CDKN2A Germline Mutations in Individuals with Cutaneous Malignant Melanoma

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Cyclin-dependent kinase inhibitor type 2A (*CDKN2A*) has been identified as a major melanoma susceptibility gene based on the presence of germline mutations in high-risk melanoma families. In this study, we sought to identify and characterize the spectrum of *CDKN2A* mutations affecting p16 inhibitor of cyclin-dependent kinase type 4 (INK4a) in individuals with melanoma using a population-based study design. DNA samples from 1189 individuals with incident multiple primary melanoma (MPM) and 2424 with incident single primary melanoma unselected for family history of melanoma were available for screening of *CDKN2A* (p16INK4a) mutations. Variants were classified for functional impact based on intragenic position, existing functional data, sequence, and structural analysis. The impact of individual mutations and functional groupings was assessed by comparing frequencies in cases of MPM versus cases with a single first primary melanoma, and by comparing the reported incidence rates in first-degree relatives. Our results show that mutations occur infrequently in these high-risk groups, and that they occur mainly in exons 1α and 2. Rare coding variants with putative functional impact are observed to increase substantially the risk of melanoma. With the exception of the variant in position −34 of *CDKN2A* of known functional consequence, the remaining rare variants in the non-coding region have no apparent impact on risk.

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Abbreviations: CDKN2A, cyclin-dependent kinase inhibitor type 2A; INK4a, inhibitor of cyclin-dependent kinase type 4; MPM, multiple primary melanoma; SPM, single primary melanoma

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INTRODUCTION

The cyclin-dependent kinase inhibitor type 2A (*CDKN2A*) gene is located on human chromosome 9p21 and encodes two protein products of great interest owing to biological functions that suggest they are putative tumor suppressors (Serrano *et al.*, 1993; Kamb *et al.*, 1994; Nobori *et al.*, 1994; Hara *et al.*, 1996; Kamijo *et al.*, 1997). The first one, p16 inhibitor of cyclin-dependent kinase type 4 (INK4a) (MIM 600160), is a product of the splicing of exons 1α, 2, and 3 (Serrano *et al.*, 1993). Splicing of exons 1β and 2 in an alternative reading frame produces p14ARF (Mao *et al.*, 1995; Quelle *et al.*, 1995; Stone *et al.*, 1995). During the cell cycle, p16INK4a regulates the G1–S transition by preventing the hyper-phosphorylation of pRB and the consequent release of transcription factors involved in DNA synthesis (S-phase). This is accomplished by direct binding of p16INK4a to and inhibition of the cyclin-dependent kinases 4 and 6 (Cdk4,6) (Serrano *et al.*, 1993). p14ARF is capable of inducing cell cycle arrest by inhibiting MDM2-mediated degradation of p53 (Pomerantz *et al.*, 1998).

CDKN2A and *CDK4* have been identified as melanoma susceptibility genes based on the discovery of germline mutations in multiple-case families (Hussussian *et al.*, 1994;

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Kamb *et al.*, 1994): only six melanoma families harboring *CDK4* mutations have been identified worldwide to date (Zuo *et al.*, 1996; Soufir *et al.*, 1998, Molven *et al.*, 2005). Thus, the major gene known to be associated with the development of melanoma in families is *CDKN2A*. The prevalence of mutations in this gene among incident cases of melanoma has been estimated previously to be 0.2% (Aitken *et al.*, 1999). However, this estimate was obtained by screening six specific *CDKN2A* mutations in a selected subset of melanoma cases. There are few studies in which *CDKN2A* mutations have been screened throughout the entire coding region to determine the risk associated with the development of sporadic melanoma (MacKie *et al.*, 1998; Monzon *et al.*, 1998; Burden *et al.*, 1999; Hashemi *et al.*, 2000; Tsao *et al.*, 2000; Berwick *et al.*, 2004; Peris *et al.*, 2004) and the numbers of participants in these studies were small.

Our study involved population-based ascertainment of patients with an incident second- or higher-order primary melanoma (denoted MPM for multiple primary melanoma) and population-based ascertainment of incident cases diagnosed with a single first primary melanoma (SPM). All patients were screened for mutations affecting p16INK4a. In previous reports from this study we have shown that there is a strong association between melanoma risk and p16INK4a mutations that we have classified as ''functional'' (Berwick *et al.*, 2006), and we have also shown that the incidence of melanoma in first-degree relatives is higher for MPM than SPM cases (Begg *et al.*, 2005). In this article, we present a detailed accounting of all of the variants identified in the study, and we use both of these types of comparison to provide predictions of which types of variants provide the strongest association with risk of the disease, by grouping the variants on the basis of functional characteristics and bioinformatics tools.

RESULTS

Screening of mutations

The total number of eligible individual subjects with DNA was 3,680. Of these, a total of 67 did not amplify *CDKN2A* after multiple attempts, leaving 3,613 for analysis (2,424 SPM and 1,189 MPM). A total of 93 of these participants are reported in our results as both SPM and MPM in view of the fact that both the first and the subsequent primaries occurred during our case accession periods (see Begg *et al.* (2006) for rationale) and so the final number of individuals analyzed is 3,520.

The initial screening by denaturing high-performance liquid chromatography revealed 1,833 potentially positive PCR fragments in 1,575 study participants. Sequencing confirmed 44 different nucleotide changes identified in the following regions: 5′ UTR ($n=28$ patients), in the coding region ($n=289$ patients), in intron 1 ($n=24$ patients), and in the 3′ UTR ($n=1492$ patients). Figure 1 depicts the chromatograms and electropherograms corresponding to the six new germline variants found. These new variants have been submitted to dbSNP (reference numbers received September 2005–NCBI_ss No.: 46566490, 46566491, 46566492, 46566493, 46566494, and 46566495). The *CDKN2A* variants found, the prevalences of the variants among patients with SPM and MPM, and the numbers of first-degree affected relatives are shown in Table 1. All the nucleotide changes found in or around exons 1α, 2, and 3 are also shown.

The variants in Table 1 are also annotated on the basis of available evidence that the variant affects the normal function of p16INK4a, based on *in vitro* assays that measure binding of p16INK4a to CDK4 and CDK6, inhibition of CycD-CDK in transfected insect cells, protein–protein interactions in yeast, and cell cycle arrest in mammalian cells as previously reported by others (Koh *et al.*, 1995; Ranade *et al.*, 1995; Reymond and Brent, 1995; Parry and Peters, 1996; Harland

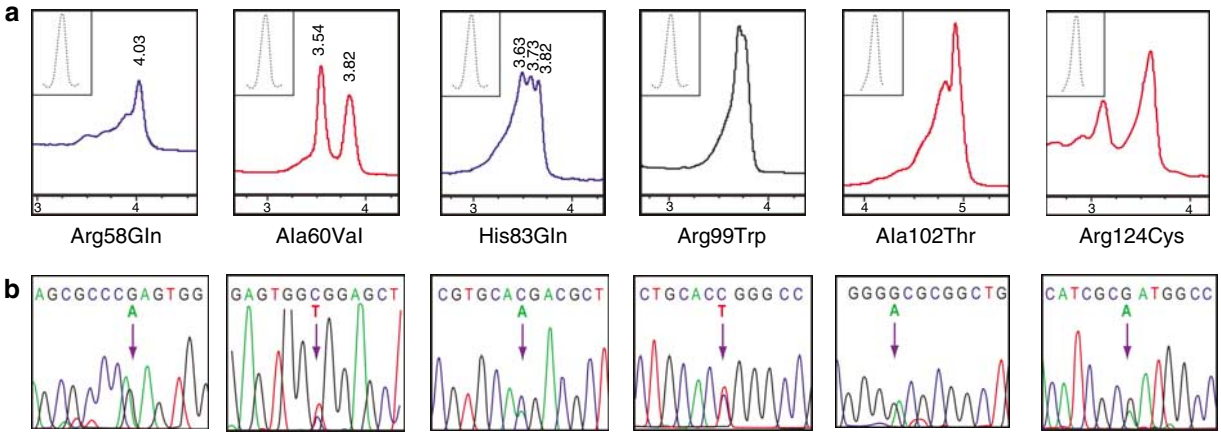

Figure 1. Detection of CDKN2A variants by denaturing high-performance liquid chromatography and direct sequencing. (**a**) Top row, depicts the chromatographic heteroduplex profiles obtained in six samples bearing novel variants. The windows highlight the homoduplex or normal profile obtained from wild-type internal controls run at the same gradient and temperature conditions (see Materials and Methods). (**b**) Bottom row, corresponds to the electropherograms obtained in the same samples, confirming the presence of nucleotide changes (arrows). Note that for the variant Arg124Cys, reverse sequencing is shown and corresponds to the forward C→T change in c.370.

Table 1. Position, functional characteristics, and prevalence of the variants identified in GEM

CDKN2A nucleotide changes[1]	Effect on p16INK4A	Effect on p14ARF[2]	*In vitro* and other supporting evidence[3]	Frequency in SPM, *N* (%)	Frequency in MPM, *N* (%)	Number of first-degree relatives with melanoma	Variant found in familial melanoma[4]
5′UTR							
(−) 70 G/A	No change	N/A		0	1 (0.08)	0/5	
(−) 34 G>T	**Novel AUG (−35)**	N/A	**Yes[5]**	**2 (0.08)**	**6 (0.50)**	**7/52**	1 family
(−) 34 G>T+c.442 G>A	**Novel AUG (−35)**+Ala148Thr	N/A	**Yes[5]**	**0**	**1 (0.08)**	**1/6**	1 family (−34 G>T)
(−) 33 G>C	No change	N/A	No change[6]	8 (0.33)	6 (0.52)	0/118	
(−) 33 G>C and c.50+37 G>C	No change	N/A		1 (0.04)	0	0/8	
(−) 25 C>T	No change	N/A		2 (0.08)	0	0/13	
(−) 14 C>T	No change	N/A	No change[6]	2 (0.08)	1 (0.09)	0/10	
Exon 1α							
c.8_9insGG CGGCGG GGAGCAG CATGG AGCC	**In-frame insertion 8 aa duplication[7]**	N/A	**Yes[8]**	**6 (0.25)**	**0**	**2/40**	
c.8_33del	**In-frame deletion 8 aa**	N/A		**0**	**2 (0.17)**	**1/21**	
c.47 T>G	**Leu16Arg**	N/A		**0**	**2 (0.17)**	**2/23**	1 family
c.67 G>A	**Gly23Ser**	N/A		**0**	**1 (0.08)**	**1/5**	
c.67 G>C+c.442 G>A	**Gly23Arg**+Ala148Thr	N/A		**1 (0.04)**	**0**	**1/10**	
c.71 G>C	**Arg24Pro**	N/A	**Yes[8,9,10]**	**2 (0.08)**	**0**	**2/13**	4 families
c.87_89delG	**Ala30fs, stop 52**	N/A	**Yes, derived[11]**	**1 (0.04)**	**0**	**0/5**	
c.87_89delG+c.442 G>A	**Ala30fs, stop 52**+Ala148Thr	N/A	**Yes, derived[11]**	**1 (0.04)**	**0**	**0/8**	
c.95 T>C	**Leu32Pro**	N/A		**0**	**2 (0.17)**	**0/12**	2 families
c.123 G>A	Pro41Pro	N/A		4 (0.16)	0	1/31	
c.123 G>A+**c.146 T>C**	Pro41Pro+**Ile49Thr**	N/A	**Yes[9,10,11]**	**0**	**1 (0.08)**	**0/6**	
c.131_132insA	**Tyr44stop**	N/A	**Yes, derived[10]**	**0**	**1 (0.08)**	**0/5**	
c.132 C>A	**Tyr44stop**	N/A	**Yes, derived[10]**	**0**	**1 (0.08)**	**1/6**	
c.136 C>A	Arg46Arg	N/A		1 (0.04)	**0**	0/5	
c.146 T>C	**Ile49Thr**	N/A	**Yes[8,9,10,12]**	**1 (0.04)**	**0**	**0/9**	
c.146 T>G	**Ile49Ser**	N/A		**0**	**1 (0.08)**	**2/7**	1 family
c.149 A>C	**Gln50Pro**	N/A	**Yes[9,13]**	**1 (0.04)**	**1 (0.08)**	**2/7**	
Intron 1							
c.50+21 G/A	No change	N/A		1 (0.04)	0	0/6	
c.50+37 G/C	No change	N/A		14 (0.58)	6 (0.52)	2/113	
c.50+54 G/A	No change	N/A		2 (0.08)	0	0/13	
c.51–5 G>A	No change	N/A		1 (0.04)	0	0/5	
Exon 2							
c.159 G>A	**Met53Ile**	**Asp109Asn**	**Yes[8,9,10]**	**1 (0.04)**	**2 (0.17)**	**0/9**	
c.159 G>C	**Met53Ile**	**Asp109His**	**Yes[8,9,10]**	**1 (0.04)**	**1 (0.08)**	**0/15**	8 families
c.170 C>T	**Ala57Val**	Arg112Arg		**0**	**2 (0.17)**	**0/11**	
c.173 G>A	**Arg58Gln**	Pro113Pro		**1 (0.04)**	**0**	**1/5**	
c.174 A>C	Arg58Arg	**Ser114Arg**		**1 (0.04)**	**0**	**0/5**	
c.179 C>T	**Ala60Val**	Gly115Gly		**1 (0.04)**	**0**	**0/6**	
c.247 C>T	**His83Tyr**	**Ala138Val**	**Yes[9,10,12,13]**	**0**	**1 (0.08)**	**0/5**	
c.249 C>A	**His83Gln**	Arg139Arg		**1 (0.04)**	**0**	**0/6**	

Table 1 continued on the following page

Table 1. continued

CDKN2A nucleotide changes[1]	Effect on p16INK4A	Effect on p14ARF[2]	*In vitro* and other supporting evidence[3]	Frequency in SPM, *N* (%)	Frequency in MPM, *N* (%)	Number of first-degree relatives with melanoma	Variant found in familial melanoma[4]
c.295 C>T	**Arg99Trp**	**Pro154Leu**		**0**	**1 (0.08)**	**0/6**	
c.301 G>T	**Gly101Trp**	**Arg156Leu**	**Yes[8,9,10,12,13]**	**1 (0.04)**	**5 (0.42)**	**7/47**	16 families
c.304 G>A	**Ala102Thr**	**Gly157Asp**		**0**	**1 (0.08)**	**0/4**	
c.306 G>A+**c.322 G>A**	Ala102Ala+ **Asp108Asn**	**Ala158Thr+ Arg163Gln**	**No change[6]**	**1 (0.04)**	**0**	**0/5**	1 family (Asp108Asn)
c.318 G>A+c.442 G>A	Val106Val+ Ala148Thr	**Ala162Thr**		**2 (0.08)**	**1 (0.08)**	**1/22**	
c.322 G>A	**Asp108Asn**	**Arg163Gln**	**No change[6]**	**1 (0.04)**	**0**	**1/2**	1 family
c.334 C>G	**Arg112Gly**	**Pro167Arg**		**0**	**2 (0.17)**	**1/13**	1 family
c.370 C>T	**Arg124Cys**	N/A		**1 (0.04)**	**0**	**0/3**	
c.373 G>C	**Asp125His**	N/A		**3 (0.12)**	**0**	**0/18**	1 family
c.384 G>A	Arg128Arg	N/A		1 (0.04)	0	0/9	
c.427 G>A+c.442 G>A	Ala143Thr+ Ala148Thr	N/A		1 (0.04)	0	0/5	
c.442 G>A	Ala148Thr	N/A	No change[6] predominantly nuclear staining[14]	159 (6.6)	70 (6.1)	45/1445	
3′ UTR							
*29 CC	N/A	N/A		1769 (73%)	876 (74%)	490/17084	
*29 CG	N/A	N/A		628 (26%)	294 (25%)	164/5844	
*29 GG	N/A	N/A		26 (1.07%)	20 (1.68%)	10/310	
*69 CC	N/A	N/A		1957 (81%)	950 (80%)	515/18740	
*69 CT	N/A	N/A		425 (18%)	212 (18%)	132/4033	
*69 TT	N/A	N/A		29 (1.20%)	22 (1.86%)	13/353	

GEM, Genes Environment and Melanoma Study; fs, frameshift; N/A, not applicable.
Note that mutations defined as ''functional'' (see text) are highlighted in bold type.
[1]Nucleotide 1 is the A of the ATG-translation initiation codon of p16INK4A.
[2]Changes occurring outside the p14ARF coding region (human p14ARF numbering) or downstream of stop codon.
[3]Includes any known report of *in vitro* assays performed.
[4]Comparison made with results obtained with the largest study conducted in melanoma families to date (Bishop *et al.*, 2002).
[5]Decreases transcription of wild-type p16 (Liu *et al.*, 1999).
[6]Existing *in vitro* data shows no change from wild type (Koh *et al.*, 1995; Ranade *et al.*, 1995; Reymond and Brent, 1995).
[7]Amino acids inserted: AAGSSMEP.
[8]Nuclear mislocalization and/or abnormal staining pattern, functional impairment (Walker *et al.*, 1999; Ghiorzo *et al.*, 2004; Becker *et al.*, 2005).
[9]Decreased or null-binding affinity to CDK4/6 (Ranade *et al.*, 1995; Reymond and Brent, 1995; Harland *et al.*, 1997; Monzon *et al.*, 1998; Loo *et al.*, 2003).
[10]Partial or total lack of growth arrest/inhibition (Koh *et al.*, 1995; Parry and Peters, 1996; Walker *et al.*, 1999; Yarbrough *et al.*, 1999; Becker *et al.*, 2001).
[11]p16 proteins with equal or less than 120 residues lack CDK-binding capacity (Parry and Peters, 1996).
[12]Inhibition of Rb phosphorylation (Ranade *et al.*, 1995; Parry and Peters, 1996).
[13]Protein instability and/or temperature sensitive (Parry and Peters, 1996; Ruas *et al.*, 1999; Loo *et al.*, 2003).
[14]Pattern of staining in p16 variant-transfected cells (Walker *et al.*, 1999).

et al., 1997; Monzon *et al.*, 1998; Liu *et al.*, 1999; Ruas *et al.*, 1999; Walker *et al.*, 1999; Yarbrough *et al.*, 1999; Becker *et al.*, 2001; Loo *et al.*, 2003; Ghiorzo *et al.*, 2004).

Classification of GEM variants

Consistent with our earlier reports examining relative risk and penetrance (Begg *et al.*, 2005; Berwick *et al.*, 2006) we use the term ''functional'' to define rare mutations in the coding region that change the amino acid for either p16INK4a or p14ARF, regardless of whether *in vitro* evidence of functionality is available. We analyze separately the single common non-synonymous variant, the previously well-documented Ala148Thr polymorphism, and the common changes in positions 29 and 69 after the stop codon, also referred to as ''nt 500'' and ''nt 540''. We included in the ''functional'' category the G>T alteration at position −34 in the 5′UTR which is known to decrease translation of the wild-type p16 on the basis of *in vitro* studies (Liu *et al.*, 1999). These mutations are highlighted in bold type in Table 2. Variants with low frequency presumed to be ''non-functional'' comprise silent mutations and those changes found in intron 1 and in the 5′UTR that do not alter or are not known to alter p16INK4a based on the literature. We also elected to classify the Ala143Thr variant as ''non-functional'' as this variant lies outside the ankyrin repeat motifs within a region that is not required for the protein to bind and inhibit CDK4. As this variant was observed in only a single patient, its exclusion from the ''functional'' category has no appreciable effect on

Table 2. Correlation of biological subgroups of variants with risk

	SPM	MPM	Melanoma in relatives	Odds ratio MPM versus SPM[1]	Rate ratio in relatives[2]
Functional mutations[3]	30	35	33/417 (7.9%)	3.9 (2.3–6.7)	3.0 (1.8–5.0)
Previously identified in melanoma-prone families[4]	11	20	23/213 (10.8%)		
Not previously identified in melanoma-prone families	19	15	10/204 (4.9%)		
In vitro evidence of functionality	17	20	22/233 (9.4%)		
No *in vitro* evidence of functionality	13	15	11/184 (6.0%)		
Non-functional mutations	37	15	3/336 (0.9%)	0.8 (0.4–1.6)	0.4 (0.1–1.2)
5′ UTR region (excluding SNP in −34)	13	8	0/154 (0.0%)		
Intron 1	18	6	2/137 (1.5%)		
Silent	6	0	1/45 (2.0%)		
Non-functional frequent polymorphisms	1028	522	301/9874 (3.1%)		
Ala148Ala wild type	2234	1084	589/21373 (2.8%)		
Ala148Thr heterozygote[5]	160	70	45/1450 (3.1%)	1.0 (0.8–1.1)	1.2 (0.8–1.7)
*29 (nt 500) CC reference	1769	876	490/17049 (2.9%)		
*29 (nt 500) CG	628	294	164/5840 (2.8%)	1.0 (0.9–1.1)	1.0 (0.8–1.3)
*29 (nt 500) GG	26	20	10/310 (3.2%)	2.0 (1.1–3.9)	0.9 (0.4–2.4)
*69 (nt 540) CC reference	1957	950	515/18700 (2.8%)		
*69 (nt 540) CT	425	212	132/4034 (3.3%)	1.0 (0.8–1.2)	1.2 (0.9–1.5)
*69 (nt 540) TT	29	22	13/353 (3.7%)	1.5 (0.8–2.7)	1.6 (0.8–3.2)
All GEM Study participants with successful sequencing	2424	1189	667/23241 (2.9%)		
All GEM Study participants	2470	1210	680/23905 (2.9%)		

MPM, multiple primary melanoma; SPM, single primary melanoma.
[1]Odds ratio from a logistic regression model comparing the MPM versus SPM frequencies in the given category, using all other patients as the referent category, adjusted for patient age at diagnosis of the index melanoma and center.
[2]Rate ratio from a Poisson regression analysis in which the first-degree relatives are the units of analysis, and age at diagnosis is the outcome, adjusted for patient age, sex, and center.
[3]Mutations present in the coding region or known to change the transcription.
[4]Bishop *et al.*, 2002.
[5]There is one patient that had Ala143Thr+Ala148Thr with a single primary tumor and no family history of melanoma.

the results of our statistical analyses. This variant was previously identified as a germline variant in an indolent case of chronic lymphocytic leukemia (Pinyol *et al.*, 2000). We recognize that our classification of rare variants as ''functional'' or ''non-functional'' is a combination of definitive categorization (in terms of changes in amino acid and the restriction to rare variants) and availability of extraneous evidence from *in vitro* studies, but we believe that this represents the most appropriate categorization to highlight the magnitude of *CDKN2A* on disease risk based on current knowledge.

Rare variants

We found 33 functional mutations in 65 patients (Table 1). A number of silent mutations were identified. Eleven of the variants observed had been previously identified in melanoma-prone families (Bishop *et al.*, 2002; Table 1, column 8). In some instances we identified different changes from those reported in melanoma-prone families affecting the same residue such as the Gly23Ser and Gly23Arg (Table 1) versus the Gly23Asp (Bishop *et al.*, 2002). The variants appear to be randomly distributed throughout exons 1α and 2, but none were identified in exon 3.

Frequently occurring variants

Most variants are rare, appearing in just one or a few study participants. The exceptions are the common Ala148Thr polymorphism present in 234 (6.9%) individuals, and the C→G and C→T variants found in the 3′ UTR (positions 29 and 69 after the stop codon, or nt 500 and 540), which were

identified in 945 (27%) and 671 (19%) of study participants, respectively.

Mutations affecting p14ARF

Table 1 shows the p14ARF amino-acid changes using the numbering for the human protein as annotated in GenBank (NP478102.1) and referred to by some investigators (Laud *et al.*, 2005; Zhang *et al.*, 2005), although mutants have been frequently reported using the murine numbering (Weber *et al.*, 2000; Bishop *et al.*, 2002). Most of the Genes Environment and Melanoma Study (GEM) *CDKN2A* exon 2 nucleotide changes translated into missense mutations in p14ARF (Table 1), and one of our mutants (Ala138Val) resides in the nucleolar localization site. Our study did not include genotyping of exon 1β, and so we do not have a full census of p14ARF variants.

Variants and risk for melanoma

As originally reported in Berwick *et al.* (2006), the risk of melanoma was greatly increased in individuals with a functional mutation (Table 2). MPM was more frequent in patients with variants previously identified in melanoma-prone families, and the frequency of occurrence of melanoma in relatives of these probands was more than twice as great, though neither of these differences reaches statistical significance. However, there was essentially no difference for either of these measures when mutations with prior *in vitro* evidence of functionality were compared with those with no such evidence. The frequency of occurrence of non-functional mutations was similar in MPM and SPM cases, 1.2 versus 1.5%, respectively, and no increase in melanoma risk among relatives was observed (relative risk = 0.4, 95% confidence interval: 0.1–1.2). For the three polymorphisms, heterozygous variants have essentially identical risk to the wild type. There is suggestive but inconclusive evidence that homozygous variants may exhibit modestly increased risk (for both polymorphisms in the 3′ UTR region). Comparisons of the functional variants on the basis of bioinformatic predictors were hindered by the fact that bioinformatic scores are available only for mutations occurring at residues that have homology with other protein families, and so these analyses are hindered by small sample sizes and relatively low statistical power. However, there is suggestive evidence that *Poly*morphism *Phen*otyping (Polyphen) and the *P*osition *S*pecific *S*coring *M*atrix difference may have value in predicting which variants confer the greatest risk. Details are available in Table S2 and S3.

DISCUSSION

Cancer genes are initially identified from studies of families that exhibit multiple cases of the disease. However, to obtain a comprehensive, representative view of the impact of such genes on risk, population-based studies are necessary. In this study, we examined rare variants in the tumor suppressor gene *CDKN2A* by identifying a large population-based sample of incident cases of melanoma (SPM), and a large population-based sample of patients experiencing a subsequent primary melanoma (MPM). The results show that mutations occur infrequently even in these high-risk groups, and that mutations affecting p16INK4a occur mainly in exons 1α and 2.

In addition to providing a comprehensive catalogue of the variants observed in the study (Table 1), we sought to examine the evidence regarding the degree to which melanoma risk may vary on the basis of the nature of the mutation. The results support the following conclusions. Rare p16INK4a variants that change the amino-acid residue or for which there exists *in vitro* evidence of a functional effect (for variants outside the coding regions) demonstrate a substantial increase in risk of melanoma, whereas non-functional rare variants demonstrate no evidence for an increase in risk. Results from comparisons of MPM versus SPM cases and from the analyses of familial aggregation were consistent with these conclusions. Common polymorphisms demonstrate no impact on the risk of melanoma, with the proviso that there is a suggestion of a modest increase in risk for individuals with homozygous variants. Comparisons on the basis of subgroupings of the rare ''functional'' variants were generally either not statistically significant or inconsistent, although we caution that our data are too sparse to address the issue of risk variation among these functional variants reliably.

Several groups have addressed the functional properties of p16INK4a-mutated alleles using various *in vitro* assays that in general measure binding of p16INK4a to CDK4 and CDK6, inhibition of p16 by CycD-CDK in transfected insect cells, protein–protein interactions in yeast, and cell cycle arrest in mammalian cells. Most reports concluded that the majority of the missense mutations cause loss of function, and the loss of function in some cases has a range of effects (Parry and Peters, 1996). Among the other mutants identified in our study, the c.87_89delG produces a frameshift in codon 30 that leads to a stop codon in codon 52. This mutant has not been directly tested in laboratory assays but its functional relevance is inferred based on the knowledge that p16INK4a proteins with less than 120 residues lack the capacity to bind to and inhibit CDK (Parry and Peters, 1996). Similarly, the C to A transversion on the third position in codon 44 and the c.131_132insA gives rise to a stop codon (Tyr44stop). As indicated above, this truncated p16INK4a mutant is not capable of inhibiting CDK4.

We identified several new point mutations: Arg58Gln, Ala60Val, His83Gln, Arg99Trp, Ala102Thr, and Arg124Cys (Figure 1). Of these, the Arg58Gln, Ala60Val, and Arg124Cys variants do not alter p14ARF and no additional information from *in vitro* studies is currently available to assess the impact of these changes on p16. His83 interacts with the Arg31 in the CDK6 molecule and other previously reported changes in this position (His to Tyr) showed a defective binding of p16 to Cdk6 and failure to induce cell cycle arrest (Yarbrough *et al.*, 1999). The substitution in position 99 consists of a charge change (positive to neutral), whereas Ala102Thr may disrupt the intra ankyrin III interaction. Additionally, the transitions c.295 C→T and c.304 G→A identified in GEM cases produce changes in p14ARF.

The prevalence of the previously well-documented Ala148Thr polymorphism is similar to previous reports in

melanoma cases, and based on *in vitro* experiments it is functionally indistinguishable from the wild-type p16INK4a (Koh *et al.*, 1995; Ranade *et al.*, 1995; Reymond and Brent, 1995). Although this variant has been recently associated with increased risk of breast cancer (Debniak *et al.*, 2005a), it does not seem to segregate with melanoma (Hussussian *et al.*, 1994; Walker *et al.*, 1995; Harland *et al.*, 1997; Debniak *et al.*, 2005b) and this is confirmed in our study. Interestingly, when melanoma cells were transfected with this variant, cells showed a predominantly nuclear staining by immunohistochemistry compared to an even nuclear/cytoplasmic staining observed in wild-type p16INK4a-transfected cells (Walker *et al.*, 1999). The other common polymorphisms present at nt 500 and at nt 540 (*29 and *69) were found among GEM participants with higher frequencies than the 12–15% reported by others ((Ruas and Peters, 1998); National Institute of Environmental Health Sciences NIEHS http://egp.gs.washington.edu – accessed June 2005). Although neither of these variants causes a change in the amino-acid sequence of p16INK4a and the functional importance is unknown, our interest in targeting this region was motivated by the fact that 3′ UTR regions may be involved in the regulation of gene expression by controlling nuclear export, polyadenylation, translation rates, and messenger RNA degradation. These variants do not segregate with melanoma in melanoma prone families according to some reports (Harland *et al.*, 1997). Others have found that the G variant in position 500 (*29) appeared associated with familial risk to melanoma, although the degree of ethnic contribution remains to be established (Aitken *et al.*, 1999). Interestingly, in a small study, Straume *et al.* (2002) found that presence of the C→T (*69) transition might be associated with low-grade vertical growth phase melanomas and might improve survival in melanoma carriers. Our results appear to suggest modest elevation of risk in carriers of the homozygous variants. This increase in risk is statistically significant only for the nt500 variant, but there is no confirmatory evidence of elevation of risk among relatives.

In summary, we identified 44 different variants in *CDKN2A*(p16INK4a) in a population-based study. Of these, 33 were functionally relevant and were identified in 35 cases of MPM and 30 cases of SPM. To the best of our knowledge, six have not been previously reported: Arg58Gln, Ala60Val, His83Gln, Arg99Trp, Ala102Thr, and Arg124Cys. Rare functional variants increased the risk of melanoma significantly. Further subclassification of these variants failed to identify strong evidence of differences in melanoma risk between the variants.

MATERIALS AND METHODS

Study participants

Subjects were recruited through an international multi-center population-based case–control study of melanoma, the GEM. The GEM population consists of incident cases of melanoma identified in eight population-based registries and one hospital center (that sees about 50 percent of the melanoma diagnosed in the state of Michigan) in nine geographic regions of the world: New South Wales (Australia); Tasmania (Australia); British Columbia (Canada); Ontario (Canada); Turin (Piemonte, Italy); California (Orange County and San Diego/Imperial Organization for Cancer Control, USA); MIUSA; NJUSA; and NC. The study was approved by the Institutional Review Board at Memorial Sloan-Kettering Cancer Center (MSKCC) and at each participating institution. The study was conducted according to the Declaration of Helsinki Principles. All participating subjects signed informed consent.

Participants were individuals diagnosed with either a single first invasive primary melanoma (SPM) or with a second- or higher-order invasive or *in situ* melanoma (MPM). All subjects completed a telephone interview in English (or in Italian in Turin). The study was approved by the institutional review board in all centers. All study participants signed informed consent, provided a sample of buccal cells or blood for DNA extraction, and completed an interview, which included the collection of detailed information on the occurrence of melanoma in the participant's first-degree relatives. Further details of the study design and its rationale can be found in Begg *et al.* (2006).

Laboratory methods

DNA was collected from subjects by means of buccal swabs (88.5%), which were mailed to the participants, or from blood samples (in 11.5% of the participants). Our methods for identifying sequence variation have been described in detail in a previous article (Begg *et al.*, 2005). Briefly, PCR products spanning exons 1α, 2, and 3 plus the adjacent intronic regions of all samples were initially screened by denaturing high-performance liquid chromatography analysis (Orlow *et al.*, 2001). Samples were amplified using primers described previously (Hussussian *et al.*, 1994; Kamb *et al.*, 1994) with few modifications. For interested readers, these are defined in Table S1. All samples showing an altered denaturing high-performance liquid chromatography chromatographic profile were re-amplified from genomic DNA in an independent PCR reaction for DNA sequencing. Sequencing was accomplished for exons 1α, 2, and 3 of the *CDKN2A* gene, the exons that code for the p16INK4a protein. We did not sequence exon 1β, which, together with exons 2 and 3, codes for the p14ARF protein. Mutations were considered ''functional'' if they were in the coding region and if the replacement, deletion, or insertion of one or more nucleotides changed the amino-acid sequence for p16INK4a or p14ARF or both, or if they were in a non-coding region and were known to inhibit transcription of wild-type p16INK4a based on previous *in vitro* studies (e.g., Liu *et al.*, 1999). The primers extended beyond either end of the coding regions. However, the primers did not extend to the deep intronic mutation IVS2-105, which has recently been associated with melanoma in some melanoma-prone families (Harland *et al.*, 2005).

An additional exon 3 fragment was tested by pyrosequencing to determine presence of variants in position 540 (Ronaghi, 2003). Fragments were amplified in a reaction containing 0.2 μM each of the following primers: forward 5′-NCCCGATTGAAAGAACCAGAGA-3′ and reverse 5′-biotin-AAAGCGGGGTGGGTTGTG-3′, 1.5 mM $MgCl_2$, 200 μM dNTP and 0.05 U/μl Taq Polymerase, and PCR buffer. The cycling consisted of a denaturation at 95°C for 5 minutes followed by 45 cycles of 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 15 seconds, with a final extension at 72°C for 7 minutes. Products were immobilized and denatured at 80°C for 2 minutes. The sequencing primer (5′-AGTCACCGAAGGTCC-3′) was added to

the single-stranded DNA and nucleotides dispensed automatically by a Pyrosequencing AB PSQ96MA instrument and software (Uppsala, Sweden). All reactions contained known controls and pyrograms were read twice.

Statistical analysis

Empirical evidence of the likely functional relevance of individual mutations and relevant groupings of similar types of mutations was obtained through two separate analyses. The first analysis compared the relative frequencies of the mutation(s) among cases of MPM versus cases of SPM, on the premise that mutations that increase the risk of melanoma will occur with a greater relative frequency among MPMs (Begg and Berwick, 1997; Monzon *et al.*, 1998; Bernstein *et al.*, 2004). These analyses involved the use of logistic regression, adjusted for age at diagnosis of the index lesion, and study center. Relevant groupings of mutations with different characteristics were compared using Fisher's exact test or with the Cochran–Armitage test in the case of ordered categories, as implemented in StatXact© software. The second analysis involved comparison of the reported frequencies of melanoma in first-degree relatives of probands. Probands with a mutation that increases the risk of melanoma are more likely to report a history of melanoma in their first-degree relatives (Begg *et al.*, 2005). For these comparisons Poisson regression was used to estimate rate ratios and 95% confidence intervals, with adjustment for age and center.

Bioinformatics tools

Various sequence-based software applications were used to predict the probability that observed variants could have a functional impact on the protein. The first one, *S*ort *I*ntolerant *f*rom *T*olerant, is based on the premise that changes in conserved amino acids within a family of proteins or across species are more likely to have an important impact on function (Ng and Henikoff, 2001, 2002). The *S*ort *I*ntolerant *f*rom *T*olerant scores range from 0 to 1 and low scores (<0.05) represent high probabilities that the amino acid change at a given position affects protein function. The *P*osition *S*pecific *S*coring *M*atrix score is expressed in terms of the observed frequency of an amino acid at each position within a block of proteins, using homology models by aligning the change to a block. Large changes from the alignment, or larger positive *P*osition *S*pecific *S*coring *M*atrix differences indicate that the variant is likely to have a deleterious effect. For our prediction of these scores using PARSESNP (Taylor and Greene, 2003, http://www.proweb.org/parsesnp), the first three best blocks family hits were chosen and included: IPB002110 (Ankyrin repeat signature), IPB010660 (NOD), and IPB000906 (ZU5 domain) with *E*-values equal or smaller than 9×10^{-9}. The third prediction tool, PolyPhen, uses protein structure, sequence and phylogenetic information to predict the impact on the 3D-structure and the consequences of amino-acid changes on protein function (Sunyaev *et al.*, 2000, 2001; Ramensky *et al.*, 2002). PolyPhen returns a prediction of probably damaging, possibly damaging, benign, or unknown, derived from a numeric (PSIC) score. For the prediction of our GEM *CDKN2A* mutants on the structure and/or function of p16INK4a by PolyPhen we used web-based tools (http://www.bork.embl-heidelberg.de/PolyPhen/), chose the Protein Data Bank as structural database, and selected the following options: map to mismatch (no), and calculate contacts (for all hits). Default options and values were used for the sorting of hits, calculation of structural parameters, minimal identity and maximal gap in alignment, and threshold for contacts. We were unable to obtain *S*ort *I*ntolerant *f*rom *T*olerant, *P*osition *S*pecific *S*coring *M*atrix, and PolyPhen scores for variants affecting p14ARF because of lack of family blocks with high homology to this protein. Similarly, scores were unavailable for p16 variants located in positions with no homology with the family blocks (see Table S3).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Primer sequences, PCR, and denaturing high-performance liquid chromatography conditions used for the mutation screening of the CDKN2A gene.

Table S2. Classification of CDKN2A-p16 variants using bioinformatics tools.

Table S3. Correlations of Bioinformatics subgroups of variants with risk.

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