

RESEARCH ARTICLE

Haplotype Analysis of Two Recurrent *CDKN2A* Mutations in 10 Melanoma Families: Evidence for Common Founders and Independent Mutations

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Germ-line mutations in *CDKN2A* have been shown to predispose to cutaneous malignant melanoma. We have identified 2 new melanoma kindreds which carry a duplication of a 24bp repeat present in the 5' region of *CDKN2A* previously identified in melanoma families from Australia and the United States. This mutation has now been reported in 5 melanoma families from 3 continents: Europe, North America, and Australasia. The M53I mutation in exon 2 of *CDKN2A* has also been documented in 5 melanoma families from Australia and North America. The aim of this study was to determine whether the occurrence of the mutations in these families from geographically diverse populations represented mutation hotspots within *CDKN2A* or were due to common ancestors. Haplotypes of 11 microsatellite markers flanking *CDKN2A* were constructed in 5 families carrying the M53I mutation and 5 families carrying the 24bp duplication. There were some differences in the segregating haplotypes due primarily to recombinations and mutations within the short tandem-repeat markers; however, the data provide evidence to indicate that there were at least 3 independent 24bp duplication events and possibly only 1 original M53I mutation. This is the first study to date which indicates common founders in melanoma families from different continents. *Hum Mutat* 11:424-431, 1998. © 1998 Wiley-Liss, Inc.

KEY WORDS: *CDKN2A*; melanoma; familial; mutation; founder

INTRODUCTION

The isolation of *CDKN2A*, a melanoma predisposition gene mapping to 9p21-22 (Nobori et al., 1994; Kamb et al., 1994a) has resulted in the identification of a large number of families with germline *CDKN2A* mutations (reviewed in Foulkes et al., 1997; Dracopoli and Fountain 1996; Hayward, 1996). The product of the *CDKN2A* gene, p16, inhibits progression of cells through the G1 phase of the cell cycle by binding to cyclin-dependent kinase 4 (CDK4) and

cyclin D complexes (or CDK6 and cyclin D) and blocking the kinase activity of this enzyme (Serrano,

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Hannon, and Beach 1993; reviewed in Sherr and Roberts, 1995). Loss of functional p16 results in hyperphosphorylation of the retinoblastoma protein (pRb) leading to unrestrained entry of cells into S phase and uncontrolled proliferation. The *CDKN2A* gene appears to act as a classical tumour suppressor where inactivation of both alleles has been detected frequently in sporadic melanoma cell lines (Nobori et al., 1994; Kamb et al., 1994a; Ohta et al., 1994; Liu et al., 1995; Luca et al., 1995; Pollock et al., 1995, 1996; Flores et al., 1996) and to a lesser extent in uncultured metastatic melanomas (Ohta et al., 1996; Flores et al., 1996; Platz et al., 1996) as well as melanoma cell lines established from patients with inherited mutations (Hussussian et al., 1994; Gruis et al., 1995a).

Approximately 50% of all melanoma families show linkage to 9p21–22 and mutations in the coding region of *CDKN2A* have been reported in approximately half of these 9p-linked families (reviewed in Hayward, 1996; Dracopoli and Fountain, 1996). To date multiple mutations have been reported in both exon 1 α and exon 2 of *CDKN2A* in melanoma families from the United States, England, Canada, Australia, Italy, The Netherlands, and Sweden (reviewed in Foulkes et al., 1997). In contrast, there have been no documented mutations in the alternative exon 1 β (E1 β) of *CDKN2A* in a total of 157 families examined; however, the 9p linkage status for some of these families was unknown (Stone et al., 1995; Fitzgerald et al., 1996; Flores et al., 1997; Liu et al., 1997; Platz et al., 1997). Some mutations in *CDKN2A* have been reported multiple times, but where kindreds in geographically isolated regions carry identical mutations, haplotype analysis has invariably revealed a common founder rather than a mutation hotspot within *CDKN2A*. For example, 13/15 Dutch melanoma kindreds were found to carry an identical 19bp deletion removing nucleotides 225–243 from exon 2, and to share a common haplotype of 9p markers, indicating a common founder (Gruis et al., 1995b). In Sweden, 2 independent reports have documented 2 and 4 families, respectively, that carry a 3bp insertion in exon 2 that results in the insertion of an arginine in p16 (Borg et al., 1996; Platz et al., 1997). Two of these families have been shown to share a common haplotype (Borg et al., 1996), making it likely that the other 4 Swedish families also share the same common ancestor. Eight of 16 Italian melanoma families studied share a G101W mutation and D9S171/D9S942 haplotype (Ciotti et al., 1996; Ghiorzo et al., 1996), also suggestive of a common founder. This G101W mutation has also been seen in several apparently unrelated U.S. families

(Hussussian et al., 1994; Kamb et al., 1994b; Whelan et al., 1995); however, the possibility of a shared founder (possibly of Italian origin) in these families has not been examined to date. In the United States, 2 melanoma families (Ohta et al., 1994; Goldstein et al., unpublished observations) and another 2 apparently unrelated individuals with melanoma (Fitzgerald et al., 1996) carry identical 14bp deletions—although the possibility of a common founder has not been investigated. In melanoma kindreds from Australia, 2 mutations have been documented to occur multiple times; a C>G transversion in exon 2 resulting in an M53I substitution, which has been reported in 3 families, and a 24bp duplication present in the 5' region of the *CDKN2A* gene identified in 2 families (Walker et al., 1995; Flores et al., 1997). The M53I mutation has also been identified in a small Canadian family with multiple cancers including melanoma (Sun et al., 1997) and a U.S. family (M3/M6) with 2 affected brothers (Fitzgerald et al., 1996). The 24bp duplication has also been reported in a U.S. family (Goldstein et al., 1995).

In this paper, we present 2 new kindreds containing the 24bp duplication from the United Kingdom. One of these, MEL 13, was documented by MacGeoch et al. (1994) as carrying no exon 2 mutation (but at that time exon 1 α was not screened). The other family, MEL 29, has not been reported previously. We have investigated whether M53I and the 24bp duplication in the *CDKN2A* gene represent multiple independent mutational events, or, since all families in this study are of European descent, they reflect the presence of common founders. Haplotype analysis of 11 markers flanking the *CDKN2A* locus revealed evidence to indicate that there were at least 3 independent 24bp duplication events and possibly only 1 original M53I mutation.

MATERIALS AND METHODS

Subjects

DNA from at least 2 affected individuals from melanoma families in which either the M53I mutation or the 24bp duplication in *CDKN2A* has been identified were sent to the one laboratory (NKH and PMP) where all haplotyping analysis was carried out. Families carrying the M53I mutation included 41001 and 60001, for which the pedigrees are published in Walker et al. (1995)—41031 (Flores et al., 1997), 231 (Sun et al., 1997), and M3/M6—which is described in Fitzgerald et al. (1996). Families carrying the 24bp duplication in *CDKN2A* included 40582 and MEL 13, for which the pedigrees are published in Walker et al. (1995) and MacGeoch et al. (1994), respec-

tively, and 41119, 0255, and MEL 29, for which the details are presented in Table 1. Identification of the 24bp duplication in the 0255, 40582, and 41119 families has been reported previously (Goldstein et al., 1995; Walker et al., 1995; Flores et al., 1997).

A panel of 46 control individuals was used for allele frequency estimation and consisted of unaffected spouses collected from a series of Australian melanoma families. All were of European ancestry. As described previously (Nancarrow et al., 1993), bloods were obtained from family members by venepuncture from which lymphoblastoid cell lines (LCLs) were established and genomic DNA was extracted using standard methods.

Genotyping

Simple tandem repeat polymorphisms were typed for the D9S162, IFNA, D9S736, D9S1749, D9S974, D9S1604, D9S942, D9S1748, D9S171, D9S126, and D9S169 loci using PCR. Primer sequences were obtained from the Genome Database and reactions consisted of 100 ng template DNA, 5–10 pmol each primer, 200nM dATP, dGTP, dTTP, 2nM dCTP, 10mM Tris-HCl, 1.5mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, 0.5 U DynaZyme, and 1 μCi of α³²P-dCTP. PCR conditions consisted of an initial denaturation step at 94°C for 180 s, followed by 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s. For the D9S974 locus the reaction, conditions included 5% DMSO, and a 58°C annealing temperature; otherwise the PCR was performed similarly. For the D9S1748 locus, which is situated 236bp 3' to E1β (Mao et al., 1995: Figure 1), the forward primer was 5' end-labelled with γ³²P-dATP according to the protocol supplied with T4 polynucleotide kinase from New England Biolabs (Beverly, MA). PCR reactions contained 10 pmol of the forward primer (1 pmol labelled and 9 pmol unlabelled), 10 pmol of the re-

verse primer, and 200 nM of each dNTP; otherwise PCR conditions and cycling were the same as above. Products were denatured at 95°C and electrophoresed through 7 M urea –6% polyacrylamide gels, which were typically run for 3–6 hours at 1,000–1,500 V, dried, and exposed to X-ray film for 1–18 hr. For those markers where allele sizes were unknown (D9S736, D9S1749, D9S974, D9S1604, and D9S1748), several alleles were run alongside a size standard generated from M13mp18 DNA using the –20 sequencing primer (Pharmacia Biotech, Uppsala, Sweden). Allele 1 was designated the largest allele seen in all control individuals and family members.

RESULTS

Marker Order

The map order (Fig. 1) of the polymorphic markers used in this study is based on that published by Cairns et al. (1995). In addition, the marker D9S974 was reported by Ohta et al. (1996) to lie within 20 kb of *CDKN2A* and 50 kb of *CDKN2B*, therefore we have placed this marker centromeric to D9S1749 and telomeric of *CDKN2A*. The D9S1748 locus reported by Cairns et al. (1995) has been further localized by the cloning of *CDKN2A* exon 1β (E1β) and the identification of the forward primer 236bp 3' to E1β (Mao et al., 1995: Figure 1). The most likely order of D9S1604 and D9S942 with respect to D9S1748 was determined by mapping these markers in a panel of 12 melanoma cell lines for which the deletion status of E1β, exon 1α and exon 2 of *CDKN2A* was known (Castellano et al., 1997). Eight lines were deleted for exon 1α but not E1β (group 1), and the other 4 lines were deleted for exon 2 but not exon 1α nor E1β (group 2). D9S942 was deleted in 1 cell line from group 1 and was not deleted in any line from group 2, whereas D9S1604 was deleted in 3 lines, all from group 1. These data suggest

TABLE 1. Summary of Melanoma Family Information

Family no.	Origin	Ancestry	Mutation	No. affected	No affecteds used to determine haplotype ^a	Reference
60001	Australia	Irish	M531	15	12	Walker et al., 1995
41001	Australia	Scottish	M531	11	11	Walker et al., 1996
41031	Australia	English	M531	5	3	Flores et al., 1997
M3/6	USA	English	M531	3 ^b	2	Fitzgerald et al., 1996
231	Canada	Scottish	M531	2 ^c	2	Sun et al., 1997
41119	Australia	Scottish	24bp dup.	8	5	Flores et al., 1997
40582	Australia	Scottish/English	24bp dup.	6	4	Walker et al., 1995
MEL 13	UK	English	24bp dup.	3	2	MacGeoch et al., 1994
MEL29	UK	English	24bp dup.	2	2	
0255	USA	Scottish/English/German	24bp dup.	7	4	

^aIncludes inferred genotypes of obligate carriers.

^bThere was possibly a fourth member of this family affected, but pathology confirmation was not available.

^cOne of these was an unaffected carrier.

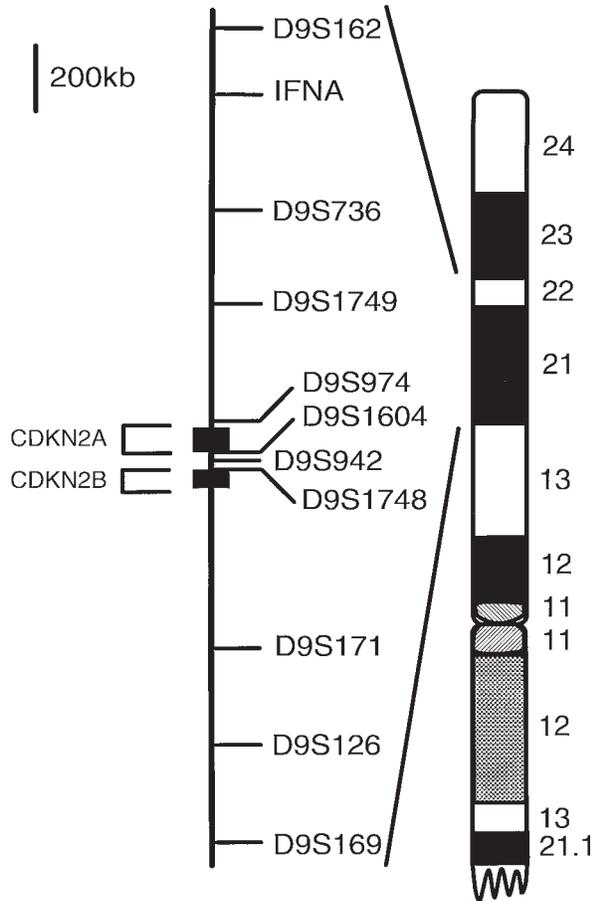


FIGURE 1. Schematic representation of the 11 polymorphic markers used in haplotype analysis. The location of *CDKN2A* and *CDKN2B* with respect to these markers is shown. Approximate distances between markers have been taken from Cairns et al. (1995) and Ohta et al. (1996).

that D9S1604 is the closest marker to exon 1 α , although its exact location (centromeric or telomeric) relative to this exon could not be determined. Similarly, D9S942 is most likely to map between exon 1 α and E1 β .

Haplotype Analysis

Figure 2 shows the segregating haplotype identified in each of the families with the M53I mutation in *CDKN2A*. In each case the the segregating haplotype was determined by analyzing at least 2 affected individuals, but in some families the segregating alleles could not be unequivocally determined, therefore both alleles are indicated for these markers. This was particularly evident in the M3/M6 family where the affected individuals were brothers and had identical genotypes for all markers used. Figure 3 shows the segregating haplotype identified in each of the families with the 24bp duplication in exon 1 α of

	60001	M3/6	41031	41001	231
D9S162	6	12/7	12	12	12
IFNA	3	4	2	3	3
D9S736	6	5/7	6	6	6
D9S1749	1	10/19	10/9	10	9
D9S974	8	8	8	8	8
D9S1604	2	2/1	2	2	2
D9S942	9	7/6	7	7	7
D9S1748	4	4/7	4	6	6
D9S171	7	10	6	7	7
D9S126	6	6/1	6	4	4
D9S169	1	13/5	6	10	10/11

FIGURE 2. 9p haplotype analysis in families carrying the M53I mutation. The position of *CDKN2A* relative to the markers is shown. The minimum region of haplotype sharing is indicated by a solid shaded box. More extended regions of haplotype sharing between families are indicated within the dashed line. Alleles which have undergone replication slippage are contained within small dotted boxes.

CDKN2A. Similarly, where the segregating allele could not be determined, both alleles are provided.

Each marker used in this study was also amplified in a panel of control individuals to determine the relative frequencies of alleles in the general population (Table 2), as well as the relative frequencies of the segregating haplotypes observed in Figures 2 and 3 (data not shown). For those markers where allele sizes were unknown (D9S736, D9S1749, D9S974, D9S1604, and D9S1748), the size of allele 1 is provided in Table 2.

Families With the M53I Mutation

There was clear haplotype sharing in all 5 melanoma families carrying the M53I mutation in *CDKN2A* (Fig. 2). The 2 families which demonstrated the most haplotype sharing (10/11 loci examined) were the Australian family (41001) and the Canadian family (231). Family 231 differed at the D9S1749 locus by 1 repeat unit but shared the same haplotype for the 3 markers telomeric to D9S1749; therefore, the difference at this locus has been presumed to be due to replication slippage. Family 41031

	41119	MEL29	40582	0255	MEL13
D9S162	9	12	12	12/15	1/12
IFNA	6	3	3	4	2/3
D9S736	6	8	5	7	5
D9S1749	15	22	12	13	31
D9S974	5	10	8	8	8
D9S1604	2	2	1	1	1
D9S942	9	5	8	3	3
D9S1748	8	6	7	7	7
D9S171	6	6	10	2	6
D9S126	6	6	1	3	3
D9S169	9	10/12	5	10	10

FIGURE 3. 9p haplotype analysis in families carrying the 24bp duplication mutation. Legend as for Figure 2.

TABLE 2. Allele Frequencies as Percentages in a Panel of Control Individuals^a

Allele	D9S162	IFNA	D9S736	D9S1749	D9S974	D9S1604	D9S942	D9S1748	D9S171	D9S126	D9S169
1	1	2	1	–	6	35	2	1	6	8	1
2	9	17	–	–	–	65	1	1	17	2	–
3	4	51	–	–	14	–	5	10	10	3	–
4	3	8	1	–	2	–	2	10	1	34	4
5	2	1	43	2	7	–	7	11	7	1	5
6	15	21	38	–	16	–	1	5	31	50	26
7	22	–	11	–	26	–	9	14	7	–	14
8	–	–	4	–	19	–	8	24	1	–	4
9	1	–	1	2	4	–	10	20	3	–	5
10	–	–	–	2	4	–	5	5	17	–	11
11	–	–	–	1	3	–	9	–	1	–	7
12	34	–	–	4	3	–	–	–	–	–	20
13	7	–	–	7	–	–	3	–	–	–	2
14	2	–	–	7	–	–	–	–	–	–	–
15	–	–	–	8	–	–	5	–	–	–	–
16	–	–	–	5	–	–	7	–	–	–	–
17	–	–	–	4	–	–	9	–	–	–	–
18	–	–	–	4	–	–	5	–	–	–	–
19	–	–	–	–	–	–	11	–	–	–	–
20	–	–	–	6	–	–	2	–	–	–	–
21	–	–	–	4	–	–	3	–	–	–	–
22	–	–	–	5	–	–	–	–	–	–	–
23	–	–	–	10	–	–	–	–	–	–	–
24	–	–	–	12	–	–	–	–	–	–	–
25	–	–	–	4	–	–	–	–	–	–	–
26	–	–	–	3	–	–	–	–	–	–	–
27	–	–	–	–	–	–	–	–	–	–	–
28	–	–	–	2	–	–	–	–	–	–	–
29	–	–	–	–	–	–	–	–	–	–	–
30	–	–	–	–	–	–	–	–	–	–	–
31	–	–	–	6	–	–	–	–	–	–	–
N ^b	92	92	90	82	90	88	92	92	90	92	92
Size of allele 1 ^c	196	152	128	171	221	193	131	181	177	248	285

^aDue to rounding off, totals may not equal 100%.

^bTotal number of chromosomes analysed.

^cSize is given in base pairs; allele 1 has been designated the largest allele observed in either the control individuals or family members. Allele numbers have been allocated based on 2 base pair repeat spacings irrespective of whether intervening alleles were observed.

has 5 alleles in common with the segregating haplotype shared by the former 2 families (Fig. 2). The segregating allele at D9S1749 locus cannot be determined in this family due to replication slippage: 41031-001 carried alleles 10 and 19 while 41031-005 (the paternal aunt of 41031-001) carried alleles 9 and 22. As the unaffected mother of 41031-001 carried allele 19 and 31 (data not shown), the segregating allele was assumed to be either allele 9 or 10 (inherited from the deceased affected father). As allele 10 occurs more frequently in the M53I families studied, this is likely to be the segregating allele (Fig. 2). The replication slippage resulting in the loss of 1 repeat unit evident in the 41031 family supports the hypothesis of replication slippage at the same locus in the 231 family. Due to the shared genotypes of the 2 affected individuals (brothers) in the M3/M6 family, the segregating haplotype cannot be conclusively determined; however, it is likely that this family also shares the 10-8-2-7-4 haplotype with the 41031 family (Fig. 2). The Australian melanoma

families 41001 and 60001 were both reported to carry the M53I mutation by Walker et al. (1995) and initial haplotype analysis with the markers IFNA-D9S942-D9S171 revealed these 2 families did not share the segregating allele at D9S942, the marker closest to *CDKN2A*. It was thus concluded in that study that these families likely arose from independent mutational events. The availability of new markers flanking *CDKN2A* has enabled this original analysis to be extended. Figure 2 shows haplotype sharing flanking the D9S942 locus and encompassing both exon 1 α and exon 2 of *CDKN2A*, indicating that perhaps these 2 families do share a common founder and the difference in D9S942 alleles can be explained by replication slippage of 2 repeat units in family 60001. Providing for the replication slippage in the 60001 family, the minimum region of haplotype sharing in all 5 families carrying the M53I mutation encompasses the markers immediately flanking the *CDKN2A* locus, D9S974-D9S1604-D9S942. This haplotype, 8-2-7, was observed in

at most 3/46 control individuals (data not shown), thus the probability that 5/5 M53I-carrying melanoma families would share the 8-2-7 haplotype by chance is $(3/92)^5$ (i.e., less than 3.7×10^{-8}), suggesting that this haplotype-sharing is indeed due to a common founder in all families. This degree of significance is tolerant to extreme variation in allele frequencies; e.g., assuming that the haplotype frequency was 10-fold more prevalent in the population (i.e., 30/46 controls carry the 8-2-7 haplotype), the probability of the 5 M53I families sharing a common haplotype is still low (i.e., $(30/92)^5$, or 0.0037). However, it should be noted that the values calculated above represent probabilities, not significance levels (P-values).

Families With the 24bp Duplication Mutation

There was much less evidence for haplotype sharing in the 5 melanoma families carrying the 24bp duplication (Fig. 3). Families MEL 13 from England and 0255 from the USA shared a haplotype spanning the region D9S974-D9S1604-D9S942-D9S1748, while family 40582 shared the more limited D9S974-D9S1604 haplotype. The common (8-1) haplotype for the latter 2 markers encompasses the *CDKN2A* gene and was found to occur only twice in 46 control individuals (data not shown). The probability therefore that this haplotype has occurred in 3 families by chance is $(2/92)^3$ (i.e., less than 1×10^{-5}), strongly suggesting they share a common founder. Once again, a potential 10-fold misspecification of the haplotype frequency would still yield significant evidence for this conclusion (i.e., $(20/92)^3$, or 0.01). Families MEL 29 from England and family 41119 from Australia both carried unique haplotypes for this region of 9p, indicating that the 5 families have arisen from at least 3 independent mutational events.

DISCUSSION

In this paper, we report 2 new families which carry a duplication of a 24bp repeat present in the 5' region of *CDKN2A* previously identified in several melanoma kindreds. Moreover, we have performed extensive haplotype analysis in all 5 families reported to carry this duplication, in addition to 5 families reported to carry an M53I mutation in exon 2. Haplotype analysis in the 5 families carrying the 24bp duplication suggests that there were at least 3 independent mutational events. A schematic representation of the 24bp repeat present in the 5' end of the *CDKN2A* gene and the duplication identified in these families has been published previously (Walker et al., 1995: Figure 4). This duplication is hypothesised to have arisen due to an unequal crossing over between the two 24bp repeats naturally present

in the wildtype sequence, or possibly through polymerase slippage during replication. Further evidence that this repeat region is unstable and therefore prone to both meiotic and mitotic slippage is provided by the identification of a somatic 24bp deletion of 1 of these normally occurring repeats in a prostate tumour (Komiya et al., 1995).

All 5 families carrying the M53I mutation show evidence of a common founder: The differences observed in the segregating haplotypes are due either to multiple recombinations and/or mutation at 1 of 2 microsatellite markers. Simple tandem repeat sequences are mutated by gaining or losing 1 or more repeat units thought to result from replication slippage. Gain or loss of 1 repeat unit is much more likely than gain or loss of 2 repeat units; however, larger mutations do occur (Weber and Wong, 1993; Primmer et al., 1996). The discovery that expansion of trinucleotide repeats are associated with various diseases has resulted in several studies into mutational bias and the directional evolution of repeat sequences. Several reports have suggested that mutations occur disproportionately in heterozygotes whose alleles differ greatly in length; i.e., they have a greater 'span' (Amos et al., 1996). Although we cannot know the allele sizes of the individuals in which these mutations originally occurred, it is interesting to note that both the mutations we observed (family 41031) and those we hypothesize (family 231 and 60001) (Fig. 3) occur at D9S942 and D9S1749, the 2 markers used in this study with both the highest level of heterozygosity and the largest span of alleles. Several studies have also indicated that the gain of 1 repeat unit is the most common mutation to occur (Weber and Wong, 1993; Primmer et al., 1996). The mutations at the D9S1749 locus in both the 41031 and 231 families (Fig. 3) involve a change from allele 10 to allele 9, which represents a gain of 1 repeat unit as allele 1 has been designated the largest allele. As the loss of 2 repeat units we hypothesize to occur in family 60001 occurs more rarely, it is possible that the M53I mutation has arisen independently in this family and the haplotype sharing we observe around *CDKN2A* occurred by chance. However, the low frequency of this haplotype in the control individuals argues strongly against this occurrence.

Although 3 of the families carrying the M53I mutation were Australian, it was a Canadian family (231) which demonstrated the most haplotype sharing with 1 of the families from Australia (41001). Likewise in those families which carried the 24bp duplication, haplotype sharing was evident between one of the English families, a U.S. family, and, to a more limited extent, one of the Australian families.

In contrast, the two English families (MEL 29 and MEL 13) had little in common with each other as did the 2 Australian families (41119 and 40582). Thus, country of origin is not always a good predictor for identifying a common founder in melanoma kindreds.

The M53I mutation in *CDKN2A* has recently been shown to be functionally deleterious (Sun et al., 1997). Functional analysis for the 24bp repeat has indicated that at least in vitro this variant form of p16 is capable of binding CDK4 (Parry and Peters, 1996). However, this does not rule out the likelihood that this variant is functionally compromised in vivo. The absence of this mutation in control individuals suggests it is likely to be disease-causing, or at least in linkage disequilibrium with another disease causing mutation at this locus.

Prior to this study, we hypothesized that all families carrying the M53I mutation were likely to have arisen from a common ancestor, in keeping with other documented *CDKN2A* mutations where the presence of founders had been demonstrated. In contrast, we anticipated that not all families carrying the 24bp duplication would share a common founder, since this mutation is more likely to recur due to the inherent instability of tandem repeat regions. Our findings support both our original hypotheses since the most likely explanation for the haplotype data in the 5 families with the M53I mutation is that they share a common ancestor, and we have demonstrated that the 24bp duplication has arisen at least 3 times in the 5 families studied.

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