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BRIEF COMMUNICATION

Mutation Analysis of the *CDKN2A* Promoter in Australian Melanoma Families

Pamela M. Pollock, Mitchell S. Stark, Jane M. Palmer, Marilyn K. Walters, Joanne F. Aitken, Nicholas G. Martin, and Nicholas K. Hayward*

Joint Experimental Oncology Program of the Queensland Institute of Medical Research, University of Queensland, and the Queensland Cancer Fund, P.O. Royal Brisbane Hospital, Brisbane, Australia

Approximately 50% of all melanoma families worldwide show linkage to 9p21-22, but only about half of these have been shown to contain germ line *CDKN2A* mutations. It has been hypothesized that a proportion of these families carry mutations in the noncoding regions of *CDKN2A*. Several Canadian families have been reported to carry a mutation in the 5′ UTR, at position −34 relative to the start site, which gives rise to a novel AUG translation initiation codon that markedly decreases translation from the wild-type AUG (Liu et al., 1999). Haplotype sharing in these Canadian families suggested that this mutation is of British origin. We sequenced 1,327 base pairs (bp) of *CDKN2A,* making up 1,116 bp of the 5′ UTR and promoter, all of exon 1, and 61 bp of intron 1, in at least one melanoma case from 110 Australian families with three or more affected members known not to carry mutations within the p16 coding region. In addition, 431 bp upstream of the start codon was sequenced in an additional 253 affected probands from two-case melanoma families for which the *CDKN2A* mutation status was unknown. Several known polymorphisms at positions −33, −191, −493, and −735 were detected, in addition to four novel variants at positions 120, −252, −347, and −981 relative to the start codon. One of the probands from a two-case family was found to have the previously reported Q50R mutation. No family member was found to carry the mutation at position −34 or any other disease-associated mutation. For further investigation of noncoding *CDKN2A* mutations that may affect transcription, allele-specific expression analysis was carried out in 31 of the families with at least three affected members who showed either complete or "indeterminate" 9p haplotype sharing without *CDKN2A* exonic mutations. Reverse transcription polymerase chain reaction and automated sequencing showed expression of both *CDKN2A* alleles in all family members tested. The lack of *CDKN2A* promoter mutations and the absence of transcriptional silencing in the germ line of this cohort of families suggest that mutations in the promoter and 5′ UTR play a very limited role in melanoma predisposition.

Roughly half of all melanoma families worldwide show linkage to 9p21-22, although the majority of these 9p-linked families do not carry germ line mutations in *CDKN2A* (Dracopoli and Fountain, 1996; Hayward, 1996). Several reasons have been put forward to explain the low frequency of coding region mutations in *CDKN2A* in those families that have haplotype sharing on 9p. These include the possibility that some coding mutations have been missed or the presence of mutations outside the coding sequence. A recent study has identified a mutation in the 5′ UTR of *CDKN2A*, at position −34 relative to the start site, in several Canadian families. This mutation gives rise to a novel AUG translation initiation codon that markedly decreases translation from the wild-type AUG (Liu et al., 1999).

Haplotype analysis of these families revealed a common founder, possibly of British origin, raising the possibility that this mutation might account for a large number of 9p-linked but *CDKN2A*-mutation-negative Australian melanoma families. An alternative explanation for the low incidence of *CDKN2A* mutations currently identified in melanoma families sharing a common 9p21-22 haplotype is the presence of another melanoma predisposition locus mapping to 9p21-22. Potential candidates for this locus include the *CDKN2B* gene, a *CDKN2A* homologue located < 35 kb proximal to *CDKN2A* which encodes the p15 protein, and exon 1β (E1β) of *CDKN2A*, which encodes the alternative reading frame product $p14^{ARF}$ generated from alternative splicing at this locus (Fig. 1). Extensive screening of *CDKN2B* and E1β in several large cohorts of families nevertheless has shown no mutation in either gene (Kamb et al., 1994; Stone et al., 1995; FitzGerald et al., 1996; Flores et al., 1997; Liu et al., 1997; Platz et al., 1997; Fargnoli et al., 1998).

Our aim in this study was to determine whether mutations in the *CDKN2A* promoter, or possibly other noncoding regions of the gene, are prevalent

*Correspondence to: Dr. Nick Hayward, Queensland Institute of Medical Research, P.O. Royal Brisbane Hospital, Brisbane QLD 4029, Australia. E-mail: nickH@qimr.edu.au

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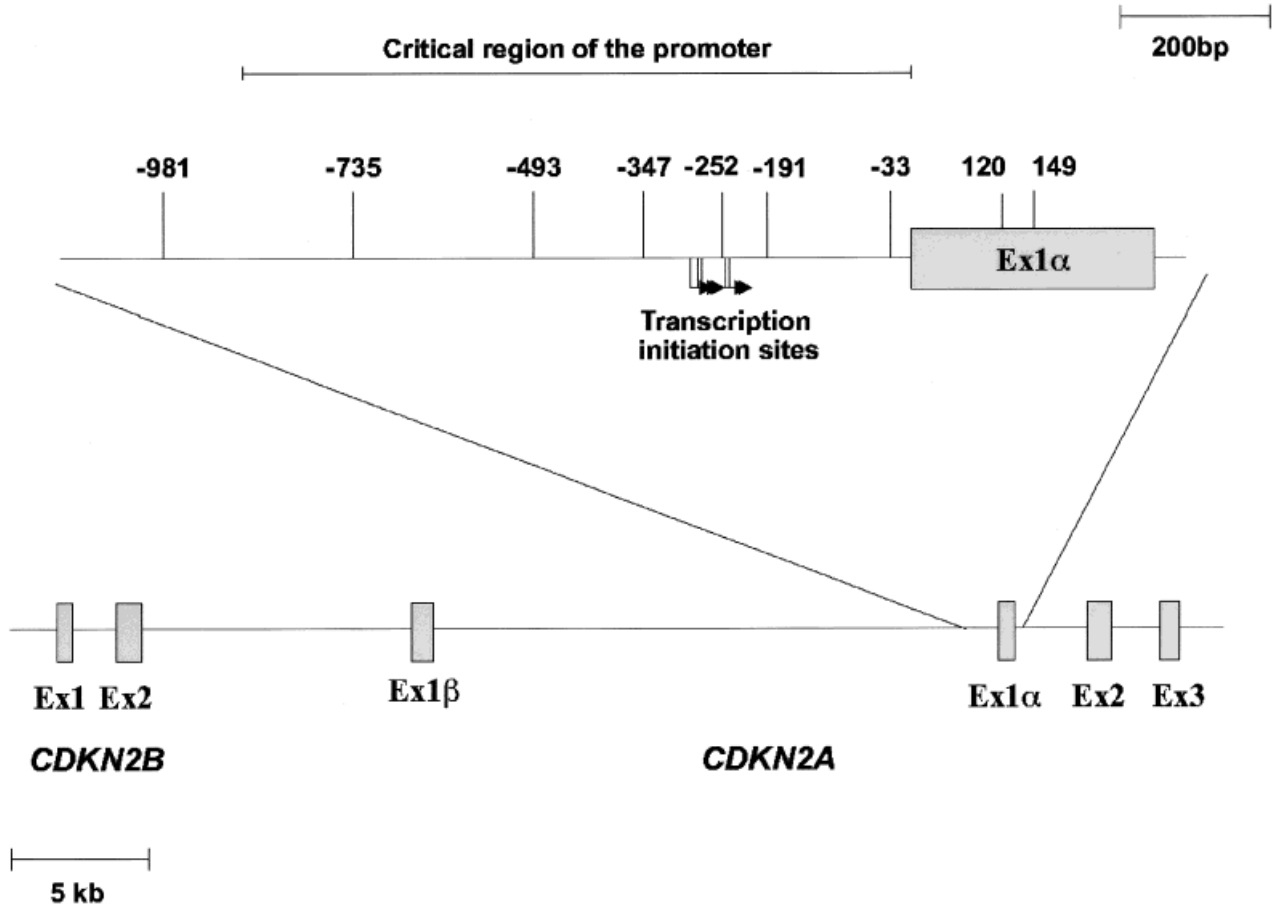

Figure 1. Schematic representation of the genomic region encompassing *CDKN2A* and *CDKN2B* (adapted from Figure 3 in Ruas and Peters, 1998). The upper, expanded portion depicts the region screened for mutations in this study and the variants identified. The critical region of the promoter and the multiple transcription start sites identified previously are provided (Hara et al., 1996).

in Australian melanoma families. We previously reported the screening of 48 Australian melanoma families for mutations in all four coding exons of *CDKN2A*, in addition to exon 2 of *CDK4* (Walker et al., 1995; Flores et al., 1997). Allele-specific expression analysis was performed in 12 families and showed that melanoma did not segregate with a nontranscribed *CDKN2A* allele in these families. *CDKN2A* mutations also have been analyzed in 481 melanoma families ascertained from a population-based cancer registry (Aitken et al., 1994, 1999). These people were stratified into three groups (high, intermediate, and low risk) according to their melanoma risk by use of a permutation procedure described previously (Aitken et al., 1994).

In the present study, mutation screening of the *CDKN2A* promoter was carried out in at least one person from each of the previously reported *CDKN2A* mutation-negative high-risk (i.e., those with at least three affected members) melanoma families from the population-based study, as well as our ad hoc, clinically ascertained cohort of "high-risk" families (Flores et al., 1997; Aitken et al., 1999). To avoid mutation screening in possible phenocopies, affected members either with the earliest age at onset or with an affected child were chosen for analysis.

The critical region of the *CDKN2A* promoter lies within 869 bp directly upstream of the *CDKN2A* coding domain. Several transcription start sites have been determined by RNAse protection assays, and disruption of these sites has been shown to abolish promoter activity (Hara et al., 1996). We amplified a 1,327 bp region of *CDKN2A* amplified with two overlapping primer pairs: 96F, 968 R, and 781F, 1,424R (primers, product length, and annealing temperatures are provided in Table 1). In all cases, polymerase chain reaction (PCR) amplification involved a "touchdown" thermal cycling routine of two cycles at each annealing temperature,

TABLE 1. Primers Used for Amplification and Sequencing of *CDKN2A* Genomic DNA and cDNA*

Amplified fragment	Primers	Sequence	Annealing temperature[a]	Product size (bp)
Genomic promoter fragment	P96F	AAAGCAGGGGGCACTCATATTC	65–55°	893
	P968R	TCCGAGCACTTAGCGAATGT		
Genomic promoter fragment	P781F	ACGCACTCAAACACGCCTTTG	65–59°	642
	P1424R	CAAACTTCGTCCTCCAGAGTC		
Sequencing primer	P427F	TGCCCCAGACAGCCGTTTTAC	61°	na
Exon 2	42F	GGAAATTGGAAACTGGAAGC	60–55°	509
	551R	TCTGAGCTTTGGAAGCTCT		
Exon 3	X3P2F	GACGGCAAGAGAGGAGGG	60–55°	206
	X3P2R	AAAACTACGAAAGCGGGGTGG		
RT-PCR promoter fragment	RT957F	TTCAGGGGTGCCACATTC	60–55°	844
	346R	CCAGGTCCACGGGCAGA		
RT-PCR 3′ UTR fragment	200F	AGCCCAACTGCGCCGAC	60–55°	777
	X3P2R	AAAACTACGAAAGCGGGGTGG		

*RT-PCR, reverse transcription polymerase chain reaction; na, not applicable.
[a]Temperature range (°C) indicates upper and lower limits on the touchdown PCR cycling conditions.

decreasing by 2°C steps, followed by 25 cycles at the lowest temperature. Each cycle consisted of 45 sec at 94°C, 90 sec at the annealing temperature, and 90 sec at 72°C. A 12-min initial denaturing step at 94°C and a 3-min final extension step at 72°C were employed. PCR reactions consisted of 100–200 ng DNA, 10–30 pmol of each primer, and 200 μM dNTPs (Promega, Madison, WI) and were performed with 1.25 U of AmpliTAq Gold (Hoffman-La Roche, Basel, Switzerland) in 100 mM Tris-HCl at pH 8.3, 500 mM KCl, and 1.5 mM $MgCl_2$. Because of the high GC content of the *CDKN2A* gene, reactions were performed at a final concentration of 500 mM Betaine (Sigma Chemical Company, St Louis, MO). All products were electrophoresed on a 1.5% TAE agarose gel, excised, and purified using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The fragments were sequenced with primers 96F, 427F, 781F, and 1,424R using Applied Biosystems Incorporated (ABI) dye terminator sequencing kits according to the manufacturer's specifications. Reactions were run on an ABI 377 automated sequencer (PE Applied Biosystems, Foster City, CA).

In addition to analysis of 1,116 bp of the 5′ UTR and promoter, all of exon 1, and 61 bp of intron 1 in 110 probands from melanoma families with at least three affected members, 431 bp immediately upstream of the start codon, together with exon 1 and 61 bp of intron 1, were analyzed in 253 affected individuals from two-case melanoma families. We noticed three discrepancies with respect to the sequence of Hara et al. (1996), which contained two thymine insertions and a cytosine replacement of thymine at positions −628, −605, and −1,085, respectively (Genbank entry X94154). Because these differences were observed in a homozygous state in all individuals, we concluded that they probably represent errors in the sequence of Hara et al. (1996). Consequently, we have numbered all variants in this article with respect to the corrected sequence.

Several known polymorphisms at positions −33, −191, −493, and −735 (Soufir et al., 1998; Harland et al., 2000) were detected, in addition to four novel variants at positions 120, −252, −347, and −981 relative to the start codon (Fig. 1, Table 2). The variant at position 120 (A > C) did not lead to an amino acid change and was not studied further. The variant at position −981 was found in two people, but RNA was not available for allele-specific expression analysis. DNA from other affected members of these families also was not available, and thus it is not known whether this polymorphism segregates with the disease. The variant is located upstream of the critical promoter region identified by Hara et al. (1996), and we have detected it in several healthy individuals (unpublished observations); therefore, it is likely that this variant represents a rare novel polymorphism.

The variant at position −252 occurred in two families with three (family 60007) and five (family 41110) affected members, respectively. In family 60007, the variant was present in the two affected members tested (a parent and child), but in family 41110, the variant was identified in only two of five affected members. The fact that the variant at −252 did not segregate with the disease in family 41110 suggests that it is also a novel rare polymorphism. Similarly, the −347 substitution was ob-

TABLE 2. Frequencies of Sequence Variants in the Promoter, 5′ UTR and Exon 1 of *CDKN2A*

		Promoter				5′ UTR					Exon 1	
							−191A → G					
	N	−981G → T	−735G → A	−493A → T	−347G → C	−252A → T	AA	AG	GG	−34G → T	−33G → C	120A → C (A40A)	149A → G (Q50R)
Families with at least 3 cases	110	2	5	5	1	1	33 (30%)	61 (55%)	16 (14%)	0	1	0	0
Families with at least 2 cases	253	ND*	ND	ND	0	0	89 (35%)	135 (53%)	29 (11%)	0	1	2	1

*ND, not determined.

served in one person. An affected brother of this person did not carry the allele, indicating that it was not segregating with melanoma in this family. A proband from a two-case intermediate-risk melanoma family carried a nucleotide 149 A > G substitution, which leads to an arginine replacement of glutamine at residue 50 of p16 (Q50R). We have reported this melanoma-associated mutation previously (Walker et al., 1995) in a large kindred (14 cases); however, there was no known relationship between the proband in the present study and this family, though we presume that they share a common ancestor. No person was found to carry the previously characterized mutation at position −34 or any other disease-associated mutation.

To investigate further the noncoding *CDKN2A* variants that may affect transcription, we carried out allele-specific expression analysis in a subset of 31 families that had between three and 11 affected members. Data on 12 families had been published previously (Flores et al., 1997). Nine of these families showed complete haplotype sharing on chromosome arm 9p and has between three and eight affected members. Another 16 families with between three and 11 affected members showed "indeterminate" 9p haplotype sharing (where all but one person haplotyped shared the same chromosome arm 9p haplotype), though only three to five members were haplotyped in the majority of families. Another six families were included; in these families there was less convincing haplotype sharing (e.g., three of five affected members shared the same haplotype). It should be noted that deciding whether a family is 9p-linked is difficult in Queensland, where the sporadic rate of melanoma is high. The lifetime risk of melanoma in this state is estimated at 1 in 17 for females and 1 in 12 for males (MacLennan et al., 1992).

To identify affected persons heterozygous for known *CDKN2A* polymorphisms, we amplified exons 2 and 3 as described previously (Castellano et al., 1997). The promoter fragment was amplified with primers 781F and 1,424R as described earlier. Diagnostic restriction enzyme digestions were performed to detect germ line polymorphisms in *CDKN2A* at nucleotide positions −191 relative to the start site, 442 in exon 2, and 500 and 540 in the 3′ UTR. For those people identified as heterozygous for at least one of these polymorphisms and carrying the common haplotype, RNA was isolated from lymphoblastoid cells by use of a QIAquick RNeasy kit (QIAGEN) and reverse-transcribed using Superscript II (GIBCO BRL, Grand Island, NY) according to the manufacturer's guidelines.

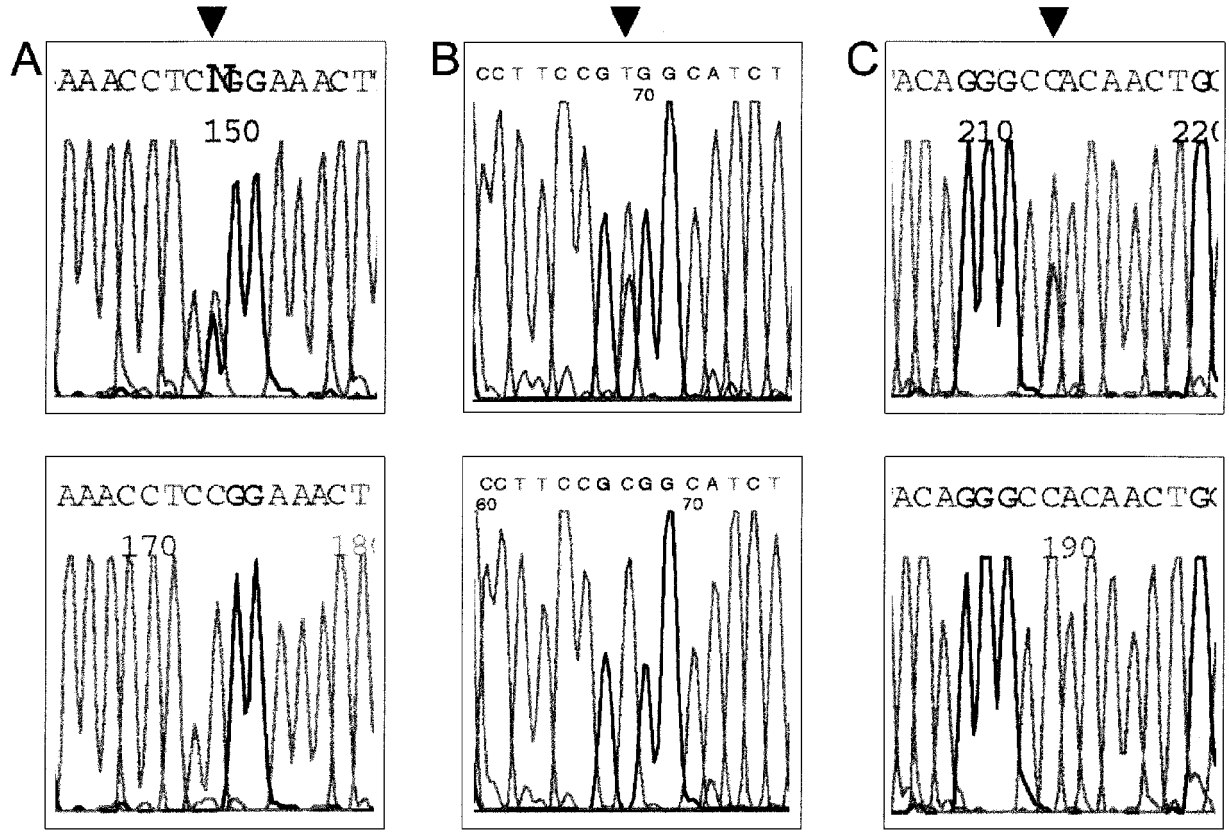

Figure 2. Representative sequence chromatograms of RT-PCR products from people heterozygous at nucleotide 442, 500, or 540 of *CDKN2A*. In each case, the upper panel is generated from an affected member of a melanoma family carrying a germ line polymorphism, whereas the lower panel is from a wild-type family member. **A:** The sequence of the reverse strand. **B,C:** The sequence of the forward strand. Arrowheads indicate the polymorphisms. A: Expression of both alleles is evident at nucleotide 442 in individual 40872-08. B: Expression of both alleles is evident at nucleotide 500 in individual 41142-03. C: Expression of both alleles is evident at nucleotide 540 in individual 40354-07.

The reverse transcription–PCR (RT-PCR) for detection of polymorphisms at positions 442, 500, and 540 was done with primers mapping to exon 2, 206F (Hussussian et al., 1994), and the 3′ UTR, X3P2R. RT-PCR for detecting the polymorphism at position −191 was performed with primers mapping 62 bp upstream of the −191 polymorphism (RT957F) and within exon 2 (346R) (Table 1). For RT-PCRs using RT957F, a longer extension step of 120 sec was employed, and optimal amplification was achieved with Elongase (GIBCO BRL) in the buffer supplied by the manufacturer.

Sequencing of the RT-PCR fragments from all individuals heterozygous for one or more of the polymorphisms at nucleotide position −191, 442 (A148T), 500, or 540 showed expression of both alleles in all 31 families analyzed (Fig. 2). We noted that the sequence data obtained at position −191 were sometimes equivocal, and a comparison with the data obtained at other polymorphic loci for the same people suggested that this polymorphism is not well suited to routine screening for allele-specific loss of expression, possibly because of secondary DNA structures that lead to unequal amplification of the two alleles (data not shown). It also should be noted that variants that confer only a small effect on promoter efficiency or variants that affect the temporal or spatial expression of *CDKN2A* would not be detected, but such variants may confer a small increase in the relative risk of melanoma.

The lack of germ line *CDKN2A* promoter mutations noted in this study and the absence of transcriptional silencing suggest that mutations in the promoter and 5′ UTR of this gene play a minor role in melanoma predisposition. This is in agreement with results of a similar study in which approximately 1 kb of the *CDKN2A* promoter upstream of the start codon was screened for mutations in 107 melanoma families collected from the United Kingdom, Italy, and Australia. Only one family (from Australia) was found to carry the nucleotide

−34 mutation (Harland et al., 2000). It is notable that this mutation was not seen in affected individuals from 363 Australian melanoma families with two or more affected members screened in our study.

These data suggest that *CDKN2A* promoter mutations are not responsible for melanoma predisposition in this series of families, and they support the hypothesis that another melanoma predisposition locus may map to chromosome bands 9p21-22. This accounts for the absence of *CDKN2A* mutations in some 9p-linked families. Zhu et al. (1999) have detected a quantitative trait locus (QTL) accounting for one-third of the population variance in nevus density linked to *CDKN2A*. This amount of genetic variance cannot be due to rare exonic mutations of this locus or to polymorphisms, unless they have large phenotypic effects. The fact that none of the *CDKN2A* promoter variants reported here and by others appear to have any significant effect on transcription would argue against their possible role in nevus development. Thus, the combination of evidence for a major nevus QTL in the region and against a role for variants in the promoter and exons of *CDKN2A*, and the findings of the present study, points to an undiscovered gene in this region with major influence on melanoma risk. It is possible that it is a melanoma predisposition gene with a pleiotropic effect on nevus development.

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