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Mutation testing in melanoma families: INK4A, CDK4 and INK4D

JA Newton Bishop[2], M Harland[1], DC Bennett[3], V Bataille[4], AM Goldstein[5], MA Tucker[5], BAJ Ponder[6], J Cuzick[7], P Selby[2] and DT Bishop[1]

[1]ICRF Genetic Epidemiology, Leeds, UK; [2]ICRF Cancer Medicine Research Unit, St James's University Hospital, Beckett Street, Leeds LS9 7TF, UK; [3]St George's Hospital Medical School, London, UK; [4]ICRF Skin Tumour Laboratory, Royal London Hospital, London, UK; [5]Genetic Epidemiology Branch, National Cancer Institute, Bethesda, MD, USA; [6]CRC Human Cancer Genetics Group, Box 238, Addenbrooke's Hospital, Cambridge, UK; [7]ICRF Mathematics Statistics and Epidemiology, Lincolns Inn Fields, London, UK

Summary The *INK4A* gene which codes for the cyclin-dependent kinase (CDK) inhibitor INK4A or p16 underlies susceptibility to melanoma in some families. Germline mutations in the gene that codes for the target protein of p16, CDK4, underlie susceptibility in very rare families. We report mutation screening of the *INK4A* and *CDK4* genes in 42 UK families. A total of nine families were identified with *INK4A* mutations and none with *CDK4* exon 2 mutations. These mutations were in 8/22 (35%) families with three or more cases of melanoma and 1/20 (5%) families with only two cases. In one of these nine families a novel single base pair substitution was identified, Gly67Arg. In an attempt to identify another melanoma susceptibility gene, a member of the *INK4* family, the *p19 INK4D* gene has been studied. The *p19* gene was sequenced in DNA from the 42 UK families and six additional US families. No mutations were identified.

Keywords: INK4A; INK4D; familial melanoma

Rare families exist in which there appears to be a predisposition to cutaneous melanoma (Clark et al, 1978; Newton, 1994). In some families, there is an apparent susceptibility to other tumours, particularly to pancreatic (Lynch and Fusaro, 1991) and other gastrointestinal cancers (Bergman et al, 1990), but in most families the susceptibility appears to be to melanoma alone. Early linkage data suggestive of linkage to chromosome 1 (Bale et al, 1989; Goldstein et al, 1993) have not been substantiated (Gruis et al, 1990; Nancarrow et al, 1992), but strong evidence of linkage to chromosome 9 (Cannon-Albright et al, 1992; Nancarrow et al, 1993) was quickly followed by the identification of germline mutations in the *INK4A* (*CDKN2*) gene (on chromosome 9p) which codes for the p16 protein, in melanoma families (Hussussian et al, 1994; Kamb et al, 1994). Mutations in this gene have been identified in the germline of families world-wide (Dracopoli and Fountain, 1996), mutations that co-segregate with cases and for which abnormal CDK4 binding has been demonstrated (Harland et al, 1997). The proportion of families with mutations, however, remains moderate, at around 40% of families with three or more cases overall. In our initial UK series, five of 12 families of this type, and one of 12 families with two cases of melanoma had identifiable mutations in the *INK4A* gene (Harland et al, 1997).

There is some evidence for linkage to chromosome 9 even in families without identifiable *INK4A* mutations (Hussussian et al, 1994; Liu et al, 1997) so that there may be additional mutations yet to be identified in the promoter region of the gene, or in other linked genes. No significant contribution has been identified from two candidate genes in this region, coding for the protein p15 and p14ARF (Fitzgerald et al, 1996). There are, however, haplotype data to suggest that there are likely to be other melanoma susceptibility genes elsewhere (Goldstein et al, 1994; MacGeoch et al, 1994).

Zuo et al (1996) have identified one such gene. They reported mutations in the gene that codes for the target protein of p16, *CDK4*. Three families have now been reported world-wide in which germline *CDK4* mutations have been detected in melanoma families (Zuo et al, 1996; Bressac-de-Paillerets et al, 1997). In these families, single base pair substitutions were detected at the same point in the p16-binding domain, residue 24 and, more recently, mutations have been detected in a second domain also important in binding to cyclin D1 (Coleman et al, 1997), strengthening considerably the significance of the p16–CDK4–RB pathway in melanoma carcinogenesis. Several groups have now looked at their melanoma families without identifiable *INK4A* mutations and have failed to identify additional *CDK4* mutations. It is clear, therefore, that *CDK4* mutations are rare (Harland et al, 1997) and that there are other melanoma susceptibility genes still to identify.

p16 is a member of a family of CDK inhibitors, the INK4 family. The two cyclin D-dependent CDKs, CDK4 and CDK6, induce phosphorylation of the retinoblastoma protein (RB1), thereby releasing RB1 from its growth inhibitory functions. In turn, the proteins from the INK4 family inhibit this process and act as brakes to phase G1 of the cell cycle (Hirama and Koeffler, 1995; Sherr and Roberts, 1995; Sherr, 1996). There are at least four members of the family, p16INK4A, p15INK4B, p18INK4C and p19INK4D (to be distinguished from p14ARF). The

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Correspondence to: JA Newton Bishop

The first two authors contributed equally to this work.

Table 1 *p19 INK4D* primers used

Primer	Sequence	Annealing temp (°C)	Usage
p19-N1F	GCCAGTGTCGACATGCTG	55	Long-range PCR (F) Intronic sequencing (F) PCR amplification, exon 1 (F)
p19-N2R	TGTCCAACACACCAAAAGGA	55	Long-range PCR (R)
p19-2R	CGGAGGTGTCCTGGACATTG	55	Intronic sequencing (R)
p19-in1F	CTGGGAACCGGTCCTTCTCT	55	Intronic sequencing (F)
p19-in1R	AGGGGTCACTGGAAAGAGAA	55	Intronic sequencing (R)
p19-ex1R	CTGGGAACCGGTCCTTCTCT	55	PCR amplification, exon 1 (R)
p19-143F	ACTTTCCTGGGGGCTTATGC	55	PCR amplification, exon 2 (F)
p19+98R	CTATAAGCCACAAACTGTGC	55	PCR amplification, exon 2 (R)

expression of the *INK4* genes shows tissue specificity suggesting lack of redundancy (Sherr, 1996). The polypeptides are composed of 4–5 ankyrin repeats, and mutagenesis studies have suggested that mutations are likely to affect function only if they occur in the region coding for these ankyrin repeats (Yang et al, 1996). There is a little information which suggests that the INK4 family members may have a role in cellular differentiation via effects of the CDKs on phosphorylation of substrates other than RB; for example, p18 and p19 which are widely expressed during embryogenesis (Zindy et al, 1997).

All members of the family bind to CDK4 and 6 (Hirai et al, 1995; Guan et al, 1996). They have a moderate degree of homology. p19INK4D, for example, has 48% amino acid homology with p16INK4A. All members of the family compete with cyclin D1 for its binding site on CDKs (Guan et al, 1996). The presence of each member of the family in phase G1 will induce G1 arrest, so that at a cellular level at least all members of the family are potential tumour suppresser genes. We chose *p19 INK4D* as a candidate gene because of its chromosomal location at 19p13.2, a reported site of repeated translocation in melanoma tumours (Parmiter et al, 1986).

MATERIALS AND METHODS

DNA from 42 UK families at increased risk of melanoma was screened for germline mutations. The families have been recruited in the period since 1989 in England and Wales. Ethical Committee approval for this study was obtained from all institutions involved. Twenty-two of these families have been previously described (Harland et al, 1997). Wherever possible, DNA from two cases from each family was screened. Six additional US families were screened for *INK4D* mutations. These families were selected because they had sarcoma and/or haematological malignancies in addition to melanoma. Three of these families have been previously described (Goldstein et al, 1994; Liu et al, 1997).

DNA extraction and PCR

DNA extraction from peripheral blood, and the polymerase chain reaction (PCR) amplification of the three coding exons of *INK4A*, and exon 2 of *CDK4*, was carried out as described previously (Harland et al, 1997).

Analysis of *p19 INK4D*

Previously, only the cDNA sequence has been available for *p19 INK4D* (GenBank accession numbers U40343, U20498 and U49399). Studies using primers designed from this sequence have been able to cover, at best, 85% of the coding sequence of *INK4D* (Shiohara et al, 1996; Miller et al, 1997). In order to examine the terminal amino acids in each of the two exons of *INK4D*, and also the splice donor and acceptor sites, it was important to determine the intronic sequence of *INK4D*, so that intronic primers flanking the two coding exons could be designed. Primers flanking the cDNA sequence were designed from the available sequence for the 3′ and 5′ non-coding regions (Guan et al, 1996) (GenBank accession number U40343). Long-range PCR across the two exons and the intron, in genomic DNA samples, produced a PCR product approximately 1100 bp longer than would be predicted from the cDNA sequence. Sequencing through the coding regions and into the intron has enabled us to determine the complete intronic sequence of *INK4D*, 1091 bases in total. This sequence, which has been submitted to GenBank (accession number AF061327), has enabled us to design primers flanking the two exons of p19, and so allowed us to investigate the complete coding region of *p19 INK4D* in our families.

Long-range PCR of *p19 INK4D*

Primers were based on the *p19 INK4D* cDNA sequence (GenBank accession number U40343) (Table 1). A total of 100 µl long-range PCR reactions were carried out using 250 ng genomic DNA, 0.2 mM dNTPs, 50 µM each primer, 10% dimethyl sulphoxide (DMSO), and 5 units Deep Vent DNA polymerase (New England Biolabs), in the reaction buffer provided by the supplier. Thermal cycling conditions were as follows: an initial denaturation at 94°C for 5 min; followed by 35 cycles of denaturing at 94°C (30), annealing at 55°C (30), and extension at 72°C (6′), with a final 10-min extension at 72°C. PCR fragments were isolated by agarose gel electrophoresis and purified prior to sequencing using the QIAquick Gel Extraction Kit (Quiagen). Sequencing of the intronic region was performed as described below, using forward and reverse primers designed from the known cDNA sequence, and also primers designed from generated intronic sequence (Table 1). PCR amplification for the screening of the two exons of *p19 INK4D*, was carried out as described previously for *INK4A* and *CDK4* (Harland et al, 1997), the primers used are shown in Table 1.

Sequencing

Sequencing reactions were carried out using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer) and the

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products were analysed on an ABI 377 DNA sequencer. DNA sequencing was performed in both directions, initiated from the forward and reverse primers used in the initial PCR amplification of each exon. In kindreds where sequence variation was found in one or both of the initial samples selected for investigation, all members of that family, for whom DNA was available, were sequenced for the putative mutation identified in the initial samples, in order to study the segregation of the mutation with melanoma.

RESULTS

Table 2 shows the characteristics of the families screened. Forty-two UK families were mutation tested (Table 3). Partial testing was reported in 22 of these families previously (Harland et al, 1997). Six additional US families were screened for *INK4D* mutations in whom significant *INK4A* and *CDK4* mutations had previously been excluded (Hussussian et al, 1994). In one of these families, Asn71Ser was identified. This mutation has not yet been shown to have functional implications (Ranada et al, 1995). In total, 26 families with three or more cases, and 22 with only two cases of melanoma were screened. In nine of the 22 families with two cases of melanoma, one of those cases had proven multiple primary tumours, as additional evidence for an underlying genetic susceptibility to melanoma.

The three UK families not previously reported with germline mutations had Arg24Pro (in common with another UK family and several others world-wide), Met53Ile (again in common with a second UK family and others world-wide) and a novel single base pair substitution Gly67Arg (Figure 1).

A previously reported polymorphism in exon 1 of *INK4D*, was detected in codon 30 (Miller et al, 1997) in cases from five families with three or more cases of melanoma and one family with two cases only. No mutations were identified in either *CDK4* exon 2 or the *INK4D* genes.

DISCUSSION

INK4A (*CDKN2*) clearly underlies susceptibility to melanoma in a significant proportion of melanoma-prone families, and germline mutations in the *CDK4* gene underlie susceptibility in a small minority of families. We report three new families with germline mutations. Two of these have been widely reported in other centres, and in all families the mutations co-segregate with the tumours. Furthermore, functional assays have shown these mutations to be significant (Harland et al, 1997). The novel mutation Gly67Arg has not, to our knowledge, been reported before, but it occurs at a site predicted by the ankyrin structure of the p16 protein, to be critical to function. A similar, single base pair substitution at the same codon has been detected in an Australian pedigree (Mann et al, 1997).

In this series of 42 UK families recruited since 1989, 36% of families with three or more cases and 5% of families with only two cases had identifiable *INK4A* mutations. The prevalence of germline *INK4A* mutations in the larger families is consistent with that reported world-wide. Some authors have suggested that approximately 25% of families with two or more cases in Australia, Europe and the USA have identifiable mutations, and in a population-based study in Sweden by Platz et al the frequency of these mutations was estimated to be even lower (Platz et al, 1997).

Table 2 The family characteristics of those families screened in which there were three or more cases of melanoma. The number of cases reported is shown, and whether or not the family is known to have had cases with multiple primaries

Pedigree number	Number of cases	Cases with multiple primaries	*INK4A* exon 1	*INK4A* exon 2	*INK4A* exon 3	*CDK4* exon 2	*INK4D* exon 1	*INK4D* exon 2
MEL21	10	Yes	Arg24Pro					
NIH 1	10	Yes		*Ala148Thr*				
MEL07	5	Yes						
MEL09	5	Yes		Met53Ile			*90C/G*	
MEL32	5	Yes		Met53Ile				
MEL48	5	Not known						
NIH 2	5	Yes					*90C/G*	
MEL02	4	Not known					*90C/G*	
MEL03	4	No						
MEL06	4	Yes						
MEL13	4	Yes	23ins24					
MEL15	4	Yes		Ala118Thr				
MEL26	4	Yes						
MEL37	4	Not known					*90C/G*	
MEL39	4	Not known		Gly67Arg				
MEL40	4	Yes	Arg24Pro					
MEL49	4	Yes					*90C/G*	
MEL24	3	No	88delG					
MEL28	3	No						
MEL30	3	No						
MEL35	3	No					*90C/G*	
MEL44	3	Yes					*90C/G*	
MEL50	3	No					*90C/G*	
MEL53	3	Yes					*90C/G*	
NIH 3	3	Yes		*Asn71Ser*				
NIH 4	3	No						

Polymorphisms are indicated in italics. The protein produced by mutation Asn71Ser has been normal in vitro testing.

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Table 3 The family characteristics of those families screened in which there were two cases of melanoma. All families have been screened for exons 1–3 of *INK4A*, exon 2 of *CDK4* and exons 1 and 2 of *INK4D*. Only positive findings are indicated.

Pedigree number	Number of cases	Cases with multiple primaries	*INK4A* exon 1	*INK4A* exon 2	*INK4A* exon 3	*CDK4* exon 2	*INK4D* exon 1	*INK4D* exon 2
MEL01	2 (1st)	Yes						
MEL04	2 (2nd)	Yes						
MEL11	2 (1st)	Yes						
MEL14	2 (1st)	Yes						
MEL16	2 (1st)	Yes						
MEL19	2 (1st)	Yes						
MEL20	2 (2nd)	No						
MEL22	2 (1st)	Yes						
MEL23	2 (1st)	Yes						
MEL25	2 (2nd)	Not known						
MEL29	2 (1st)	Not known	23ins24					
MEL34	2 (1st)	No						
MEL38	2 (1st)	No						
MEL41	2 (2nd)	No						
MEL42	2 (1st)	No						
MEL43	2 (1st)	No						
MEL46	2 (1st)	No						
MEL54	2 (1st)	Not known						
MEL55	2 (1st)	No						
MEL56	2 (1st)	No						
NIH 5	2 (1st)	Yes						
NIH 6	2 (1st)	No						

[a] Figure in brackets denotes the degree of relatedness between affected individuals.

Table 4 A summary of the findings in 48 families. The decreasing likelihood of identifying a mutation with decreasing numbers of cases is illustrated

Number of cases in family	Number of families	Number *INK4A* mutations found	Families with mutations (%)
10	2	1	50
5	5	2	40
4	10	3	30
3	9	2	22
2	22	1	5

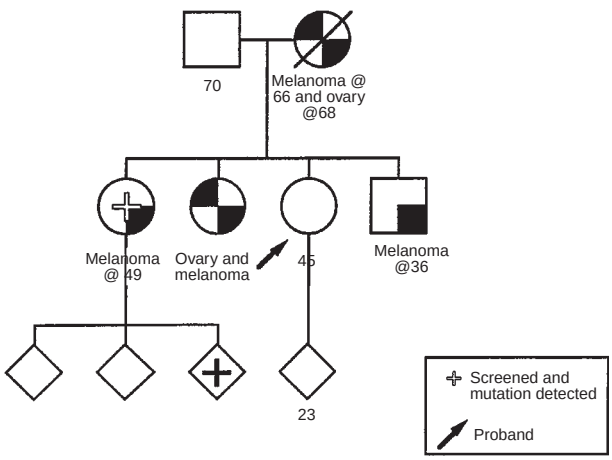

Figure 1 Pedigree of family in which a germline mutation Gly67Arg was identified. In all, there were four cases of melanoma. The mutation was detected in two samples tested, one of which was taken from a melanoma case

The reported studies are difficult to compare, as noted by Goldstein and Tucker (1997), because different groups have been variably stringent in their selection criteria. Some stipulate that the number of cases reported should be in first-degree relatives, others do not. Furthermore, groups appear to vary in how rigorously they sought mutations. In this study, there appears to be a clear difference in the likelihood of identifying germline *INK4A* mutations in families of different sizes. Mutations were significantly more likely in families with larger numbers of cases (Table 4). This might imply that the families with larger numbers of cases are less likely to represent clustering of cases by chance alone, or that they carry more penetrant genes.

Overall, in the families with three or more cases, 64% did not have identifiable *INK4A* mutations, nor did they have the rarer *CDK4* exon 2 mutations. There is some evidence from other groups that some families without detectable *INK4A* mutations still have evidence of linkage to chromosome 9 (Walker et al, 1995) and it may be that, in these families, there are still to be found mutations in intronic sequences or the promoter that have an effect on p16 transcription.

There is some evidence for linkage to other chromosomes (Bale et al, 1989; Walker et al, 1994) and we have previously reported suggestive haplotype data (MacGeoch et al, 1994) so that it also seems likely that there are other high penetrance melanoma susceptibility genes to be identified. We have taken a candidate gene approach to the identification of such genes. It would seem that the p16/RB1 pathway is of critical importance in melanoma carcinogenesis and therefore we chose to look at another member of the INK4 family, *p19 INK4D*.

The four members of the INK4 family appear to function in a similar way in terms of binding to CDK4/6 and inducing G1 arrest. The role of members of the INK4 family, other than INK4A, in carcinogenesis is not yet clear. Mutations in all four members of

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the family have been sought in cell lines. In one study of 100 cell lines for example, 35 homozygous deletions and seven intragenic mutations were detected in *INK4A*, and 29 lines had homozygous deletions in *INK4B*. Germline mutations have been sought both in the *INK4B* gene coding for p19 and in p14ARF (whose transcript is derived in part from an alternative codon reading frame of p16) but no evidence has yet been found for such mutations in melanoma families (Fitzgerald et al, 1996; Liu et al, 1997). INK4D is the most recently described member of the INK4 family and the gene is on chromosome 19 (19p13). Structural chromosome breakpoints are reported in around 4% of short-term melanoma cultures on 19p (Nelson et al, 1996). The chromosomal location of *INK4D* is, however, of most interest because of a report by Parmiter et al (1986) of a translocation involving chromosome 19 at 19p13 in three cases of melanoma. In only one line, an ovarian cancer cell line SKOV3, was there a single base pair deletion in exon 1 of *INK4D* (Gemma et al, 1996). In tumour samples, abnormalities have rarely been reported (Shiohara et al, 1996; Miller et al, 1997). These studies in cell lines and tumour samples suggest a possible role for *INK4D* in some specific tumour types but suggest that *INK4D* is not a tumour suppresser gene of such fundamental importance as *INK4A*.

In summary, we have not found any significant *INK4D* mutations in the germline of 48 families with a susceptibility to melanoma. Its similar function to that of INK4A and its chromosomal location at a site of translocations in melanoma patients, made it a candidate gene for genetic susceptibility to melanoma. To date we have no evidence for such a role, but it might reasonably be argued that if p19 does underlie susceptibility in some families, these families are likely to be rare and screening of samples from other continents should also be carried out.

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