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## Genotype–Phenotype Relationships in U.S. Melanoma-Prone Families With CDKN2A and CDK4 Mutations

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**Background:** Two genes have been implicated in the development of cutaneous malignant melanoma (CMM). CDK4 (the gene encoding cyclin-dependent kinase 4, an oncogene) has exhibited germline mutations found in only three melanoma-prone families to date. CDKN2A is a tumor suppressor gene that encodes p16 (which inhibits activity of the cyclin D1–CDK4 complex) with germline mutations detected in 10%–25% of melanoma-prone families, some of whom are also prone to pancreatic cancer. **Methods:** We compared 104 CMM patients from 17 CDKN2A families and 12 CMM case subjects from two CDK4 families. We used nonparametric statistics to test for differences in median age at first CMM diagnosis, numbers of CMMs, and numbers of nevi. The three recurrent mutations were haplotyped. All *P* values were two-sided. **Results:** The median age at CMM diagnosis (*P* = .70) and the median numbers of CMMs (*P* = .73) did not differ between CMM case subjects from CDKN2A versus CDK4 families. Assessment of CMM case subjects from CDKN2A families with and without pancreatic cancer revealed no statistically significant differences in median age at diagnosis (*P* = .80) or in tumor number (*P* = .24). There was, however, a statistically significant difference in age-adjusted median numbers of nevi (*P* = .004), and CMM case subjects from CDKN2A families without pancreatic cancer had greater numbers of nevi. Recurrent CDKN2A mutations were a change from valine to aspartic acid at codon 126 (*n* = 3) and from glycine to tryptophan at codon 101 (*n* = 3). Six CDKN2A families had pancreatic cancer. Both CDK4 families carried a mutation resulting in an arginine-to-cysteine substitution at codon 24. Analyses of recurrent CDKN2A

and CDK4 mutations suggested common haplotypes. **Conclusions:** The recurrent CDKN2A mutations were observed in families with and without pancreatic cancer, which suggests that other factors may be involved in the development of pancreatic cancer. Despite hypothetical differences in the mechanisms of action between CDKN2A and CDK4, clinical factors were indistinguishable between CMM case subjects from CDKN2A versus CDK4 families. [J Natl Cancer Inst 2000;92:1006–10]

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Cutaneous malignant melanoma (CMM) is a potentially fatal form of skin cancer whose etiology is heterogeneous and complex. In the United States, the age-adjusted incidence rate for melanoma in whites (1990 through 1996) was 13.9 per 100 000. During the same period in the United States, the mortality rate was 2.5 per 100 000 (1). Approximately 10% of malignant melanomas develop in individuals with a familial predisposition and often in association with clinically dysplastic or atypical nevi (2).

To date, two genes have been implicated in melanoma pathogenesis. The first, CDKN2A, located on chromosome 9p21, encodes a low-molecular-weight protein, p16, that inhibits the activity of the cyclin D1–cyclin-dependent kinase 4 (CDK4) complex (3). This complex phosphorylates the retinoblastoma protein, allowing the cell to progress through the G<sub>1</sub> cell-cycle checkpoint. Thus, p16 acts as a tumor suppressor and negatively regulates cell growth by arresting cells at G<sub>1</sub>. Germline CDKN2A mutations have been detected in 10%–25% of melanoma-prone families from North America, Europe, and Australia (4). In addition, some CDKN2A melanoma-prone families also have pancreatic cancer. Several studies (5–8) have demonstrated an increased risk of pancreatic cancer among CDKN2A melanoma-prone families, although the precise relationship between the CDKN2A gene and pancreatic cancer remains unknown.

In contrast, the second melanoma gene CDK4, located at 12q13, acts as an oncogene (9), and germline mutations have been detected in only three melanoma-prone families worldwide (10,11). The Arg24Cys germline mutation, identified in two families (10), was first described as a tumor-specific antigen in sporadic melanoma; the alteration produced a mutated

protein that prevented binding of the CDK4 protein to p16 (9). The second germline mutation, Arg24His, which occurred in the same codon as the first alteration, has been observed in one family (11). Other genetic factors remain to be identified.

In this study and in previous work, we identified germline mutations in 19 melanoma-prone families. Seventeen families had CDKN2A mutations [(12–14); current study], and two families had CDK4 mutations (10). Recurrent CDKN2A mutations were Val126Asp (three families) and Gly101Trp (three families). In addition, six CDKN2A families, with five different mutations—Arg87Pro, Gly101Trp, Val126Asp (*n* = 2), IVS2 + 1, and 234del14—had at least one case of pancreatic cancer. Both CDK4 families carried the Arg24Cys mutation. Given the different mechanisms of action of the tumor suppressor CDKN2A and the dominant oncogene CDK4 plus the observation of pancreatic cancer in only a subset of CDKN2A families, we hypothesized that clinical characteristics in CMM case subjects might differ in the various sets of families. Thus, in the present study, we used nonparametric statistics to compare the median age at first diagnosis of invasive melanoma, numbers of melanomas, and total numbers of nevi in CMM case subjects from CDKN2A versus CDK4 families and in CMM case subjects from CDKN2A families with and without pancreatic cancer. We also haplotyped the three recurrent mutations to assess whether the alterations occurred *de novo* or were founder mutations.

## SUBJECTS AND METHODS

### Family Data

Families were recruited if there was a history of invasive melanoma in at least two first-degree rela-

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tives. The subjects for this study were drawn from families in which a CDKN2A or a CDK4 mutation had been identified. The original identifications of the mutations in 12 CDKN2A families and in two CDK4 families were previously published (10,12–14) (Table 1). The 19 families ranged in size from eight to 80 members with three to 12 CMM patients. The families were referred by health-care professionals or through self-referrals. All of the families were Caucasian and resided in various regions of the United States. The families have been followed prospectively for 4–22 years starting in the mid-1970s.

Written informed consent was obtained from the subjects prior to participation under an institutional review board-approved protocol. All family members willing to participate in the study were clinically evaluated. Clinical evaluation of family members and spouses included complete skin examination, routine medical history, and phlebotomy to obtain lymphocytes. Variables recorded during the clinical examination included the type, distribution, and total number of nevi. Dysplastic or atypical nevi were not enumerated separately; therefore, total nevi included both clinically banal and atypical nevi. Total numbers of nevi were available only for patients who were clinically examined. Thus, patients who were deceased at ascertainment of their families did not contribute to the analyses involving numbers of nevi. There were no differences in total numbers of nevi expected between patients who were deceased at their family's ascertainment and those who were clinically examined. All diagnoses of melanoma were confirmed by histologic review of pathologic material, pathology reports, or death certificates. For each case of invasive melanoma, the following information was obtained: the patient's age at diagnosis, the thickness of each tumor (in millimeters), and the total number of invasive melanomas. All diagnoses of pancreatic cancer were also confirmed by review of histologic ma-

terials, local pathology reports, medical records, or death certificates.

### Mutation Analyses

A total of 13 new families (23 melanoma patients) were analyzed for CDKN2A mutations as follows: Eleven individuals with melanoma from nine families were examined with the use of single-strand conformation polymorphisms under previously described conditions (12). In addition, exons 1–3 of CDKN2A were sequenced in 12 affected individuals from four other families. Exons were amplified with the use of polymerase chain reaction (PCR) with the following primer pairs: exon 1—1.102F(agagggtggggcggac)/1.63R(tcgaacttcgtcctcca); exon 2—2.62F/2.42R; and exon 3—3.90F/530R (12). The PCR products were sequenced with the use of dye-terminator sequencing on an ABI 310 fluorescent sequencer (PE Applied Biosystems, Foster City, CA). Sequencing primers included those used in the PCR reactions as well as primers 1.63R, 1.26R, 200F(agcccaactgcgcccgcac), 436R(cggcatctatgcccga), and 505R(tctaagttccc-gaggttctcaga) (12).

A subset of mutation carriers was confirmed by sequencing under a contract with Gene Logic (Gaithersburg, MD). The DNA sequence of exons 1 through 3 of the CDKN2A gene was analyzed in both forward orientation and reverse orientation.

### Haplotype Analysis

Families with recurrent mutations were haplotyped by typing family members with marker loci flanking the gene of interest; i.e., we determined which alleles from closely linked loci were transmitted with melanoma in each of the families with a recurrent mutation. For CDKN2A, seven markers with the following order were used: IFNA, D9S736, and D9S1749 (located 40+ kilobase (kb) distal to exon 3 of CDKN2A), D9S942 and D9S1748 (lo-

cated between exon 1 $\alpha$  and exon 1 $\beta$ ), D9S1604 (located 2–5 kb proximal to exon 1 $\beta$ ), and D9S171, centromere. Allele sizes for all markers except D9S736 are comparable to those from the haplotype study conducted by Pollock et al. (14). The largest observed allele is designated allele 1. For CDK4, the following seven markers were used in this order: centromere–D12S96–D12S103–CDK4–D12S90–D12S305–D12S72–D12S104–AFMA122YC5.

### Statistical Methods

The mean and median ages at first diagnosis of invasive melanoma and the number of invasive melanomas were estimated for each subject with CMM. The mean and median numbers of nevi for each clinically examined CMM patient were also estimated. We used the exact form of the nonparametric Wilcoxon Mann–Whitney test as implemented in the computer program StatXact-4 (15) to test for differences in the medians of the above variables. Two comparison groups were evaluated: 1) CMM patients from CDKN2A families versus CMM patients from CDK4 families and 2) CMM patients from CDKN2A families with pancreatic cancer versus CMM patients from CDKN2A families without pancreatic cancer. Because of the relatively small numbers of patients available for analysis, we assumed independence of CMM patients within families. Since observations from family members might be correlated, we conducted a second analysis in which a summary measure for each variable within each family was created. The results from the second analysis were consistent with the results from the original analysis (data not shown). All *P* values were two-sided and were considered significant at the .05 level.

Precise determination of the melanoma ascertainment event was not possible. However, comparison of analyses restricting results to the prospective period (i.e., after ascertainment of the family) showed

**Table 1.** Germline mutations in CDKN2A and CDK4 in melanoma-prone families

Family	Confirmed cases of		Exon	Description of mutation	
	Melanoma	Pancreatic cancer		Alteration	Nucleotide/effect
CDKN2A families*					
A	8		1	23ins24	24-base-pair duplication
B	3		1	Leu16Arg	T47 → G
C	5		2	Met53Ile	G159 → C
D	6		2	Arg58Ter	C172 → T
E	3		2	Asn71Ser	A212 → G
F	10	1	2	Arg87Pro	G260 → C
G	5		2	Gly101Trp	G301 → T
H	3	2	2	Gly101Trp	G301 → T
I	4		2	Gly101Trp	G301 → T
J	6		2	Val126Asp	T377 → A
K	5	3	2	Val126Asp	T377 → A
L	10	1	2	Val126Asp	T377 → A
M	12		2	Ala148Thr	G442 → A
N	6		2	167del31	Stop at codon 122
O	7		2	225del19	Stop at codon 140
P	8	1	2	240del14	Stop at codon 118
Q	3	2	Intron 2	IVS2 + 1	Splice aberration
CDK4 families					
R	7		2	Arg24Cys	C297 → T
S	5		2	Arg24Cys	C297 → T

\*Mutations in families D–K, M, and Q were reported in (12); mutations in families R–S were reported in (10); mutation in family P was reported in (13); mutation in family A was reported in (14).

results similar to those obtained from analyses with the use of data from the entire period (i.e., before and after ascertainment of the family), although with substantially less power. Therefore, the latter analysis was presented.

## RESULTS

CDKN2A mutations were identified in five of the 13 new families studied. The mutations included two deletions, 167del31 (family N) and 225del19 (family O), and three missense mutations, Leu16Arg (family B), Met53Ile (family C), and Val126Asp (family L). Table 1 shows the numbers of patients with confirmed melanoma and pancreatic cancer and the mutation in each family. There were 104 CMM patients in the 17 CDKN2A families and 12 CMM patients in the two CDK4 families. Only two CMM patients were known not to carry their family's mutation (families F and N). All other CMM patients were mutation carriers (n = 76) or obligate mutation carriers (n = 20) or had unknown mutation status (n = 18). Family M had the well-characterized Ala148Thr polymorphism; all available CMM patients (n = 8) in family M carried this alteration. Exclusion of this family from the analyses did not alter the results (data not shown).

### CDKN2A Versus CDK4 Analysis

The median age at CMM diagnosis ( $P = .70$ ) and the median numbers of CMM tumors ( $P = .73$ ) were indistinguishable

between CMM case subjects from CDKN2A versus CDK4 families (Table 2). For CMM case subjects from both sets of families, the median age at first diagnosis of invasive melanoma was 34.2 years; the median number of CMM tumors was 1.0. There were no statistically significant differences in the median numbers of nevi (banal and atypical) in CMM case subjects between the two types of families ( $P = .11$ ).

### Analysis of CMM Case Subjects in CDKN2A Families With and Without Pancreatic Cancer

Six CDKN2A families had at least one case of pancreatic cancer (Table 1). Four of the 10 pancreatic cancer patients also had had prior invasive or *in situ* melanoma. Six had a CDKN2A mutation or were obligate mutation carriers. Mutation status could not be determined in the other four patients. The median age  $\pm$  standard deviation at pancreatic cancer diagnosis was 70.5 years (mean, 67.6 years  $\pm$  12.2 years), similar to that in the U.S. general population (median age, 71 years) (16).

Comparison of CMM case subjects in CDKN2A families with and without pancreatic cancer showed no statistically significant differences in median age at diagnosis ( $P = .80$ ) or tumor number ( $P = .24$ ) between CMM case subjects from families with pancreatic cancer (33.8 years; 1.0 tumor) versus families without

pancreatic cancer (36.0 years; 1.0 tumor). There was, however, a statistically significant difference in the median numbers of nevi without ( $P = .005$ ) and with ( $P = .004$ ) adjustment for age. CMM case subjects from families without pancreatic cancer had greater numbers of nevi than CMM case subjects from families with pancreatic cancer (Table 2). Restriction of this analysis to known mutation carriers had little effect on the results (data not shown).

### Haplotype Analysis

Analysis of markers flanking the CDKN2A locus suggested common haplotypes for families with the Val126Asp and Gly101Trp mutations (Table 3). Table 3 shows the alleles that segregated with melanoma in the families for each marker studied. Allowing for replication slippage at D9S1749, a marker shown to have a high occurrence of replication slippage because of its high heterozygosity and large allele span (14), the Val126Asp families J and L shared a common IFNA-D9S171 haplotype. Family K shared a more limited haplotype from D9S1749-D9S942-D9S1604. The Gly101Trp families G and I shared a common haplotype from IFNA-D9S171. Family H shared a more limited haplotype with its segregating allele at D9S1748 2 base pairs (bp) different and its D9S1749 allele 6 bp different from that segregating in families G and I. None of 26 control haplotypes con-

**Table 2.** Clinical and genetic epidemiologic characteristics of cutaneous malignant melanoma (CMM) patients

Characteristic	No. of CMM patients	Mean $\pm$ standard deviation, y	Median, y	Range, y	$P^*$
CDKN2A versus CDK4					
Age at first CMM diagnosis					
CDKN2A	104	36.3 $\pm$ 12.8	34.2	14.2-68.8	.70
CDK4	12	38.9 $\pm$ 15.0	34.2	23.6-64.4	
No. of melanomas					
CDKN2A (n = 199)	104	1.9 $\pm$ 2.1	1.0	1-14	.73
CDK4 (n = 29)	12	2.4 $\pm$ 3.4	1.0	1-13	
No. of nevi					
CDKN2A	63	137.0 $\pm$ 108.5	100	4-653	.11
CDK4	10	132.3 $\pm$ 121.5	56	36-328	
CDKN2A families with versus without pancreatic cancer (PC)					
Age at first CMM diagnosis					
With PC	39	35.8 $\pm$ 11.9	33.8	18.0-68.8	.80
Without PC	65	36.0 $\pm$ 13.4	36.0	14.2-67.8	
No. of melanomas					
With PC (n = 63)	39	1.6 $\pm$ 1.2	1.0	1-5	.24
Without PC (n = 136)	65	2.1 $\pm$ 2.4	1.0	1-14	
No. of nevi					
With PC	22	95.0 $\pm$ 75.8	75	4-247	.005†
Without PC	41	159.5 $\pm$ 117.3	118	21-653	.004‡

\*Two-sided  $P$  value for differences between medians, by exact form of the Wilcoxon Mann-Whitney test.

†Without age adjustment.

‡With age adjustment.

**Table 3.** Haplotype analysis in families with recurrent CDKN2A and CDK4 mutations

Mutation	Family	Alleles segregating with melanoma in each family* by CDKN2A haplotype†						
		IFNA	D9S736	D9S1749 □ D9S942		D9S1748	D9S1604	D9S171C
Val126Asp	J	6	1	16	11	9	2	1
	K	6	3	17/18‡	11	9	2	5
	L	6	1	17	11	9	2	1
Gly101Trp	H	2	4	21	9	9	2	1
	G	6	4	18	9	10	2	1
	I	6	4	18	9	10	2/1‡	1
		Alleles segregating with melanoma in each family* by CDK4 haplotype†						
		CD12S96	D12S103 □ D12S90		D12S305	D12S72	D12S104	AFMA122YCS
Arg24Cys	S	1	2	2	2	2	1	1
	R	1	2	2	2	2	1	2

\*Allele numbers were assigned on the basis of actual length and 2-base-pair repeat spacings. The largest observed allele was designated allele 1. Allele sizes for all markers flanking CDKN2A except D9S736 are comparable to those used by Pollock et al. (14).

†□ = location of gene, CDKN2A or CDK4. C indicates centromere location relative to other markers.

‡Both alleles are presented when the segregating allele could not be unambiguously determined because of replication slippage (D9S1749) or because of insufficient data in the family (D9S1604).

tained either mutation-specific haplotype. On the basis of control allele frequencies, the D9S942–D9S1604 haplotype observed in the Val126Asp families would be expected in six of 1000 individuals and that observed in the Gly101Trp families would be expected in two of 100 individuals in the general population. The CDK4 families also shared a common haplotype from D12S96–D12S104, approximately a 5-centimorgan region (Table 3).

## DISCUSSION

Consistent with their positive versus negative roles in cell cycle control, CDK4 behaves as a proto-oncogene, whereas p16 acts as a tumor suppressor. Despite the hypothetical differences in action between the tumor suppressor CDKN2A and the dominant oncogene CDK4, clinical characteristics, such as the median age at CMM diagnosis, numbers of CMM tumors, and total numbers of nevi, were indistinguishable between CMM case subjects from CDKN2A families versus CDK4 families.

The comparisons of CMM case subjects in the CDKN2A and CDK4 families lacked power because of the small numbers of melanoma patients from the CDK4 families. However, since, to date, only three such families have been identified, the current analyses allowed a preliminary evaluation of CMM case subjects with different types of melanoma susceptibility genes. Direct assessment of CDK4 families will remain limited because of their rarity.

Because of the association between

pancreatic cancer and CDKN2A mutations, we examined clinical characteristics in melanoma patients from CDKN2A families with and without pancreatic cancer. We observed no statistically significant differences in ages at melanoma diagnosis or numbers of melanomas. Total numbers of nevi, banal and atypical/dysplastic, were, however, statistically significantly different between CMM case subjects in the two sets of families with CMM case subjects in CDKN2A families with pancreatic cancer having fewer nevi. It remains to be seen whether this observation represents a chance finding in relatively small numbers of CMM case subjects or whether it is associated with relevant genetic and/or environmental characteristics and may help disentangle the CDKN2A mutation–pancreatic cancer relationship.

Pancreatic cancer has been observed in CDKN2A families with insertions, deletions, missense mutations, splice site alterations, and 5' untranslated region mutations (5,8,17–19). There is little evidence for a direct genotype–phenotype association between pancreatic cancer and specific CDKN2A mutations. At present, we cannot identify the genotype or phenotype that predisposes individuals to pancreatic cancer. In addition, the two CDKN2A founder mutations (Val126Asp and Gly101Trp) were observed in families with and without pancreatic cancer, which suggests that other factors (genetic and/or environmental) may be involved in the development of pancreatic cancer. These findings are consistent with observations in Dutch p16 melanoma-prone

families in whom only a subset of large melanoma-prone families had any excess of pancreatic or other gastrointestinal tumors (7,18).

In contrast to the dramatically earlier age at melanoma diagnosis in CDKN2A families (current study: 34.2 years) compared with the U.S. general population (median, 54 years), pancreatic cancer does not appear to occur at an earlier age (current study: 70.5 years) in CDKN2A families compared with the U.S. general population (median, 71 years) (16). Screening for melanoma, which is extremely effective, should begin at a young age, since family members may develop melanoma in their teen years and melanoma remains the principal cause of morbidity and mortality in these families. In contrast, given the inability to identify those at (highest) risk for developing pancreatic cancer, the late age at diagnosis, the lack of effective screening programs, and poor outcome regardless of stage, additional studies and improvements in screening, surveillance, and treatment are required before such programs could be implemented.

To date, many different germline CDKN2A mutations have been identified in families from North America, Europe, and Australasia. Some mutations have been observed only once (e.g., Arg58Ter and Arg87Pro); other mutations have been observed multiple times (e.g., Val126Asp, 23ins24, Met53Ile, and Gly101Trp). Haplotype analyses of recurrent mutations from geographically isolated regions have consistently revealed evidence for common founders rather

than mutation hotspots in the CDKN2A gene. For example, a 19-bp deletion removing nucleotides 225–243 in exon 2, named the “p16-Leiden” mutation, has been shown to be a Dutch founder mutation (18,20). Similarly, a 3-bp insertion (113insArg) that was observed in Sweden (17,21) has been shown to have a common haplotype in the examined families (17).

Recently, two recurrent mutations (Met53Ile and 23ins24) from geographically diverse populations (North America, Great Britain, and Australia) were examined to determine whether the mutations occurred *de novo* or were founder mutations (14). The results showed a common haplotype in all five families with the Met53Ile mutation. The same haplotype appeared in two additional Canadian families, which suggests that the common founder was of British origin (14,19). In contrast, there were at least three independent 24-bp duplication (23ins24) events, as would be expected because it was hypothesized to have arisen as a result of unequal crossing over between the two 24-bp repeats, which occur naturally in the wild-type sequence. Thus, this mutation would be more likely to recur because of the inherent instability of tandem repeat regions (14). Finally, a recently described recurrent mutation of the 5' untranslated region of the CDKN2A gene, noted G-34T, was also shown to have arisen from a common founder in the British population (19).

The two recurrent CDKN2A mutations in our study have been observed in other North American and European families (8,11,22,23). Additional studies are under way to determine whether the two mutations result from common founders across geographically diverse populations and to estimate the ages of the mutations. In contrast, to date, the recurrent CDK4 mutation has been observed only in the two families in our study. Analysis of markers flanking the CDKN2A locus and the CDK4 locus suggested common haplotypes for the families with recurrent mutations. Therefore, the three recurrent missense mutations observed in these U.S. melanoma-prone families—the

Val126Asp and Gly101Trp CDKN2A mutations and the Arg24Cys CDK4 mutation—each showed evidence for a common founder, regardless of the mechanism of action of the respective gene.

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## NOTES

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