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Germline mutations of the CDKN2 gene in UK melanoma families

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Germline mutations in CDKN2 on chromosome 9p21, which codes for the cyclin D kinase inhibitor p16, and more rarely, mutations in the gene coding for CDK4, the protein to which p16 binds, underlie susceptibility in some melanoma families. We have sequenced all exons of CDKN2 and analysed the CDK4 gene for mutations in 27 UK families showing evidence of predisposition to melanoma. Five different germline mutations in CDKN2 were found in six families. Three of the mutations (Met53Ile, Arg24Pro and 23ins24) have been reported previously. We have identified two novel CDKN2 mutations (88delG and Ala118Thr) which are likely to be associated with the development of melanoma, because of their co-segregation with the disease and their likely functional effect on the CDKN2 protein. In binding assays the protein expressed from the previously described mutation, Met53Ile, did not bind to CDK4/CDK6, confirming its role as a causal mutation in the development of melanoma. Ala118Thr appeared to be functional in this assay. Arg24Pro appeared to bind to CDK6, but not to CDK4. No mutations were detected in exon 2 of CDK4, suggesting that causal mutations in this gene are uncommon. The penetrance of these mutant CDKN2 genes is not yet established, nor is the risk of non-melanoma cancer to gene carriers.

INTRODUCTION

The CDKN2 gene on chromosome 9p21 has been suggested as a candidate gene for predisposition to melanoma (1). The evidence for the involvement of the CDKN2 gene in melanoma is strong; it encodes the p16 protein, which binds to the cyclin dependent kinases (CDK4 and CDK6), inhibiting their ability to phosphorylate the retinoblastoma (Rb) protein and so controls passage through the G1 checkpoint of the cell cycle (2).

The CDKN2 gene is located on chromosome 9p21, which has been shown, in a number of studies, to be linked to familial melanoma (3–9). The presence of mutations and deletions in CDKN2 in a wide range of tumours provides evidence for its role as a tumour suppressor gene (10–12). Recently CDKN2 knock out mice were reported to develop tumours; predominantly sarcomas, providing further evidence that CDKN2 deficiency facilitates tumour development in mammals (13).

Approximately half the melanoma families reported have been shown to have mutations in the CDKN2 gene, which segregate with melanoma cases within the families (14). To date a total of 17 different germline mutations, thought to be involved in the development of melanoma, have been found in the coding region of CDKN2 (14,15).

Another candidate melanoma susceptibility gene is the gene which codes for CDK4, the protein to which p16 binds, on chromosome 12q13. An identical mutation has been found in two unrelated melanoma pedigrees in the CDKN2 binding domain of CDK4 (16). The mutation is a C→T transition at nucleotide 297 in exon 2, which causes a replacement of an arginine with a cystine at amino acid 24 (Arg24Cys). This variant has been shown to have arisen as a somatic mutation in a human melanoma (17). Recently a second mutation, Arg24His, has been identified in exon 2 of CDK4 (18). The CDKN2 gene product would normally inhibit the protein encoded by CDK4, but mutations at the CDKN2 binding site render the CDK4 protein resistant.

We have previously reported the absence of germline mutations in exon 2 of CDKN2 in six UK melanoma families, three of which showed evidence of linkage to 9p21 (8). Here we report on the

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expansion of our investigation to include a further 21 families with evidence of predisposition to melanoma. In addition to exon 2, exons 1 and 3 of CDKN2 and also the CDK4 gene were analysed for mutations.

It is important to consider the significance of individual sequence variants in relation to their possible effect on cell cycle regulation. The functionality of mutations identified was analysed by *in vitro* CDK binding assay, using components synthesised in rabbit reticulocyte lysates. This assay has previously been shown to be a rapid and effective method for assessing the effects of CDKN2 mutations (19). Mutants that show impaired binding to CDK4 and CDK6 in this assay generally fail to inhibit the enzymatic activity of cyclin D-CDK complexes expressed from baculoviruses and show a reduced ability to induce G1 arrest in transiently transfected cells (19).

RESULTS

Sequence variants

Six families were found to contain germline mutations in the coding region of CDKN2 which could affect the function of the gene. The presence of each mutation was verified by sequencing in both forward and reverse directions (Fig. 1). Three unrelated base pair substitutions, a deletion and two (identical) insertions were detected (Table 1). These putative causal mutations were shown to segregate with disease in each family (Fig. 2).

Table 1. Summary of UK melanoma kindreds

Kindred[a]	Number of cases[b]	Multipoint LOD score[c]	Mutations detected	Polymorphisms detected
A				
MEL 21	8 (2)		Arg24Pro	540C/T
MEL 07	5 (1)	–2.01		540C/T
MEL 09	5 (2)	–1.42	Met53Ile	
MEL 02	4 (1)	0.21		
MEL 03	4 (2)	0.61		
MEL 06	4 (1)	0.43		
MEL 13	4 (2)	0.8	23ins24	
MEL 15	3 (3)		Ala118Thr	
MEL 24	3 (2)		88delG	540C/T
MEL 25	3 (2)			500C/G, 540C/T
MEL 26	3 (2)			
MEL 28	3 (2)			540C/T
B				
MEL 01	2 (2)			540C/T
MEL 04	2 (1)			
MEL 11	2 (2)			Ala148Thr, 500 C/G
MEL 14	2 (1)			500C/G
MEL 16	2 (2)			540C/T
MEL 19	2 (2)			540C/T
MEL 20	2 (2)			
MEL 22	2 (1)			500C/G
MEL 23	2 (2)			500C/G, 540C/T
MEL 27	2 (2)			Ala148Thr, 500C/G, 540C/T
MEL 29	2 (2)		23ins24	
MEL 30	2 (2)			
MEL 05	1 (1)			500C/G
MEL 10	1 (1)			Ala148Thr
MEL 12	1 (1)			540C/T

[a]Kindreds are listed in order of number of affected individuals in each family. The table is split into (**A**) families with ≥3 melanomas (the generally accepted criteria for a melanoma family) and (**B**) families with ≤2 melanomas.
[b]Numbers in parentheses indicate the number of samples from cases sequenced.
[c]Multipoint LOD score for linkage to chromosome 9p ($\Theta = 0{,}0$) determined by MacGeoch *et al*. (8). The presence or absence of atypical naevi was ignored in this analysis. Susceptibility to melanoma was assumed to be due to a partially penetrant dominant oncogene with a lifetime penetrance of 0.6.

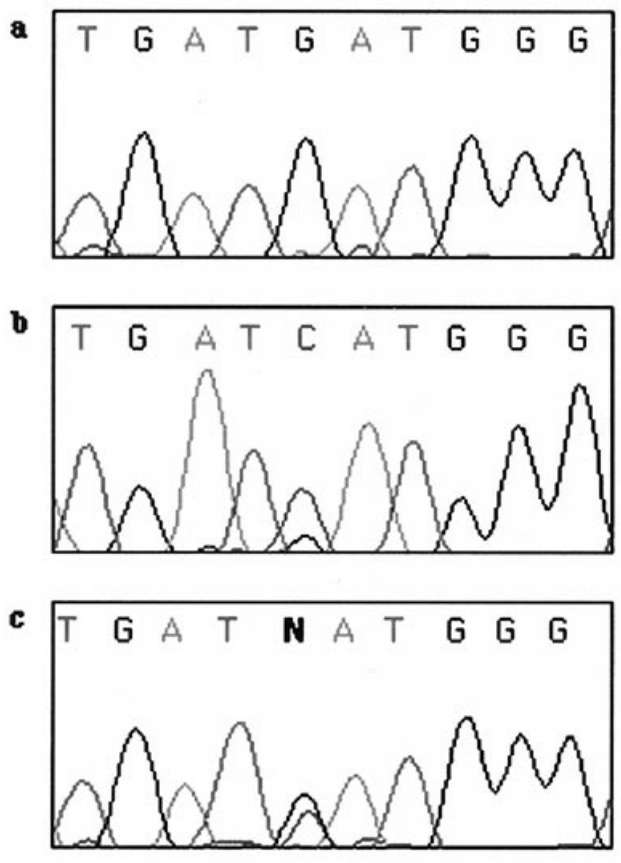

Figure 1. Sequence analysis of CDKN2, exon 2, in kindred MEL 09. (**a**) A wild-type sample, sequenced in the reverse direction, is homozygous for guanidine at nucleotide 159. (**b**) A sample from an affected individual appears homozygous for cytosine at nucleotide 159 when sequenced in the forward direction. (**c**) The same sample appears heterozygous G/C at nucleotide 159 when sequenced in the reverse direction.

The 9p linkage status of six of the 27 families has been previously determined (8). Family MEL 07, for which linkage to 9p21 has been excluded, was not shown to carry any germline CDKN2 or CDK4 mutations. A previously described mutation, Met53Ile, was found to be present in family MEL 09, which was originally reported to show evidence against linkage to chromosome 9p (8). Re-examination of the haplotyping data for this family has revealed that this was based on an incorrect assignment of alleles, due to inaccuracies in the clinical data available at the time.

A CDKN2 mutation was detected in one (MEL 13) of the four families in which some evidence of linkage to 9p21 has previously been demonstrated. The linkage status of the remaining four families, in which germline CDKN2 mutations were detected, has not been determined.

Three previously described polymorphisms were identified in this study (Table 1). Ala148Thr, which was found to be present in 4% of the Utah population (20) and 3% of CEPH parents (21), was found to be present in three UK melanoma families (11%) (MEL 10, MEL 11 and MEL 27). As reported in previous studies (15,21,22) this CDKN2 variant was found not to segregate with disease.

A G→C transversion at base 500 in the 3′ untranslated region (UTR) within exon 3 was identified in seven of the 27 UK families (26%). This polymorphism has previously been reported in three out of eight Australian kindreds (38%) (22) and was found to be present in 11% of CEPH parents (23).

Another polymorphism was detected at base 540 in the 3′ UTR. Eleven of the 27 families studied (41%) had a C→T transition at this nucleotide. As with the two other polymorphisms detected, this sequence change did not segregate with melanoma, and is therefore unlikely to predispose to disease.

The previously described Arg24Cys mutation in exon 2 of CDK4 was not detected by *Stu*I restriction digestion in any of the 27 UK melanoma families. No mutations were demonstrated in any of the seven coding regions of CDK4, in the 17 families (MEL 1–20) in which all coding exons were sequenced. No mutations were detected in exon 2 of CDK4 in the remaining 10 families (MEL 21–30) in which exon 2 alone was sequenced.

Protein binding studies

The critical test for a mutation versus a linked polymorphism is its functional activity and one of the most direct tests is whether the protein retains the capacity to bind to CDK4 and CDK6. This was examined in an established *in vitro* assay based on expressing the proteins by coupled transcription and translation. As previously shown (24) wild-type p16 binds selectively to CDK4 and CDK6, whereas a known mutant (Pro114Leu) does not. Significantly, the Met53Ile mutation, detected in kindred MEL 09, scored as negative for CDK binding, whereas the Ala118Thr mutant, in family MEL 15, appeared to bind to CDK4 and CDK6 as effectively as wild-type. The Arg24Pro variant, identified in kindred MEL 21, appeared to be defective in binding to CDK4, but virtually normal for binding to CDK6 (Fig. 3).

DISCUSSION

We have investigated 27 UK families, all of whom had evidence of predisposition to melanoma, either in terms of multiple cases and/or multiple primaries per case. The incidence of melanoma in the UK is low, ~10 per 100 000 per annum and therefore the possibility of clustering of sporadic cases by chance is unlikely. Six of the UK families investigated were found to carry germline CDKN2 mutations that appeared to segregate with melanoma. Of the five different mutations found, three (Met53Ile, Arg24Pro and 23ins24) have previously been shown to segregate with melanoma risk in large pedigrees (15,22,25 and Hussussian, unpublished).

The majority of mutations were detected in families with larger numbers of cases of melanoma (MEL 09, 13, 15, 21 and 24), and applying the generally accepted criteria for a melanoma family (≥3 affecteds), 42% (5/12) of the melanoma families investigated carried germline CDKN2 mutations. In this study we have also included 12 families with two cases of melanoma and three families with only one case, but multiple primaries. This was based on the assumption that, due to the low incidence of melanoma in the UK, families which do not fit the ‘accepted criteria’ may still be hereditary melanoma families. It is interesting to note that a previously reported causal mutation (23ins24) was detected in one of the UK families with only two cases of melanoma (MEL 29), highlighting the importance of

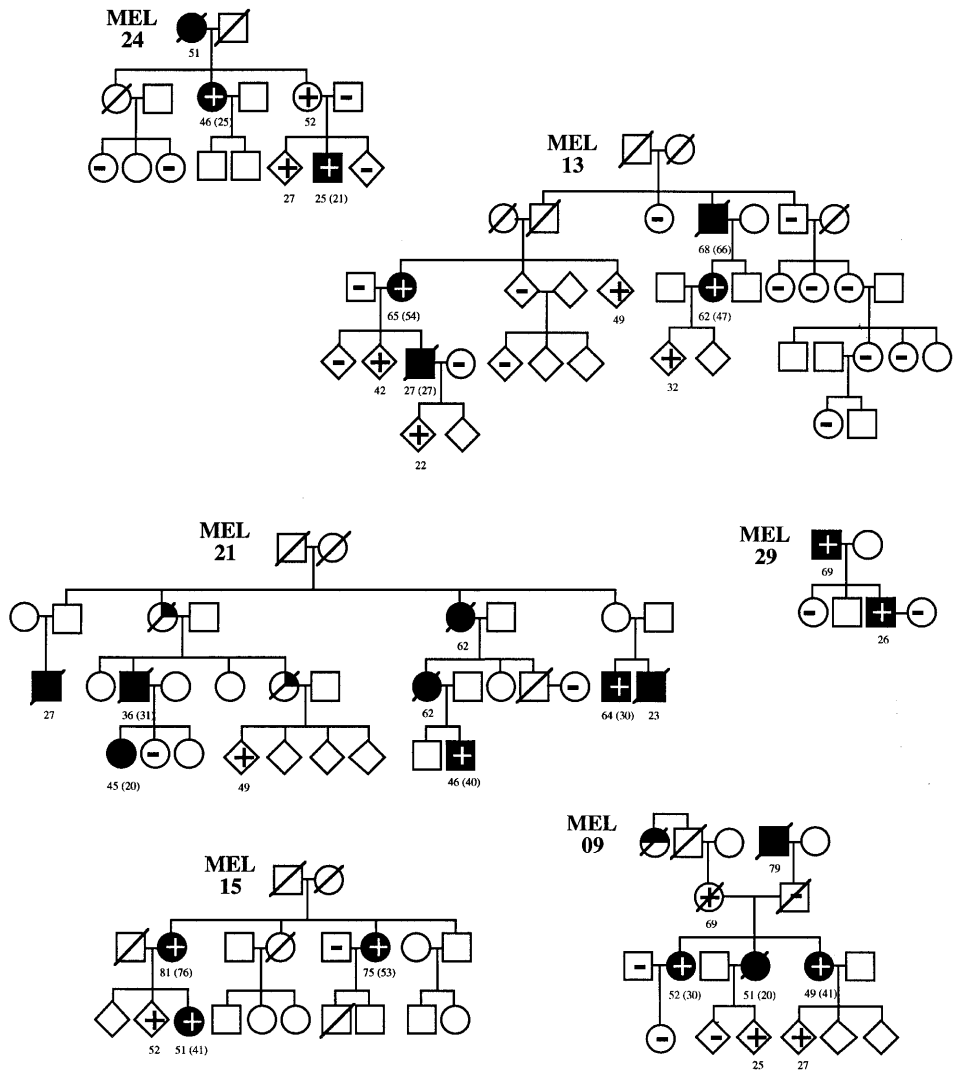

Figure 2. Segregation of detected CDKN2 mutations with melanoma. Pedigree diagrams have been simplified and adjusted to protect the identity of families. Mutations are as follows; kindred MEL 09, Met53Ile; MEL 13, 23ins24; MEL 15, 88delG; MEL 21, Arg24 Pro; MEL 24, Ala118Thr; MEL 29, 23ins24. Solid symbols represent individuals affected with cutaneous melanoma, half-filled symbols represent ocular melanoma, quarter-filled symbols represent other cancer. (+) Indicates the presence of mutation, (–) indicates absence, other individuals were not tested. The age at last contact, or age at death, is shown for all affected individuals and for all individuals that were found to carry a mutation. Also, shown in parentheses, is the age at first diagnosis of melanoma, where known.

investigating families with lower numbers of cases in countries with a lower incidence of melanoma.

The identification of germline CDKN2 mutations in families world-wide provides good evidence that CDKN2 is an uncommon, high penetrance melanoma susceptibility gene. In eight independent studies world-wide 104 families showing evidence of predisposition to melanoma have been screened for germline CDKN2 mutations. Of these, 39 (37.5%) have been identified as having mutations in CDKN2 which are likely to be involved in the development of melanoma (14,15). The observation that the CDKN2 binding site on CDK4 is also mutated in some families (16) suggests that regulation of the G1 checkpoint of the cell cycle is critical in melanoma carcinogenesis.

The segregation of individual CDKN2 mutations with melanoma in some large pedigrees is suggestive that these mutations are causal; however, the significance of each CDKN2 mutation observed must be assessed in terms of its likely functional implications. The CDKN2 protein contains four ankyrin-like repeats, spanning amino acids 11–142, which have been proposed to function as generalised protein-binding motifs (26). Deletion studies have shown that mutations in the conserved areas of the ankyrin repeats, which disrupt the CDKN2 protein primary structure, result in loss of its inhibitory function (27,28). All of the mutations identified in this study lie in conserved areas of the ankyrin-like repeats.

The 24 base insertion (23ins24) segregates with melanoma risk in two UK families (MEL 13 and MEL 29) and a large Australian pedigree (22). However, the exact effect of the mutation on the inhibitory function of the CDKN2 protein remains unclear. The insertion of the 24 bases does not cause a frameshift, and in CDK4 binding studies the mutant protein, eight amino acids longer than wild-type, appears to be functional (19).

The Met53Ile amino acid substitution occurs at a highly conserved site in ankyrin repeat II, and would therefore be

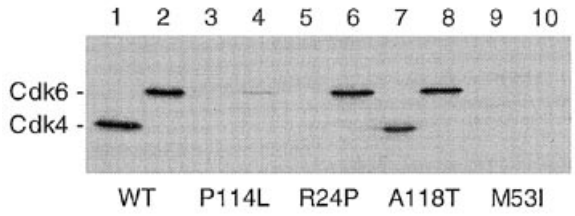

Figure 3. CDK binding activity of p16 mutants. Three variant forms identified in this study (R24P, A118T and M53I), together with wild-type (WT) and mutant (P114L) controls, were synthesized by coupled transcription and translation in rabbit reticulocyte lysates. Equivalent amounts were mixed with [^{35}S]methionine-labelled CDK4 (lanes 1, 3, 5, 7 and 9) or CDK6 (lanes 2, 4, 6, 8 and 10) and immunoprecipitated with a polyclonal antiserum against p16. The precipitates were analyzed by SDS-PAGE in a 12% polyacrylamide gel and the labelled products were visualised by autoradiography.

expected to alter the structure and function of the CDKN2 protein; this is confirmed by the inability of the mutant protein to bind to CDK4/CDK6. This, together with the fact that the mutation segregates with melanoma in family MEL 09 and several other families world-wide (20), indicates that Met53Ile is a disease-associated mutation.

The Arg24Pro amino acid substitution occurs at a conserved site in ankyrin repeat II and segregates with disease in kindred MEL 21. This single base pair substitution has been reported before in an Australian kindred (25). Interestingly, *in vitro* protein binding assays appear to show that this variant is defective for binding to CDK4, but virtually normal for binding to CDK6. A similar observation has previously been made for the variant Gly101Trp, which appeared less impaired for CDK6 binding in a two hybrid screen (29). Clearly further investigation is required to determine the significance of these findings.

Two of the mutations detected in this study have not previously been reported. 88delG appears very likely to be associated with the development of melanoma, because of its co-segregation with the disease in the families studied and its likely functional effect on the CDKN2 protein. The deletion of a single G at base 88 (88delG) results in a frameshift which causes truncation of the protein after 51 residues. The effect of this mutation on the CDKN2 protein would appear to be the most severe of the five mutations detected in the UK melanoma families; ankyrin repeat I is seriously disrupted and repeats II–IV are lost suggesting that the associated disease risks should be high. Protein binding assays have previously shown that mutations which place a stop codon upstream of codon 131 are likely to impair or abolish the inhibitory function of p16 on CDK4, thus contributing to oncogenesis (27). However, as far as can be inferred from family studies, this and a similar mutation in an Australian melanoma kindred 46delC (previously reported as 40delC), which also results in the truncation of the CDKN2 protein at amino acid 53, do not appear to have a notably higher penetrance than the other mutations discussed here (22).

The significance of the remaining previously unreported mutation Ala118Thr is unclear. Binding assays have shown the mutant protein to be functional. However, the amino acid substitution occurs at a highly conserved region of ankyrin repeat IV (27) and segregates with disease in family MEL 15. In addition this mutation has not previously been reported as a polymorphism and has not been detected in any other melanoma kindred or spouses investigated, suggesting that the Ala118Thr variant is a causal mutation.

A number of published mutations in CDKN2, particularly those recorded in sporadic tumours, show no impairment in CDK binding in this assay, suggesting that they are innocuous polymorphisms or that they affect some other properties of the protein (19). Shapiro (30) has shown that some of these mutants have dramatically reduced stability *in vivo* that will not be apparent in the *in vitro* assays employed here. It is becoming increasingly clear that among the various approaches currently used to test the function of p16 variants, no single assay is robust enough to give a definitive answer. Thus, although the *in vitro* binding or kinase inhibition assays readily identify mutants that are incapable of binding to CDK4 and CDK6, they cannot address issues such as reduced stability, aberrant sub-cellular localization or interactions with other as yet unidentified cellular proteins. Conversely, techniques dependent on ectopic expression of p16 in transfected cells are compromised by the variable levels of expression achieved and the need to distinguish the transfected and untransfected cells. Moreover, the readouts from these assays, such as reduced numbers of cell colonies, an increased proportion of cells in the G1 phase, or decreased phosphorylation of pRB, cannot discriminate between the effects of p16 on CDK4 versus CDK6. This could prove particularly relevant for variants such as Arg24Pro which may inhibit Cdk6 but not Cdk4. Further work will be required to confirm these preliminary data under different assay conditions. It will also be important to test the *in vivo* stability of variants such as Ala118Thr and the 24 base insertion which retain apparently wild-type properties in the *in vitro* binding assays.

The Arg24Cys mutation in CDK4, reported in two melanoma pedigrees which were not linked to 9p21 (14), was not identified in any of the 27 UK melanoma families. It is clear that the presence of this mutation, which renders CDK4 resistant to inhibition by p16, is important in the development of melanoma, but only in a very small number of kindreds; only 6% of families not linked to 9p21 were shown to carry this mutation (14) and in an investigation of 28 US families, none were found to have the Arg24Cys mutation (15). Other mutations in the p16 binding site in CDK4 could also potentially be involved in oncogenesis. The absence of mutations in exon 2 in the 27 families investigated indicates that causal mutations in this gene are uncommon.

CDKN2 germline mutations are clearly causal for familial melanoma. The clinical implications of these findings for the families concerned are, however, not clear. The effect of specific mutations on the development of melanoma needs to be studied further. There is evidence that the presence of CDKN2 mutations may give rise to other forms of cancer in the melanoma families, for example in kindred MEL 21, two obligate carriers of the mutation were affected with breast cancer and myeloma. The link between CDKN2 and other forms of cancer needs to be investigated and much better estimates of penetrance need to be obtained before the presence of CDKN2 mutations can be used to provide useful genetic counselling in the affected families.

MATERIALS AND METHODS

Pedigrees

Twenty seven families were studied. Twelve families had at least three cases of melanoma, the generally accepted criteria for a

melanoma family. Twelve of the families had two cases of melanoma, and in three families only one individual was affected, but with multiple primaries. Due to the relatively low incidence of melanoma in the UK it was assumed likely that these cases represented a familial disposition to melanoma, rather than a sporadic acquisition of the disease.

Where possible, two melanoma cases were screened from each of the 27 familial melanoma pedigrees; however, in 10 of the pedigrees it was only possible to obtain a sample from one affected individual.

DNA extraction and PCR

DNA was extracted from peripheral blood using the Nucleon II Genomic DNA extraction Kit (Scotlab). The three exons of CDKN2 were amplified from genomic DNA by PCR, using the primers described by Hussussian (21) and also those described by Kamb (20). 100 µl PCR reactions were carried out under standard conditions, containing 10 µl genomic DNA (250 ng), 10 µl 10× PCR buffer [1× = 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl pH 8.8, 2 mM $MgSO_4$, 0.1% Triton X-100], 2 µl dNTPs (200 µM each dGTP, dATP, dTTP, dCTP), 2 µl forward primer (50 µM), 2 µl reverse primer (50 µM), 10 µl dimethyl sulfoxide (DMSO) (10%), 0.25 µl DeepVent DNA polymerase (5 U) (New England Biolabs). PCR amplification conditions were as follows. An initial denaturation at 94°C for 5 min followed by 30 cycles of denaturing at 94°C (30 s), annealing at 55°C (30 s), and extension at 72°C (30 s), 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

Sequencing reactions were carried out using the ABI PRISM Dye Terminator Cycle Sequencing Kit (Perkin Elmer) and the products were analysed on an ABI 373 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.

Analysis of CDK4

The presence of the Arg24Cys mutation in exon 2 of the CDK4 gene in our 27 families was initially investigated by digestion of the CDK4 exon 2 PCR product with the restriction enzyme *Stu*I, which cleaves the mutant, but not the wild-type allele (16). On the assumption that other mutations in the CDK4 gene may have an effect on the ability of CDKN2 to bind, thus impairing regulation of the G1 phase of the cell cycle, we sequenced all seven coding exons of CDK4 in 17 families (MEL 1–20) and exon 2 alone in the remaining 10 families (MEL 21–30). These exons were amplified using the primers described by Zuo *et al.* (16). PCR reactions and sequencing were carried out as described above.

Protein binding assays

The Met53Ile and Ala118Thr mutations were introduced into the wild-type CDKN2 cDNA in the pRSET-A vector (Invitrogen) using the QuickChange site-directed mutagenesis system, following the protocols recommended by the supplier (Stratagene). The mutations were verified by DNA sequencing. The *in vitro* binding assays were performed essentially as previously described (31). Briefly, [^{35}S]methionine-labelled CDK4 and CDK6 were synthesized by coupled transcription and translation of plasmid DNAs in reticulocyte lysates (Promega TNT system). The wild-type and mutant forms of the CDKN2 protein were produced in the same way, but without labelling. Samples (2 µl) of the reaction products were mixed and incubated at 30°C for 30 min, diluted to 1 ml, and precipitated with a polyclonal antiserum against human CDKN2 protein. The immune complexes were collected on protein A-Sepharose beads and analysed by SDS-polyacrylamide gel electrophoresis in a 12% gel. The labelled proteins were visualised by autoradiography.

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