

# CDKN2A Variants in a Population-Based Sample of Queensland Families With Melanoma

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**Background:** Mutations in the CDKN2A gene confer susceptibility to cutaneous malignant melanoma (CMM); however, the population incidence of such mutations is unknown. Polymorphisms in CDKN2A have also been described, but it is not known whether they influence melanoma risk. We investigated the association of CDKN2A mutations and polymorphisms with melanoma risk in a population-based sample of families ascertained through probands with melanoma. **Methods:** The 482 Queensland, Australia, families in our sample were characterized previously as having high, intermediate, or low family risk of CMM. Unrelated individuals ( $n = 200$  families/individuals) drawn from the Australian Twin Registry served as control subjects. For individuals in the high-risk group, the entire CDKN2A gene coding region was screened for mutations by use of the polymerase chain reaction, agarose gel electrophoresis, allele-specific oligonucleotide (ASO) hybridization, and single-strand conformation polymorphism analysis. The intermediate- and low-risk families and control subjects were analyzed by ASO hybridization for a total of six recurring mutations as well as for polymorphisms at nucleotides (Nts) 442, 500, and 540. **Results:** CDKN2A mutations were found only in the high-risk families (nine [10.3%] of 87). The prevalence of the Nt500G (guanosine) polymorphism increased linearly with increasing familial risk (two-sided  $P = .02$ ) and was highest in the nine (primarily Celtic) families with CDKN2A mutations. After adjustment for ethnic origin, the relationship between risk group and the frequency of the Nt500G allele was weakened ( $P = .25$ ); however, there was no relationship between ethnic origin and Nt500-polymorphism frequency among the control subjects. **Conclusions:** CDKN2A mutations are rare in this population (approximately 0.2% of all melanoma cases in Queensland) and appear to be associated with melanoma in only the most affected families. The Nt500G allele appears to be associated with familial risk, but this association probably reflects Celtic ancestry. [J Natl Cancer Inst 1999;91:446–52]

Germline mutations of the CDKN2A gene, which encodes the cyclin-dependent kinase 4 (CDK4) inhibitor p16<sup>INK4A</sup>, have been detected with varying frequencies (6%–50%) in small samples of Australian (1–3), Dutch (4), English (5), French (6), Italian (7), North American (8–12), and Swedish (13,14) families who were selected because of their high incidence of cutaneous malignant melanoma (CMM). The population frequency of CDKN2A mutations is unknown, although a recent observation (14) that only 7.8% of 64 Swedish melanoma-prone kindreds who have two or more affected family members carry a germline CDKN2A mutation suggests that such mutations probably account for only a small fraction of familial melanoma. Predisposition to melanoma has also been linked to germline

mutations within the CDK4 gene on chromosome 12, although inactivation of this locus appears to be a much rarer event, since only three such mutations have been documented to date (6,15).

Within the CDKN2A gene, there are several apparently benign sequence polymorphisms [e.g., (16) and references therein; (2)], the two most common of which occur in the 3' untranslated region (3' UTR) and lead to a cytosine-to-guanine substitution at nucleotide (Nt) position 500 (Nt500C>G) numbering from the initiation codon and a cytosine-to-thymidine change at position 540 (Nt540C>T). Because neither of these polymorphisms causes a change in the amino acid sequence of p16<sup>INK4A</sup>, it is not known whether they have any effect on the function or expression of this protein. The third most common polymorphism within CDKN2A is a guanine-to-adenine substitution at position 442 (Nt442G>A) that results in replacement of an alanine residue at position 148 in p16<sup>INK4A</sup> by a threonine residue (A148T). Functional analysis indicates that this amino acid substitution does not appear to adversely affect the ability of this variant protein to inhibit CDK4 enzymatic activity, with inhibition comparable to that of wild-type p16<sup>INK4A</sup> (17,18). Germline mutations within CDKN2A, however, result in p16<sup>INK4A</sup> variants that are functionally compromised (5,17–22).

Because the overall importance of alterations in the CDKN2A gene in the causation of melanoma in the general population is unknown, we sought to examine the association between mutations and polymorphisms in CDKN2A and melanoma risk in a population-based sample of families ascertained through probands with melanoma (23) and in random control subjects from the same population.

## SUBJECTS AND METHODS

### Study Subjects

**Patients.** Family ascertainment and data collection have been described in detail previously (24). Briefly, we ascertained all 12 006 first incident cases of histologically confirmed cutaneous melanoma (invasive and *in situ*), diagnosed in residents of Queensland, Australia, during the period from 1982 through 1990 and reported to the Queensland Cancer Registry. A check of records of pathology laboratories throughout Queensland for 1984 and 1987 showed that approximately 95% of melanomas diagnosed in Queensland are recorded by the cancer registry; from this check, we also identified additional patients. We approached 10 407 patients for whom doctor's permission was obtained; 7784 (75%) of these patients completed a brief family history questionnaire, indicating any first-degree relatives (parents, siblings, or children) with a diagnosis of melanoma. A more detailed questionnaire on family history of melanoma was sent to a sample of these patients, including all who had claimed a positive family history ( $n =$

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1529) and a 20% sample of the remainder ( $n = 1391$ ); this questionnaire asked for the names and addresses, dates of birth, and ages of their first-degree relatives and whether there was any history of melanoma among these relatives. A total of 2118 (73%) of the index patients responded. Medical confirmation was sought for all relatives reported to have had melanoma (25). First-degree relatives of confirmed case patients were in turn ascertained according to the sequential sampling scheme of Cannings and Thompson (26). In total, 16 128 relatives belonging to 1912 separate families were ascertained through the index patients or other relatives. A total of 886 relatives for whom we had no information on age were excluded, which resulted in a final sample of 15 242 relatives in 1897 separate families (15 complete families were excluded because age was unknown for all relatives). Questionnaires about standard melanoma risk factors, including propensity to burn in the sun, pigmentation (skin color, hair color at 21 years of age, and eye color), total freckling in summer, and density of melanocytic nevi, were mailed to 7619 living relatives for whom the index patient provided a name and contact address; of these 7619 living relatives, 5158 (68%) responded after intensive telephone follow-up. Index patients and other family members provided proxy reports [on which we have previously reported the quality (27)] for an additional 4588 relatives, giving a total of 9746 relatives with risk factor information. Only medically verified cases among relatives were included in the analysis.

**Control subjects.** Control subjects comprised 200 unrelated individuals sampled at random from 3300 participants in a twin study of the inheritance of alcohol use, who had been drawn from the Australian Twin Registry (28). Ancestry of all four grandparents was asked both for patients and control subjects. The two samples did not differ significantly in this respect, both being more than 95% of northern European extraction.

## Familial Melanoma Risk

Strength of family history is traditionally defined according to the number of affected relatives in a family (29–31). However, this does not take into account the differences between families in family size and in the age, sex, and birth years of family members or, most importantly, the age of onset in affected individuals. These are strong determinants of a family's melanoma risk that may confound comparisons between families. To overcome this problem, we based our measure of strength of family melanoma history on a standardized family risk index,  $T$ , calculated as the number of cases of melanoma in the family beyond that expected, given the number of relatives in the family and their ages, sex, and birth cohorts. The total sample of 1897 families was divided into three strata of familial melanoma risk—low, intermediate, and high—by use of a permutation procedure described previously (23). High-risk families were defined arbitrarily as those whose family risk index fell at or above the 97.5 percentile rank in 1000 random permutations constructed by replacing each member of the family with an approximate age-, sex-, and birth cohort-matched individual selected at random from the total sample of relatives. Intermediate-risk families were defined as those whose index fell between the 50<sup>th</sup> and 97.5 percentile ranks, and low-risk families comprised the remainder of the sample (i.e., those whose index fell below the 50<sup>th</sup> percentile rank). Of the total 1897 families, 91 were classified as high risk, 414 as intermediate risk, and 1392 as low risk.

## Sampling Families and Relatives for Blood Collection

We attempted to obtain blood samples from two case patients in each of the 91 high-risk families, sampled at random from all patients in the family, and from one case patient in each of 200 intermediate-risk families and 200 low-risk families, selected at random from all intermediate-risk and low-risk families. If no case patients were available in a selected intermediate-risk or low-risk family, the next random number was chosen until 200 families in each group had been recruited. Ultimately, we had data on patients from 482 families.

We used a stratified random sampling scheme to achieve approximately equal numbers of patients who were aged under 50 years and at least 50 years at diagnosis in each group, to allow stratification by age at onset in later analyses. Blood collection was not attempted among patients older than 90 years of age. In the high-risk group, a total of four families could not be sampled because all case patients either were deceased or refused to participate, and in another 32 families only one case patient was available and agreed to participate. Blood had already been drawn in previous studies (1,3) from some high- and intermediate-risk families in our sample, and these families were included in the analysis; thus, some families contributed more than the prescribed one or two case patients. The total number of patients sampled per family is shown in Table 1.

**Table 1.** Numbers of individuals genotyped from three melanoma risk groups and a control group

| Risk group*  | No. of families | No. of individuals |
|--------------|-----------------|--------------------|
| High         | 87              | 167                |
| Intermediate | 194             | 214                |
| Low          | 201             | 201                |
| Control      | 200             | 200                |

\*See "Subjects and Methods" section for description of risk groups.

Selected case patients were first approached by us by letter and then telephoned by a research nurse, who explained the study and asked if the subject would agree to provide a blood sample. Blood was drawn by the subject's local doctor or by a pathology laboratory after written informed consent had been obtained from the participants, and samples were returned to our laboratory by courier. This project had ethics approval from our Institutional Ethics Committee.

## DNA Extraction

Blood samples (10 mL) were frozen until ready for use. After thawing, 40 mL of a solution of 10 mM Tris–1 mM EDTA (TE) was added, and the samples were centrifuged at 700g for 10 minutes at room temperature. The supernatants were decanted, and the white blood cell pellets were resuspended in 50 mL TE before being pelleted again as described above. The cell pellets were washed once more with TE, and then each was taken up in 3 mL of lysis buffer (135  $\mu$ g/mL proteinase K, 0.68% sodium dodecyl sulfate [SDS], 1.87 mM EDTA, 324 mM NaCl, and 8.1 mM Tris–HCl [pH 8]) and DNA was extracted according to the "salting out" method of Miller et al. (32).

## Genotyping

**Overview of molecular analysis.** The whole of the CDKN2A coding region was screened for mutations in patients from the high-risk group, with the use of a combination of agarose gel electrophoresis, allele-specific oligonucleotide (ASO) analysis, and single-strand conformation polymorphism (SSCP) analysis, as detailed below. The intermediate- and low-risk families were analyzed for a total of six mutations, one of which (a 24-base-pair [bp] duplication) was assessed by gel electrophoresis; the other five mutations (R24P [arginine to proline at position 24], L32P [leucine to proline at position 32], M53I [methionine to isoleucine at position 53], G101W [glycine to tryptophan at position 101], and V126D [valine to aspartate at position 126])—which represent some of the most common CDKN2A mutations reported to date—were analyzed by ASO hybridization.

**Polymerase chain reaction (PCR) and agarose gel electrophoresis.** To 6  $\mu$ L (50 ng) of template DNA was added 3.75  $\mu$ L of 10 $\times$  manufacturer's PCR buffer, 3.75  $\mu$ L of dimethyl sulfoxide (DMSO), 3.75  $\mu$ L of deoxynucleoside triphosphates (dNTPs) (i.e., 2 mM each of deoxyadenosine triphosphate [dATP], deoxythymidine triphosphate [dTTP], deoxyguanosine triphosphate [dGTP], and deoxycytidine triphosphate [dCTP]), 0.75  $\mu$ L of forward primer (20- $\mu$ M stock), 0.75  $\mu$ L of reverse primer (20- $\mu$ M stock), and 0.375  $\mu$ L of Dynazyme DNA polymerase (Finnzymes, Espoo, Finland), and the reaction mixture was made up to a final volume of 37.5  $\mu$ L with distilled water. For exons 1, 2, and 3, respectively, primers 2F/1108R (33), 42F/551R (33), and X3.P2F/X3.P2R (5'-GACGGCAAGAGAGGAGGG-3' and 5'-AAACTACGAAAGCGGGTGG-3') were used. Samples were overlaid with two drops of mineral oil, and amplification was carried out by use of a 65 °C–55 °C "touchdown PCR" protocol in a thermal cycler (The Perkin-Elmer Corp., Foster City, CA), after which 10  $\mu$ L of the PCR products was run on 2% agarose gels. The volume of PCR product blotted for ASO depended on the amount of product visualized on the gels. Moreover, the gels were scrutinized for samples that showed more than one band, which would be indicative of a major deletion or insertion within one of the CDKN2A alleles.

**ASO analysis.** The appropriate amount of each PCR product to ensure equal loading of the filter was added to the wells of a 96-well plate, and to each sample was added an equal volume of 20 $\times$  standard saline citrate (SSC; 3 M NaCl and 0.3 M trisodium citrate). The plate was then placed in a thermal cycler, and samples were denatured at 94 °C for 5 minutes. A slot blot apparatus (Schleicher & Schuell, Dassel, Germany) was used to prepare the ASO filters. A piece of blotting paper prewetted with 10 $\times$  SSC was placed below the nylon membrane

(Hybond N; Amersham, Aylesbury, England) to reduce seepage, and each slot was rinsed with 10× SSC. The denatured PCR products were then loaded into individual slots and filtered under mild vacuum onto the membrane, followed by two washes with 10× SSC. Membranes were subsequently removed from the apparatus and placed in a solution of 0.5 M NaOH, 2 M NaCl, and 25 mM EDTA for 5 minutes to maintain the DNA in a denatured state. Filters were then neutralized by being washed in a solution of 1 M Tris-HCl (pH 8) and 1.5 M NaCl for 1 minute before they were air dried, and the DNA was cross-linked onto the membrane by UV irradiation. Membranes were prehybridized (by use of 0.2 mL/cm<sup>2</sup> of filter) in 5× Denhardt's solution (1× Denhardt's solution is 0.2 g/L each of bovine serum albumin, polyvinyl pyrrolidone-40, and Ficoll-400), 5 mM EDTA, 1% SDS, 0.75 M NaCl, and 0.05 M NaPO<sub>4</sub> (pH 7.2) for at least 2 hours at the appropriate temperature for the oligonucleotide (Table 2). Hybridization was carried out for 2–6 hours under the same conditions as for prehybridization, with the addition of end-labeled oligonucleotide and with the volume of solution being reduced to 2.5 mL/100 cm<sup>2</sup> of filter. Oligonucleotides (100 ng [roughly 18 pmol]/18 mL of hybridization solution) were end labeled in a 20-μL reaction volume by the addition of 2 μL of 10× kinase buffer (New England Biolabs, Beverly, MA), 1.5 μL of T4 polynucleotide kinase, and 5 μL of [<sup>32</sup>P]dATP and then incubated for 30 minutes at 37 °C. The kinase was then inactivated by incubation of the reaction mixture at 65 °C for 10 minutes. Unincorporated label was removed by passage of the sample through a column of Sephadex G-25 (suspended in 10 mM Tris-HCl and 1 mM EDTA [pH 8]). After hybridization, the membranes were washed twice in 2× SSC–0.1% SDS at room temperature and then washed at progressively higher temperatures or in lower concentrations of SSC to reduce nonspecific binding. Filters were exposed to x-ray film for 2–16 hours.

**SSCP analysis.** Reaction components (15-μL reaction mixtures) for each PCR fragment were as follows: 3 μL of DNA, 1.5 μL of 10× *Taq* buffer, 1.5 μL of DMSO, 1.5 μL of dNTPs (2 mM each of dATP, dTTP, and dGTP and 0.1 mM dCTP—except for the exon 2A fragment for which the dCTP concentration was 0.02 mM), 0.9 μL of MgCl<sub>2</sub> (1.5 mM final), 0.15 μL of *Taq* polymerase (Promega Corp., Madison, WI), 0.15 μL (0.1 μCi/μL) of [<sup>32</sup>P]dCTP, 15 pmol of each primer, and distilled water up to 15 μL. Primers specific for different fragments of CDKN2A (8) were as follows: *exon 1*, X1.31F and X1.26R; *exon 2a*, X2.62F and 286R; *exon 2b*, 200F and 346R; and *exon 2c*, 305F and X2.42R. The translated part of exon 3 encodes only four amino acids and was thus not analyzed by SSCP. Amplification of all fragments was done by use of a 65 °C–55 °C touchdown protocol. SSCP analysis was carried out by use of 0.5× MDE gels (FMC BioProducts, Rockland, ME) that were pre-run at 9 W for at least 20 minutes in 54 mM Tris-HCl, 54 mM boric acid, and 1.2 mM EDTA (pH 8.3). Then 30 μL of stop solution (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, and 0.25% xylene cyanol) was added to PCR products and heated

at 94 °C for 5–10 minutes and snap cooled on ice for 5 minutes before 2.5 μL of each sample was loaded onto the gel. Electrophoresis was done at room temperature by use of 9-W constant power for 7–8 hours or, alternatively, at 5 W overnight, after which gels were transferred onto filter paper and dried for 1 hour before being exposed to x-ray film for 2–16 hours.

**Sequencing.** Those samples that showed aberrant band mobilities by SSCP analysis were reamplified and run on a 2% agarose gel. The appropriate fragments were excised and purified by passage through a QiaQuick gel extraction column (Qiagen, Hilden, Germany); 50–250 ng was subjected to “cycle sequencing” by use of a dye-terminator sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. PCR products were analyzed on a model 377 automated DNA sequencer (Applied Biosystems).

## Statistical Analysis

**Parametric linkage and disequilibrium analysis.** As a test for errors, MLINK (34) was used to try to detect apparent recombination events between the three polymorphisms in the high-risk families. Disequilibrium was measured by use of the program ASSOCIAT (34), with the use of all genotyped founders or a single nonfounder per pedigree where no founder was available. Disequilibrium has been expressed as the *D'* measure (35).

**Nonparametric affected-pedigree-member linkage analysis.** The affected pedigree member (APM) method for nonparametric linkage analysis was applied to the high-risk pedigrees because this method can be used to detect both linkage and association. To increase this technique's power to detect association, we included parent-offspring pairs, as described by Weeks and Lange (36). Increased identity-by-state sharing in parent-offspring pairs can only reflect association. The *P* values are one-sided and are considered significant for *P* < .003.

**Tests for allelic association.** Because multiple family members were genotyped in a proportion of families, the calculation of appropriate test statistics for allelic association becomes complicated. For comparison of allele frequencies in CMM case patients, unaffected relatives, and control subjects, a “gene dropping” approximate-randomization test was used. Gene dropping refers to the simple technique of simulating pedigrees by drawing founder (and marry-in) genotypes from the estimated population genotypic distribution. We have used the naive estimates of polymorphism frequencies and have assumed Hardy-Weinberg equilibrium and panmixia. The descendants' genotypes are simulated by randomly drawing one allele from each parental genotype. The observed phenotype for each individual remains the independent variable in each replicate, allowing the null distribution of a statistic testing association between trait and marker genotype to be estimated. In the present case, the simple Pearson contingency chi-squared statistic is used. This Monte Carlo approach deals correctly with family structure.

Two approaches to testing for a linear trend in the allele frequencies in the four risk categories have been used. In the first approach, allele frequencies for each subgroup have been calculated by use of the subroutine USERM13 supplied with the MENDEL package (37,38). The Fisher information has been used to calculate an effective sample size that allows for nonindependence between allele in members of the same family, applied as the weights for a linear logistic regression testing for a linear trend versus an indicator of risk group membership (“control” = 0; “low familial risk” = 1; “moderate familial risk” = 2; “high familial risk” = 3). In the second approach to a trend test, a Monte-Carlo test was used. The same linear score (i.e., 0–3) was treated as the independent variable in a linear regression model versus number of increasing alleles in the individual's genotype. The residual sum of squares from this model was used as the test statistic, and the null hypothesis distribution was simulated via gene dropping.

Testing for confounding by ethnicity was performed by standard logistic regression in which all subjects were treated as unrelated. Individuals were scored from 0 to 1 on the proportion of grandparents originating from England, Scotland, or Ireland—the three most common regions of origin and those most likely to be associated with increased risk of CMM. Thus, an individual reporting two English, one Scottish, and no Irish grandparental ancestry would have scores of 0.5, 0.25, and 0 on these variables. We also created a Celtic ancestry variable by adding the Scottish and Irish scores. The *P* values are two-sided and considered statistically significant for *P* < .05.

## RESULTS

### Mutations

Mutations were found only in the group at high familial risk. Overall, nine (10.3%) of 87 high-risk families carried germline

**Table 2.** Oligonucleotides for allele-specific hybridization analysis

| Oligonucleotide specificity* | CDKN2A exon | Oligonucleotide sequence (5'–3') | Hybridization temperature† °C |
|------------------------------|-------------|----------------------------------|-------------------------------|
| R24P mut                     | 1           | CCGGGTCCGGTAGAGG                 | 53                            |
| R24P poly                    | 1           | CCGGGTCCGGTAGAGG                 | 53                            |
| L32P wt                      | 1           | GGCTGCTGGAGGCGGT                 | 49                            |
| L32P mut                     | 1           | GGTGCCGGAGGCGGT                  | 51                            |
| M531 wt                      | 2           | AGTCATGATGATGGGCA                | 47                            |
| M531 mut                     | 2           | AGTCATGATCATGGGCA                | 47                            |
| G101W mut                    | 2           | ACCGGCCTGGGCGCGG                 | 57                            |
| G101W wt                     | 2           | ACCGGCCGGGGCGCGG                 | 59                            |
| V127D mut                    | 2           | TCGCGATGACGCACGGT                | 49                            |
| V127D wt                     | 2           | TCGCGATGTCGCACGGT                | 49                            |
| A148T poly                   | 2           | TAGATGCCACGGAAGGT                | 45                            |
| A148T wt                     | 2           | TAGATGCCGCGGAAGGT                | 47                            |
| Nt500 poly                   | 3           | GAAACCTCGGAAACTTAG               | 49                            |
| Nt500 wt                     | 3           | GAAACCTCCGAAACTTAG               | 49                            |
| Nt540 poly                   | 3           | TACAGGGCTACAACCTGG               | 43                            |
| Nt540 wt                     | 3           | TACAGGGCCACAACCTGG               | 41                            |

\*mut = mutant allele; poly = polymorphic allele; wt = wild-type allele; Nt = nucleotide.

†Temperatures were set at 7 °C below the *T<sub>m</sub>* (melting temperature) of the oligonucleotide, calculated using the formula:  $T_m = 4(G + C) + 2(A + T)$ .



CDKN2A mutations. Of these, seven mutations have been reported previously from a subset of 27 of the total of 87 high-risk families (1,3). These families had been screened with the use of SSCP analysis and direct sequencing, and the mutations detected consisted of the following: one family with each of the L32P, G35A, D108N, Nt46delC, and 24-bp duplication mutations and two families with the M53I mutation. Two additional germline CDKN2A mutations were found in the remaining 60 high-risk families not previously analyzed: a 24-bp deletion (detected by agarose gel electrophoresis) (Fig. 1) at the beginning of the coding region, which would result in the deletion of the first eight amino acids of p16, and a guanine-to-cytosine Nt change at position 71 (determined by SSCP and ASO analysis), which would lead to the substitution of an arginine for a proline residue at position 24 (R24P) in p16 (Fig. 2).

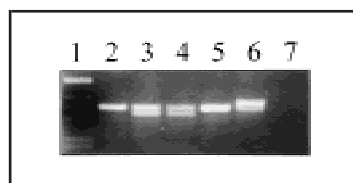
The mutations that we had detected in previous studies (1,3), along with the findings from other studies (2,5-9,11,12,22) indicating that several of the CDKN2A mutations were recurrent, influenced the choice of mutations that we screened for in the intermediate- and low-melanoma-risk groups and control subjects. None of the five mutations examined by ASO analysis (R24P, L32P, M53I, G101W, and V126D) was detected in these groups. In addition, selected members of nine families in the intermediate-risk group and one member of a low-risk family had been analyzed previously for mutations in the entire coding region of CDKN2A, but no such mutation was found (1,3).

### Polymorphisms

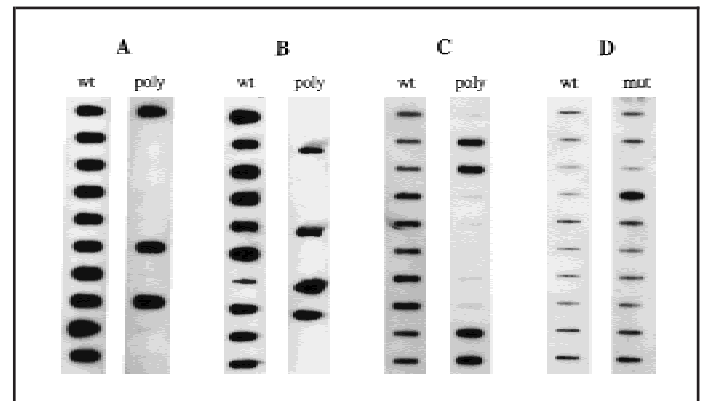
The three most common CDKN2A polymorphisms were assessed by ASO analysis (Fig. 3) in each of the different familial melanoma risk groups and control subjects. No recombination events between the three polymorphisms were detected. There was strong linkage disequilibrium between the two 3' UTR polymorphisms and between the Nt500G polymorphism and the Nt442A polymorphism (Table 3).

The prevalence of the Nt500G allele was higher among CMM case patients than among control subjects (14.2% and 11.1%, respectively) (Table 4), although this was not statistically significant (likelihood ratio test,  $P = .12$ ; empirical  $P = .12$ ). Among CMM case patients, the prevalence of this polymorphism increased linearly with increasing familial risk (Table 4) and was highest in the nine families with CDKN2A mutations. The trend tests (ordinal values: high = 3; intermediate = 2; low = 1; and control subject = 0) were statistically significant (maximum likelihood [ML] method,  $P = .02$ ; empirical  $P = .02$ ) with these families included, but they were not statistically significant when they were excluded (ML method,  $P = .07$ ; empirical  $P = .08$ ). The similar trends observed for the Nt540T and Nt442A polymorphisms were not statistically significant (ML method,  $P = .73$ ; empirical  $P = .74$ ). The nine families

**Fig. 1.** Agarose gel showing the 24-base-pair (bp) deletion mutation of the CDKN2A gene. **Lane 1:** molecular size markers (from top to bottom: 506, 396, 344, 298, 220, and 201 bp); **lane 2:** wild-type control DNA sample; **lanes 3, 4, and 5:** samples from three melanoma case patients in family 002622 (the individual whose sample is in lane 5 does not carry the mutation); **lane 6:** a reference sample from an unrelated melanoma patient who carries a 24-bp insertion mutation.



**Fig. 2.** Autoradiograph of a representative single-strand conformation polymorphism gel showing aberrant band mobility for exon 1 of the CDKN2A gene in affected members of family 001790, who carry the R24P (arginine to proline at position 24) mutation (**lanes 4 and 6**). **Lanes 1, 2, 3, 10, and 11** contained wild-type samples; **lanes 5, 7, 8, and 9** contained reference DNAs with a variety of other mutations within exon 1.



**Fig. 3.** Representative autoradiographs from allele-specific oligonucleotide hybridization analysis of CDKN2A gene polymorphisms and mutations. **Panel A:** Nt442G>A (A148T [alanine to threonine at position 148]) polymorphism. **Panel B:** Nt500C>G polymorphism. **Panel C:** Nt540C>T polymorphism. **Panel D:** Nt159G>C (M53I [methionine to isoleucine at position 53]) mutation. Nt = nucleotide; wt = wild-type allele; poly = polymorphic allele.

**Table 3.** Normalized disequilibrium coefficient ( $D'$ ) for selected CDKN2A variants

| First variant | Frequency, % | Second variant |               |
|---------------|--------------|----------------|---------------|
|               |              | Nt442A         | Nt500G        |
| Nt442A        | 2.4          | —              | —             |
| Nt500G        | 13.3         | 0.913 (19.3*)  | —             |
| Nt540T        | 10.4         | 0.140 (2.5*)   | 0.200 (11.1*) |

\*Chi-squared statistic for testing the null hypothesis ( $D' = 0$ ) with 1 degree of freedom.

with mutations were more likely to be of Scottish or Irish descent (mean Celtic ethnicity score for mutation-positive families = .55; that for non-mutation-positive high-risk families = .26; that for intermediate-risk families = .32; and that for control subjects = .25; Kruskal-Wallis test,  $P = .01$ ). After adjustment for ethnic origin, the relationship between risk group and the Nt500G allele frequency was weakened ( $P = .25$ ). However, there was no relationship between ethnic origin and Nt500G-polymorphism frequency among the control subjects (logistic regression for frequency versus percentage Celtic grandparents,  $P = .13$ ).

None of these variants was linked or associated with CMM on APM analysis of the high-risk families alone (Table 5).

### Proportion of CMM Due to CDKN2A Mutations

One estimate for the proportion of CMM in the population due to detectable mutations may be calculated as follows: The

**Table 4.** Frequency of three CDKN2A polymorphisms in different melanoma risk groups and in control subjects

| Subgroup*                     | No. of individuals genotyped | Nt500G allele frequency × 100 (95% CI)† | Nt540T allele frequency × 100 (95% CI)† | Nt442A allele frequency × 100 (95% CI)† |
|-------------------------------|------------------------------|---|---|---|
| High risk (mutation positive) | 34                           | 30.4 (10.8–50.0)                        | 18.9 (0–42.8)                           | 5.0 (0–11.9)                            |
| High risk (mutation negative) | 133                          | 16.8 (11.1–22.5)                        | 10.7 (6.0–15.4)                         | 3.4 (1.0–5.8)                           |
| Medium risk                   | 214                          | 13.9 (10.4–17.4)                        | 11.1 (8.0–14.2)                         | 2.7 (1.1–4.3)                           |
| Low risk                      | 201                          | 12.7 (9.4–16.0)                         | 10.5 (7.6–13.4)                         | 3.0 (1.4–4.6)                           |
| All melanoma case patients    | 514                          | 14.2 (12.0–16.4)                        | 10.9 (8.9–12.9)                         | 3.0 (2.0–4.0)                           |
| Control subjects              | 200                          | 11.1 (8.0–14.2)                         | 9.5 (6.6–12.4)                          | 1.8 (0.4–3.2)                           |

\*Risk groups are defined in the “Subjects and Methods” section.

†95% CI = 95% confidence interval. Nt = nucleotide.

**Table 5.** Affected pedigree member (APM) analysis of melanoma in high-risk families

| Polymorphism* | No. of families (No. of affected individuals genotyped) | $1/\sqrt{p}$ † | $P$ ‡ |
|---------------|---|----------------|-------|
| Nt442A        | 67 (158)  | –1.7           | .95   |
| Nt500G        | 51 (110)  | –1.7           | .96   |
| Nt540T        | 48 (103)  | 0.5            | .30   |

\*Nt = nucleotide.

†APM  $t$  statistic with the use of the inverse square root weighting of allele frequencies.

‡ $P$  values are one-sided and are considered statistically significant for  $P < .003$  (Bonferroni correction for three markers with the use of  $\alpha_1 = .01$ ).

number of CMM patients responding to the initial questionnaire was 7784; in the 91 high-risk families, 151 were probands (i.e., patients who had age of disease onset in the period 1982 through 1990). There were 46 families with one proband, 34 families with two, seven families with three, and four families with four. If we assume that all probands from high-risk families were sampled and nonresponders do not differ from responders with respect to familial risk, then 151 of 7784 come from a high-risk family. Only 87 of 91 high-risk families were genotyped for mutations, and nine mutations were detected. Hence,  $(151 \times 9/87)/7784 = 0.2\%$  of all CMM cases in Queensland are due to CDKN2A mutations.

## DISCUSSION

Although several reports (12,14,39,40) have determined the incidence of CDKN2A mutations in various cohorts of CMM cases, none of these studies has estimated a true population-based frequency of CDKN2A mutations. In all instances, these studies have focused on relatively small numbers of patients with high *a priori* probabilities of carrying a CDKN2A mutation—i.e., those individuals with a family history of CMM, multiple CMMs, or early age of onset of CMM—rather than on the general population. For the long-term design of adequate screening, surveillance, and treatment strategies for CMM, it would be beneficial to know the frequency of CDKN2A mutations both in the population at large (with or without having developed CMM) and in all CMM case patients (with or without a family history of the disease). Although we have not strictly done such analyses here, to our knowledge, we have gotten closer than others to this goal. In the present study, we sought to examine the association between mutations and melanoma risk in a population-based sample of Queensland families characterized according to their family history of melanoma and subdivided into

groups of high-, intermediate-, or low-risk families (23). Furthermore, we determined the prevalence of three CDKN2A polymorphisms to ascertain whether any of these might also contribute to melanoma risk.

A relatively low frequency (nine [10.3%] of 87) of the germline CDKN2A mutation carriers was found, and these individuals were only from the high-risk group. This figure is likely to be an underestimate, since the methodologies used to search for mutations in this study would not have detected changes in the promoter region or introns of CDKN2A, which might in some way abrogate expression of this gene. To date, only one melanoma family of 20 (including seven from the high-risk group in the present study) who were analyzed for transcriptional silencing of one allele of CDKN2A has been found to carry such a defect (3,11), although the exact nature of the mutation was not elucidated (11). Large deletions of one entire allele, as have been described recently for families with melanoma and tumors of the nervous system (41), would not have been detected in our study because Southern blot analysis and hemizygoty mapping were not employed (on the complete sample). However, such gross deletions of CDKN2A are unlikely to contribute significantly to the overall mutation frequency of this locus. In a cohort of 48 melanoma-prone families (27 of whom belong to the high-risk group of the present study) on which we have previously reported (3), all families contained at least one affected case patient who was heterozygous for the marker D9S942, which lies between the alternate first exons of CDKN2A (Hayward N: unpublished observations). These data thus indicate that there were no hemizygous deletions of the CDKN2A locus in that cohort. Furthermore, it is unlikely that we have missed many, if any, mutations by the SSCP screening approach, since this methodology works extremely well for the CDKN2A locus. We have previously reported SSCP analysis of the CDKN2A gene in 34 melanoma cell lines that were identified as carrying at least one allele of this locus and found 15 mutations (42). The entire coding region was sequenced in the remaining 19 cell lines, and we found no further mutations, thus indicating a 100% success rate for mutation detection by SSCP in this series of samples for this particular gene.

Although none of the six mutations that we assessed was detected in the intermediate- or low-risk melanoma groups or in control subjects, mutations other than these are likely to exist in these groups. Full screening of these individuals was not considered cost-effective in this study, given the low yield of mutations in the high-melanoma-risk subgroup.

The striking message from our study is that CDKN2A mutations may account for quite a small proportion of the spectacular (but rare) dense familial clusterings of melanoma, and

the contribution of such mutations to the population burden of CMM is trivial—only one fifth of 1%, estimated from our sample. It is possible that a greater proportion of melanoma risk might be attributable to more common, but less dramatic, genetic risk factors than rare CDKN2A mutations. This is why we chose to examine the three common polymorphisms in this gene. Each of these showed a trend toward higher frequency of the rarer allele with increasing familial risk category. This trend verged on significance for the Nt500G allele, but the frequency of this allele was highest in the nine families in whom mutations were also detected. Because this group of families also had the highest degree of Celtic ancestry, the association most probably reflects a high-susceptibility genetic background. The only way to resolve this issue is by use of within-family tests [such as the Transmission Disequilibrium Test (43)] rather than relying on group comparisons. In any event, it is clear that any association of CDKN2A polymorphisms and melanoma risk is not strong. It is possible, however, that in conjunction with other genetic risk factors, e.g., certain MC1R alleles (44,45), a more pronounced effect may be seen.

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## NOTES

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