Detection of p53 Mutations in Benign and Dysplastic Nevi

David B. Levin,2 Kenneth Wilson, Giovana Valadares de Amorim, Jane Webber, Patrick Kenny, and Wolfgang Kusser

Department of Biology, Centre for Environmental Health, University of Victoria, Victoria [D. B. L., G. V. d. A., J. W., W. K.]; British Columbia Cancer Agency, Victoria [K. W.]; and University of British Columbia, Vancouver [W. K.].

Abstract

We have examined melanocytic cells derived directly from fresh biopsy tissue for the presence of p53 mutations. Using selective media that permits growth of melanocytes and inhibits growth of fibroblasts and keratinocytes, we established short-term, primary cultures of melanocytes from skin biopsies of common acquired nevi, dysplastic nevi, and from metastatic melanoma. Using PCR-single-stranded conformational polymorphism analysis, we have detected p53 mutations in 2 of 11 benign compound nevi and 2 of 5 dysplastic nevi. All nevi positive for p53 mutations were derived from patients who previously had cutaneous moles and three of the four had a family and/or personal history of melanoma.

Introduction

Melanoma is thought to develop through a multistep process characterized by distinct histopathological stages (1). The first stage, the common acquired nevus, consists of a focal proliferation of morphologically normal cutaneous melanocytes. Dysplastic nevi, which constitute the second stage, exhibit cytological and morphological abnormalities. Neoplastic transformation of melanocytes begins with an initial phase of radial growth, which may give rise to vertical growth, invasiveness, and metastasis.

The molecular mechanisms by which malignant transformation of melanocytes occur are not well understood. The most common genetic aberrations in human malignancies are point mutations in the tumor suppressor gene p53. Mutations in the p53 gene can alter the presence, frequency, and role of p53 mutations at different stages in the development of melanoma (4–10).

To evaluate the role of p53 in the development of melanoma, we analyzed p53 mutations in different stages of melanoma tumourigenesis. Using selective media that eliminate fibroblasts and keratinocytes, we established primary cultures of melanocytes from skin biopsies of 11 common acquired (benign) nevi and 5 dysplastic nevi, as well as from biopsies of 8 metastatic melanoma. Exons 5 to 9 of the p53 gene were amplified by PCR directly from genomic DNA of the cultured melanocytes, the PCR products were screened for the presence of mutations by SSCP (Single-Stranded Conformational Polymorphism Analysis), and the putative mutations were identified by DNA sequence analysis of the cloned PCR products.

Materials and Methods

Biopsy Specimens. Biopsy specimens of benign and dysplastic nevi were provided by two practicing dermatologists in Victoria, British Columbia: Dr. P. Kenny, FRCP, and Dr. G. Telford, FRCP. Needle aspirate biopsies of metastatic melanoma were provided by Dr. K. Wilson, FRCP, at the British Columbia Cancer Agency, Victoria Clinic. Newborn foreskin was supplied by Dr. J. Popkin, FRCP, Victoria General Hospital, Victoria, British Columbia. The excised specimens were washed briefly in 70% ethanol, rinsed in sterile PBS, placed in MCDB-153 medium containing antibiotics (11), stored at 4°C, and transported to the laboratory within 24 h.

Primary Culture of Melanocytes from Benign and Dysplastic Nevi and Metastatic Melanoma. Melanocytes derived from benign and dysplastic nevi, as well as from neonatal foreskin (which served as a “normal” melanocyte control), were cultured as follows (11). Biopsy tissues were transferred to sterile PBS containing 0.25% trypsin and incubated at 4°C for 48 h. Tissues were then placed into MCDB-153 medium (GIBCO-BRL, Grand Island, New York) in sterile 25-cm² culture flasks and incubated at 37°C in the presence of 5% CO₂. The MCDB-153 medium was supplemented with 0.9 ng/ml basic fibroblast growth factor (GIBCO-BRL), 5 μg/ml insulin (GIBCO-BRL), 5 μg/ml transferrin (GIBCO-BRL), 1 μg/ml α-tocopherol (Sigma Chemical Co., St. Louis, MO), 30 μg/ml crude pituitary extract (GIBCO-BRL), 0.5 μg/ml hydrocortisone (Sigma), and 5% heat-inactivated fetal bovine serum (GIBCO-BRL). Eight mg 12-O-tetradecanoylphorbol-13-acetate (Sigma) and 20 μg/ml catalase (GIBCO-BRL) were added to the first two passages. Fibroblasts were eliminated by incubating the cultures 3 to 4 days in the presence of 100 μg/ml gentamicin (GIBCO-BRL). Cells derived from metastatic melanoma proliferate in the absence of any exogenous growth factor (12) and thus were cultured in the same medium as defined for normal and dysplastic melanocytes without basic fibroblast growth factor.

PCR Amplification of p53 Exons 5 to 9. PCR amplification of p53 exons 5 to 9 from genomic DNA was performed using established protocols (13, 14). Primers were synthesized, which permit the recovery of these exons plus the neighboring intron sequences. The primer sequences were analyzed by specialized software (OLIGO) for optimum amplification. One-tenth of the PCR reaction volume was subjected to electrophoresis on 1% agarose gels containing 250 ng/ml ethidium bromide to confirm success of the DNA amplification. The remainder of the sample was washed in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) in Centricon-100 (Amicon Corp., Beverly, MA) tubes to remove the primers and concentrate the product.

End-labeling of PCR Products and SSCP. PCR products of p53 exons 5 to 9 from melanocytes of neonatal foreskin, common and dysplastic nevi, metastatic melanoma, as well as positive control (1067, a PCR fragment derived from a bladder carcinoma biopsy containing a known mutation in exon 6) were end-labeled as follows. [γ-32P]ATP (0.5 μC) (New England Nuclear, DuPont Canada, Ltd.) was mixed with 11 units of T4 polynucleotide kinase (Pharmacia, Upsala, Sweden) in 5× T4 kinase buffer (50 mM Tris-Cl pH 9.5, 10 mM MgCl₂, 5 mM DTT, and 5% glycerol) in a ratio of 1:2:2, respectively. An equal volume of label mixture and sample (2.5 μl) were incubated at 37°C for 1 h. One μl of each sample was subjected to nuclease acid precipitation with trichloroacetic acid (15) and counted in a scintillation

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2 To whom requests for reprints should be addressed, at Department of Biology, Centre for Environmental Health, University of Victoria, P.O. Box 1700, Victoria, British Columbia, V8W 2Y2 Canada.
3 The abbreviations used are: SSCP, single-strand conformational polymorphism analysis; FRCP, fellow of the Royal College of Physicians of Canada.
counter. The labeled PCR products were then mixed with SSCP loading dye (95% deionized formamide, 10 mM EDTA, and 0.01% SDS) in a ratio of 1:2 and if necessary, diluted further in SSCP loading dye to equalize the number of copies in each sample. Samples were denatured (95°C for 15 min), immediately placed in liquid nitrogen for 10 s, and then placed on ice until loaded on a 24-cm gel. MDE polyacrylamide gel (Hydrolink MDE; J. T. Baker, Inc., NJ). Electrophoresis was conducted in SSCP TBE running buffer (54 mM Tris-borate, 1.2 mM EDTA, pH 8.3) for approximately 4 h at 20°C at 50 W (16, 17). After electrophoresis, gels were transferred to Whatman 3M filter paper and dried. DNA bands were visualized by autoradiography following standard procedures (15). The intensity of the bands on the autoradiograms was determined by scanning densitometry with an LKB Laser Densitometer.

Molecular Cloning of Putative Mutants and DNA Sequence Analysis. PCR products of putative mutants were cloned into a pBlueScript SK+ plasmid (Stratagene Corp., La Jolla, CA) that had been modified to facilitate cloning of PCR products generated by Taq DNA polymerase. The vector was cut at the EcoRV site, and diTPP was added to the 3'-ends with TdT. Ligation reactions (15) were incubated for 1 h at 16°C. Escherichia coli strain XLl-Blue (Stratagene Corp.) was transformed with the ligation products by standard procedures (15) and plated on Luria Broth/agar plates containing ampicillin (0.05 mg/ml), tetracycline (0.0125 mg/ml), X-Gal (0.05 mg/ml) and isopropylthio-ß-D-galactopyranoside (0.5 mM). Plasmid DNAs were extracted from recombinant colonies by the manufacturer's instructions (Stratagene).

a "cycle sequencing" protocol using fluorescent primers (Table 1) according to the manufacturer’s instructions (Pharmacia Corp., Upsala, Sweden). Both strands of each cloned fragment, and at least five clones of each putative mutant, were sequenced. Sequencing reactions were carried out with 100–200 ng of plasmid template in a "cycle sequencing" protocol using fluorescent primers (Table 1) according to the manufacturer’s instructions (Stratagene).

Results

Selective Culture of Melanocytes from Skin Biopsies. We successfully established short-term cultures of melanocytes from neonatal foreskin, common and dysplastic nevi, as well as from metastatic melanoma. Pure cultures of melanocytes were obtained after approximately 10 days in culture. Melanocytes derived from newborn foreskin were typically spindle-shaped and bipolar (Fig. 1A). The majority of cells were elongate and narrow, with large nuclei surrounded by melanin and sharply defined dendrites. Melanin was also visible in melanophores distributed along the dendrites (Fig. 1A, mel). These cells proliferated well in vitro, growing to confluence in about 10 days, and underwent several passages. Similar morphologies were observed in cultures of melanocytes from common nevi (Fig. 1B), but a small proportion of tripolar and multipolar cells were also observed. Melanocytes from dysplastic nevi (Fig. 1C) were entirely multipolar. Melanocytes from common and dysplastic nevi did not proliferate extensively, undergoing only two to three cell divisions, resulting in 100 to 1000 cells/flask, depending upon the sample. Melanocytes from metastatic melanoma (Fig. 1D) consisted of a mixture of pigmented and nonpigmented cells with varying morphologies. Some cells were round, darkly pigmented, and grew only in suspension (Fig. 1D, drc). Other cells that attached to the culture flask were bipolar or multipolar with extremely long, sinuous dendrites that appeared to lack melanophores. Many of these cells were binucleated (Fig. 1D).

PCR Amplification of p53 Exons and SSCP. The expected sizes of amplified p53 exons 5 to 9 were obtained (Reference 14: exon 5, 252 bp; exon 6, 224 bp; exon 7, 237 bp; exon 8, 288 bp; exon 9, 221 bp) and subjected to SSCP. We detected bands with altered electrophoretic mobility, indicative of point mutations, in 2 of 11 (18%) benign compound nevi, 2 of 5 dysplastic nevi (40%), and 1 of 8 (12.5%) metastatic melanoma (Fig. 2). All four nevi that were positive for p53 mutations were derived from patients who previously had moles removed from their skin, and three of these four had either a personal history or a family history of melanoma (Table 2; see discussion below). The band shifts detected in melanocytes from benign and dysplastic samples were faint in comparison to the wild-type bands, less than 10% of wild-type band intensity as determined by scanning densitometry (data not shown), suggesting that only a subset of cells carried mutated p53 sequences. No band-shifts were detected in p53 exons derived from cultured neonatal foreskin.

DNA Sequence Analysis. The mutations detected by DNA sequence analysis of the mutant p53 exons are presented in Table 2. Melanocytes derived from one benign compound nevus contained a A → G transversion in p53 exon 6 (codon 222). This was a silent mutation (CCG → CCC) that does not alter the amino acid sequence of the P53 protein. Melanocytes derived from the other benign compound nevus showed a deletion of a G-residue in p53 exon 8 (codon 302) plus an A → G transition in the intron preceding exon 8 (position 14,395 of the p53 sequence). The deleted G of codon 302 induces a frame-shift mutation, which would result in an altered P53 protein. Melanocytes derived from dysplastic nevi also had two mutations each. One dysplastic nevus had a deleted G-residue (codon 144) in exon 5 (frame-shift) plus a G → T transversion in the intron between exon 5 and exon 6 (position 13,249 of the p53 sequence), while the other dysplastic nevus had T → G transversions both within exon 5 (codon 166, TCA → GCA, SER → ALA) and in the intron between exon 5 and exon 6 (position 13,275 of the p53 sequence), and a deleted C-residue within exon 5 (codon 138, frame-shift). The mutation detected by SSCP in the metastatic melanoma sample was revealed by DNA sequence analysis to reside within the intron sequence of exon 8.

Discussion

We have detected p53 mutations in melanocytes from benign and dysplastic nevi derived from patients with either a personal history of atypical nevi or melanoma or with a family history of melanoma. Although our sample size is small, our results suggest that p53 mutations may occur in melanocytes from benign and dysplastic nevi and that these mutations may be an early event in the development of melanoma in some patients who may have a particular genetic background. It is of interest to note that all samples that were positive for p53 mutations were derived from female patients between 27 and 32 years of age, all four nevi were derived from patients who had previously had moles removed from their skin, and three of the four had a personal (1) or a family history of melanoma (2), both of which are established risk factors for malignant melanoma. The malignant potential of these benign and dysplastic lesions, however, is speculative because they were removed.

Table 1. Nucleotide sequence of primers and primer positions

<table>
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<tr>
<th>Primers</th>
<th>Nucleotide sequence</th>
<th>Position</th>
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<td>p53 exon 5</td>
<td>5' - TTTCACAATCTGCTCTCTTCAC-3'</td>
<td>13024-13043</td>
</tr>
<tr>
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<td></td>
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<tr>
<td>Reverse</td>
<td>5' - GAGAGACGAGCGGCTGTGT-3'</td>
<td>13256-13275</td>
</tr>
<tr>
<td>p53 exon 6</td>
<td>5' - GCCAGGGTCCCCGCGGCCTC-3'</td>
<td>13276-13295</td>
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<tr>
<td>Reverse</td>
<td>5' - GCTGGCTACTGGCAGGCCC-3'</td>
<td>13476-13495</td>
</tr>
<tr>
<td>p53 exon 8</td>
<td>5' - AAAGGCGACCAGCGGTGGTG-3'</td>
<td>14346-14365</td>
</tr>
<tr>
<td>Forward</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>5' - GAGGAGGACCGAGTGCGCAG-3'</td>
<td>14614-14633</td>
</tr>
<tr>
<td>M13</td>
<td>5' - CGCCAGGGTTTCCCCAGTCAGC-3'</td>
<td></td>
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</tr>
<tr>
<td>Reverse</td>
<td>5' - CAGGAAAACGGCTTGAG-3'</td>
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patient with a personal history of both moles and melanoma (two previous melanomas removed), and the other was derived from a patient who had a personal history of atypical nevi and a family history of melanoma (mother, maternal grandmother, and maternal grandfather). Of the two dysplastic nevi, both were derived from patients with previous moles removed, and one patient had a family history of melanoma. The metastatic melanoma sample was derived from an elderly patient (84 years of age) with no personal or family history of moles or melanoma.

Prior immunohistochemical studies have provided evidence for elevated levels of p53 protein in both primary and metastatic melanomas (4–10). However, there are discrepancies in the data concerning the presence of p53 mutations in malignant and metastatic melanoma tissues. Four studies (4–7) found a high percentage of primary and metastatic melanomas (97, 92, 60, and 47%, respectively) have p53 immunoreactive cells, suggesting mutations in p53 occur frequently in these stages of tumorigenesis. However, three studies (8–10) found significantly lower percentages of p53 immunoreactivity in primary melanoma (30, 27, and 5%, respectively) and higher frequencies of p53 immunoreactivity in metastatic melanoma. Christofolini et al. (9) detected immunoreactive p53 in 27% primary melanomas versus 85% of metastatic melanomas. Lassam et al. (10) detected p53 immunoreactive cells in 5% of malignant melanoma versus 20% of metastatic melanoma samples. These data suggest that p53 mutation is a late event in the development of melanoma.

These two studies (9, 10) also attempted to detect p53 immunoreactive cells in premalignant melanocytes from common acquired (benign) and dysplastic nevi. Lassam et al. (10) did not detect p53 immunoreactive cells in benign and dysplastic cells, supporting the idea that p53 mutations occur late in the development of melanoma. Christofolini et al. (9), on the other hand, detected p53 immunoreactive cells in 12 of 75 (16%) of benign nevi (as well as in 27% of primary melanomas and 85% of metastatic melanomas), suggesting that p53 mutations may also occur very early in the development of melanoma.

These discrepancies may be explained in part by the variety of methodologies used in the experiments. Tissue studied ranged from fresh frozen sections to formalin-fixed, paraffin-embedded biopsies that were up to 13 years old, to melanoma cell lines. Formalin-fixed tissues are less reactive to antibodies than alcohol-fixed or frozen.
sections (7), and different antibodies have different sensitivities (8). Moreover, the p53 protein may complex with various cellular proteins that can impair the binding of antibodies in different ways (19). Thus, immunostaining for p53 proteins with altered stability may not reflect the presence of p53 mutations.

Furthermore, there is poor concordance between the high percentage of p53-positive cells detected by immunohistochemistry and the frequency of mutations detected in the p53 gene. Castresana et al. (20) failed to detect any genetic alterations in 51 formalin-fixed, paraffin-embedded malignant melanoma sections examined by PCR-SSCPA. Volk and et al. (21) examined nine metastatic melanoma cell lines and two melanocyte cell lines for point mutations in the p53 gene by reverse transcription-PCR and direct DNA sequencing and found that only one of the nine metastatic melanoma cell lines had a point mutation (a C → T transition at codon 248 in exon 7). Weiss et al. (8) detected p53-immunoreactive cells in 12 of 13 melanoma cell lines. However, the p53 protein could be immunoprecipitated from only 4 of the 12 reactive cell lines, and only these 4 cell lines contained mutations in the p53 gene. All four mutations were C → T transitions in exon 6 (codon 220), exon 7 (codon 241), or exon 8 (codon 278). Fodstad et al. (22) detected loss of heterozygosity (46%) and point mutations (23%) in malignant melanomas, whereas Dockhorn-Dworniczak et al. (23) detected p53 mutations in only 1 of 49 primary melanoma tumors (this tumor had two point mutations in p53).

To date, no genetic events, inherited or acquired, have been universally associated with melanoma development. Many genetic pathways appear to be involved (24), and the role of p53 mutations in the development of melanoma is not clear. In our study, p53 mutations were detected in codons 138 (ΔC, frame-shift), 144 (ΔG, frame-shift), 166 (T → G transversion, SER → ALA substitution), 222 (G → C transversion, silent mutation), 302 (ΔG, frameshift). The pattern of mutations detected in our study gave no clear "mutational fingerprint" such as observed for other skin cancers where UV sunlight exposure has been implicated (25). However, at least one mutation detected in a dysplastic nevus sample (codon 166, T → G) is consistent with the type of mutation induced by UVA radiation (26). Comparison of these mutations to p53 mutations in other cancers reveals similar frameshift mutations in codon 138 (head and neck cancers; prostate cancers) and codon 144 (breast cancer). Although mutations in codon 166 have been detected in bladder and lung cancer, these were C → T transitions. Thus, the T → G transversions detected in this study may represent a new class of mutation at this position.

The cells used in the study were subjected to short term culture in vitro, under conditions that eliminated the vast majority of other cell types (fibroblasts, keratinocytes, and infiltrating leukocytes), thus enriching for the target cells. We find it unlikely that the p53 mutations detected occurred during culture because no mutations were detected in melanocytes cultured from newborn foreskin, which proliferated to a much

<table>
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<tr>
<th>Tissue</th>
<th>Sex</th>
<th>Age</th>
<th>PHMole</th>
<th>PHMel</th>
<th>FHMel</th>
<th>Mutation</th>
<th>Codon</th>
<th>Exon</th>
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<tr>
<td>Benign compound nevus</td>
<td>F</td>
<td>31</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>G → C (CCG → CCC, silent)</td>
<td>222</td>
<td>Exon 6</td>
</tr>
<tr>
<td>Benign compound nevus</td>
<td>F</td>
<td>27</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>ΔG (Frame shift)</td>
<td>302</td>
<td>Exon 8</td>
</tr>
<tr>
<td>Dysplastic nevus</td>
<td>F</td>
<td>31</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>ΔG (Frame shift)</td>
<td>144</td>
<td>Exon 5</td>
</tr>
<tr>
<td>Dysplastic nevus</td>
<td>F</td>
<td>30</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>ΔC (Frame shift)</td>
<td>138</td>
<td>Exon 5</td>
</tr>
</tbody>
</table>

Table 2 Source of tissue, patient history, and p53 mutations detected

a PHMole, patient had personal history of mole(s); PHMel, patient had personal history of melanoma; FHMel, patient had family history of melanoma; Exon, p53 exon in which mutation was detected.

Fig. 2. SSCP analysis. A, SSCP analysis of melanocytes from control cells (foreskin; Lane 1) and dysplastic nevi (Lanes 2–5). B, SSCP analysis of melanocytes from control cells (foreskin; Lane 1), metastatic melanoma (Lanes 2, 4, and 5), and a control DNA containing a known mutation (Lane 3). M, molecular size marker, 1 kilobase ladder. Arrows, bands with altered electrophoretic mobility; double-stranded DNA.
greater extent than melanocytes from nevi (which underwent two to three cell divisions only). The number of p53 immunoreactive cells detected by Christofolini et al. (9) in benign nevi was less than 1%. Thus, only a very small number of melanocytes in a benign nevus may carry p53 mutations, which may explain why other studies failed to detect them (particularly in formalin-fixed tissues). Mutations in p53 prevent cell cycle arrest, and selective short-term culture of melanocytes from biopsy tissues may permit those few cells with p53 mutations to enjoy a growth advantage and thus increase their numbers sufficiently to permit detection of the mutations. Finally, although PCR-SSCPA is a rapid and sensitive method for the detection of mutations, it may not detect all mutations (27), and the number of premalignant nevi with p53 mutations may be underestimated.

Acknowledgments

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References

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