Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 8541–8545, August 1996
Genetics

Prevalence of germ-line mutations in p16, p19ARF, and CDK4 in familial melanoma: Analysis of a clinic-based population

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Contributed by Kurt J. Isselbacher, May 9, 1996

ABSTRACT **Five to ten percent of individuals with melanoma have another affected family member, suggesting familial predisposition. Germ-line mutations in the cyclin-dependent kinase (CDK) inhibitor p16 have been reported in a subset of melanoma pedigrees, but their prevalence is unknown in more common cases of familial melanoma that do not involve large families with multiple affected members. We screened for germ-line mutations in p16 and in two other candidate melanoma genes, p19ARF and CDK4, in 33 consecutive patients treated for melanoma; these patients had at least one affected first or second degree relative (28 independent families). Five independent, definitive p16 mutations were detected (18%, 95% confidence interval: 6%, 37%), including one nonsense, one disease-associated missense, and three small deletions. No mutations were detected in CDK4. Disease-associated mutations in p19ARF, whose transcript is derived in part from an alternative codon reading frame of p16, were only detected in patients who also had mutations inactivating p16. We conclude that germ-line p16 mutations are present in a significant fraction of individuals who have melanoma and a positive family history.**

The incidence of melanoma in the United States has increased dramatically during the last 50 years, with the lifetime risk approaching 1% of the population (1). Two major predisposing factors have been identified, excessive sun exposure and genetic susceptibility. Approximately 5–10% of individuals who develop melanoma have an affected close relative, raising the possibility of familial transmission of cancer predisposition (2). Within large genetically defined pedigrees, transmission of susceptibility to melanoma is inherited as an autosomal dominant trait, with a calculated penetrance of 53% by age 80 (3). Genetic linkage analyses have identified a locus on chromosome 9p21 coseggregating with melanoma susceptibility and have suggested a potential second genetic locus on chromosome 1p (4, 5).

The p16 gene (also called CDKN2, MTS-1, and INK4a) has recently been implicated as the melanoma susceptibility gene on chromosome 9p21 (6). p16 encodes a cyclin-dependent kinase (CDK) inhibitor (CDKI), initially identified by its ability to bind CDK4, the kinase thought to be responsible for the inactivating phosphorylation of the retinoblastoma protein pRB (7). The ability of p16 overexpression to induce cell cycle arrest is consistent with its potential function as a tumor suppressor gene (8–10). This is supported by the presence of mutations and deletions in the p16 gene in a number of primary tumors, including pancreatic cancer, esophageal cancer, glioblastoma, and T-cell leukemia, and in a large number of immortalized cell lines derived from melanoma, leukemia, lung, breast, brain, bladder, kidney, and ovarian cancers (11–14).

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The identification of p16 as the melanoma susceptibility gene has been the subject of some controversy (15). Germ-line p16 gene mutations have been reported in melanoma patients belonging to genetically defined pedigrees in some studies but not in others. For example, Dracopoli and coworkers (16) reported potential disease-causing mutations occurring in 9 of 18 melanoma pedigrees, of which 4 mutations were found in 6 pedigrees showing genetic linkage to chromosome 9p. Kamb and coworkers (17) found only two potential missense mutations in 13 families with genetic linkage to chromosome 9p and none in 38 families where linkage was not established. These apparently contradictory results may be explained at least in part by geographic or ethnic bias in the collection of large cancer pedigrees. Analysis of Dutch melanoma pedigrees has revealed a single mutation that is shared by 13 of 15 presumably related families (18). Constitutional p16 mutations were detected in 7 of 18 Australian pedigrees in one study (19) but only in 1 in 17 other Australian melanoma families (20), and no mutations were detected in a limited study of 6 English melanoma pedigrees (21).

In addition to the p16 gene, two other genes have recently been implicated in genetic predisposition to melanoma. p19ARF (ARF is an acronym for alternative reading frame) is a transcript that is derived in part from alternative splicing of the p16 gene (22–24). The first exon of p19ARF is unique, whereas its second exon is derived from p16, although processed with a different codon reading frame. Thus, although p16 and p19ARF are derived in part from the same gene and are deleted together in multiple tumors, they encode completely distinct proteins (24). The ability of p19ARF, like p16, to inhibit cell cycle progression in cultured cells has raised the possibility that it might constitute a separate melanoma tumor suppressor gene at the 9p21 locus (24). The other candidate melanoma predisposition gene, CDK4, encodes a protein that is normally inhibited by the p16 gene product (25). A specific mutation in CDK4, R24C, which appears to render the protein resistant to inhibition by p16, has been reported in two melanoma pedigrees (26). While the different genetic causes of predisposition to melanoma have been studied in melanoma pedigrees, the majority of patients who have a positive family history do not belong to large genetically defined cancer pedigrees with many affected members over numerous generations. To determine the contribution of these genes to their apparent genetic predisposition, we undertook a germ-line mutational analysis of p16, p19ARF, and CDK4 in consecutive patients with melanoma and a positive family history who presented to a referral dermatology center.

Abbreviations: CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; ARF, alternative reading frame.

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MATERIALS AND METHODS

Subjects. Medical records of 3500 consecutive patients treated at the Massachusetts General Hospital Pigmented Lesion Clinic from 1984 to 1991 were retrospectively reviewed, and 1400 individuals with melanoma were identified. Of these, 125 patients reported a positive family history, which was confirmed by the presence of a first or second degree relative with biopsy-proven melanoma in 68 cases. The histopathological and clinical features of these familial cases are described elsewhere (27). Thirty-three patients were available to participate in this study. Patients were contacted according to procedures approved by the Institutional Review Board and asked to donate blood for mutational analysis without disclosure of results.

Mutational Analysis. Genomic DNA and mRNA were prepared from peripheral blood mononuclear cells isolated by centrifugation through a Ficoll gradient.

DNA-Based Analysis. Each of the three p16 exons were amplified by PCR from genomic DNA, using primers complementary to their flanking intron sequences (16). For exon 1, 10% dimethyl sulfoxide (DMSO) was added to the reaction buffers for both optimal PCR amplification and nucleotide sequencing. To facilitate automated sequencing analysis, compound PCR primers containing the standard M13-derived sequencing primers at their 5′ end were synthesized. The alternatively spliced exon 1β of p19ARF was amplified from genomic DNA using primers complementary to flanking intron sequences (p19X1SF, 5′-TGCGTGGGTCCCAGTCTGCA-3′; p19X1SR, 5′-CGCGGTTATCTCCTCCTCCTCC-5′). PCR fragments were resolved by gel electrophoresis, and sequencing analysis was performed using *Taq* polymerase cycle sequencing with dye-labeled dideoxyterminators (Applied Biotechnology, Foster City, CA). The use of uncloned PCR products ensured representation of both p16 alleles and minimized the possibility of artefactual errors that might be present within an individual PCR product. Base positions where the height of a secondary peak was ≥50% that of the primary peak were marked as heterozygous and confirmed by analysis of both sense and antisense strands. To test for gross germ-line deletions affecting p16, germ-line DNA was digested with *Eco*RI or *Hin*dIII, and Southern blots were hybridized with a p16 cDNA or actin control probes, followed by densitometric analysis. DNA specimens were also screened for the R24C CDK4 mutation using digestion of the appropriate PCR fragment with *Stu*I, which specifically cleaves the mutant allele (26).

RNA-Based Analysis. Total cellular mRNA was extracted using standard methods, reverse transcribed using random hexamers, and subjected to two rounds of PCR amplification in the presence of 10% DMSO, using nested primers complementary to the 5′ and 3′ noncoding regions of the p16 or p19ARF transcripts (9). After electrophoresis, abnormally migrating PCR products were purified and sequenced.

Statistical Considerations. The association between the mutation and family and health characteristics were tested using the Fisher exact test. The comparison of the two populations on the basis of age was made using a Wilcoxon nonparametric test. The estimated rate of gene mutation in the population and the exact confidence interval for this estimate are reported.

RESULTS

A retrospective review of medical records at the Pigmented Lesion Clinic of Massachusetts General Hospital identified 33 melanoma patients (from 28 independent families) who had a documented first or second degree relative with melanoma and were available to provide a blood sample for mutational analysis. Clinical characteristics for all patients (n = 33) and for unrelated patients (n = 28) are shown in Table 1. The age of diagnosis ranged from 21 to 65 (median age 39), compared with a median age of 53 for melanoma in the general population (1). In addition to a positive family history, a number of individuals had other markers of genetic susceptibility to melanoma: nine patients (27%) had a second primary melanoma, developing from 1–17 years after the initial cancer; four (12%) had more than one relative affected with melanoma; and two (6%) had relatives who themselves had two primary melanomas. In addition, 19 patients (58%) had dysplastic nevi, potential precursor lesions commonly seen in individuals at risk for melanoma. The histology, invasiveness, and geographic location of the primary tumor was not different from that commonly seen with cutaneous melanoma (27).

Germ-Line p16 Mutations. To search for germ-line mutations in p16, we first analyzed DNA specimens for gross deletions or genomic rearrangements using Southern blotting. None were found. To detect smaller deletions or point mutations, each of the three exons that encode p16 was then analyzed by PCR amplification and nucleotide sequencing (Table 2). A nonsense mutation converting a tryptophan to a stop codon was detected in exon 1 in two related patients (patient 30, father; patient 12, son); a deletion of 58 nucleotides (patient 9) and one of 14 nucleotides (unrelated patients 26 and 24) were present in exon 2; a missense mutation, converting a methionine to isoleucine in exon 2, was detected in two brothers (patients 3 and 6). This specific mutation has previously been reported in two Australian melanoma pedigrees with a total of 25 affected members (19), confirming its identity as a definite disease-associated mutation. As expected, all these germ-line mutations were heterozygous. When compared with patients with familial melanoma who had wild-type p16 alleles, individuals bearing a p16 mutation showed trends suggesting a higher incidence of multiple primary tumors (P = 0.07) and a higher likelihood of multiple affected family members (P = 0.12) (Table 1). Other characteristics, such as young age at diagnosis and presence of dysplastic nevi were observed in most familial melanoma patients, irrespective of p16 mutations. While recent studies have reported an increased incidence of pancreatic cancer in some p16-associated

Table 1. Clinical characteristics of melanoma patients with a germline p16 mutation

Germline p16 mutation*	No. of patients	Median age at first diagnosis	No. of patients with >1 primary melanoma	No. of patients with dysplastic nevi	No. of patients with >1 affected relative/no. of unrelated patients†
+	7	33	4 (57%)	5 (71%)	2/5 (40%)
−	26	41‡	5 (24%)§	14 (53%)¶	2/23 (9%)‖
Both	33	39	9 (27%)	19 (58%)	4/28 (14%)

*Definite mutations only, including deletions and nonsense mutations and missense mutations that are definitively linked to transmission of susceptibility to melanoma by pedigree analysis.
†n = patients from independent families; five patients who had other relatives in the study are excluded.
‡P = 0.17.
§P = 0.07.
¶Not significant.
‖P = 0.12.

Table 2. Definite germline p16 mutations in familial melanoma

	Exon	Codon*	Nucleotide change and position*	Effect on p16 protein	Effect on p19ARF protein
Deletions					
Patient 9	2	56	58 bp deletion at 166	Truncation at codon 145	Altered C terminus†
Patient 16	2	80	14 bp deletion at 240	Truncation at codon 118	Altered C terminus‡
Patient 24	2	80	14 bp deletion at 240	Truncation at codon 118	Altered C terminus
Nonsense					
Patient 12	1	15	G to A at 44	Trp to Stop at codon 15	None
Patient 30§	1	15	G to A at 44	Trp to Stop at codon 15	None
Missense¶					
Patient 3	2	53	G to C at 159	Met to Ile at codon 53	Asp to His at codon 68
Patient 6‖	2	53	G to C at 159	Met to Ile at codon 53	Asp to His at codon 68

*Codons and nucleotide positions are derived from the revised p16 sequence (24), which identifies the first Met as eight codons upstream of that initially reported (11).
†This deletion alters the reading frame of p19ARF, removing the normal downstream stop codon and returning it to the p16 reading frame. The resulting chimeric p19ARF-p16 protein contains the 82 C-terminal amino acids of p16 substituted for the terminal 62 amino acids of p19ARF.
‡This deletion and the resulting shift in reading frame lead to the substitution of 60 novel amino acids for the 38 C-terminal amino acids of p19ARF. Patients 16 and 24 are unrelated.
§Patients 12 and 30 are father and son.
¶The Met to Ile substitution at codon 53 has been linked to familial transmission of the disease in two Australian melanoma pedigrees with a total of 25 affected members (reported as Met45Ile; ref. 28).
‖Patients 3 and 6 are brothers.

melanoma pedigrees (29, 30), the two melanoma patients in our study who had a family history of pancreatic cancer (one with two second degree relatives with pancreatic cancer and one with a first degree relative who had both melanoma and pancreatic cancer) did not have a germ-line p16 mutation. In addition to disease-associated mutations in p16, other nucleotide substitutions were detected that did not alter the encoded amino acid (i.e., silent polymorphisms; patients 19, 21, and 31); encoded an alanine to threonine substitution at codon 148, which has been previously identified as a common variant in the general population (patients 10 and 31; refs. 27 and 29); or led to an amino acid change that was not shared by a relative who also had melanoma (i.e., absence of linkage; patients 10, 21, and 31) (Table 3). Finally, one novel missense mutation was detected in a patient whose affected relative was not available for testing (patient 19). The functional significance of this missense mutation is therefore unknown.

Analysis of the p19ARF Transcript. To screen for mutations in the p19ARF transcript, its unique first exon, known as exon 1β, was PCR amplified and sequenced. No mutations in this exon were detected in any familial melanoma patients. The second exon of p19ARF is identical to that of p16, but its codon reading frame is shifted following splicing to exon 1β. The small deletions described in exon 2 of the p16 transcript (patients 9, 16, and 24) also disrupted the coding region of the p19ARF transcript, resulting in a larger predicted protein with a novel C terminus (Table 2). The melanoma-associated methionine to isoleucine change in p16 (patients 3 and 6) was also accompanied by an aspartic acid to histidine substitution in p19ARF. In these cases, genetic predisposition to melanoma may therefore result from disruption of either p16, p19ARF, or both genes. The shift in the codon reading frame of exon 2 between p16 and p19ARF has also led to the suggestion that nucleotide substitutions that cause silent polymorphisms in the p16 transcript might cause genuine mutations in the p19ARF transcript (24). Four such silent polymorphisms in p16 (one in patients 19 and 31, and two in patient 21) resulted in an amino acid change in the p19ARF transcript (Table 3). However, all four were missense mutations in p19ARF rather than definitive nonsense mutations. Furthermore, for patients 21 and 31, these substitutions were not shared by a relative who also had melanoma and, hence, did not show linkage with the disease.

Table 3. Polymorphisms and novel p16 missense mutations in familial melanoma

	Exon	Codon	Nucleotide change and position	Effect on p16 protein	Effect on p19ARF protein
Missense*					
Patient 19	2	107	C to T at 319	Arg to Cys at codon 107	Ala to Val at codon 121
Polymorphisms†					
Patient 10‡	2	85	G to A at 253	Ala to Thr at codon 85	Arg to His at codon 99
	2	148	G to A at 442	Ala to Thr at codon 148	None
Patient 19	2	64	G to A at 192	None	Ala to Thr at codon 79
Patient 21§	2	68	C to T at 203	Ala to Val at codon 68	None
	2	98	C to A at 267	None	Ile to Met at codon 104
	2	113	G to A at 339	None	Ala to Thr at codon 128
Patient 31¶	2	106	G to A at 318	None	Ala to Ser at codon 121
	2	148	G to A at 442	Ala to Thr at codon 148	None

*The Arg to Cys substitution at codon 107 has not been previously reported. It could represent either a new disease-associated mutation or a rare polymorphism.
†Polymorphisms are identified by the absence of an amino acid substitution resulting from the nucleotide change in the p16 gene, by the frequent observation of an amino acid change within the general population, or by the presence of a missense mutation in only one of two family members with melanoma (absence of linkage to disease).
‡Both nucleotide changes in patient 10 are homozygous, and neither is present in the patient's brother, who is also affected with melanoma. The Ala to Thr at codon 148 is a common polymorphism in the general population (reported previously as Ala140Thr; refs. 27 and 29).
§The Ala to Val at codon 68 is absent in the brother of patient 21, who is also affected with melanoma.
¶Both nucleotide substitutions are absent in the patient's brother, who also has melanoma. Ala to Thr at codon 148 is a common polymorphism.

Thus, no mutations were detected that specifically inactivated the p19ARF gene without also disrupting the p16 gene.

Analysis of p16 Pre-mRNA Splicing Variants. In testing the integrity of the p16 transcript by reverse transcriptase-PCR, abnormally truncated products were detected along with the expected wild-type product in numerous cases, both in melanoma patients (11 in 30 cases) and in normal controls (4 in 30 cases). Sequencing analysis revealed two classes of abnormal p16 mRNA species. Class 1 transcripts had aberrant splicing of exons 1 and 2, with loss of the 3′ end of exon 1 and the 5′ end of exon 2. Class 2 transcripts contained an aberrant fusion of exons 1 and 3, with loss of the 3′ end of exon 1, the entire exon 2, and splicing of the residual exon 1 to intron sequences upstream of exon 3. The novel splicing junctions utilized in these aberrant p16 transcripts were unique to each case. Analysis of genomic DNA revealed no mutations or deletions within the p16 coding sequences or their surrounding intron sequences. These aberrant transcripts, detected in both melanoma patients and controls, therefore do not result from mutations in the p16 gene. They may instead reflect the amplification by reverse transcriptase-PCR of aberrantly spliced p16 pre-mRNA, which is expressed at very low levels in peripheral blood mononuclear cells. Thus, in mutational analyses based on blood specimens, the presence of such abnormally processed p16 transcripts should not be taken as indicative of a p16 mutation. In contrast to p16, the p19 ARF transcript did not show any aberrant pre-mRNA splicing, even in specimens expressing a truncated p16 transcript.

Infrequent Germ-Line Mutations in CDK4. A specific mutation in CDK4, R24C, has recently been reported in two melanoma pedigrees in which linkage studies had excluded the chromosome 9p21 locus, identifying CDK4 as a second melanoma predisposition gene (26). This potentially dominant CDK4 mutation, which renders its encoded protein resistant to inhibition by p16 (25), can be readily tested using a a specific PCR-based assay (26). None of our familial melanoma patients had this mutation. The R24C mutation thus appears to be an infrequent cause of genetic predisposition to melanoma.

DISCUSSION

We found evidence of a mutated germ-line p16 allele in 5 of 28 (18%) unrelated melanoma patients who had at least one affected first or second degree relative (95% confidence interval: 6%, 37%). Since 5–10% of all individuals with melanoma have such a family history, our observations confirm the contribution of p16 to genetic susceptibility in this relatively common subset of melanoma patients. In contrast, mutations were not detected in other candidate melanoma predisposition genes. The R24C mutation in CDK4 may contribute to rare melanoma pedigrees, but it does not appear to be implicated in more common forms of familial melanoma. The alternative product of the p16 gene, p19ARF, has been proposed as a second tumor suppressor gene at the chromosome 9p21 melanoma locus, but we did not detect definite mutations that inactivated p19ARF without also disrupting the p16 transcript. It is therefore unclear whether p19ARF is independently associated with genetic predisposition to melanoma.

The prevalence of germ-line p16 mutations that we observed in individuals with familial melanoma is lower than that reported for North American melanoma pedigrees. While this prevalence has been subject to conflicting reports, recent studies indicate that approximately 50% of melanoma pedigrees harbor p16 mutations (16). Our study differs from such analyses in that melanoma families presenting to a dermatology service commonly have only a small number of affected relatives, unlike the large families with multiple affected members that are selected as melanoma pedigrees for genetic analysis. Some of our cases may therefore have resulted not from inheritance of a dominant-acting cancer predisposition gene, but rather from other family-associated characteristics with lower penetrance, such as excessive sun exposure during childhood in fair-skinned individuals. Such heterogeneity would be consistent with the observation that patients with a p16 mutation showed a trend toward characteristic clinical features of genetic predisposition to cancer (early age of onset, multiple primary tumors, and multiple affected family members), compared with familial cases who had wild-type p16 alleles. However, larger studies will be required to confirm this potential association, and clinical criteria alone are unlikely to reliably predict p16 mutation carriers.

Our results have implications for genetic testing and counseling in melanoma patients who have a positive family history. Identification of individuals carrying a mutation confering predisposition to cancer offers the possibility for enhanced screening and early detection. This is particularly true in melanoma, where avoidance of excessive sun exposure and frequent skin examinations could lead to reduced mortality in affected individuals. Guidelines for accuracy in p16 mutational testing will be important, given the conflicting results previously obtained in different laboratories, and issues of confidentiality (28) will need to be addressed before such testing can become generally available.

We are indebted to the patients who participated in this study and to their referring physicians. We also thank Drs. Rhoda Alani, Stephen Friend, and Bruce Chabner for helpful comments and Dr. Robert Chadwick for technical advice. This work was supported by a grant from the Massachusetts General Hospital to the Center for Cancer Risk Analysis.

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