Lack of Genetic and Epigenetic Changes in *CDKN2A* in Melanocytic Nevi

To the Editor:

The strongest known epidemiologic risk factor for melanoma is a large number of melanocytic nevi (Swerdlow and Green, 1987), whereas the most important genetic risk factor is germline mutation of the *CDKN2A* gene, which encodes the cell cycle inhibitor p16 (Kamb *et al*, 1994; Nobori *et al*, 1994). *CDKN2A* mutations exist in some melanoma-prone families (reviewed in Hayward, 1996; Foulkes *et al*, 1997; Ruas and Peters, 1998), but account for only a small fraction of all familial melanoma (Platz *et al*, 1997; Aitken *et al*, 1999).

Some melanoma kindreds include individuals with a high prevalence of ''dysplastic'' (Greene *et al*, 1985) or ''atypical'' (Gruis *et al*, 1995) nevi, but this phenotype does not segregate with *CDKN2A* (reviewed in Hayward, 1996); however, evidence for linkage of ''common'' nevus count with *CDKN2A* has been found in three melanoma pedigrees, only one of which carried an exonic mutation, suggesting that other variants in the region, outside the sequence encoding p16, affect nevus density (Cannon-Albright *et al*, 1994). We have shown by sib-pair linkage analysis that ~30% of variance in moliness is due to genetic variation at or close to the marker D9S942 (Zhu *et al*, 1999), 15 kilobases upstream of *CDKN2A*, and we surmise that D9S942 is in disequilibrium with a functional polymorphism nearby. We hypothesize that as germline mutations in the exons of *CDKN2A* are rare, it is likely that variants in the noncoding regions of this gene are responsible for this major determinant of nevi, and by inference, of melanoma. Somatic changes and loss of heterozygosity (LOH) of *CDKN2A* support a role for this gene in nevus etiology (Healy *et al*, 1996a; Lee *et al*, 1997). Given the above links between *CDKN2A*, melanoma, and nevi, we looked for chromosomal loss, structural and epigenetic changes in the *CDKN2A* gene in benign melanocytic lesions.

A 4 mm punch was used to separate neval from stromal tissue from 10 g paraffin-embedded sections of 25 intradermal nevi and 25 compound nevi. Sections of nevi and stroma were incubated in 80 μl of lysis buffer (10 mM Tris-HG, pH 8.3; 50 mM KCl; 2.5 mM $MgCl_2$) plus 4.4 μl of 10 mg per ml proteinase K and 4 μl of 10% Tween 20. Samples were incubated at 55°C for 18–48 h then boiled for 15 min, adjusted to 1 mM EDTA, and centrifuged for 1 min at 5000 r.p.m. LOH at *CDKN2A* was assessed by comparing genotypes at D9S942 as previously described (Pollock *et al*, 1998).

CDKN2A was screened for mutations in nevus samples using SSCP analysis as described (Aitken *et al*, 1999). Samples that showed aberrant band mobility were re-amplified and run on a 2% agarose gel. Appropriate fragments were excised and purified by passing them through a QiaQuick (Qiagen) gel extraction column, then 50–250 ng were sequenced as described (Aitken *et al*, 1999). Matching stromal DNA was analyzed when variants were detected.

The CpG island domain of *CDKN2A*, encompassing the promotor and exon 1 (nucleotides 28602–28856 in GenBank entry AC000048), was screened for methylation using the bisulfite sequencing method under conditions described in Clark *et al* (1994). The control primers used for the standard bisulfite p16 amplification were: outer, p16–4 (28844–28819) and p16–6 (28602–28579), and inner, p16–4 and p16–5 (28516–28541) (Huschtscha *et al*, 1998). The MSPPCR p16 primers were: outer, p16CG-4 (28962–28939) and p16CG-6 (28641–28616), and inner, p16CG-4 and p16CG-5 (28880–28856); sequence coordinates are from GenBank entry AC000048. Reaction conditions for the standard bisulfite polymerase chain reaction (PCR) and methylation-specific primers PCR (MSP-PCR) were as described (Huschtscha *et al*, 1998).

DNA was successfully extracted from 45 pairs of specimens, of which 41 were constitutionally heterozygous for D9S942. In all nevus samples bands corresponding to each allele were seen at equal intensity to matching somatic DNA, indicating no LOH. No nevus had acquired a somatic change in the protein-coding region of *CDKN2A*, although four individuals carried germline *CDKN2A* variants. Individual 22 was homozygous for the nucleotide 442 G to A variant (ala148thr), and individuals 29 and 43 were heterozygous for this polymorphism. One case possessed a heterozygous nucleotide 412 A to G mutation (arg138gly) (**Fig 1**). To our knowledge this is the first report of an individual with a germline *CDKN2A* mutation who does not have melanoma or a family history of melanoma.

No hypermethylation of the *CDKN2A* promoter, which might lead to loss of p16 expression in the nevus samples, could be

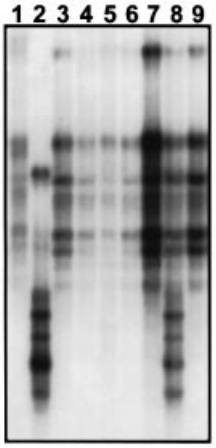

Figure 1. Autoradiograph of a representative SSCP gel showing aberrant band mobility for exon 2 of *CDKN2A*. *Lane 1*, sample 21 (heterozygous for arg138gly mutation); *lane 2*, sample 22 (homozygous for ala148thr polymorphism); *lanes 3*–7 and *9*, control samples (homozygous for wildtype alleles); *lane 8*, sample 29 (heterozygous for ala148thr polymorphism).

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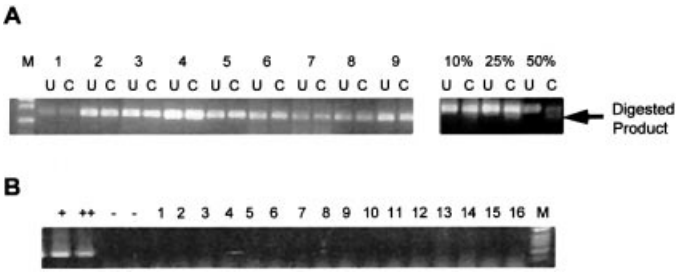

Figure 2. Methylation analysis of the p16 promoter region. (*A*) DNA from nevus samples (1–9) was bisulfite-treated and amplified with primers that amplify methylated and unmethylated DNA in proportion. The PCR was either undigested (U) or digested with *Taq*I (C). Methylated p16 DNA was amplified as a control from bisulfite-treated human DNA that had been spiked at 10%, 25%, and 50% levels (Warnecke *et al*, 1997). *Lane M*; DNA size markers. (*B*) DNA from nevus samples (1–16) was bisulfite-treated and amplified using MSP-PCR primers. Methylated p16 DNA was amplified as a control from bisulfite-treated human DNA that had been spiked at 0.1% (+) and 1% (++) and not spiked as a negative control (–).

detected in any of the 40 nevi tested, either by *Taq*I digestion (**Fig 2*a***) or by MSP-PCR (**Fig 2*b***).

Our results suggest little or no involvement of genetic or epigenetic alterations of *CDKN2A* in nevus etiology, and thus support the data of others who have failed to detect *CDKN2A* mutation (Healy *et al*, 1996b) or methylation (Gonzalgo *et al*, 1997) in various types of nevi; however, our findings do not rule out mutations in noncoding regions nor factors other than methylation affecting expression of this gene. Alternatively, variations in noncoding regions of *CDKN2A* or in a gene adjacent to *CDKN2A* may account for the variance in nevus count we have shown to be linked to this region (Zhu *et al*, 1999).

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