



SHORT REPORT

Lack of germline *CDK6* mutations in familial melanoma

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Germline mutations in genes encoding several components of the retinoblastoma pathway have been linked with inherited predisposition to melanoma. Most commonly, such mutations involve *CDKN2A*, a cyclin-dependant kinase inhibitor of two kinases, *CDK4* and *CDK6*, which phosphorylate the retinoblastoma protein (pRB) and thereby promote passage through the G₁/S cell-cycle restriction point. Less frequently, germline mutations in the *CDK4* gene have also been linked with an increased risk of melanoma. Despite the sequence and functional homology between *CDK4* and *CDK6*, the role of germline mutations in *CDK6* in melanoma predisposition is unknown. We detected no *CDK6* mutations within the p16 (*CDKN2A*) binding domain in index cases from 60 melanoma-prone kindreds that lacked germline mutations in the coding regions of either *CDKN2A* or within the entire *CDK4* coding region. We conclude that germline mutations in *CDK6* do not make a significant contribution to melanoma predisposition. *Oncogene* (2000) 19, 1849–1852.

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Between 8 and 12% of melanoma cases arise in families with a genetic predisposition to the disease (Tucker and Goldstein, 1995). In a subset of these kindreds, germline coding mutations of the *CDKN2A* gene (on human chromosome 9p21) co-segregate with cases of melanoma (Hussussian *et al.*, 1994; Walker *et al.*, 1995; Dracopoli and Fountain, 1996; FitzGerald *et al.*, 1996; NL and DH, unpublished). The *CDKN2A* gene product – designated p16 – is a cyclin-dependent kinase (CDK) inhibitor (CDKI). p16 plays a key regulatory role in the retinoblastoma (Rb) pathway at the G₁/S cell-cycle restriction point by inhibiting the phosphorylation of Rb via *CDK4/6* (Kato *et al.*, 1993; Weinberg, 1995; Ruas and Peters, 1998). The genetic basis for melanoma in families and individuals that lack coding mutations of *CDKN2A* is not well understood at this time. Possible explanations include non-coding mutations of *CDKN2A*; alterations in other

genes located at 9p21; and mutations of other major or minor melanoma predisposition genes located elsewhere in the human genome.

Genetic alterations of two other members of the Rb pathway in addition to *CDKN2A* have been linked with an increased risk of melanoma. Individuals harbouring germline mutations in the Rb gene and who have retinoblastoma as children show an increased risk of melanoma as adults (Draper *et al.*, 1986; Traboulsi *et al.*, 1988; Eng *et al.*, 1993; Bataille *et al.*, 1995). In addition, three melanoma prone families have been reported to possess germline mutations in the *CDK4* gene that co-segregate with the disease. Two of these families possess a cysteine rather than the wild-type arginine at amino acid 24 (R24C; Zuo *et al.*, 1996). More recently, an additional mutation at position 24 (R24H) was identified in a single French kindred (Soufir *et al.*, 1998). Moreover, the R24C (and presumably the R24H) mutant protein has a reduced affinity for p16, resulting in relaxation of control at the G₁/S restriction point. *CDK6* shows considerable amino acid homology with *CDK4* (Figure 1) and both proteins possess very similar 3D structure (Brotherton *et al.*, 1998; Russo *et al.*, 1998). Functionally, both proteins bind cyclin D1 and are specifically inhibited by *CDKN2A* (Serrano *et al.*, 1993). We therefore hypothesized that germline mutations in *CDK6* might predispose to melanoma.

We used direct sequencing and SSCP analysis to screen a single affected member from 60 melanoma-prone families in which a minimum of two individuals had been diagnosed with either melanoma or pancreatic carcinoma (Table 1). Prior to the sequence analysis of *CDK6*, we screened each of the 60 DNA samples for *CDKN2A* and *CDK4* germline mutations. The *CDKN2A* gene was analysed as outlined previously (Liu *et al.*, 1995, 1999). All of the patients analysed in this study lacked any functional mutations within 1 kilobase (kb) of the promoter and 5'UTR sequences directly upstream of the ATG initiation codon and in exons 1 and 2 of the *CDKN2A* gene (data not shown). To determine whether any of these families harboured germline *CDK4* mutations, we first used PCR to generate a DNA fragment of approximately 1.4 KB, which encompassed exons 2–5 of *CDK4*, and then analysed this fragment by direct cycle sequencing. We found no evidence of germline mutations within the sequenced product (corresponding to amino acids 1–72 in the translated protein); specifically, we did not observe the R24C and R24H alterations described previously (Zuo *et al.*, 1996; Soufir *et al.*, 1998).

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Mutations at various sites within the *CDK4* coding sequence have been found to decrease the affinity to *CDK4* for p16 in *in vitro* binding assays. Among these are variations at amino acids 22, 25, 34–35, 56, 95–101, 181/184 and 281–283 (Coleman *et al.*, 1997; Luh *et al.*, 1997; Byeon *et al.*, 1998; Ceha *et al.*, 1998). These data suggest that at least some p16 binding sites reside outside of the established p16-binding pocket (amino acids 22–25) or that mutations in these other regions result in conformational changes in *CDK4*, thereby prohibiting *CDKN2A* from accessing the binding pocket. In order to include these regions in our *CDK4* screening, we looked for sequence variations in the six remaining translated exons (3–8) of *CDK4* using PCR-SSCP (Zuo *et al.*, 1996). We observed no variation in the banding pattern relative to wild-type (unaffected) control DNA samples for any of the 60 patients across the six exons screened in this manner, including the putative p16-interactive motifs found in exon 3 (amino acids 95–97; Luh *et al.*, 1997; Ceha *et al.*, 1998), exon 5 (amino acids 181 and 184; Coleman *et al.*, 1997) and exon 8 (amino acids 281–283; Coleman *et al.*, 1997; Ceha *et al.*, 1998).

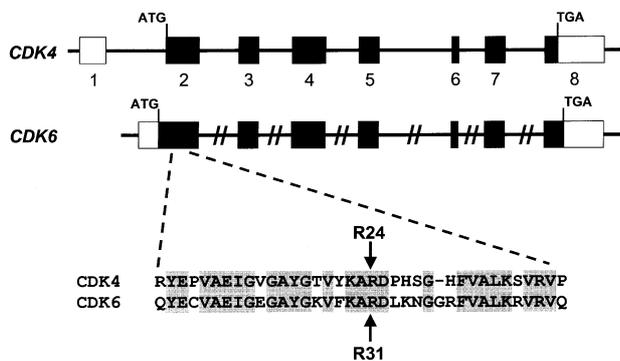


Figure 1 Genomic structural homology of *CDK4* and *CDK6*. *CDK4* structure is taken from Zuo *et al.* (1996) and deduced *CDK6* structure is shown for comparison. Note that *CDK6* intronic sizes are not known. The enlargement at the bottom shows amino acid homology between *CDK4* and *CDK6* within the region of *CDK4* involved in binding to *CDKN2A*, with the position of R24/R31 indicated

The fact that both *CDK4* and *CDK6* bind cyclin D and p16 implies that these two CDKs share structural homology. Although the translated proteins are of slightly different lengths (*CDK4* is 303 amino acids in length, whereas *CDK6* is 326 amino acids), the two kinases exhibit 64.4% homology across the entire length of their peptide sequences. Notably, within the R31 region of *CDK6* (R24 of *CDK4*) these two proteins show somewhat higher homology (67.6%; Figure 1). Since this region also corresponds to the *CDKN2A*-binding site in *CDK4*, we decided to examine the R31 region of *CDK6*.

As the genomic DNA sequence of *CDK6* was unknown at the time we began this study, it was first necessary to identify the putative intron/exon boundaries within the gene region, using the amino acid and cDNA nucleotide homology between *CDK4* and *CDK6* as a reference. Human genomic sequences corresponding to *CDK6* were identified from a BLAST search of GenBank (NCBI, NIH) using the published *CDK6* cDNA sequence (also known as PLSTIRE; GenBank Accession Number X66365). Sequences corresponding to the putative first exon of *CDK6* were then compared to the nucleotide sequence of *CDK4* via the CLUSTAL sequence alignment program to identify intron/exon boundaries. Exon 1 and the flanking intronic sequences of *CDK6* were identified within human BAC clone GS119P05 from chromosome 7q21 (Genbank Accession Number AC004011; Figure 2). When the sequences of *CDK4* and *CDK6* were compared, we found that the two kinases were highly homologous in their exon structure, with very similar sequence at the intron/exon boundaries. As shown schematically in Figure 1, the region encoding the putative *CDKN2A*-binding site in *CDK6* was found to lie within exon 1 (whereas in *CDK4* this region is found in exon 2). This difference reflects the fact that the first exon of *CDK4* is entirely untranslated, whereas in *CDK6*, the published untranslated sequence upstream of the initiation codon is found directly 5' to the ATG. In order to screen the R31 (R24) region, we again employed direct sequencing of PCR products. Oligonucleotides for PCR (Table 2) were designed in the 5' untranslated region and intron 1 so as to ensure the

Table 1 Breakdown of 60 kindreds analysed in this study, using similar criteria to Soufir *et al.* (1998).

At least one individual with multiple primary melanomas	At least two affected individuals are first degree relatives	At least one individual diagnosed with melanoma prior to age 30 years	At least one individual with pancreatic carcinoma	Number of families with two melanomas or pancreatic carcinomas	Number of families with three or more melanomas or pancreatic carcinomas
X	X	X		1	9
X	X			7	4
X	X		X	0	1
X		X		1	1
X				1	0
	X	X		7	5
	X			15	5
	X		X	0	2
		X		1*	0
Totals				33	27

*Patient sampled would be an obligate carrier of any familial mutation found. All patients lacked coding or splice site mutations in exons 1 and 2 of the *CDKN2A* gene as well as within 1 kilobase (KB) upstream of the ATG initiation codon that comprises the promoter and 5' untranslated region (5'UTR). Most of the patients (53/60) had visited the Familial Melanoma Clinic at the Toronto-Sunnybrook Regional Cancer Centre (Toronto, Ontario, Canada) between March 1996 and March 1999, where they were questioned in detail regarding their family history of malignant melanoma, and had provided written informed consent to participate in the study. These patients ranged in age from 22–70 years at the onset of the study, with the age at the first diagnosis of melanoma ranging from 19–63 years. In addition, seven families residing in various regions of the United States were included. The families had been followed prospectively for 4–22 years and had consented to participate under an NIH IRB-approved protocol

amplification of the entire first exon, including the region corresponding to codon 24 of *CDK4* (codon 31 of *CDK6*), the putative *CDKN2A* binding site. By this

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GTCCCGCGCCGCTCGCTCATCCCCGAGGGGGCCCCGCAACCTCTCCGCGC
GAAGACGGCTTCAGCCCTGCAGGAAAGAAAAGTAACTTCGCTTTCTCG
GAGGAACAGGAAGGATTAAGCGGCTTGGGAGAGGGCAGGAGCGCGCGGA
GGGTAGCGATGGAGGCTTCGTAAAGAGGAGGAGGGAGTCTGGAGGAAC
CCCGAGGAAGGCTTCTGGGCTGTCTGATTGCACTTTCTTCTTATCTCCC
GTCTCTCTCTTTAGGTGCAATGATTCGGACTGAGACCGCTTGGGCAGA
GGCTATGTAATCGTGTCTGTGTTGAGACTTCGCTTCGAGGAGGGAAGAG
GAGGGATCGGCTCGCTCCTCCGCGCGGGCGGGCGGGCGGACTGTCAGG
CGGAGTTTCGCGGGCGGCGCACCCAGGGTTACGCCAGCCCCGCGGGGAGGT
CTCTCCATCCAGCTTCTGCAGCGGGCAAGCCCCAGCGCCGAGCGCCTG
AGCCCGCGGGGAGCAACTAAAGCTAGACCGATCTCCGGGAGCCCGGAG
TAGGGCAGCGGGCGGGCCAGCTAGTTGAGCGCACCCCCGCGCGCCCA
GCGGCGCGGGCGGGGGCGGCTCCAGGCGGATGAGAGAAGGACGGCCCTGT
GCCCCTGACAGCAGTACGAATGCGTGGCGGAGATCCGGGAGGGCGCC
TATGGGAAGGTGTTCAAGGCCCGGCACTTGAAGAACGGAGGCGCTTTCGT
GGCGTTGAAGCGCGTCCGGGTGCAGACCGGGGAGGAGGCATCCCGCTCT
CCACCATCCCGAGGTGGGGTGGTGGAGCACCTGGAGACCTTCGAGGC
CCCAACGTTGTCAGGTGAGCCAGGGAGCTGCGCCCTCGCCATCTGGGGCC
TCGCGCGCGGGGAGGGCCAGCCGGGAATCTCAGGCCAGAAAGGTG
CGGGGCGAGGGGCGACTGGGGAGGTCCTGCGCGACCTGTTGGTCTTACAG
AGTGAGAGTTAAGTTTGTGTCAGACAGCCGGAGCCAGAGCCGGTGACCT
TGCCAGACAGCGAGCCGAAAGACAAGTCAAAAAACATGACCTGAGCAGG
CTTCCCTGCAGCTGTGCACTTAGAAGCGTTTCTCCCTGCCACCCCTGA
AGGAGGGTCTGGGGGAAGTGGGTGCGTTTTAGGGACAGGATAAGCCAACC
TGAGACATGGCCCGCTAGCCCCGCAATTTTCTGTCGCATGCCGTTGG
GGAATTTTGAAGACAGAGTAGGTGAGACTGGAGATGAGGATGCCGTG
TGATAATCAGTGGGAACCTT
    
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Figure 2 Region of Human BAC clone GS119P05 corresponding to exon 1 and surrounding intronic sequences of *CDK6*. Light grey shaded area denotes exon 1 sequence. Bold sequence shows positions of PCR oligonucleotides used in this study. Dark shaded area indicates position of Cy5.5-labelled sequencing primer used. Positions of initiation codon (ATG) and R31 (CGC; italicized) are also shown

method, we were able to sequence the entire length of exon 1 of *CDK6* (encompassing amino acids 1–77) and found no evidence of mutations in this region. Due to the lack of germline mutations described in the literature in exons 3–8 of *CDK4*, we elected not to proceed with SSCP analysis of the remaining exons of *CDK6*.

Considering the frequency and functional consequences of the inactivating *CDKN2A* mutations found in melanoma-prone individuals, the very low frequency of germline mutations found in the two downstream targets of this cyclin-dependant kinase inhibitor is perhaps surprising. As the majority of these mutations have the net effect of increasing *CDK4* and *CDK6* protein activity (via reduced p16 binding), it would follow that mutations in the p16-interactive coding regions could lead to similar cell cycle deregulation as do mutations in *CDKN2A*. We found this not to be the case. Several other mechanisms (e.g. enhancer mutations, alternative RNA splicing) may ultimately prove to offer a means of inactivation of *CDKN2A* and provide the hereditary basis of melanoma in kindreds linked to 9p21, but without *CDKN2A* mutations. However, in the absence of accurate linkage data to 9p21 in all kindreds, the possibility still exists that another as yet unidentified melanoma susceptibility locus is responsible for the melanoma susceptibility in these families.

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Table 2 PCR and sequencing primers used in this study

Primer name	Sequence	Product length
A. PCR primers		
CDK4-AF	5'-CATTGTGTTATCTCTGGGGCCGGC-3'	1483 base pairs
CDK4-AR	5'-ACTAGCCAAGCATGGAGGTGCGTG-3'	
CDK6-F	5'-GCTAGACCGATCTCCGGGGA-3'	432 base pairs
CDK6-R	5'-GGCGTGAAAGACCCGGACTC-3'	
B. Cy5.5-labelled sequencing primers		
CDK4-F*	5'-GGTCATACCATCCTAACTCT-3'	
CDK6-F*	5'-TCTCCGGGGAGCCCGGAGTA-3'	

Leukocyte DNA was extracted from peripheral blood samples using standard methods (Miller *et al.*, 1988). Exon 1 of *CDK6* was analysed by direct cycle sequencing. The reaction mixture for the *CDK6* PCR contained 2.5 units of Thermus aquaticus (Taq) DNA polymerase, 1 × Taq DNA polymerase reaction buffer, 2 mM MgCl₂ (Gibco/BRL), 0.2 mM dNTPs (Amersham Pharmacia Biotech, Piscataway, NJ, USA), 0.5 mM of each of the forward and reverse primers and 50 ng of patient DNA. The samples were denatured at 98°C for 5 min and then subjected to 30 cycles of: denaturation at 98°C for 30 s; annealing at 62°C for 30 s; extension at 72°C for 1 min and a 5 min 72°C final extension in a GeneAmp 2400 thermal cycler (Perkin-Elmer, Foster City, CA, USA). The quality and quantity of all PCR products was evaluated on a 1% agarose gel containing 1 µg/ml ethidium bromide. PCR reaction products were purified using the QIAquick PCR purification kit (Qiagen, Chatsworth, CA, USA) prior to sequencing, and 30–50 ng of DNA were subsequently subjected to cycle sequencing using a fluorescence-tagged primer (CDK6F*) and the Thermo Sequenase Cycle Sequencing Core Kit (Visible Genetics Inc., Toronto, Ontario). The samples were denatured at 94°C for 2 min, then subjected to 35 cycles consisting of: denaturation at 94°C for 30 s, annealing at 62°C for 30 s; and extension at 72°C for 1 min. After a final 2 min extension at 72°C, 4 µl of loading dye (Visible Genetics) was added to each reaction tube. The samples were heated at 95°C for 2 min and placed on ice. Aliquots were then loaded onto a MicroGene Blaster™ automated sequencing system (Visible Genetics) for analysis. We assayed for germline mutations in *CDK4* using either sequencing (exon 2), or SSCP (exons 3–8) (exon 1 of *CDK4* was not sequenced as it is non-coding). For *CDK4* analysis, a similar PCR cycling protocol to that used for *CDK6* was employed, but with an annealing temperature of 63°C. Sequencing reactions were also performed as outlined above, using the fluorescence-tagged primer CDK4F* and with an annealing temperature of 55°C. Exons 3–8 of *CDK4* were analysed by SSCP using oligonucleotides and PCR protocols described by Zuo *et al.* (1996). After purification using the QIAquick PCR purification kit (Qiagen, Chatsworth, CA, USA), the DNA fragments were subjected to end-labelling with 1.5 units of T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA), and 5 µCi of [γ-³²P] ATP (ICN Biomedicals, Costa Mesa, CA, USA) for 45 min at 37°C. Aliquots (3–5 µl) of the labelled PCR fragments were denatured for 3–5 min at 95°C in an equal volume of stop solution (0.3% Bromphenol Blue, 0.3% Xylene Cyanol FF, 10 mM EDTA (pH 7.5), 97.5% deionized formamide). For analysis, 3–5 µl of the denatured samples were loaded onto a non-denaturing gel containing 10% glycerol (1 × TBE, 6% acrylamide) and electrophoresed at 6 W at room temperature for approximately 16 h. The gels were then dried and exposed to X-ray film at –70°C for 2–24 h

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