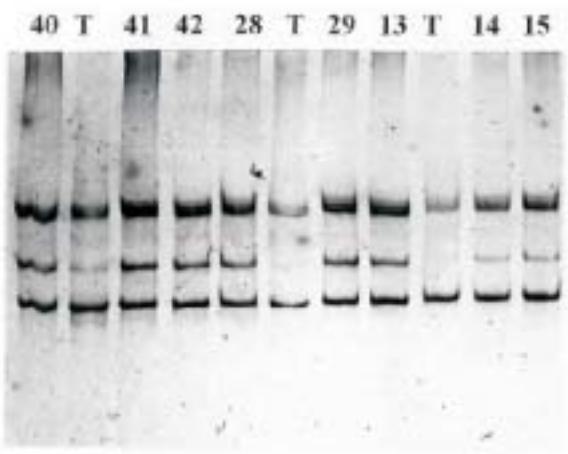

Figure 2. Single strand conformational polymorphism (SSCP) analysis of CDKN2A exon 2 in constitutional DNA of the three suspect families selected for further analysis, and in tumor DNA ("T") of their affected members.

the other two families showed a number of hemizygous constitutional alterations (Fig. 3, Table 4).

In both families, alterations found in the peripheral blood DNA of the affected members suggested

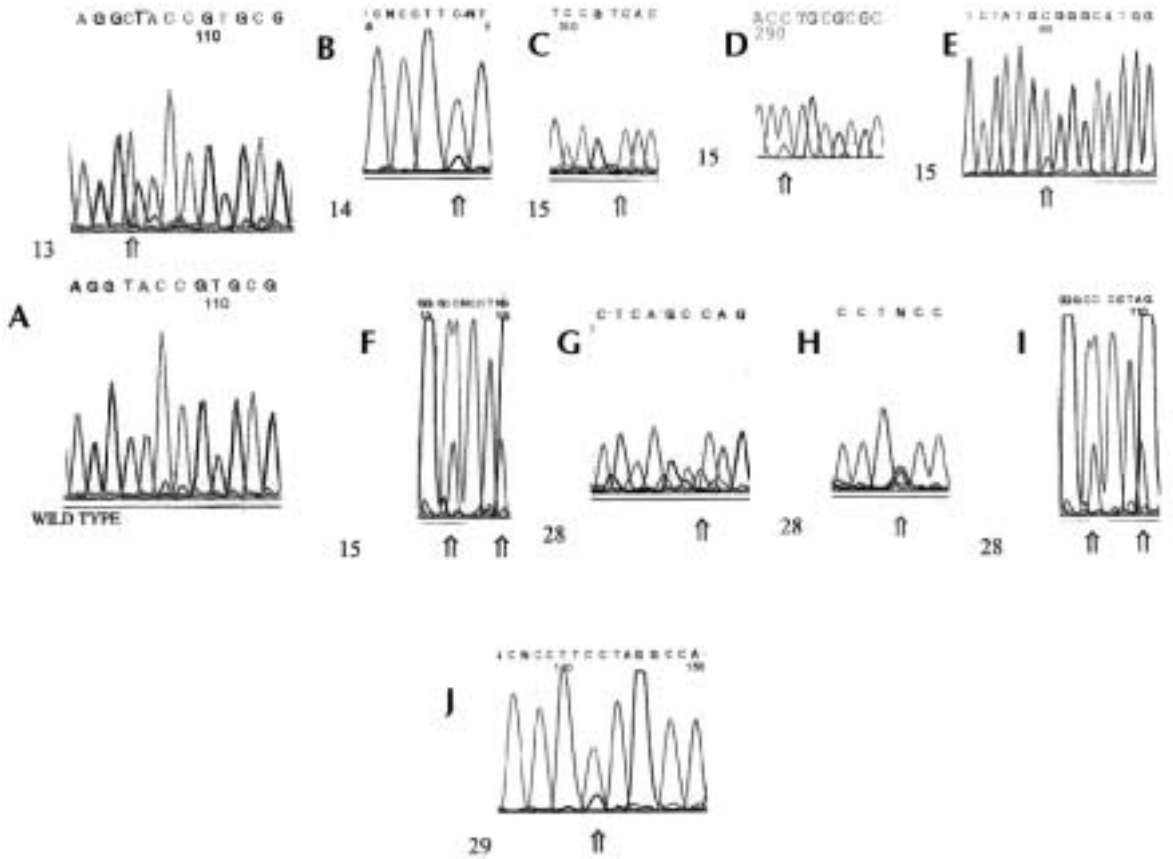

Figure 3. Sequence analysis of CDKN2A alleles in the peripheral blood. **A.** Individual 13, exon 2: insertion of G at nucleotide 387 (upper) and wild-type sequence (lower), both shown on antisense strand. **B.** Individual 14, exon 3: C → G at 268. **C.** Individual 15, exon 2: deletion of G at 178. **D.** Individual 15, exon 2: C → T at 388. **E.** Individual 15, exon 2: G → A at 432 **F.** Individual 15, exon 3: C → T at 249 and G → A at 252. **G.** Individual 28, exon 2: insertion of T at 352. **H.** Individual 28, exon 2: G → C at 262. **I.** Individual 28, exon 3: C → T at 249 and G → A at 252. **J.** Individual 29, exon 3: C → G at 268.

CDKN2A heterozygosity, with one allele constitutionally inactivated by an early stop codon in exon 2 (at 129 for the subject No. 13, and at 119 for the subject No. 28). The subject No. 28 seemed to have additional potentially inactivating mutations in exon 2. If the same hemizygous CDKN2A defect was found in some unaffected relative, it would indicate a clear genetic predisposition to melanoma.

However, no cosegregating mutations were found in the unaffected members of either family. In the unaffected subjects No. 14 and 29, we detected only single nucleotide polymorphisms in the postcoding region of exon 3, and a suspect insertion of A at 398 in exon 2 of the subject No. 14 (data not shown, because it was too close to the noise level). Only the unaffected subject No. 15 had definite alterations in the coding region, all found in exon 2, among which the frameshift after codon 60 (with a premature stop at 145) indicated a hemizygous CDKN2A inactivation.

Discussion

We believe that our study delineated a reasonable procedure for the early detection of melanoma predisposition among blood relatives of a patient with malignant melanoma. In the small group of families screened in this study, a single case of predisposition was identified (the subject No. 15). More than that would have been well above the statistical expectation for the group (37).

Whereas comparable CDKN2A changes have been noted on the same codons for all three inactivating insertions/deletions we found, we are not aware that these mutations have been previously reported. Our subject No. 13 had an insertion leading to Y129stop; in the literature, only the change Y129C (28) and a bigger deletion of 23 bp at that locus (29) have been described. The change A60T at the codon 60 has been described (30), but not a deletion resulting in A60R, as in our subject No. 15. In our subject No. 28, the insertion lead to A118C, whereas only A118T at the same locus has been described so far (31).

Although they did not cosegregate with the affected father, CDKN2A alterations in our subject No. 15 constitute a convincing indicator of melanoma predisposition. However, it is not yet fully understood how CDKN2A mutations predispose for melanoma. They are found only in a small subset of tumor samples and seem to be far more abundant in cultured melanoma cells. For example, mutations or homozygous deletions of CDKN2A region have been reported in more than 75% of sporadic melanoma cell lines (32). Therefore, melanoma should perhaps be associated with sequential accumulation of genetic alterations in the 9p21 region rather than with the mutations of CDKN2A exons alone.

No other candidate melanoma suppressor has been identified in this region, and there is only limited evidence of the involvement of other pathway genes, such as CDK4 gene (33). CDKN2A exon 2 also codes (in conjunction with a nearby coding region 1β) for another protein, p14ARF (alternate reading frame), implicated in some tumors and apparently within the p53 pathway. However, this protein does not seem to have an appreciable independent role in human melanoma (34). Presently, only CDKN2A mutations and less specific 9p21 region defects appear to be well-established genetic features of this malignancy.

Non-cosegregating CDKN2A alterations in our 13-15 family (the evidence for the subject No. 14 was inconclusive) may perhaps be interpreted as a familial case of instability at this locus. This would be consistent with the overall picture of 9p21 region in melanoma samples, in which loss of heterozigosity for various and frequently distant markers seems to be far more common than CDKN2A mutations.

On the other hand, a recent hypothesis includes the possibility that even the polymorphisms or heterozygosity in noncoding regions may somehow have direct or epigenetic influence in the gene inactivation (35). Furthermore, some authors argue that a single mutated CDKN2A allele in constitutional DNA may be sufficient for tumorigenesis (36).

Relatively recent arguments of this kind generally soften typical requirements on tumor suppressor models, but they are not needed to support our melanoma predisposition finding for the subject No. 15. At least one of his genetic alterations qualifies for haploinsufficiency, and it did not cause any malignancy. It is a typical predisposition case, because a somatic hit on the remaining functional CDKN2A allele can fully inactivate this suppressor. Also, the number of his sequence alterations, compared with other unaffected members, indicates a more general instability in the 9p21 region of this subject.

All arguments suggesting that mutations in CD KN2A exons should not be the only indicator of melanoma predisposition support our stepwise approach to the screening of unaffected family members. In particular, high incidence of loss of heterozygosity in these tumors justifies cosegregation analysis of the LOH-retained alleles as the first step in the screening.

In our small sample of eight families, no case of cosegregating constitutional mutations in CDKN2A exons had been statistically expected and none was found. With less than 10% chance of undetected familial melanoma and the likelihood of germline CDKN2A mutations about four times smaller, any screening of this kind, even on much larger samples, should include all potential indicators of melanoma predisposition rather than focus only on cosegregating exon mutations.

Melanoma predisposition screening has not yet become common practice, not even for the relatives of affected persons. If strategies for such screenings should be considered in the near future, the approach we described here could prove not only more affordable, but also more informative than straightforward CDKN2A sequencing for all subjects.

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