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Research Article

High-risk Melanoma Susceptibility Genes and Pancreatic Cancer, Neural System Tumors, and Uveal Melanoma across GenoMEL

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Abstract

GenoMEL, comprising major familial melanoma research groups from North America, Europe, Asia, and Australia has created the largest familial melanoma sample yet available to characterize mutations in the high-risk melanoma susceptibility genes *CDKN2A/alternate reading frames* (*ARF*), which encodes p16 and p14ARF, and *CDK4* and to evaluate their relationship with pancreatic cancer (PC), neural system tumors (NST), and uveal melanoma (UM). This study included 466 families (2,137 patients) with at least three melanoma patients from 17 GenoMEL centers. Overall, 41% (n = 190) of families had mutations; most involved p16 (n = 178). Mutations in *CDK4* (n = 5) and *ARF* (n = 7) occurred at similar frequencies (2-3%). There were striking differences in mutations across geographic locales. The proportion of families with the most frequent founder mutation(s) of each locale differed significantly across the seven regions (P = 0.0009). Single founder *CDKN2A* mutations were predominant in Sweden (*p.R112_L113insR*, 92% of family's mutations) and the Netherlands (*c.225_243del19*, 90% of family's mutations). France, Spain, and Italy had the same most frequent mutation (*p.G101W*). Similarly, Australia and United Kingdom had the same most common mutations (*p.M53I, c.IVS2-105A>G, p.R24P*, and *p.L32P*). As reported previously, there was a strong association between PC and *CDKN2A* mutations (P < 0.0001). This relationship differed by mutation. In contrast, there was little evidence for an association between *CDKN2A* mutations and NST (P = 0.52) or UM (P = 0.25). There was a marginally significant association between NST and *ARF* (P = 0.05). However, this particular evaluation had low power and requires confirmation. This GenoMEL study provides the most extensive characterization of mutations in high-risk melanoma susceptibility genes in families with three or more melanoma patients yet available. (Cancer Res 2006; 66(20): 9818-28)

Introduction

The etiology of cutaneous malignant melanoma (CMM) is heterogeneous and complex. To date, two high-risk melanoma susceptibility genes *CDKN2A* (MIM 600160) and *CDK4* (MIM 123829) have been identified. Causal mutations in both genes cosegregate with melanoma and are inherited in a dominant pattern. The *CDKN2A* gene, located on chromosome 9p21, is the major known high-risk melanoma susceptibility gene (1, 2). *CDKN2A*, a tumor suppressor gene, encodes two distinct proteins translated, in alternate reading frames (ARF), from alternatively spliced transcripts. The α transcript, comprising exons 1α, 2, and 3, encodes a low molecular weight protein, p16. The p16 protein regulates G_1-phase exit by inhibiting the *CDK4*-mediated phosphorylation of the retinoblastoma protein (3, 4). The smaller β

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transcript, comprising exons 1β, 2, and 3, specifies the alternative product p14ARF. p14ARF acts via the p53 pathway to induce cell cycle arrest or apoptosis (5, 6). For ease of presentation, we use *CDKN2A* to represent mutations in the α transcript; thus, all *CDKN2A* mutations involve the p16 protein. For mutations of exon 1β and large deletions that involve the p14ARF protein, we use *ARF*. In contrast to *CDKN2A*, few families with cosegregating *CDK4* germ-line mutations have been identified (7–9). To date, all cosegregating *CDK4* germ-line mutations have been identified in exon 2, which codes for the p16 binding site (7–9).

In addition to melanoma, other cancers have been observed in some *CDKN2A* melanoma-prone families. Several studies have shown an increased risk of pancreatic cancer (PC) among *CDKN2A* melanoma-prone families (10–16). However, the precise relationship between the *CDKN2A* gene and PC remains unknown. Neural system tumors (NST) have also been reported to be associated with large deletions of *CDKN2A/ARF* and/or mutations that affect p14ARF but these studies are based on very small numbers of patients/families (17–21). Uveal melanoma (UM) occasionally also occurs in families with multiple CMM patients, suggesting the existence of possible common genetic factors, but to date, only one family with both UM and CMM and a *CDKN2A* germ-line mutation (*p.G67S*) has been reported (22).

The International Melanoma Genetics Consortium (GenoMEL), comprising major familial melanoma research groups from North America, Europe, Australia, and the Middle East, reports analyses of the largest familial melanoma sample yet available to examine the characterization of mutations in the three major known high-risk melanoma susceptibility genes. A second goal of the study was to evaluate the relationship between the susceptibility genes and the occurrence of PC, NST, and UM in GenoMEL families.

Materials and Methods

Families from 17 GenoMEL centers were pooled for this study. Families with at least three CMM patients that were collected by each of the 17 centers were eligible for the study. Diagnoses were confirmed by review of histologic materials, pathology reports, medical records, or death certificates. To be eligible, families also had to have been evaluated for mutations in the *CDKN2A* gene (exons 1α, 2, and 3; n = 466 families). Mutation evaluation (predominantly sequencing and/or denaturing high-performance liquid chromatography) was conducted at each center on available subjects. In 89% (n = 414) of families, at least 2 CMM patients were screened for *CDKN2A* mutations. Table 1 presents the number of eligible families and total number of melanoma patients by study center. For all centers, written informed consent was obtained from the subjects before participation in the study under Institutional Review Board–approved protocols. Although the ascertainment of families differed between groups due to variation in local health care procedures and/or approaches for accruing families, the eligibility criteria for inclusion in this study were uniform as described above. Details of the participating families from most of the 17 centers are described elsewhere (see Table 1 for references). Most of the 466 participating families (n = 410 families) were also tested at each center for *CDK4* (exon 2) and *ARF* (exon 1β) mutations. Evaluation of *CDK4* was restricted to exon 2 because no causal mutations have been identified outside of this exon. In addition, 253 of these families were evaluated using real-time quantitative PCR methods or multiplex ligation-dependent probe amplification for large deletions of the *CDKN2A* and *ARF* regions. Because of the screening protocols at many centers, many patients/families tested for *CDK4* and *ARF* mutations and/or large deletions had previously identified *CDKN2A* mutations. For each family, the absence or presence of a mutation was reported. For mutation-positive families, the type of *CDKN2A* (exons 1α, 2, and 3), *ARF* (exon 1β), or *CDK4* (exon 2) mutation was also recorded. Other variables for study included number of CMM patients in each family, age at first melanoma diagnosis for each CMM patient, and number of melanoma patients and first-degree relatives of melanoma patients with PC, NST, or UM. NST included acoustic neuroma, astrocytoma, ependymoma, glioblastoma, medulloblastoma, meningioma, neuroblastoma, neurofibroma, or neurolemmoma.

The distribution of the identified mutations and the types and frequencies of the different mutations across all 17 GenoMEL groups (total) and within specific geographic regions were evaluated. For this study, seven geographic locales were defined as follows: United Kingdom (Leeds and Glasgow), Mediterranean Europe (Genoa, Italy; Emilia-Romagna, Italy; and Barcelona, Spain), France (Paris), the Netherlands (Leiden), Sweden (Lund and Stockholm), Australia (Brisbane and Sydney), and North America [Boston, Philadelphia, National Cancer Institute (NCI), Utah, and Toronto]. The nonparametric Wilcoxon-Mann-Whitney, Jonckheere-Terpstra, or Kruskal-Wallis test, as implemented in the computer program StatXact (version 4.0.1), were used to test the hypothesis of no difference in the distributions of the variables being compared. All statistical tests were two sided.

Evidence for causality for many of the identified mutations, from, for example, segregation with disease, absence of mutations in control samples, and functional studies has been presented previously (see Table 1 for references). To further examine the potential functional consequences of the GenoMEL-reported *CDKN2A* missense mutations, we evaluated the biochemical severity of the identified *CDKN2A* missense mutations using the Grantham scale (43) and BLOSUM62 matrix (44). For the Grantham scale, missense mutations were classified as radical or conservative. Using the BLOSUM62 matrix, missense mutations were categorized as nonconservative or conservative. Further, to determine which amino acids in *CDKN2A* were evolutionarily conserved, the human protein sequence was compared with that of nine other animal species identified using tBLASTn (45). Clustal W (46) was used to create a multispecies alignment of the *CDKN2A* sequence.

Results

Table 1 presents the number of families, melanoma patients, and mutations detected by participating center. There were 466 families with 2,137 CMM patients (1,867 confirmed CMM cases) in this study. The total number of melanoma patients (confirmed and not confirmed) per family ranged from 3 to 54 with a median number of melanoma cases per family of 4. Overall, 41% of families (n = 190) had mutations; of these, 178 families had *CDKN2A* mutations involving p16. The *CDKN2A* mutation frequency varied from <25% in Boston, Emilia-Romagna, Philadelphia, Brisbane, Tel Aviv, and Sydney to >50% in Genoa, Glasgow, Leeds, Leiden, Lund, and Toronto. Similar frequencies of mutations (2-3%) were observed for *CDK4* (5 of 271) and *ARF* (7 of 277). The identified mutations included four large deletions of *CDKN2A* (n = 1) or *ARF* (n = 3; total, 2%).

Table 2 shows the types and number of mutations and number of families with mutations overall and by geographic locale. There were 66 different mutations (57 *CDKN2A* mutations) detected in 190 families (178 families with *CDKN2A* mutations). Sixty-five percent of the mutations were observed only once; the remainder was seen in more than one family. Missense mutations in the same codon of *CDK4* were detected in five families, two from North America (both *p.R24C*) and one each from France, Australia, and United Kingdom (all *p.R24H*). The seven *ARF* mutations included four novel splice site mutations (47) observed in families from the Netherlands, United Kingdom (n = 2), and North America and three different large deletions observed in United Kingdom (n = 2) and North American families. Sixty-five percent of the *CDKN2A* mutations were missense mutations; 16%, deletions; 7%, insertions or duplications; and 5% each, nonsense or splicing mutations. Overall, 40% of the *CDKN2A* mutations were in exon 1α, whereas 53% occurred in exon 2.

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The Grantham scale was used to evaluate the biochemical severity of the *CDKN2A* missense mutations; the results suggested that many missense changes (35%) were radical (Table 3). Additionally, the majority of the missense mutations that altered p16 (76%) were considered to be nonconservative amino acid replacements using the BLOSUM62 matrix (Table 3). The proportion of radical (based on the Grantham scale) or nonconservative (based on the BLOSUM62 matrix) substitutions was similar in exons 1α and 2 of *CDKN2A*. Furthermore, the proportion of radical or nonconservative amino acid changes that involved p14ARF was similar to the proportion that altered p16; 43% of the amino acid substitutions were expected to radically alter p14ARF function using the Grantham scale, and 71% were predicted to be nonconservative using the BLOSUM62 matrix.

The most frequent *CDKN2A* mutations were *c.225_243del19* (also known as *p16-Leiden*), *p.M53I, p.G101W, c.331_332insGTC* (more commonly presented as *p.R112_L113insR*), *c.-34G>T*, and *c.IVS2-105A>G*. The distribution of mutations differed by geographic locale (Fig. 1; Table 2). The proportion of families with the most frequent founder mutation(s) of each locale differed significantly across the seven regions ($P = 0.0009$). The majority of families with mutations from the Netherlands and Sweden each had a single founder mutation and very few other mutations (*c.225_243del19*, 90% of families with mutations and *p.R112_L113insR*, 92% of families with mutations, respectively). Australia and United Kingdom shared the same common recurrent mutations (*p.M53I, c.IVS2-105A>G, p.R24P*, and *p.L32P*) involving 42% and 50% of reported families with mutations, respectively. *p.G101W* was the most frequent mutation in Mediterranean Europe (60% of families with mutations) and France. North America had the greatest number of different mutations, possibly reflecting the ancestry of its inhabitants. Two of the frequent recurrent mutations in North America (*p.G101W* and *p.M53I*) were the most frequent mutations observed in Mediterranean Europe/France (*p.G101W*) and United Kingdom/Australia (*p.M53I*). The most frequent mutation in North America (*c.-34G>T*) was observed in Australia and United Kingdom, reflecting its likely British origin (48). The age at origination of the most frequent North American missense mutation *p.V126D* predates the colonization of North America (49). Given its almost complete absence from all other studied geographic locales, it suggests that this mutation likely originated in European region(s) not represented in GenoMEL.

Figure 2 shows the human protein sequence for *CDKN2A* along with that of nine other animal species identified using tBLASTn (45). Each of the 30 missense mutations observed in the current study is enclosed in a box to more clearly show amino acids across the tested species. For both exons 1α and 2, GenoMEL-reported missense mutations occurred in ~20% of the total codons. Four (*p.M53I, p.G101W, p.V126D*, and *p.L32P*) of the five most frequent missense mutations (*p.M53I, p.G101W, p.R24P, p.V126D*, and *p.L32P*) all occurred at codon positions that were perfectly conserved across the tested species. The multispecies alignment generated by Clustal W (46) showed that 48% of all of the *CDKN2A* amino acid residues were either perfectly conserved or contained only conserved substitutions across the 10 tested species (Fig. 2). However, 80% of the 30 codons with GenoMEL-identified disease-associated missense mutations were conserved ($P = 0.006$). This significant result was primarily because of mutations in exon 2. Specifically, in exon 2, 55% of the 103 codons were perfectly conserved; however, 89% of the codons with reported

Table 1. Number of families; melanoma patients (confirmed and not confirmed); *CDKN2A, CDK4* (exon 2), and *ARF* (exon 1β) mutations; and large deletions involving the *CDKN2A/ARF* regions (exons 1α, 1β, 2, or 3) by study center and country

City/center, country	Total no. families	Total no. CMM patients	Total no. CMM confirmed	*CDKN2A* (exon 1α, 2, or 3)	*ARF* (exon 1β)	*CDK4* (exon 2)	Large deletions of the *CDKN2A* and *ARF* regions*	References for family study sample
Brisbane, Australia	102	512	450	18	0	1	0	23, 24, 25
Sydney, Australia	62	258	258	14	0	0	0	26, 27, 28
Tel Aviv, Israel	1	5	5	0	0	0	NT	
Paris, France	41	146	138	19	0	1	0	8
Genoa, Italy	13	55	35	9	0	0	0	13
Emilia-Romagna, Italy	11	36	33	1	0	0	0	29
Leiden, the Netherlands	27	159	159	19	1	0	NT	30, 31
Barcelona, Spain	12	41	40	5	0	0	0	20, 32
Stockholm, Sweden	10	37	36	4	0	0	NT	33, 34, 35
Lund, Sweden	15	60	60	8	NT	0	NT	14, 36
Glasgow,United Kingdom	10	31	31	6	NT	NT	NT	37, 38
Leeds, United Kingdom	75	293	169	35	4	1	3	28, 39
Boston, United States	6	23	13	1	0	0	NT	40
NCI, United States	42	219	219	19	2	2	1	1, 41
Philadelphia, United States	9	33	26	2	0	0	NT	
Utah, United States	11	150	150	5	0	NT	NT	42
Toronto, Ontario, Canada	19	79	45	13	0	0	NT	
Total	466	2,137	1,867	178	7	5	4	
Frequency of mutations				0.38	0.03	0.02	0.02	

Abbreviation: NT, not tested.

*Large deletions column is not mutually exclusive. That is, families with large deletions are also listed in columns for *CDKN2A* or *ARF* based on boundaries of each deletion.

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Table 2. Number of families with mutations in *CDKN2A, ARF*, or *CDK4* overall and by geographic locale

Mutation location	Gene	No. families with each mutation by geographic locale							
		United Kingdom	Mediterranean Europe	France	The Netherlands	Sweden	Australia	North America	Total
5′-UTR	*CDKN2A*								
	c.-34G>T	2					1	**8**	**11**
Exon									
1α	*p.P3T*							1	1
1α	*c.9_32del24*						1		1
1α	*c.18_19ins6*			1					1
1α	*c.32_33ins9-32*	2					2	2	**6**
1α	*p.W15X*						1		1
1α	*c.46delC*						1		1
1α	*p.L16P*			1			2		3
1α	*p.L16R*							1	1
1α	*c.52_57dup6*	1							1
1α	*p.G23D*			1				1	2
1α	*p.G23R*				1				1
1α	*p.R24P*	**3**	1	2			**3**		**9**
1α	*p.E27X*		1						1
1α	*c.88delG*	1							1
1α	*p.L32P*	**3**					**3**		**6**
1α	*p.G35A*	2					1		3
1α	*p.G35E*							1	1
1α	*p.A36P*						1		1
1α	*p.P38R*	1							1
1α	*p.P48L*					1			1
1α	*p.P48T*		1						1
1α	*p.I49S*						1		1
1α	*p.Q50R*						1		1
2	*p.V51F*						1		1
2	*p.M53I*	**10**					**5**	**4**	**19**
2	*p.M53V*							1	1
2	*c.167_197del31*							1	1
2	*p.R58X*							1	1
2	*p.V59G*		1	1					2
2	*p.L62P*			1					1
2	*p.L63P*	2							2
2	*p.L65P*		1						1
2	*p.G67R*	2							2
2	*p.G67S*			1			1		2
2	*p.A68L*			1					1
2	*p.E69G*			1					1
2	*p.N71K*			2					2
2	*p.N71S*						1	1	2
2	*c.225_243del19*	1			**18**		1	1	**21**
2	*c.240_253del14*	1						1	2
2	*p.R87P*							1	1
2	*p.L97R*			1					1
2	*p.R99P*			1					1
2	*p.G101W*		**9**	3				**4**	**16**
2	*c.307_308del2*							1	1
2	*p.D108N*						1		1
2	*p.R112G*	1					1	1	3
2	*p.R112_L113insR* (*c.331_332insGTC*)					**11**			**11**
2	*p.L113L/p.P114S*			1					1
2	*p.A118T*	1							1
2	*c.358delG*		1						1
2	*p.V126D*			1				**6**	7
2	*p.D153spl*							1	1

(*Continued on the following page*)

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Table 2. Number of families with mutations in *CDKN2A, ARF*, or *CDK4* overall and by geographic locale (Cont'd)

Mutation location	Gene	No. families with each mutation by geographic locale							
		United Kingdom	Mediterranean Europe	France	The Netherlands	Sweden	Australia	North America	Total
Intron	*CDKN2A*								
2	*c.IVS2-105A>G*	**7**					**3**	1	**11**
2	*c.IVS2+1G>T*							1	1
Exon									
1α	Deletion	1							1
2									
3									
Exon	*ARF*								
1β	*c.192A>T*	1							1
1β	*c.193+1G>A*				1				1
1β	*c.193+2T>C*							1	1
1β	*c.193G>C*	1							1
1β	Deletion	2*						1	3*
Exon	*CDK4*								
2	*p.R24C*							2	2
2	*p.R24H*	1		1			1		3
Summary									
No. families with mutations		46	15	20	20	12	33	44	190
Percentage of families with mutations (%)		54	42	49	74	48	20	51	41
No. mutations (each mutation counted once)		22	7	16	3	2	21	24	66

NOTE: Most frequent mutations for each geographic locale and total are in bold. Sequence variations are described according to their effect on the protein (p.) for missense mutations and according to the sequence change (c.) for insertion, deletion, intronic, and 5′-untranslated region variants. Numbering relates to the following reference sequences: *CDKN2A*, Genbank accession no. NM_000077; *CDK4*, Genbank accession no. NM_000075; and *ARF*, Genbank accession no. NM_058195. Note in the latter, the suggested initiation codon is incorrect; the true initiating methionine is the second of those listed in the putative translation product. Intronic mutations affecting splicing of the transcripts encoding p16 and p14ARF can be found in Genbank sequence no. AB060808.

Abbreviation: UTR, untranslated region.

*Each deletion is different/unique.

disease-associated missense mutations were completely conserved ($P = 0.005$). In contrast, for exon 1α, 40% of the 50 codons were conserved, whereas only 64% of the codons with reported disease-related missense mutations were fully conserved across the tested species ($P = 0.19$).

We estimated the median age at CMM diagnosis by gene category restricting the assessment to patients with confirmed melanoma only. The median age at CMM diagnosis was significantly different in melanoma patients from families without mutations (median, 45 years) compared with families with mutations: *CDKN2A* (36 years), *CDK4* (32 years), or *ARF* (30 years; $P < 0.0001$). Similar patterns were observed for families from all seven geographic locales. The median age at melanoma diagnosis did not differ significantly between *CDKN2A, CDK4*, and *ARF* ($P = 0.32$), although the number of patients with *CDK4* or *ARF* mutations was small.

Table 4 shows the number of families with PC, NST, or UM by gene category. Of the 66 families who had PC, 74% ($n = 49$) had a *CDKN2A* mutation. In contrast, 33% (122 of 365) of families without PC, NST, or UM had a *CDKN2A* mutation. This mutation frequency in melanoma-only families was significantly less than in families that also had PC ($P < 0.0001$); no such significant difference was observed for families with NST ($P = 0.27$) or UM ($P = 0.28$). As reported previously, the presence of PC in a family was strongly associated with the occurrence of a *CDKN2A* mutation (28% of families with a *CDKN2A* mutation had PC versus 6% of families with no mutation had PC; $P < 0.0001$). In contrast, there was no significant association between NST and *CDKN2A* mutations ($P = 0.52$). Five of the 11 families with NST and *CDKN2A* mutations also had at least one case of PC. Only 1 of 8 (12.5%) families with UM had a *CDKN2A* mutation (*p.G67S*; $P = 0.16$; ref. 22).

There were no reports of PC in families with *ARF* or *CDK4* mutations. In addition, none of the five families with *CDK4* mutations had NST. Two of the seven families with *ARF* mutations had NST (29%). Although this evaluation had low power, there was a marginally significant association between NST and *ARF* mutations ($P = 0.05$; Table 4). However, evaluation of NST and all mutations that altered p14ARF (i.e., exon 1β splice site mutations and large deletions plus *CDKN2A* mutations that involved both p16 and p14ARF; $n = 9/99$) did not show any significant association ($P = 0.12$).

The distribution of PC differed according to the specific *CDKN2A* mutation. Among the 10 most common recurrent *CDKN2A*

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mutations (comprising 66% of families with *CDKN2A* mutations), the distribution of PC differed significantly (P = 0.0003). The frequency of PC varied from >60% in *p.R112_L113insR* and *c.225_243del19* to 35% to 50% in *p.L32P, p.V126D*, and *p.G101W*, to 15% to 25% in *p.R24P* and *c.-34G>T*, and to <11% in *p.M53I, c.IVS2-105A>G*, and *c.32_33ins9-32*. For the families with other *CDKN2A* mutations (n = 60), 18% of families had PC. Evaluation of *CDKN2A* mutations that affected the p16 protein only versus p16 and p14ARF also showed differences with respect to the occurrence of PC. Whereas among families without PC, there were similar percentages of families with mutations that affected p16 only (19%) versus p16 and p14ARF (17%); among families with PC, these percentages differed substantially (26% versus 49%, respectively). However, the significance of this association varied according to whether the analysis included all reported CMM (P = 0.03) or was restricted to confirmed melanoma patients only (P = 0.15).

Discussion

GenoMEL has created the largest familial melanoma data set yet assembled to characterize mutations in the major known high-risk melanoma susceptibility genes. The data set included 466 melanoma-prone families with 2,137 CMM patients. There were 66 different mutations detected in 190 families. Overall, 41% of families had a mutation in one of the three known high-risk melanoma susceptibility genes; 38% of families (n = 178) had *CDKN2A* mutations that involved the p16 protein. Seventy percent of the *CDKN2A* mutations were missense or nonsense mutations, 23% were insertions or deletions, 5% were splicing mutations, and

Table 3. Nonsynonymous amino acid changes for p16 and p14ARF proteins of observed *CDKN2A* missense mutations with biochemical severity classification according to the Grantham scale or BLOSUM62 matrix

CDKN2A missense mutations (p16 protein)	Grantham scale	BLOSUM62 matrix	Nonsynonymous amino acid changes to p14ARF protein	Grantham scale	BLOSUM62 matrix
p.P3T	C	NC	—		
p.L16P	C	NC	—		
p.L16R	R	NC	—		
p.G23D	C	NC	—		
p.G23R	R	NC	—		
p.R24P	R	NC	—		
p.L32P	C	NC	—		
p.G35A	C	C	—		
p.G35E	C	NC	—		
p.A36P	C	NC	—		
p.P38R	R	NC	—		
p.P48L	C	NC	—		
p.P48T	C	NC	—		
p.I49S	R	NC	—		
p.Q50R	C	C	—		
p.V51F	C	NC	*p.G65V*	R	NC
p.M53I	C	C	*p.D68H*	C	NC
p.M53V	C	C	*p.D67G*	C	NC
p.R58X	—	—	*p.P72L*	C	NC
p.V59G	R	NC	*p.S73R*	R	NC
p.L62P	C	NC	—		
p.L63P	C	NC	—		
p.L65P	C	NC	—		
p.G67R	R	NC	*p.R81P*	R	NC
p.G67S	C	C	*p.R81Q*	C	C
p.A68L	C	NC	*p.R82L*	R	NC
p.E69G	C	NC	—		
p.N71K	C	C	*p.L86M*	C	C
p.N71S	C	C	—		
p.R87P	R	NC	—		
p.L97R	R	NC	—		
p.R99P	R	NC	—		
p.G101W	R	NC	*p.R115L*	R	NC
p.D108N	C	C	*p.R122Q*	C	C
p.R112G	R	NC	*p.P126R*	R	NC
p.L113L/p.P114S	C	NC	*p.A128T*	C	C
p.A118T	C	C	*p.G132D*	C	NC
p.V126D	R	NC	—		

Abbreviations: R, radical; C, conservative; NC, nonconservative.

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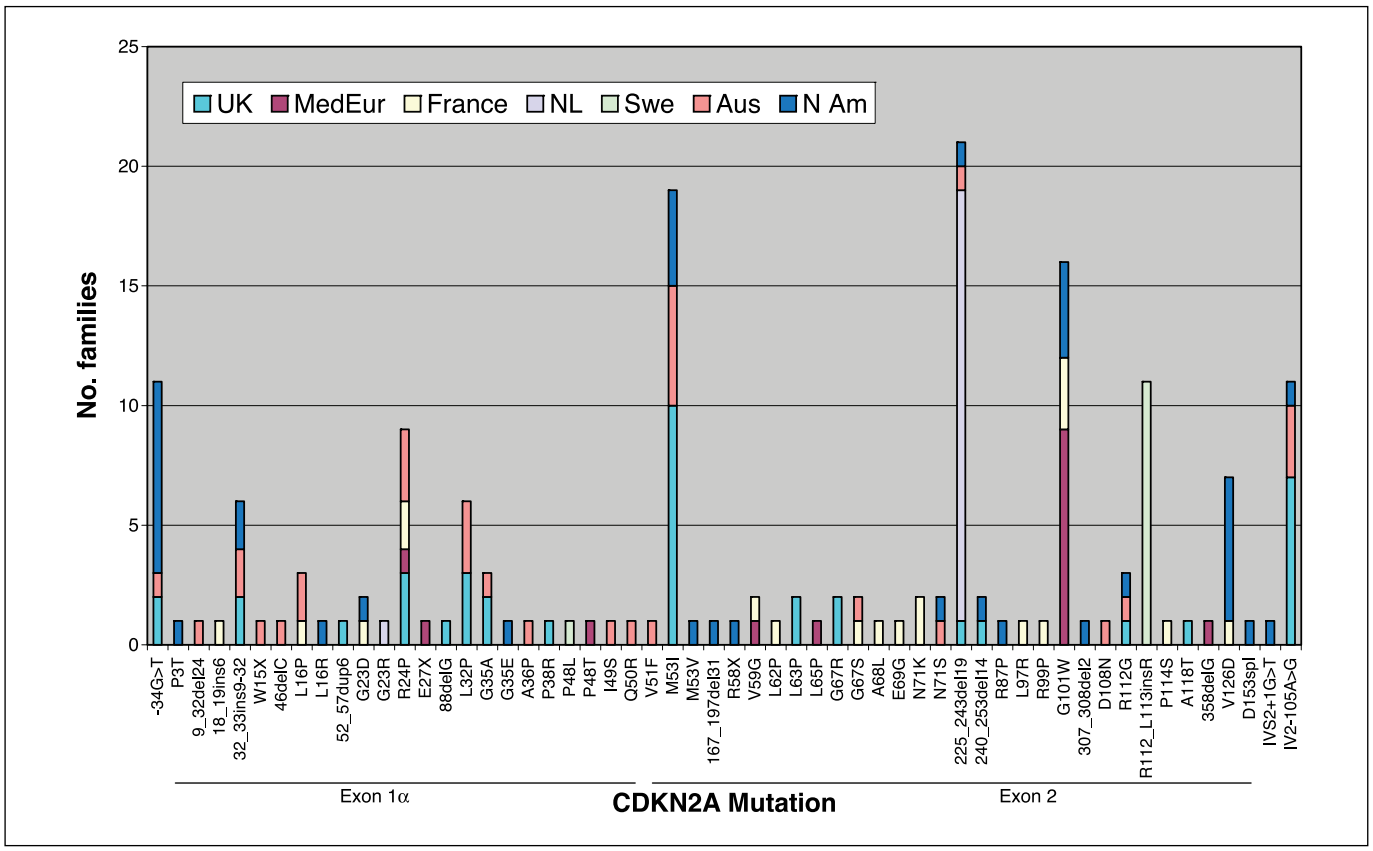

Figure 1. Number of families with *CDKN2A* mutations according to family's geographic locale. Seven locales are defined as follows: *UK*, United Kingdom; *MedEur*, Mediterranean Europe (Spain and Italy); *France*; *NL*, the Netherlands; *Swe*, Sweden; *Aus*, Australia; and *N Am*, North America. For presentation purposes, mutations are presented without leading protein (p.) or sequence change (c.) notation. Codon positions for *CDKN2A* exons 1α and 2 are noted underneath mutations.

2% were regulatory mutations. The distribution of *CDKN2A* mutation types is consistent with that observed in the Human Genome Mutation Database.[27] This finding is not surprising because the majority of disease-associated mutations identified to date are highly penetrant mutations associated with Mendelian disorders. In contrast, the seven *ARF* mutations were either novel splicing mutations (47) or large deletions (18, 50); the five *CDK4* mutations were all missense mutations that occurred in the same codon (7–9). Inheritance of the causal mutations of *CDKN2A, ARF*, and *CDK4* are consistent with autosomal dominant inheritance with incomplete penetrance (41, 51). Sixty-five percent of the GenoMEL *CDKN2A* mutations reported to be associated with familial melanoma were missense mutations that occurred at 30 different codon positions. Examination of two different measures used to predict biochemical severity of missense changes (Grantham scale and BLOSUM62 matrix) suggested that, for the *CDKN2A* gene, the BLOSUM62 matrix score was a better predictor of altered protein function assumed to be associated with mutations that segregate with familial melanoma.

Several studies have shown that disease-associated mutations are more likely to occur in perfectly conserved codon positions (52, 53). This finding was also observed for the *CDKN2A* missense mutations described in this study. Eighty percent of the disease-associated mutations occurred in amino acid residues that were perfectly conserved or contained only conserved substitutions across the 10 tested species, whereas only 48% of the total coding amino acids were conserved. This significant result was primarily because of mutations in exon 2. That is, in exon 2, there were significantly more disease-associated mutations that occurred at conserved codons (89%) relative to all conserved codons (55%); however, no such significant difference was observed for exon 1α (64% versus 40%, respectively).

There were striking differences in the frequencies and distributions of mutations across geographic locales. Single founder *CDKN2A* mutations were the predominant mutations in Sweden (*p.R112_L113insR* or *c.331_332insGTC*; ref. 54), comprising 92% of observed families with mutations, and the Netherlands (*c.225_243del19*; ref. 31), involving 90% of reported families with mutations. France and Mediterranean Europe (Spain/Italy) had the same most frequent *CDKN2A* founder mutation (*p.G101W*; ref. 55), encompassing 15% and 60% of families with mutations, respectively. Similarly, Australia and United Kingdom had the same most common recurrent *CDKN2A* mutations (*p.M53I, c.IVS2-105a>G, p.R24P*, and *p.L32P*), comprising 42% and 50% of observed families with mutations, respectively, which was not unexpected as the ancestry of long-term residents of Australia is predominantly British.[28] A recent audit, carried out to assess the

[27] http://www.hgmd.org.

[28] Australian Bureau of Statistics: http://www.abs.gov.au/Ausstats/abs@.nsf/0/b85e1eb3a2bc274aca256d39001bc337?OpenDocument.

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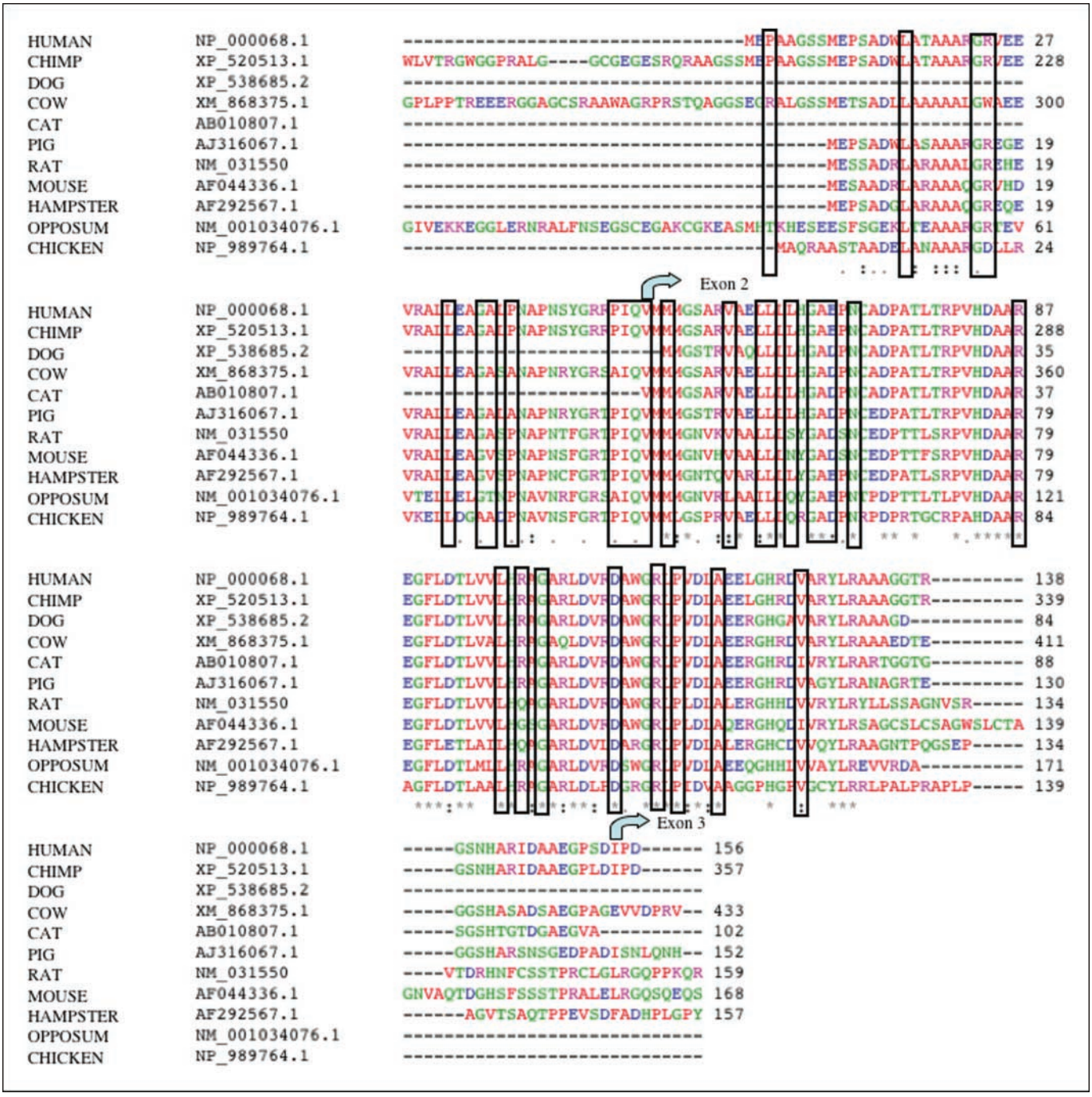

Figure 2. Comparison of the human protein sequence for *CDKN2A* with that of nine animal species generated by Clustal W (46). *Arrows,* codon positions for exons 2 and 3. Each of the 30 *CDKN2A* missense mutations observed in the current study is enclosed in a box to more clearly view amino acids across the 10 tested species. *Asterisk,* the residues or nucleotides in that column are identical in all sequences in the alignment; *colon,* conserved substitutions have been observed; *period,* semiconserved substitutions have been observed (below a column).

variability of mutation detection across GenoMEL participating centers, has shown that the divergence in mutation frequencies between centers was not a result of variation in mutation detection approaches; in fact, the standard of screening across groups was uniformly high.[29] Future studies are needed to explore whether the difference in mutation distribution leads to genotype-phenotype differences across the geographic locales.

Mutations in *CDK4* and *ARF* occurred at similar rare frequencies (2-3%). Large deletions that affected either p16 or p14ARF were also seen in 2% of the families. These findings have implications for the identification of other high-risk melanoma susceptibility genes.

[29] M. Harland, A.M. Goldstein, K. Kukalizch, C. Taylor, D. Hogg, S. Puig, C. Badenas, N.A. Gruis, J.A.C. ter Huurne, W. Bergman, N.K. Hayward, M. Stark, H. Tsao, M.A. Tucker, M.T. Landi, G. Bianchi-Scarra, P. Ghiorzo, P.A. Kanetsky, D.E. Elder, G.J. Mann, E.A. Holland, D.T. Bishop, J. Newton Bishop, and members of GenoMEL, the Melanoma Genetics Consortium. CDKN2A mutation audit within the Melanoma Genetics Consortium (GenoMEL) reveals a high standard of screening across participating centres. Submitted for publication.

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Table 4. Number of families with mutations in each gene and occurrence of mutations in families with other cancers

Gene category	No. families	No. families with PC (%)	P*	No. families with NST (%)	P*	No. families with UM (%)	P*
CDKN2A	178	49 (28)	<0.0001	11 (6)	0.52	1 (0.6)	0.16
CDK4	5	0	—	0	—	0	—
ARF	7	0	—	2 (29)	0.05	0	—
No mutations	276	17 (6)		13 (5)		7 (2.5)	
Total	466	66 (14)		26 (6)		8 (1.7)	

*Number of families with each gene category compared with families with no mutations using nonparametric Wilcoxon-Mann-Whitney test.

If other susceptibility genes are similarly rare, conventional strategies for detecting these susceptibility loci, such as linkage analysis, may be challenging because most families would be unlinked to the locus. Further, if population frequencies for *CDK4* and *ARF* mutations follow a pattern similar to that seen for *CDKN2A* (25, 56), the current results suggest that mutations in *CDK4* and *ARF* would be expected to be vanishingly rare in the general population.

As expected, we observed a significantly younger median age at melanoma diagnosis in families with mutations than in families without mutations. This pattern was observed in all seven geographic locales. Overall, families with mutations had a median age at melanoma diagnosis 9 years earlier than families without mutations. Therefore, if a high-risk/high-penetrance susceptibility gene explains melanoma incidence in most of these families, median melanoma onset would be expected to be later than that seen for *CDKN2A, ARF*, or *CDK4*.

The familial predisposition to NST in most melanoma-prone families has not been elucidated to date. NST have been stated previously to be associated with large deletions and/or mutations that involve p14ARF but these studies were based on very small numbers of patients/families (17 as reported in ref. 57, 18–21). In contrast, in a recent study of 24 Jewish, mostly Ashkenazi, melanoma families with NST, examination of germ-line point mutations and genomic deletions at the *CDKN2A/ARF* and *CDK4* loci revealed no alterations (58). Yet, the series included 10 (10 of 24) families, with at least two melanoma or two NST probands, suggesting the possibility of an inherited predisposition for development of melanoma and NST. Similarly, analysis of Swedish patients with multiple primary melanomas and NST was negative for the *CDKN2A* Swedish founder mutation *p.R112_L113insR* (or *c.331_332insGTC*; ref. 59). The current study adds to the growing evidence, indicating that there is no association between NST and *CDKN2A* mutations involving p16. However, the relationship between NST and *ARF* mutations remains unresolved. The evaluation of NST and *ARF* in the current study showed marginally significant evidence for an association between NST and *ARF* mutations ($P = 0.05$). But, evaluation of NST and all mutations that involved p14ARF (i.e., *ARF* splicing mutations and large deletions plus *CDKN2A* mutations that altered both p16 and p14ARF) did not show significant evidence for an association. Given the low frequency of *ARF* mutations, ~3% in this large GenoMEL sample, much larger studies will be required to better understand the possible association between NST and mutations that alter the p14ARF protein.

As reported previously, there was a very strong association between PC in a family and the presence of a *CDKN2A* mutation (10–16). Similarly, the distribution of PC differed according to the specific common recurrent *CDKN2A* mutations (60). We attempted to evaluate the relationship between PC and whether the *CDKN2A* mutation altered only the p16 protein or both p16 and p14ARF proteins. However, the results were inconsistent. The patterns observed for all reported melanoma patients were similar to that seen when the analysis was restricted to patients with confirmed melanomas only (and consequently families with at least three confirmed CMM patients). That is, for both samples, "all reported melanoma patients" and "confirmed melanoma patients only," there were similar percentages of families with mutations that affected p16 only versus p16 and p14ARF among the families without reported PC. In contrast, among the families with PC, there were substantially different percentages of families in the two samples with mutations that affected p16 only versus p16 and p14ARF. However, the results were significant only for the "all reported melanoma patients" comparison; among the common recurrent *CDKN2A* mutations evaluated, two mutations (*p.L32P* and *p.V126D*) that do not involve p14ARF had PC reported in >40% of observed families with those mutations; in addition, one mutation (*p.M53I*) that alters p14ARF had only 10% of families observed to have PC. Additional studies are needed to further explore this association.

The current study had several limitations. The ascertainment and sampling of families at most GenoMEL centers was not population based. In addition, follow-up and extension of families differed across research centers. Furthermore, the study was restricted to melanoma-prone families with at least three CMM patients. Thus, given the possible differences between families with several melanoma cases and those with few cases, the results from the study may not be applicable to families with only one or two melanoma patients. In addition, no information about individual mutation status was provided for CMM, PC, NST, or UM patients. Finally, ~10% of the patients with reported melanoma could not be confirmed. Similarly, 22% of the reported family members with PC, NST, or UM were not confirmed. Removal of the nonconfirmed melanoma patients (and their families) did not alter the types and/or distribution of mutations detected (data not shown). However, as discussed above, the association between mutations and PC differed depending on the inclusion of "all reported" compared with "only confirmed" cancer cases. Despite these limitations, this GenoMEL study provides the most informed assessment of the three high-risk melanoma susceptibility genes in multiple case (≥3 CMM patients) melanoma-prone families to date. Further, it shows the importance of consortium-based research for studies of rare susceptibility genes. In addition, this large familial melanoma data set is part of the ongoing search of GenoMEL for additional melanoma susceptibility genes, both high risk and low risk, using several strategies, including linkage, genome-wide association,

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and candidate gene approaches. Finally, future GenoMEL studies that include this data resource will hopefully lead to improved understanding and estimation of melanoma and nonmelanoma cancer risks in multiple case melanoma-prone families.

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