

CDKN2A MUTATIONS IN MULTIPLE PRIMARY MELANOMAS

JOSE MONZON, B.Sc., LING LIU, M.D., HERBERT BRILL, B.Sc., ALISA M. GOLDSTEIN, Ph.D., MARGARET A. TUCKER, M.D., LYNN FROM, M.D., JOHN McLAUGHLIN, Ph.D., DAVID HOGG, M.D., AND NORMAN J. LASSAM, M.D., Ph.D.

ABSTRACT

Background Germ-line mutations in the *CDKN2A* tumor-suppressor gene (also known as *p16*, *p16^{INK4a}*, and *MTS1*) have been linked to the development of melanoma in some families with inherited melanoma. Whether mutations in *CDKN2A* confer a predisposition to sporadic (nonfamilial) melanoma is not known. In some patients with sporadic melanoma, one or more additional primary lesions develop, suggesting that some of these patients have an underlying genetic susceptibility to the cancer. We hypothesized that this predisposition might be due to germ-line *CDKN2A* mutations.

Methods We used the polymerase chain reaction, single-strand conformation polymorphism analysis, and direct DNA sequencing to identify germ-line mutations in the *CDKN2A* gene in patients with multiple primary melanomas who did not have family histories of the disease. A quantitative yeast two-hybrid assay was used to evaluate the functional importance of the *CDKN2A* variants.

Results Of 33 patients with multiple primary melanomas, 5 (15 percent; 95 percent confidence interval, 4 percent to 27 percent) had germ-line *CDKN2A* mutations. These included a 24-bp insertion at the 5' end of the coding sequence, three missense mutations (Arg24Pro, Met53Ile, and Ser56Ile), and a 2-bp deletion at position 307 to 308 (resulting in a truncated *CDKN2A* protein). In three families, *CDKN2A* mutations identical to those in the probands were found in other family members. In two families with mutations, we uncovered previously unknown evidence of family histories of melanoma.

Conclusions Some patients with multiple primary melanomas but without family histories of the disease have germ-line mutations of the *CDKN2A* gene. The presence of multiple primary melanomas may signal a genetic susceptibility to melanoma not only in the index patient but also in family members, who may benefit from melanoma-surveillance programs. (N Engl J Med 1998;338:879-87.)

©1998, Massachusetts Medical Society.

SOME patients with cancer have one or more subsequent primary tumors of the same histologic subtype. This phenomenon can be explained by the long-term exposure of a particular tissue to a carcinogen ("field cancerization"), the growth of micrometastatic deposits derived from the original primary lesion, or a genetic predisposition.¹⁻³ The identification of patients with genetic lesions that pose a substantial risk of a second primary cancer could have considerable clinical value, prompt-

ing appropriate surveillance, not just for the affected patient but also for other family members.

For a white person living in North America, the estimated lifetime risk of melanoma is approximately 1 in 80.^{4,5} If the development of a single melanoma is considered to be an independent event, it follows that the probability of a second primary lesion is again 1 in 80, or 1.25 percent. In fact, about 5 percent of patients with melanoma have one or more additional primary lesions.^{6,7} This higher-than-expected prevalence of multiple primary tumors may be due to excessive exposure to an environmental factor, such as ultraviolet light. Alternatively, the phenomenon may have a genetic basis. In support of this latter possibility, it is noteworthy that 8 to 24 percent of patients with more than one melanoma have family histories of melanoma.⁸⁻¹³ Moreover, as with familial melanoma, the initial primary lesion in patients with multiple primary melanomas develops at a relatively early age.^{9,10}

Approximately 10 percent of patients with melanoma have family histories of the disease,¹⁴⁻¹⁶ suggesting a genetic susceptibility. In fact, approximately 20 percent of families with a predisposition to melanoma have germ-line mutations in the *CDKN2A* tumor-suppressor gene.¹⁷ In some of these kindreds, there is also a predisposition to pancreatic cancer.¹⁸ *CDKN2A* (also known as *p16*, *p16^{INK4a}*, and *MTS1*) encodes a cell-cycle regulator that inhibits the activities of *cdk4* and *cdk6*, two protein kinases that in turn phosphorylate the retinoblastoma protein.¹⁹⁻²¹ Since unphosphorylated retinoblastoma protein inhibits the expression of genes involved in controlling the progression of the cell cycle, the impaired function of *CDKN2A* may result in dysregulated cellular proliferation.

We hypothesized that patients with more than one primary melanoma, even in the absence of a family history of the disease, might have germ-line mutations of the *CDKN2A* gene. We studied 33 patients with two or more primary melanomas and no

From the Institute of Medical Sciences (J. Monzon, D.H., N.J.L.), the Department of Medical Biophysics (L.L., H.B., D.H., N.J.L.), and the Departments of Dermatology (L.F.) and Preventive Medicine and Biostatistics (J. McLaughlin), University of Toronto, Toronto; the Genetic Epidemiology Branch, National Cancer Institute, Bethesda, Md. (A.M.G., M.A.T.); and the Division of Medical Oncology, Toronto-Sunnybrook Regional Cancer Centre, Toronto (N.J.L.). Address reprint requests to Dr. Lassam at Toronto-Sunnybrook Regional Cancer Centre, 2075 Bayview Ave., Toronto, ON M4N 3M5, Canada.

known family histories of melanoma or pancreatic carcinoma to determine whether they had these mutations.

METHODS

Patients

We reviewed a computerized data bank and medical records at the Toronto–Sunnybrook Regional Cancer Centre to identify patients with multiple primary melanomas and no family histories of the disease. We defined multiple primary disease as either two or more biopsy-confirmed invasive melanomas or one melanoma in situ and one or more invasive melanomas. A total of 28 patients met these criteria and were available to participate in the study. Five additional patients were referred to us: three from the Hamilton Regional Cancer Centre, Hamilton, Ontario, Canada, and two from the National Institutes of Health, Bethesda, Maryland. The 33 patients were unrelated to one another, all were white, and they ranged in age from 21 to 67 years. All but two of the patients resided in the greater Toronto area. The patients were questioned in detail about a possible family history of malignant melanoma or pancreatic carcinoma, and all 33 reported no knowledge of a family history of either cancer. Written informed consent was obtained from all the patients.

Polymerase Chain Reaction and Single-Strand Conformation Polymorphism Analysis

Leukocyte DNA was extracted from peripheral-blood samples with the use of standard methods. Exons 1 α and 2 of the *CDKN2A* gene were amplified with the use of the primers listed in Table 1. The reaction mixture for the polymerase chain reaction (PCR) consisted of 0.5 U of DNA *Taq* polymerase (GIBCO BRL, Gaithersburg, Md.), 1 \times PCR buffer (20 mM TRIS–hydrochloric acid, pH 8.4, and 50 mM potassium chloride), 1.5 mM magnesium chloride, 0.2 mM deoxynucleoside triphosphate, 1 mM forward and reverse primers, 0.05 μ Ci of [α -³²P]deoxycytidine triphosphate, and 100 ng of DNA from the patient (the PCR reactions for exon 1 α also contained 10 percent dimethylsulfoxide). The samples were denatured at 95°C for 5 minutes and then subjected to 35 cycles of denaturing for 1 minute at 95°C, annealing at 58 to 61°C for 1 minute, and extension at 72°C for 2 minutes, followed by a final period of extension at 72°C for 2.5 minutes. Since the sensitivity of single-strand conformation polymorphism (SSCP) analysis in detecting mutations tends to de-

crease as the size of the PCR product increases, fragments greater than 300 bp were digested with the appropriate restriction endonucleases. The reactions were stopped by the addition of an equal volume of stop buffer (95 percent formamide, 20 mM EDTA, 0.05 percent bromophenol blue, and 0.05 percent xylene cyanol), and the samples were denatured at either 98°C (exon 1 α) or 95°C (exon 2) for five minutes and then cooled on ice. The DNA fragments were separated electrophoretically at room temperature on a 6 percent nondenaturing polyacrylamide gel (supplemented with 10 percent glycerol) at 6 W for 12 to 18 hours. After electrophoresis, the gels were dried and exposed to autoradiographic film for 8 to 48 hours.

DNA Sequencing

All DNA samples were also examined by direct sequencing. PCR was carried out as described above with the use of M13 tagged primers ME1-F and ME1-R for exon 1 α and ME2-F and ME2-R for exon 2. The PCR products were separated electrophoretically on 2 percent agarose gels and purified with the use of the Qiaex II gel extraction kit (Qiagen, Chatsworth, Calif.). Both strands of the PCR products were sequenced with the use of the appropriate set of M13 primers and analyzed with the ABI Prism 310 Genetic Analyzer (Perkin–Elmer, Foster City, Calif.). To confirm the presence of allele-specific mutations, all DNA samples displaying variant SSCP bands or heterozygous sequences or both were reamplified with the use of specific primers for exon 1 α and exon 2 (exon 1 α , E1-F and E1-R; exon 2, E2-F and E2-R). The resulting products were ligated into the pCR-Script Amp SK (+) cloning vector (Stratagene, La Jolla, Calif.) and sequenced with the use of the ABI Prism Dye Terminator kit (Perkin–Elmer).

Yeast Two-Hybrid Assay

A yeast two-hybrid assay²² was used to evaluate the interaction between the various *CDKN2A* variants and *cdk4*. As shown in Figure 1, this assay measures the reconstitution of a functional transcriptional activator from two separate fusion proteins: *CDKN2A* fused to an activation domain (derived from the herpes simplex virus VP16 protein), and *cdk4* fused to a DNA-binding domain (derived from the *Escherichia coli* LexA protein).

A second peripheral-blood sample was obtained from each patient with a germ-line *CDKN2A* mutation, and leukocyte RNA was extracted with the use of the RNeasy Total RNA kit (Qiagen). First-strand complementary DNA (cDNA) was synthesized with 3 to 5 μ g of total RNA and 500 ng of oligo-dT₁₆, as described

TABLE 1. PRIMERS USED IN THE STUDY.

PRIMER	SEQUENCE	EXON	DIRECTION	APPLICATION*	ANNEALING TEMPERATURE (°C)
E1-F	GAAGAAAGAGGAGGGGCT	1	Forward	SSCP analysis	61
E1-R	GCGCTACCTGATTCCAATTC	1	Reverse		
E2-F	TGGCTCTGACCATTCTGTTC	2	Forward	SSCP analysis	58
E2-R	TTTGGAAGCTCTCAGGGTAC	2	Reverse		
ME1-F	TGTA AACACGACGGCCAGTCTGCGGAGAGGGGAGAGCA	1	Forward	Sequencing and SSCP analysis	60
ME1-R	CAGGAAACAGCTATGACCAGAGTCGCCCGCCATCCCCCT	1	Reverse		
ME2-F	TGTA AACACGACGGCCAGTCTCTACACAAGCTTCCTTTCC	2	Forward	Sequencing	60
ME2-R	CAGGAAACAGCTATGACCGGGCTGAACTTCTGTGTGCTGG	2	Reverse		
cDNA-F	CGGAGAGGGGGAGAACAGAC	1	Forward	cDNA synthesis	58
cDNA-R	TTACGGTAGTGGGGGAAGGC	3	Reverse		
E1- <i>Bgl</i> II-F	GGAGAGGGGGAGATCTGACAAAGGGCG	1	Forward	cDNA synthesis	68
E3- <i>Nor</i> I-R	GTGGGTTGTGGCGCCGCAGTTGTGG	3	Reverse		

*SSCP denotes single-strand conformation polymorphism.

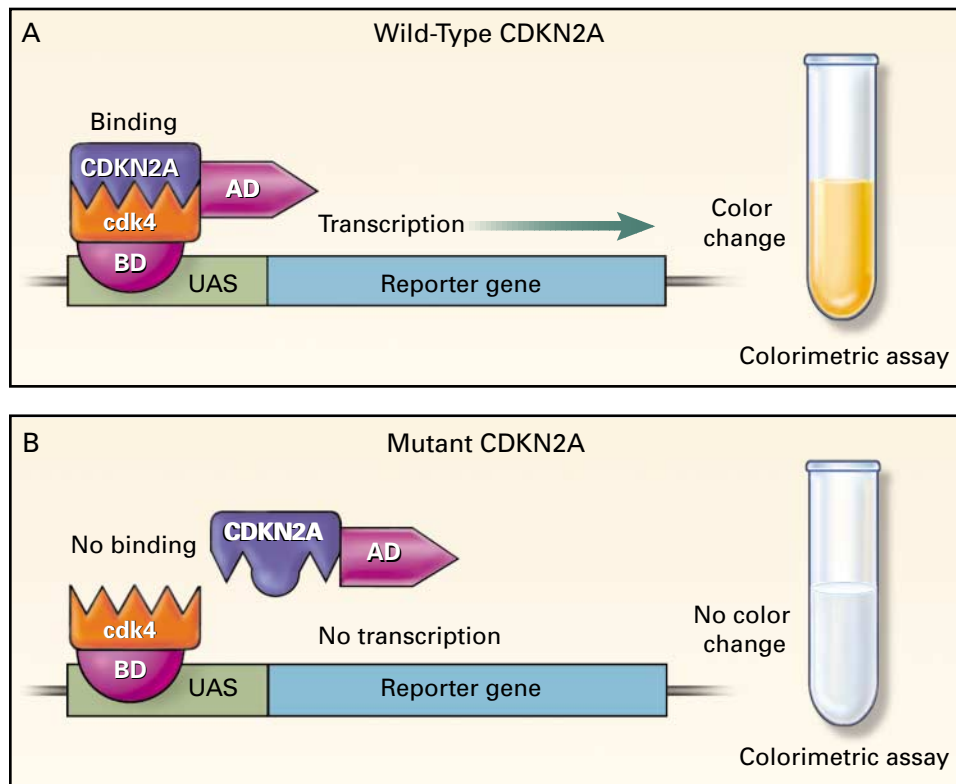


Figure 1. The Yeast Two-Hybrid Assay.

Wild-type *cdk4* was fused to a DNA-binding domain (BD) derived from the LexA bacterial transcription factor, which binds specific sequences within the upstream activation site (UAS) of the reporter gene. CDKN2A was fused to an activation domain (AD) derived from a herpes simplex virus transcription factor. Binding of *cdk4* to CDKN2A (Panel A) reconstituted the transcriptional activator and resulted in the expression of the reporter gene. The level of the reporter-gene product was then quantitated with the use of a colorimetric assay. Mutant CDKN2A lacked the ability to bind to wild-type *cdk4* and did not reconstitute the transcriptional activator, resulting in little or no expression of the reporter gene (Panel B).

by the manufacturer (GIBCO BRL). The first PCR was carried out in 1× standard PCR buffer, 2.5 mM magnesium chloride, 0.2 mM deoxynucleoside triphosphate, 5 percent dimethylsulfoxide, 0.5 mM each of cDNA-F and cDNA-R (Table 1), 0.5 U of *Taq* polymerase, and 1 μl of the cDNA mixture. The mixture was heated to 95°C for five minutes and then subjected to 30 cycles at 95°C for one minute, 58°C for one minute, and 72°C for two minutes, followed by a period of extension at 72°C for five minutes. Nested PCR was then carried out with the use of the first PCR product as the template, together with synthetic oligonucleotides containing *Bgl*III or *Not*I restriction cloning sites (0.4 mM each of E1-*Bgl*III-F and E3-*Not*I-R) (Table 1) and the same reaction concentrations used for the first PCR. The conditions for nested PCR consisted of an initial denaturing period of 95°C for five minutes, followed by 35 cycles at 95°C for one minute and 68°C for three minutes, and a final extension period at 72°C for seven minutes. The resultant DNA fragments were purified and subjected to automated sequencing before being analyzed. cDNA encoding the mutant form of *CDKN2A* with the 24-bp insertion was kindly provided by G. Peters (Imperial Cancer Research Fund Laboratories, London).

The full-length *CDKN2A* allelic variants generated by reverse-transcription PCR were digested with *Bgl*III and *Not*I, purified, and subsequently ligated in frame into a pVP16 cDNA vector (a

gift from S. Hollenberg, Fred Hutchinson Cancer Research Center, Seattle) to yield plasmid pVP16/p16.

Wild-type *cdk4* cDNA (a gift from G. Hannon and D. Beach, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) was ligated in frame with *E. coli* LexA DNA-binding domain encoded by the pBTM116 expression vector (a gift from S. Hollenberg) to yield the plasmid pBTM116/CDK4.

The pBTM116/CDK4 plasmid was introduced into L40 *Saccharomyces cerevisiae* through lithium acetate transformation. Colonies were selected on Trp(-) medium, transformed with pVP16/p16 plasmids containing either the wild-type or variant *CDKN2A* alleles, and selected on Trp(-)/Leu(-) medium. The remaining colonies were then cultured in Trp(-)/Leu(-) medium overnight at 37°C, and a colorimetric β-galactosidase assay²² was used to quantitate CDKN2A binding with CDK4. The assays were performed at least three times for all CDKN2A variants and in all cases were normalized to the binding activity of wild-type CDKN2A.

RESULTS

Study Population

Table 2 shows the clinical characteristics of the patients. The mean age at the time of the first diagno-

TABLE 2. CHARACTERISTICS OF THE 33 PATIENTS.

CHARACTERISTIC	VALUE
Sex (no. of patients)	
Men	16
Women	17
Age at first diagnosis (yr)	
Mean \pm SD	43 \pm 13.6
Range	21–67
Synchronous melanomas (no. of patients)	13
No. of melanomas excised (no. of patients)	
2	25
3	5
>3	3
Dysplastic nevi (no. of patients)	
Present	29
Absent	3
Unknown	1

sis of melanoma was 43 years. In less than half the cases (39 percent), the second melanoma was diagnosed within one year after the first (defined as synchronous). Seventy-six percent of the patients had only two melanomas removed, and 24 percent had three or more excised. In addition to multiple primary melanomas, most of the patients (91 percent) also had clinically or histologically diagnosed dysplastic nevi. The relatively young age at the time of the first diagnosis of melanoma and the presence of dysplastic nevi are typical of multiple primary melanomas.^{10,12}

Mutational Analyses

The primers used for PCR–SSCP analysis were selected so that the amplified DNA fragment encompassed the entire coding sequence and splice junctions of exons 1 α and 2 of *CDKN2A*. We did not analyze exon 3, since no mutations have been identified within this 11-bp exon.²³ Using SSCP analysis, we detected bands of aberrant mobility in 4 of the 33 patients; two bands were located in exon 1 α , and two in exon 2.

We also sequenced the genomic DNA of exons 1 α and 2 of the *CDKN2A* gene in all 33 patients. This analysis showed that five patients (15 percent; 95 percent confidence interval, 4 percent to 27 percent) had germ-line mutations in the *CDKN2A* gene. Four of these patients had abnormal SSCP banding patterns (as described above), whereas the pattern in the fifth patient was indistinguishable from that of the wild-type gene. The five mutations (Fig. 2) included a 24-bp insertion at the beginning of exon 1 that resulted in the addition of eight amino acids to the amino terminal end of the CDKN2A protein, a 2-bp deletion of nucleotides 307 to 308 that resulted in a premature termination signal at

codon 117, and three missense mutations: the substitution of C for G at nucleotide 71 (Arg24Pro), the substitution of C for G at nucleotide 159 (Met53Ile), and the substitution of T for G at nucleotide 167 (Ser56Ile). To confirm these findings, a second blood sample from each of the five patients was analyzed. The *CDKN2A* exon bearing the relevant mutation was amplified by PCR, subcloned, and sequenced. Although SSCP screening is generally considered to be a less sensitive means of detecting mutations than DNA sequencing, it is technically straightforward and has been used in a number of studies to screen for *CDKN2A* mutations. Although not unexpected, our observations indicate that direct DNA-sequence analysis of the *CDKN2A* gene gives fewer false negative results than SSCP analysis.

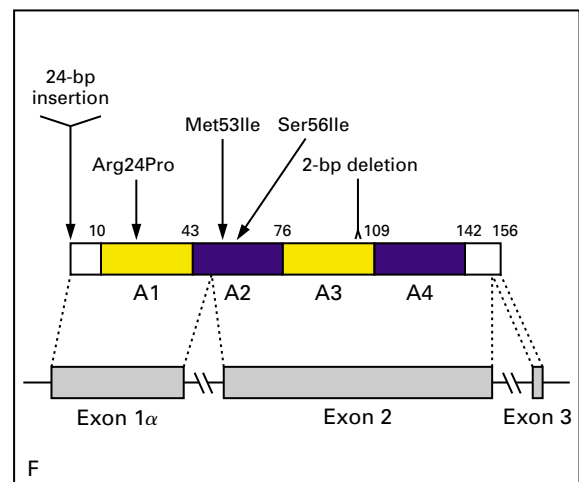
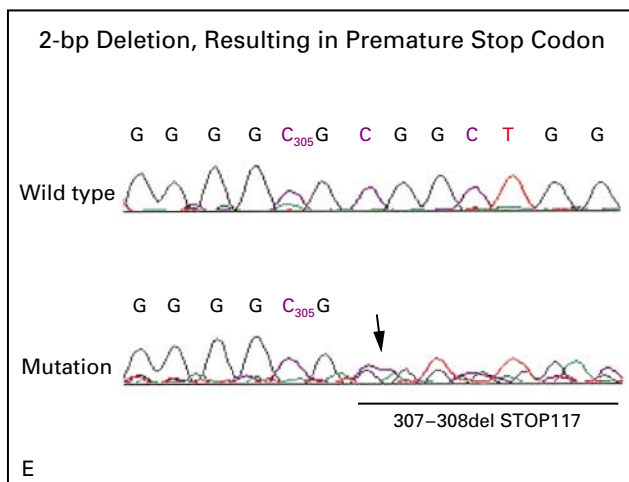
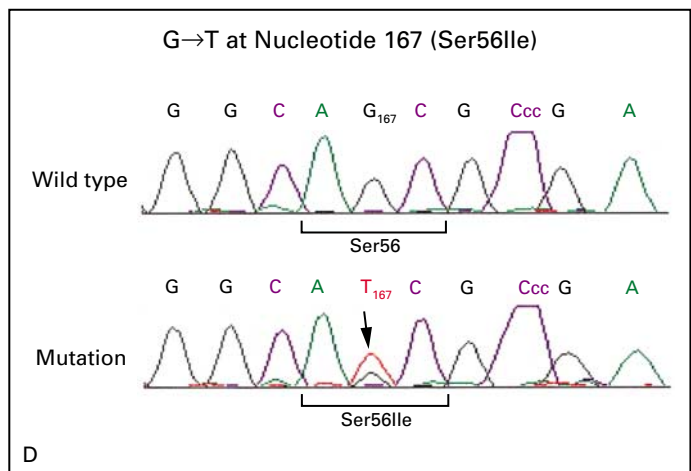
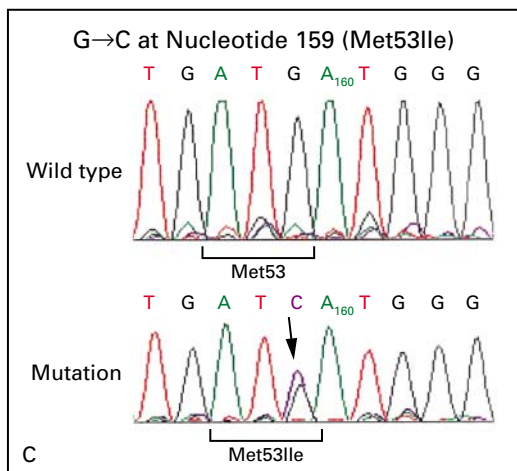
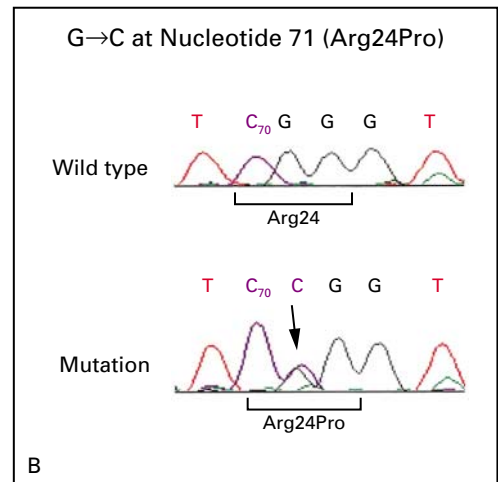
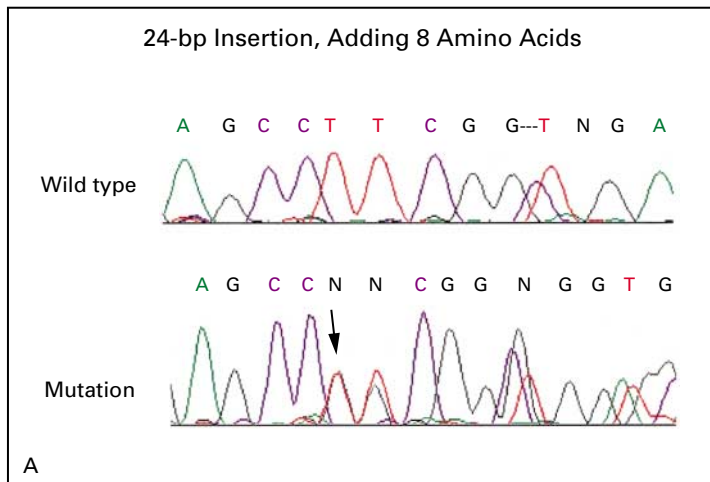
All five patients with *CDKN2A* germ-line mutations resided in the greater Toronto area. In two of these patients, one invasive melanoma and one melanoma in situ had previously been diagnosed, whereas in the other three patients, two or more invasive melanomas had been diagnosed.

Results of the Yeast Two-Hybrid Assay

An alteration in the germ-line sequence of the *CDKN2A* gene may not necessarily alter the function of the corresponding protein. To help discriminate clinically relevant germ-line mutations from polymorphic variants, which do not usually affect the function of the protein, we used a yeast two-hybrid system to measure the binding of CDKN2A to one of its targets: cdk4. The yeast two-hybrid system exploits the fact that many transcriptional activators have two distinct domains: a site-specific DNA-binding domain and an activation domain. When the two domains are separated, the transcriptional activator is inactive. To evaluate the interaction between two proteins, each domain is separated and fused to one of the two proteins. If the two proteins interact, the transcriptional activator is reconstituted, resulting in the expression of a reporter gene. In our assay, wild-type or mutant forms of CDKN2A were fused to an activation domain derived from the herpes simplex

Figure 2. Heterozygous Mutations in *CDKN2A* Found in Patients with Multiple Primary Melanomas and Their Locations on the Gene and Its Corresponding Protein.

The following heterozygous mutations, with the corresponding wild-type sequences, are shown: Panel A, an insertion of 24 bp, resulting in an additional eight amino acids on the amino terminal of CDKN2A; Panel B, the substitution of C for G at nucleotide 71 (Arg24Pro); Panel C, the substitution of C for G at nucleotide 159 (Met53Ile); Panel D, the substitution of T for G at nucleotide 167 (Ser56Ile); and Panel E, the deletion of 2 bp at nucleotides 307 to 308, resulting in a premature termination signal at codon 117. Panel F shows the locations of the mutation on the gene and the protein. The locations of ankyrin-like repeats (A1, A2, A3, and A4) are also shown.



virus VP16 protein, and wild-type cdk4 was fused to an *E. coli* LexA DNA-binding domain (Fig. 1). Binding of CDKN2A to cdk4 resulted in the expression of the reporter gene, and its product was quantitated with the use of a colorimetric assay.

Four of the five CDKN2A variants had decreased binding to cdk4, with binding activity ranging from 2 percent to 11 percent, as compared with the wild-type form of CDKN2A (Fig. 3). In contrast, the mutant CDKN2A protein with the insertion of eight amino acids showed normal binding to cdk4. This result is not entirely unexpected, since the eight-amino-acid insertion is situated outside the ankyrin motifs that are believed to play a part in cdk4 binding. Parry and Peters²⁵ analyzed the binding of this same mutant CDKN2A to cdk4 and also failed to observe a reduction in binding as compared with the wild-type form of CDKN2A. Although the functional importance of this insertional mutation is unclear, it is noteworthy that the corresponding 24-bp insertion has been observed in a number of melanoma-prone kindreds throughout the world.

Pedigree Investigation

Although all the patients in our study reported that they did not have family histories of melanoma or pancreatic carcinoma, three of the mutations we detected — the eight-amino-acid insertion, the Arg24Pro mutation, and the Met53Ile mutation — have been reported in melanoma-prone families and have been found to cosegregate with the cases of melanoma.^{18,26,27} For our five patients with *CDKN2A* mutations, we therefore made special efforts to uncover evidence of family histories of melanoma and other cancers by reviewing a computerized data base of sporadic and familial cases of melanoma at the Toronto–Sunnybrook Regional Cancer Centre and by contacting family members. We found that the father of the patient with the Met53Ile mutation had died of a “melanotic sarcoma” and that additional family members had received diagnoses of melanoma, one of whom was found to have the same mutation as the index patient (Fig. 4, Pedigree 1). The patient with the Ser56Ile mutation was also found to have a family history of melanoma (Fig. 4, Pedigree 2). This patient had an invasive melanoma removed at the age of 36 years and the following year had a melanoma in situ resected. Although the patient was unaware of any family history of melanoma, a data-base search revealed that one of his daughters (who was 4 years old at the time he received the second diagnosis of melanoma) had had a melanoma in situ excised at the age of 18 years. She also had the Ser56Ile mutation. The patient’s other two children had clinically apparent dysplastic nevi, but their *CDKN2A* status is unknown.

We were unable to uncover any evidence of a family history of melanoma in the other three patients

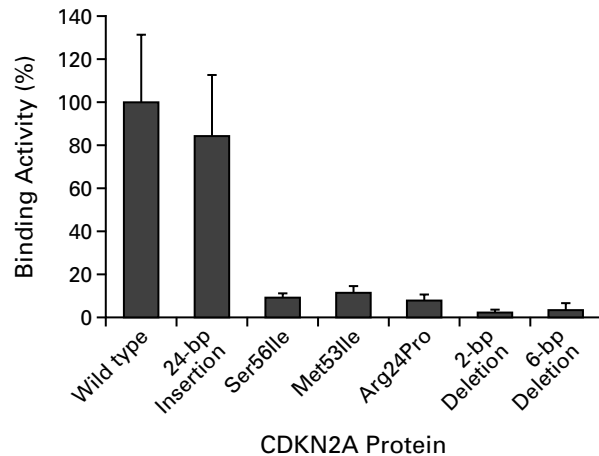


Figure 3. Binding Activity of Mutant CDKN2A Proteins in the Yeast Two-Hybrid Assay.

The binding activity of the various mutant proteins was normalized to that obtained with the wild-type construct. The 6-bp deletion is a previously described in-frame deletion²⁴ known to be associated with deficient binding to cdk4. The means (\pm SE) of three experiments are shown.

with mutations. The patient with the 24-bp insertion had a malignant melanoma removed at the age of 42 years and a melanoma in situ excised the following year. The patient with the Arg24Pro mutation had six independent primary melanomas resected: two invasive melanomas and four melanomas in situ. Finally, the patient with the 2-bp deletion had three melanomas removed: two invasive melanomas and one melanoma in situ. His father had the mutant *CDKN2A* allele but no history of any cancer. Both of the patient’s siblings had the same mutation but had not received diagnoses of melanoma. Although cancers other than melanoma have been reported in some kindreds with *CDKN2A* mutations, none of the persons with mutations in the five families we investigated had a history of any tumor other than melanoma.

DISCUSSION

Several lines of evidence demonstrate that germline mutations of the *CDKN2A* gene confer a predisposition to the development of melanoma in some kindreds with familial malignant melanoma. First, mutational analyses of the *CDKN2A* gene in such kindreds have revealed a large number of germline *CDKN2A* gene mutations that cosegregate with the cases of melanoma. Second, the *CDKN2A* germline mutations in these melanoma-prone families encode CDKN2A proteins that fail to bind to or inhibit the activity of a downstream target of CDKN2A, cdk4.^{24,28-30} Third, if loss of function of the CDKN2A protein confers a predisposition to

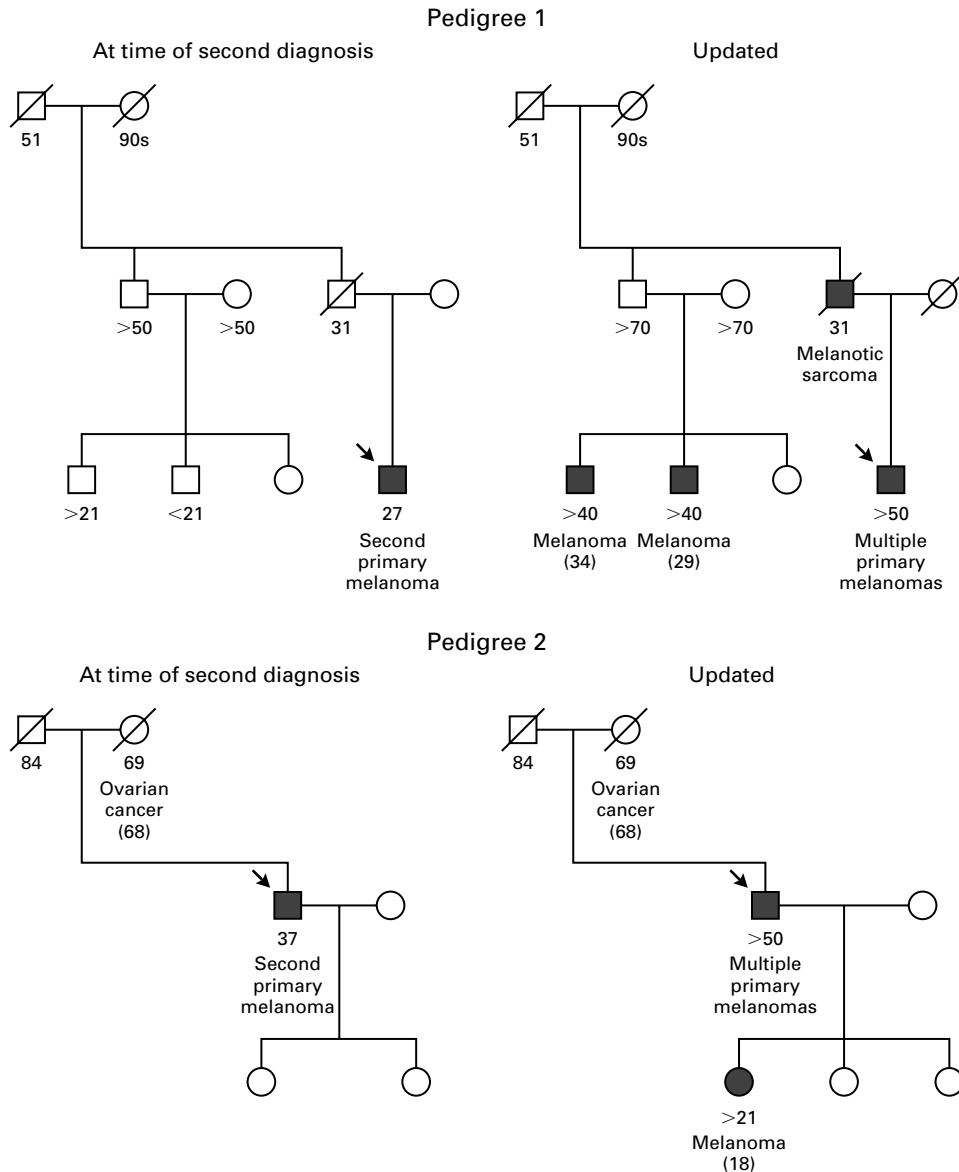


Figure 4. Pedigrees of Two Patients with Germ-Line *CDKN2A* Mutations and Multiple Primary Melanomas, According to Information Obtained at the Time of the Second Diagnosis of Melanoma in the Index Patient (Arrow) and Additional Information Obtained Subsequently.

The pedigrees have been simplified to maintain confidentiality. Circles represent female family members, squares male family members, solid symbols family members with melanoma, clear symbols family members without melanoma, and slashes dead family members. The numbers below the symbols indicate the age of the family member at the time of the history taking or at the time of death in the case of deceased family members; numbers in parentheses indicate the age at the time of the diagnosis.

malignant melanoma, it follows that tumor specimens from affected members of melanoma-prone kindreds should contain one *CDKN2A* allele with the original germ-line mutation, with the second allele either lost or bearing a mutation that results in the production of a dysfunctional protein. Loss of the wild-type allele has, in fact, been detected both

in cell lines and in tumor specimens from patients with familial melanoma.^{24,31} In addition to mutations of the *CDKN2A* gene, alterations of other components of the signaling pathway regulated by the *CDKN2A* gene product may also confer a predisposition to melanoma, although the frequency or penetrance of mutations in such genes may be lower

than that of mutations of *CDKN2A*. For example, two melanoma-prone families have been described with an identical germ-line mutation in the *CDK4* gene,³² and patients with germ-line mutations of the retinoblastoma gene, who have retinoblastoma as children, have an increased incidence of melanoma as adults.³³⁻³⁶

Several clinical observations suggest a genetic predisposition in some patients with multiple primary melanomas. The probability that more than one melanoma will develop simply by chance is far lower than the observed frequency of multiple melanomas. Moreover, the first tumor in patients with multiple primary melanomas tends to develop at a younger age than in patients with a solitary melanoma. Many patients with multiple primary melanomas also have dysplastic nevi, which are associated with an increased risk of melanoma. The genetic abnormalities conferring a predisposition to multiple primary melanomas may parallel the genetic abnormalities in the familial form of this cancer. The frequency of germ-line mutations of *CDKN2A* in our patients (5 in 33 patients, or 15 percent; 95 percent confidence interval, 4 to 27 percent) was similar to that observed among kindreds in southern Ontario with two or more first- or second-degree relatives who had diagnoses of melanoma (11 in 39 patients, or 28 percent; 95 percent confidence interval, 14 to 43 percent; data not shown). Thus, a patient with multiple primary melanomas has about the same probability of carrying a germ-line mutation in the *CDKN2A* gene as an affected member of a melanoma-prone family.

The absence of a family history in most patients with multiple primary melanomas suggests that additional genetic or environmental factors, or both, affect the phenotypic expression of *CDKN2A* mutations. It is often difficult to obtain an accurate family history of cancer, even among patients who are highly motivated to provide accurate information, but we uncovered evidence of family histories in two of our patients with multiple primary melanomas. A daughter of the patient with the Ser561Ile mutation was less than five years old at the time her father received the diagnosis of a second melanoma; 14 years later, unbeknownst to her father, she received a diagnosis of melanoma in situ (Fig. 4, Pedigree 2). Similarly, although the patient with the Met53Ile mutation had no knowledge of a family history of melanoma, we ultimately identified other family members with melanoma (Fig. 4, Pedigree 1).

Four of the five mutations we detected encoded dysfunctional *CDKN2A* protein that had reduced binding to one of its natural targets, *cdk4*. The abnormalities detected included the Arg24Pro, Met53Ile, and Ser561Ile missense mutations, as well as a 2-bp deletion at nucleotides 307 through 308 that resulted in a frame shift and a premature termination sig-

nal at codon 117. The functional consequence of the fifth mutation (the eight-amino-acid insertion) remains obscure. Despite numerous attempts, we were unable to demonstrate that this mutant form of *CDKN2A* bound to *cdk4* less avidly than the wild-type form. Our results are similar to those of Parry and Peters,²⁵ who evaluated the same mutant form using a different in vitro binding assay and also found no evidence of reduced binding to *cdk4*. These data, coupled with the fact that the insertion lies outside the ankyrin-repeat region of *CDKN2A*, which is believed to mediate binding to *cdk4*, suggest that this mutant form is functionally normal with respect to *cdk4* binding. Four melanoma-prone families with the same insertion have been reported (two in Australia, one in England, and one in the United States). In all these cases, the mutant *CDKN2A* allele cosegregated with the cases of melanoma. There is also evidence that in two of these families, the mutations did not arise from a common founder (Hayward N: personal communication). These observations suggest that *CDKN2A* mutations outside the ankyrin motifs confer a predisposition to melanoma through a mechanism that has not yet been identified. It is possible that some mutant *CDKN2A* proteins fail to bind to other downstream targets, such as *cdk6*.

We conclude that patients with multiple primary melanomas have a genetic predisposition to this cancer that resembles the genetically determined susceptibility in melanoma-prone families. Our data reinforce the importance of obtaining a detailed family history from any patient with more than one melanoma and from sources other than the patient. Furthermore, we suggest that even in the absence of a family history, other family members may benefit from ongoing clinical surveillance. Although the precise role of genetic screening in the management of melanoma is currently unclear, close relatives of patients with multiple primary melanomas may benefit from such analyses.

Supported by grants from the National Cancer Institute of Canada and the Medical Research Council of Canada.

We are indebted to Drs. A. Schub and E. Warner for critically reviewing the manuscript, to Drs. J. Rusthoven and M. McKenzie for their help in referring patients for the study, and to the patients and their families who participated in the study.

REFERENCES

1. Birch JM. Genetic determinants of cancer in man. In: Waring MJ, Ponder B, eds. *Biology of carcinogenesis*. Lancaster, Pa.: MTP Press, 1987:165-89.
2. Schottenfeld D. Multiple primary cancers. In: Schottenfeld D, Fraumeni J, eds. *Cancer epidemiology and prevention*. 2nd ed. New York: Oxford University Press, 1996:1370-87.
3. Carey TE. Field cancerization: are multiple primary cancers monoclonal or polyclonal? *Ann Med* 1996;28:183-8.
4. Rigel DS, Friedman RJ, Kopf AW. The incidence of malignant melanoma in the United States: issues as we approach the 21st century. *J Am Acad Dermatol* 1996;34:839-47.

5. Rigel DS. Malignant melanoma: incidence issues and their effect on diagnosis and treatment in the 1990s. *Mayo Clin Proc* 1997;72:367-71.
6. Giles G, Staples M, McCredie M, Coates M. Multiple primary melanomas: an analysis of cancer registry data from Victoria and New South Wales. *Melanoma Res* 1995;5:433-8.
7. Brobeil A, Rapaport D, Wells K, et al. Multiple primary melanomas: implications for screening and follow-up programs for melanoma. *Ann Surg Oncol* 1997;4:19-23.
8. Gupta BK, Piedmonte MR, Karakousis CP. Attributes and survival patterns of multiple primary cutaneous malignant melanoma. *Cancer* 1991; 67:1984-9.
9. Moseley HS, Giuliano AE, Storm FK III, Clark WH, Robinson DS, Morton DL. Multiple primary melanoma. *Cancer* 1979;43:939-44.
10. Frank W, Rogers GS. Melanoma update: second primary melanoma. *J Dermatol Surg Oncol* 1993;19:427-30.
11. Slingluff CL Jr, Vollmer RT, Seigler HF. Multiple primary melanoma: incidence and risk factors in 283 patients. *Surgery* 1993;113:330-9.
12. Kang S, Barnhill RL, Mihm MC Jr, Sober AJ. Multiple primary cutaneous melanomas. *Cancer* 1992;70:1911-6.
13. Reimer RR, Clark WH Jr, Greene MH, Ainsworth AM, Fraumeni JF Jr. Precursor lesions in familial melanoma: a new genetic preneoplastic syndrome. *JAMA* 1978;239:744-6.
14. Greene MH, Clark WH Jr, Tucker MA, Kraemer KH, Elder DE, Fraser MC. High risk of malignant melanoma in melanoma-prone families with dysplastic nevi. *Ann Intern Med* 1985;102:458-65.
15. Goldstein AM, Tucker MA. Genetic epidemiology of familial melanoma. *Dermatol Clin* 1995;13:605-12.
16. Lynch HT, Fusaro RM, Lynch J. Hereditary cancer in adults. *Cancer Detect Prev* 1995;19:219-33.
17. Goldstein AM, Tucker MA. Screening for CDKN2A mutations in hereditary melanoma. *J Natl Cancer Inst* 1997;89:676-8.
18. Goldstein AM, Fraser MC, Struewing JP, et al. Increased risk of pancreatic cancer in melanoma-prone kindreds with *p16^{INK4}* mutations. *N Engl J Med* 1995;333:970-4.
19. Sherr CJ. Cancer cell cycles. *Science* 1996;274:1672-7.
20. Herwig S, Strauss M. The retinoblastoma protein: a master regulator of cell cycle, differentiation and apoptosis. *Eur J Biochem* 1997;246:581-601.
21. Kamb A. Cell-cycle regulators and cancer. *Trends Genet* 1995;11:136-40.
22. Bartel PL, Fields S. Analyzing protein-protein interactions using two-hybrid system. *Methods Enzymol* 1995;254:241-63.
23. Smith-Sorensen B, Hovig E. CDKN2A (p16INK4A) somatic and germline mutations. *Hum Mutat* 1996;7:294-303.
24. Liu L, Lassam NJ, Slingerland JM, et al. Germline p16INK4A mutation and protein dysfunction in a family with inherited melanoma. *Oncogene* 1995;11:405-12.
25. Parry D, Peters G. Temperature-sensitive mutants of p16CDKN2 associated with familial melanoma. *Mol Cell Biol* 1996;16:3844-52.
26. Walker GJ, Hussussian CJ, Flores JF, et al. Mutations of the CDKN2/p16INK4 gene in Australian melanoma kindreds. *Hum Mol Genet* 1995; 4:1845-52.
27. Holland EA, Beaton SC, Becker TM, et al. Analysis of the p16 gene, CDKN2, in 17 Australian melanoma kindreds. *Oncogene* 1995;11:2289-94.
28. Ranade K, Hussussian CJ, Sikorski RS, et al. Mutations associated with familial melanoma impair p16INK4 function. *Nat Genet* 1995;10:114-6.
29. Lilischkis R, Sarcevic B, Kennedy C, Warlters A, Sutherland RL. Cancer-associated mis-sense and deletion mutations impair p16INK4 CDK inhibitory activity. *Int J Cancer* 1996;66:249-54.
30. Koh J, Enders GH, Dynlacht BD, Harlow E. Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. *Nature* 1995;375: 506-10.
31. Hussussian CJ, Struewing JP, Goldstein AM, et al. Germline p16 mutations in familial melanoma. *Nat Genet* 1994;8:15-21.
32. Zuo L, Weger J, Yang Q, et al. Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma. *Nat Genet* 1996;12:97-9.
33. Draper GJ, Sanders BM, Kingston JE. Second primary neoplasms in patients with retinoblastoma. *Br J Cancer* 1986;53:661-71.
34. Traboulsi EI, Zimmerman LE, Manz HJ. Cutaneous malignant melanoma in survivors of heritable retinoblastoma. *Arch Ophthalmol* 1988; 106:1059-61.
35. Eng C, Li FP, Abramson DH, et al. Mortality from second tumors among long-term survivors of retinoblastoma. *J Natl Cancer Inst* 1993;85: 1121-8.
36. Bataille V, Hiles R, Bishop JA. Retinoblastoma, melanoma and the atypical mole syndrome. *Br J Dermatol* 1995;132:134-8.

RECEIVE THE *JOURNAL'S* TABLE OF CONTENTS EACH WEEK BY E-MAIL

To receive the table of contents of the *New England Journal of Medicine* by e-mail every Thursday morning, send an e-mail message to:

listserv@massmed.org

Leave the subject line blank, and type the following as the body of your message:

subscribe TOC-L

You can also sign up through our website at: <http://www.nejm.org>
