CDKN2A Mutation and Deletion Status in Thin and Thick Primary Melanoma

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INTRODUCTION

The incidence of malignant melanoma is rising in developed countries. Investigations of potential tumor suppressor genes involved in melanoma formation have shown either hemizygosity or LOH for DNA markers within chromosome 9p21 in 34–86% of melanoma cell lines and tumors examined by several authors (1–7).

One gene within this deleted region is CDKN2A, which can produce two independent transcripts through the alternate splicing of a separate exon 1. One transcript encodes the cdk inhibitor protein, p16INK4A (p16). The p16 protein binds to and blocks the catalytic activity of two cyclin D-bound, cdk4 and cdk6. cdk4 is able to phosphorylate the retinoblastoma (pRb) protein, leading to the release of associated proteins that activate genes necessary for progression from the G1 to the S-phase of the cell cycle. It has been directly demonstrated that melanoma-associated mutant p16 proteins are defective in their ability to inhibit the catalytic activity of the cyclin D1/cdk4 complex (8–12). Loss of functional p16 protein may permit excessive cdk4 activity and therefore represents loss of a regulatory constraint on progression through the cell cycle. In addition, experiments have shown that wild-type p16 can prevent cellular transformation by H-ras and myc oncogenes (13). Thus, by these mechanisms, CDKN2A can function as a tumor suppressor gene (14–18).

The mRNA for p14ARF is generated by transcriptional splicing of exons 2 and 3 shared by CDKN2A with an alternative exon 1 (E1b; Refs. 19–21). The protein p14ARF is translated in a different reading frame from p16 and acts on a completely different pathway, the regulation of the p53 tumor suppressor. Binding of p53 to hdm2 results in its translocation to the cytoplasm and degradation; p14ARF binds to hdm2, sequesters it in the nucleolus, and thereby enables p53 levels to stabilize and rise in appropriate conditions (22).

We have performed mutational analysis of the CDKN2A gene by SSCP analysis on 39 cases of sporadic primary cutaneous malignant melanoma. Of the 11 studies to date reporting somatic CDKN2A mutations in melanoma, only five involved primary sporadic melanomas and in all of these the lesions examined were either >0.75 mm in Breslow thickness (23–25) or of unspecified thickness (26, 27). Breslow thickness is the most powerful histopathological predictor of melanoma progression currently known (28). None of the studies in which CDKN2A mutations were detected in primary melanoma examined tumors <0.75 mm thick (23, 24). Most have been on involved in the progression rather than initiation of sporadic malignant melanoma.

ABSTRACT

Human melanoma cell lines and tumor tissue from familial and sporadic melanomas have frequent, nonrandom chromosomal breaks and deletions on chromosome 9p21, a region that includes the tumor suppressor gene CDKN2A/p16INK4A. Germ-line mutations within this gene have been observed in some familial melanoma kindreds, but somatic mutation in sporadic primary melanoma is infrequent. Thirty-nine archival, paraffin-embedded, sporadic, primary cutaneous malignant melanomas (20 >3-mm-thick and 19 <0.75-mm-thick cases) were examined for mutations of the CDKN2A gene using single-strand conformational polymorphism analysis and direct sequencing. No mutations were detected. Loss of heterozygosity for the 9p21 microsatellite marker D9S942 was detected in 6 of 17 informative thick lesions (35%) but 0 of 18 thin lesions (P = 0.006). These results support other studies indicating that intragenic mutation is an infrequent mechanism of CDKN2A inactivation in primary melanoma. The finding of loss of heterozygosity for the 9p21 microsatellite D9S942 in thick but not thin primary melanoma suggests that deletion or inactivation of CDKN2A or other tumor suppressor gene(s) at this locus is

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4 The abbreviations used are: LOH, loss of heterozygosity; cdk, cyclin-dependent kinase; SSCP, single-strand conformational polymorphism.
metastatic tumors (3, 17, 29–32). To contribute information on the relative role of CDKN2A at different stages of primary melanoma tumorigenesis, we stratified our cases into two groups according to Breslow thickness and Clark level. LOH at the CDKN2A locus was assessed to determine whether there were different rates of deletion in thin versus thick melanomas and whether these rates were in line with those reported previously for sporadic primary melanoma.

**MATERIALS AND METHODS**

**Melanoma Cases.** Twenty sporadic malignant melanomas of Breslow thickness 3 mm/Clark’s level IV or thicker and 19 sporadic melanomas of Breslow thickness 0.75 mm/Clark’s level III or thinner were retrieved from the Scottish Melanoma Group archives over a 3-year period from 1993 to 1995. The main clinical and histopathological features of the two groups are summarized in Table 1. All cases had been routinely processed and paraffin embedded. Two 10-μm sections of tumor and normal skin for control were cut from the paraffin blocks in each case. Using a fresh scalpel, tumor tissue in each 10-μm section was carefully dissected from normal tissue with the aid of a dissecting microscope. Comparison to directly adjacent H&E-stained sections was used to minimize cross contamination of tumor with normal tissue, which may mask mutation detection (example dissection in Fig. 1). It is estimated after light microscopic examination of the poststained, dissected slides that contamination of dissected tumor tissue by normal stromal cells was <25% for thin lesions and <10% for thick lesions.

**DNA Preparation and PCR.** The sections were deparaffinized by sequential exposure to 600 μl each of xylene/100% ethanol/100% ethanol in 1.5-ml Eppendorf tubes with 10 min microcentrifugation at 13,000 rpm between each stage. The tissue was then incubated overnight at 55°C in 400 μl of lysis buffer [10 mM Tris (pH 8.3), 50 mM KCl, 0.45% Tween 20, and 2.5 mM MgCl₂] with 0.5 mg of proteinase K (Boehringer Mannheim, Basel, Switzerland), followed by boiling for 30 min to destroy proteinase K activity. A 10–30-μl DNA aliquot was then subjected to PCR in 10 μl of PCR buffer (Life Technologies, Inc., Gaithersburg, MD), 25 pmol of each primer, 50–75 nmol of MgCl₂ and 3 units of Taq DNA polymerase to a final volume of 100 μl. Because no disease-related mutations have been recorded in the 11-bp coding region of exon 3, this very short sequence was not examined. Four overlapping sets of primers were chosen to span all of exons 1 and 2, yielding PCR products of 203 bp or less for each set, to maximize the sensitivity of mutation detection by subsequent SSCP. PCR reactions were performed on an Omnigene (Hybaid, Middlesex, United Kingdom) automated thermal cycler. A preliminary 15-min hot start was followed by 30–40 cycles of denaturation at 94°C for 1 min, annealing for 1 min, and extension at 72°C for 2 min, with further extension for 10 min at the termination of the reaction. Primer sequences were manually and computer-matched against the CDKN2A gene sequence published by Okamoto et al. (33), which includes corrections of sequence published previously by Kamb et al. (2) and Serrano et al. (14). Primers used were: CDKN2A exon 1 sense, 5’-GGGAGCAGCATGGAGCCG- 3’ (13); CDKN2A exon 1 antisense, 5’-AGTGCAGCCGCATCCCA-3’ (13), annealing temperature (Tₘ = 63°C); CDKN2A exon 2a sense, 5’-CCTGGCTCTGCACCATTCTGT-3’ (novel, intronic); CDKN2A exon 2a antisense, 5’-GGGACGCCTGCTGACGGGT-3’ (novel, intronic); CDKN2A exon 2b sense, 5’-AAGTGACCGACGC-3’ (novel); CDKN2A exon 2b antisense, 5’-CCAGCTCTCCAGCCAGGTCCAGGG-3’ (novel, Tₘ = 54°C); CDKN2A exon 2b sense, 5’-GCATGCCGCGACGC-3’ (13); CDKN2A exon 2b antisense, 5’-GTA-3’ (novel, intronic; Tₘ = 55°C). Products were also submitted to specific restriction enzyme analysis to confirm they contained only CDKN2A sequence and not CDKN2B.

**Mutation Controls.** DNA was prepared from peripheral blood leukocytes derived from familial melanoma kindreds with known germ-line mutations (34). The DNA was amplified under...
identical PCR conditions to the test samples, and the products were used as positive controls for subsequent SSCP screening.

**SSCP Analysis.** Five to 10 µl of each PCR product were mixed with an equal volume of denaturing solution, heat denatured at 95°C for 3 min, and snap chilled in ice. Samples were loaded onto nondenaturing 10% 37.5:1 MDE polyacrylamide gels (Amersham Pharmacia Biotech, Little Chalfont, Bucks, United Kingdom), containing 5% glycerol and 0.7× 0.09 m Tris-Borate, 0.002 m EDTA (pH 8.0). Duplicate gel runs were performed at 25°C. Electrophoresis was in 1× TBE at 25 W for 90–120 min. DNA bands were visualized using standard commercial silver staining protocol and reagents (Bio-Rad Laboratories, Hemel Hempstead, Herts, United Kingdom) according to the method of Holland et al. (34).

**DNA Sequencing.** All CDKN2A PCR products with atypical band migration on SSCP were subjected to direct fluorescent dideoxy sequencing and analyzed in a Perkin-Elmer ABI 373 sequence analyzer (Merck, Poole, United Kingdom). In addition, two randomly selected products from each of the four sets of primers that showed normal migration on SSCP were sequenced as controls.

**LOH Analysis.** D9S942 is a highly polymorphic microsatellite locus located within 20 kb of the CDKN2A gene (35). PCR was performed using a 5’HEX-labeled forward primer in conditions carefully optimized to ensure that alleles of all sizes were amplified consistently. Twenty to 100 ng of DNA from tumor were added to 10 µl of a reaction mixture of 10 mM Tris-HCl (pH 10.0), 2 mM MgCl₂, 50 mM KCl, 5 pmol of primers, and 0.2 unit of Taq DNA polymerase. The PCR protocol used was 95°C for 5 min; 5 cycles of 95°C for 30 s, 48°C for 20 s, and 72°C for 20 s; followed by 40 cycles of 95°C for 20 s, 48°C for 15 s, and 72°C for 5 min; with a final extension phase of 72°C for 5 min. An equal volume of denaturing sample dye was added to the PCR products; the samples were heated to 95°C for 3 min and snap chilled before loading onto an 8% 29:1 Tris-Borate, 0.002 M EDTA (pH 8.0). Duplicate gel runs were performed at 25°C. Electrophoresis was in 1× TBE at 25 W for 90–120 min. DNA bands were visualized using standard commercial silver staining protocol and reagents (Bio-Rad Laboratories, Hemel Hempstead, Herts, United Kingdom) according to the method of Holland et al. (34).

**RESULTS**

As shown in Table 1, the thick (average, 5.5 mm) melanomas were more frequently from older males, nodular and with higher mitotic indices than the thin melanomas (average, 0.7 mm). Tumor-infiltrating lymphocytes were equally frequent in the two groups. No consistent SSCP anomalies were detected in 20 thick and 19 thin melanomas examined. Mutations were readily detected in all four positive control samples by SSCP, and all of the PCR products checked by direct sequencing showed wild-type sequence (data not shown).

Seventeen of 20 thick melanomas and 18 of 19 thin melanomas were informative at the D9S942 locus. Using the criteria for assessment of LOH cited above, 6 of 17 (35%) of the informative thick lesions showed LOH for this marker (example in Fig. 2), whereas no informative thin lesions showed LOH ($\chi^2 = 8.1; P = 0.005$). Thick lesions exhibiting LOH differed from thick lesions without LOH in that Clark level 5 invasion (2 of 6 and 2 of 11, respectively), nodularity (5 of 6 versus 6 of 11) and males (4 of 6 versus 6 of 11) appeared to be more frequent in the former group; however, none of these differences reached significance. Average Breslow thickness was identical in these two LOH-defined groups, and there were no significant differences in age, sex, type, mitotic count, or presence of tumor-infiltrating lymphocytes.

**DISCUSSION**

Our results are in agreement with the findings of most others in that CDKN2A is mutated very infrequently in sporadic primary melanoma (25–27, 29, 37–39). However, along with a recent study (39), which discriminated less sharply between thin and thick primary melanomas, we have shown that there is a significantly higher frequency of LOH at CDKN2A in thick melanomas.

SSCP screening for mutation detection has a false-negative rate of 10–20%. The CDKN2A open reading frame is comprised of nearly 75% G or C residues, a trait that further complicates mutation detection by SSCP. However, false-negative SSCP assays are unlikely to have eliminated detection of all mutations or to have obscured a true difference between the two tumor groups in this study. Mutations were detected by SSCP in all positive controls and wild-type sequence confirmed by direct sequencing of the products of all four primer sets in a sample of tumors. Another possible explanation for reports of low mutation frequency is that contamination by nontumor cells may mask mutation detection, especially in archival material (26). Careful microdissection of tumor from normal tissue minimizes but cannot eliminate this risk (Fig. 1). Given that we did not
detect any CDKN2A mutations, we cannot comment on whether CDKN2A mutation is an early or late event in the development of melanomas in which it is present. Considering alternative mechanisms to mutation for p16 inactivation, certain factors may influence the production of functional p16 protein at a transcriptional or translational level. It has been proposed that regulatory sequences upstream of exon 1 may be mutated or deleted, rendering the gene inactive (18, 40). In fact, Liu et al. (41) have demonstrated a mutation in the CDKN2A 5′ untranslated region that creates an absent initiation codon and is associated with melanoma risk. Methylation of a CpG island upstream of exon 1 has been shown to be associated with transcriptional silencing of the CDKN2A gene (42). A quantitative reduction in p16 protein due to loss of a single allele may also be influential in modifying cell cycle control (26, 37, 43).

One reason for the discrepancy between the low rates of CDKN2A mutation and the high prevalence of loss of expression of the p16 protein in tissues of advanced melanoma is that the gene may be inactivated by homozygous deletion (2, 4, 13, 16, 17, 25, 27, 31, 32, 39, 43, 44, 45). Although the significance of CDKN2A as a melanoma susceptibility and tumor suppressor locus is not in doubt, controversy regarding the importance of CDKN2A inactivation in melanoma remains. At the heart of this lies discordance between the high rate of homozygous deletion in melanoma cell lines [57 and 63% in two studies (2, 44)] and the low rate of homozygous deletion in melanoma tumors [0–20% in five studies (17, 27, 32, 39, 45)]. Just as mutation of CDKN2A has been attributed to cell culture artifacts or the failure to detect mutation in uncultured melanomas blamed on contamination by stromal cells, so have these issues been cited with regard to homozygous deletion. Although this issue awaits clarification, the importance of homozygous deletion as a method of CDKN2A gene inactivation may be that there is a concomitant requirement for inactivation of another closely linked gene to permit melanoma development or progression. This would favor a single multilocus deletional event over the simultaneous occurrence of inactivating point mutations occurring in two or more genes (13). Our finding of no CDKN2A mutations but LOH for D9S942 in 35% of thick melanomas examined does not prove the necessity of contiguous deletion of CDKN2A and neighboring tumor suppressor gene(s) in melanoma progression but would certainly be consistent with it.

In our study, 35% of thick melanomas showed 9p21 deletions that may affect both p16 and p14ARF function, if there were a dosage effect from loss of one allele, or concomitant inactivation of the second allele by a second event such as mutation, methylation, or deletion. This would be predicted to confer a greater selective advantage over a mutation affecting a single gene, because both of the central cell cycle control pathways, mediated by Rb and p53, would be affected by such a deletion.

In summary, our results suggest that CDKN2A mutation is rare in primary melanomas both thinner and thicker than 0.75 mm, which supports the concept of a limited role, if any, for mutation of this gene in the early development of sporadic melanoma. Previous studies of sporadic primary melanoma that have reported CDKN2A mutation have all been performed on melanomas either exceeding 0.75 mm in thickness or of unspecified thickness, whereas the tumors studied here were specifically stratified by Breslow thickness. The observed difference in frequency of 9p21 LOH between thick and thin melanomas is suggestive of a dosage effect from loss of one CDKN2A allele or of another putative tumor suppressor gene in this chromosomal region in advanced primary, but not early primary, melanoma. A corollary of this conclusion is that although CDKN2A is important in further melanoma progression, the earliest genetic insult(s) that permits initiation of the malignant phenotype occur outside the 9p21 region altogether. Alternatively, because five of the six thick tumors with LOH at D9S942 were nodular but none of the thin tumors were, we cannot exclude the possibility that the different rates of LOH observed in thick and thin melanomas may reflect different genetic pathways involved in the pathogenesis of nodular versus superficial spreading or lentigo maligna melanoma.

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