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 melonin 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 nevus, 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-HCl, pH 8.3; 50 mM KCl; 2.5 mM MgCl2 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 promoter and exon 1 (nucleotides 28602–28856 in GenBank entry AC000048), was screened for methylation using the bisulphite sequencing method under conditions described in Clark et al. (1999). The control primers used for the standard bisulphite 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 bisulphite 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 sequenced 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 detected.

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).
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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REFERENCES

Hayward NK: The current situation with regard to human melanoma and genetic inferences. Cur Opin Oncol 8:136±142, 1996

Figure 2. Methylation analysis of the p16 promoter region. (A) DNA from nevus samples (1–9) was bisulphite-treated and amplified with primers that amplify methylated and unmethylated DNA in proportion. The PCR was either undigested (U) or digested with TaqI (C). Methylated p16 DNA was amplified as a control from bisulphite-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 bisulphite-treated and amplified using MSP-PCR primers. Methylated p16 DNA was amplified as a control from bisulphite-treated human DNA that had been spiked at 0.1% (+) and 1% (++) and not spiked as a negative control (−).
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