p53 protects against skin cancer induction by UV-B radiation

Weidong Jiang¹, Honnavara N Ananthaswamy¹, H Konrad Muller² and Margaret L Kripke^{*.1}

¹Department of Immunology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Box 178, Houston, Texas, TX 77030, USA; ²Department of Pathology, University of Tasmania, 43 Collins Street, Hobart, Tasmania 7000, Australia

To assess the role of the *p53* tumor suppressor gene in skin carcinogenesis by UV radiation, mice constitutively lacking one or both copies of the functional *p53* gene were compared to wild-type mice for their susceptibility to UV carcinogenesis. Heterozygous mice showed greatly increased susceptibility to skin cancer induction, and homozygous *p53* knockout mice were even more susceptible. Accelerated tumor development in the heterozygotes was not associated with loss of the remaining wild-type allele of *p53*, as reported for tumors induced by other carcinogens, but in many cases was associated with UV-induced mutations in *p53*. Tumors arose on the ears and dorsal skin of mice of all three genotypes, and homozygous knockout mice also developed ocular tumors, mainly melanomas. Skin tumors in the *p53* knockout mice were predominately squamous cell carcinomas and were associated with premalignant lesions resembling actinic keratoses, whereas those in the heterozygous and wild-type mice were mainly sarcomas. These results demonstrate the importance of *p53* in protecting against UV-induced cancers, particularly in the eye and epidermis.

Keywords: sunlight; photocarcinogenesis; transgenic mice; tumor suppressor gene; mutation

Introduction

The *p53* tumor suppressor gene codes for a DNA-binding protein involved in both cell cycle arrest and apoptosis. In response to DNA damage, *p53* accumulates in cells, translocates to the nucleus, activates gene transcription, and ultimately results in cell cycle arrest, which is thought to permit repair of damaged DNA prior to cell replication (Hartwell and Weinert, 1989). In addition, *p53* activates a pathway of programmed cell death, presumably in cells with excessive DNA damage (Lane, 1992). Perturbation of either pathway is expected to contribute to neoplastic transformation by permitting replication or survival of cells with DNA damage. This prediction is supported by many reports of cancers in which *p53* is mutated or lost (Hollstein *et al.*, 1991) and by the high incidence of malignancies in people with Li–Fraumeni syndrome, who inherit a mutation in one allele of *p53* (Malkin *et al.*, 1990).

In UV-associated skin carcinogenesis in both humans and mice, mutations in *p53* are present with very high

frequency (Brash *et al.*, 1991; Kanjilal *et al.*, 1993). Molecular analysis of the mutations demonstrated that most occur at dipyrimidine sites in DNA and therefore are very likely to have arisen from mutations induced by UV-B (280–320 nm) radiation in the *p53* gene itself. In experimental animals, such mutations in *p53* occur early in the course of UV irradiation, before tumors are grossly detectable (Ananthaswamy *et al.*, 1997), and they have been found in chronically sun-damaged human skin as well (Nakazawa *et al.*, 1994). These observations suggest that mutations in *p53* are an important initial step in dysregulation of epidermal cell growth in the pathway leading to malignancy. However, the relative importance of this gene to the entire process of skin carcinogenesis is difficult to assess by simply examining its mutation frequency in the resulting tumors. Moreover, sunlight-associated skin cancers have not been noted to occur with increased frequency in persons with Li–Fraumeni syndrome (Malkin *et al.*, 1990).

A direct approach for testing the hypothesis that *p53* plays a critical role in preventing skin cancer induction by UV radiation is to irradiate mice lacking one or both functional copies of *p53*. The development of such knockout mice has provided a new tool for investigating the role of *p53* in the development of a variety of cancers. Both heterozygous (+/–) and homozygous (–/–) *p53* null animals develop a spectrum of spontaneous tumors, primarily lymphoid malignancies and various types of sarcomas (Donehower *et al.*, 1992; Jacks *et al.*, 1994). The frequency of these tumors is greatly enhanced by exposing the animals to a single dose of ionizing radiation (Kemp *et al.*, 1994), but even in such mice, skin cancers do not occur. In addition, in studies of skin cancer induction using a chemical initiation-promotion protocol, *p53* +/– and –/– mice did not show increased susceptibility to papilloma induction, although the progression from papilloma to carcinoma was enhanced compared to that in wild-type (+/+) mice (Kemp *et al.*, 1993). In spite of the strong association between *p53* mutations and UV-induced skin cancers, no studies to date have examined the role of *p53* in UV carcinogenesis using congenic *p53*–/– mice. Therefore, we investigated the rate of skin cancer development in UV-irradiated, C57BL/6 congenic *p53*–/–, +/–, and +/+ mice, the spectrum of tumors produced, and the loss of the functional *p53* allele in tumors from +/– mice.

Results

UV carcinogenesis in p53 +/– and +/+ mice

In a pilot experiment, 11 *p53* heterozygous and ten wild-type littermates from the sixth backcross genera-

*Correspondence: ML Kripke, Academic Programs, U.T.M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Box 147, Houston, Texas, TX 77030, USA
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tion onto the C57BL/6 strain were exposed to UV radiation. The animals were shaved weekly and exposed to an average of 13.3 kJ/m² UV-B radiation three times per week for 30 weeks. Most *p53* +/− mice developed skin or eye tumors within this period, with a median latency of 22 weeks (range=18–28 weeks), whereas no tumors developed in the wild-type mice during the 48th week of observation (Figure 1). No spontaneous tumors of internal organs developed in the +/− mice during this experiment. Table 1 (Experiment A) shows that nine of the 11 +/− mice developed tumors, six of them on more than one site, giving a total of 15 tumors. The most frequent site of occurrence was the ears (67%), followed by skin on the back, head, or forepaw (20%), and the eyes (13%). Histological analysis revealed that 33% of the tumors were squamous cell carcinomas, 40% were sarcomas, and 20% were melanomas. No papillomas developed, which is in agreement with our previous observation that papillomas are rarely induced by chronic UV irradiation of haired mice (Kripke, 1977). This preliminary study indicated that *p53* +/− mice were highly sensitive to UV carcinogenesis and that, in spite of the long latency of these tumors, they could be induced before spontaneous tumors appeared.

Figure 1 Probability of UV-induced tumor development in *p53* +/− and +/+ mice. Female mice in the 6th backcross generation onto the C57BL/6 strain were exposed on shaved dorsal skin to 13 kJ/m² UV-B three times per week for 30 weeks

UV carcinogenesis in congenic *p53* −/−, +/−, and +/+ mice

To carry out a comparative carcinogenesis experiment in mice lacking one or two functional *p53* alleles, it was necessary to produce congenic mice that differed only at the *p53* locus. We therefore backcrossed mice with the mutant *p53* allele onto the C57BL/6 parental strain. Mice in the 8th backcross generation were indistinguishable from C57BL/6 mice by isoenzyme analysis, and mice tested in the tenth backcross generation were histocompatible based on second-set skin grafting. Cohorts of −/−, +/−, and +/+ littermates from the 12th backcross generation were exposed to UV-B radiation three times per week for 30 weeks, beginning at 12th week of age and continuing until the mice developed tumors or died. The dose was reduced to 5 kJ/m² UV-B in this experiment because the dose used in the pilot experiment (13 kJ/m² UV-B) was lethal to the −/− mice. The rate of development of UV-associated tumors (skin or ocular) in mice of the three genotypes is shown in Figure 2. The data are presented as the probability of tumor development versus time after initiation of UV irradiation, using a life-table analysis, which permits the inclusion of animals that die during the experiment from other causes. The probability of 50% tumor development occurred at 14 weeks for the −/− mice, 24 weeks for the +/− mice, and 46 weeks for the +/+ mice.

The number and types of tumors that developed in the three groups are shown in Table 1B. Of 34 −/− mice in the study, 16 developed a total of 27 skin or ocular tumors; all of these mice also had lymphoma or leukemia at the time of death. Of the remaining 18 mice, 16 died between weeks 6 and 23 (at 18–34 weeks of age) with lymphoma or leukemia, and two died at weeks 15 and 20 of unknown causes. A similar spectrum of UV-associated tumors developed in the +/− mice, but these mice had fewer ocular tumors ($P < 0.01$, Chi-square test) and more tumors of dorsal skin than the −/− mice. In this group, five mice died of unknown causes and one died of lymphoma between weeks 17 and 38. The C57BL/6 +/+ mice developed no ocular tumors ($P < 0.02$ vs −/− mice), and the majority of tumors arose on dorsal trunk skin, rather than on the ears. Eleven mice in this

Table 1 Site and type of tumors induced by UV irradiation in *p53* −/−, −/+, and +/+ mice

	Genotype	No of mice	No with tumor	No tumors	Site	No (%)	SCC ^a	Fibrosarcoma	Melanoma	Other sarcoma	Mixed tumors
A. Pilot study	+/−	11	9	15	Eye	2 (13)	0	0	1	1	0
					Ear	10 (67)	3	2	2	2	1
					Back	3 (20)	2	0	0	1 ^b	0
					Totals (%)		5 (33)	2 (13)	3 (20)	4 (27)	1 (7)
B. Cohort study	−/−	34	16	27	Eye	11 (41)	0	1	6	4 ^c	0
					Ear	12 (44)	9	1	0	2 ^c	0
					Back	4 (15)	1	0	0	3	0
					Totals (%)		10 (37)	2 (7)	6 (22)	9 (33)	0
	+/−	31	25	34	Eye	3 (9)	0	0	1	1 ^c	1
					Ear	18 (53)	2	11	1	0	4
					Back	13 (38)	1	10	0	0	2
					Totals (%)		3 (9)	21 (62)	2 (6)	1 (3)	7 (21)
	+/+	24	13	15	Eye	0 (0)	0	0	0	0	0
					Ear	3 (20)	2	1	0	0	0
					Back	12 (80)	1	7	0	1	3
					Totals (%)		3 (20)	8 (53)	0 (0)	1 (7)	3 (20)

^aSquamous cell carcinoma; ^bTumor developed on forepaw; ^cOne unclassified malignant tumor

group remained alive and tumor-free at week 47 of the experiment.

Review of the histology of the cutaneous and ocular lesions revealed a high percentage of squamous cell carcinomas of the skin and ocular melanomas (Figure 3) in the *p53* $-/-$ mice (59% compared to 15% and 20% in the $+/-$ and $+/+$ respectively, Table 1). In addition, many *p53* $-/-$ mice exhibited solar keratoses in the treated skin, similar to those found in UV-irradiated human skin and considered to be a premalignant lesion (7/16 skin specimens examined vs 1/31 and 0/15 in the heterozygotes and wild-type mice).

Figure 2 Probability of UV-induced tumor development in *p53* $-/-$, $+/-$, and $+/+$ mice. Cohorts of C57BL/6 congenic mice of the three genotypes were exposed on shaved dorsal skin to 5 kJ/m² UV-B three times per week for 30 weeks

Premalignant dysplastic changes were also present in many of these specimens, and two of the squamous cell carcinomas were *in situ* lesions.

In contrast to the epithelial pathology observed in $-/-$ mice, sarcomas were mainly present in the $+/-$ (65%) and $+/+$ (60%), with only 40% in the $-/-$ mice. The fibrosarcomas were confirmed by immunohistochemical staining using monoclonal antibodies to vimentin and keratin. Also interesting was the observation of combined fibrosarcoma and squamous cell carcinoma in the $+/-$ (21%) and $+/+$ (20%) mice. No mixed tumors are found in the $-/-$ group (Table 1).

In this experiment, we also included control groups of *p53* $-/-$ and $+/-$ mice without UV irradiation. The rate of incidence, and histopathology of internal tumor development (mainly lymphoid malignancies) were not altered by UV irradiation in *p53* $-/-$ mice,

Figure 3 (a) Histological appearance of ocular melanoma from *p53* $-/-$ mouse. (b) Mixed tumor from *p53* $+/-$ mouse, consisting of nests of squamous cell carcinoma within fibrosarcoma. Both types were confirmed with immunohistological stains for keratin and vimentin

Table 2 *p53* mutations in UV-induced tumors from *p53* $+/-$ mice

Tumor	Affected exon	Codon	Sequence change ^a	Amino acid change
<i>Pilot study^b</i>				
A48-1 SCC	5	157–158	TACCGG→TAATGG	Met Ala→Stop
A58-2 Sarcoma	5	157–158	TACCGG→TAATGG	Met Ala→Stop
		176	cCAT→cTAT	His→Tyr
<i>Cohort study</i>				
G48 SCC	5	143	ACC→ATT	Trp→Stop
G77 FS	5	148	CCT→TTT	Pro→Phe
G191 FS	5	148	CCT→TTT	Pro→Phe
		155	cQGC→TGC	Arg→Cys
G44-2 FS+SCC	6	212	AGC→AGT	Ser→Ser
		213	DeletionGTG	Val
G14-1 FS	8	270	tCGT→tTGT	Arg→Cys
G14-2 FS	8	270	tCGT→tTGT	Arg→Cys
G25 Sarcoma	8	270	tCGT→tTGT	Arg→Cys
G35-1 FS+SCC	8	270	tCGT→tTGT	Arg→Cys
G35-2 FS+SCC	8	270	tCGT→tTGT	Arg→Cys
G42 Sarcoma	8	270	tCGT→tTGT	Arg→Cys
G44-1 SCC	8	270	tCGT→tTGT	Arg→Cys
G66 FS	8	270	tCGT→tTGT	Arg→Cys
G91 FS	8	270	tCGT→tTGT	Arg→Cys
G18 FS	–	–	–	–
G35-3 Sarcoma	–	–	–	–
G35-4 Sarcoma	–	–	–	–
G36 Melanoma	–	–	–	–
G49 FS	–	–	–	–
G54 FS	–	–	–	–
G60 FS	–	–	–	–
G96 FS	–	–	–	–
G178 FS	–	–	–	–
G202 FS	–	–	–	–
G253 FS	–	–	–	–

^aSequences of the strand containing dipyrimidine sites are shown here. Bases shown in underline represent wild-type and corresponding mutated nucleotides. Affected codon shown in capital letters, and adjacent base shown in lowercase letters. ^bOnly exon 5 of the *p53* gene was analysed

confirming the recent results of Li *et al.* (1998). However, in the *p53* $+/-$ mice, the incidence of internal tumor development was significantly increased by UV irradiation (not shown).

Status of wild-type *p53* allele in UV-induced skin cancers

Next we investigated whether skin cancer development was associated with loss of the wild-type *p53* allele from the $+/-$ mice, as was the case for chemically-induced skin tumors (Kemp *et al.*, 1993) and internal malignancies of *p53* $+/-$ mice exposed to ionizing radiation (Kemp *et al.*, 1994). Southern blot analysis of five paired samples of tumors and normal skin from the same animals revealed that both the wild-type and mutant alleles of *p53* were present (Figure 4a). Ten additional UV-induced skin tumors from *p53* $+/-$ mice in the cohort study were also analysed by both Southern blotting and PCR. None of them lost the remaining wild-type allele of the *p53* gene (not shown). Thus, of the 19 UV-induced tumors

examined, including ten fibrosarcomas, three squamous cell carcinomas (SCC), three mixed tumors (fibrosarcoma and SCC), two suspected melanomas, and one hemangiosarcoma, none showed loss of heterozygosity.

To search for mutations in the wild-type allele of *p53* in tumors from *p53* $+/-$ mice, we performed PCR-SSCP analysis on 12 of the 13 skin tumors from the pilot study and 24 of the 31 skin tumors from the cohort study. From the pilot study, six of 12 were positive and from the cohort study 13 of 24 were positive, giving an overall mutation frequency of 53%. Five of the 19 PCR-SSCP-positive tumors had alterations in exon 5. Because the mutant allele in the *p53* knockout mice contains a large insert that extends into exon 5, the primers used to detect exon 5 amplify only the wild-type allele. Therefore, we were able to sequence directly the mutations in the wild-type copy of *p53*. Seven mutations in exon 5 were detected in these five tumors (Table 2). All were C→T point mutations or CC→TT or CC→AT double tandem

Figure 4 (a) Southern blotting analysis of *Bam*HI-digested DNA from normal skin (N) or tumor (T) from UV-irradiated *p53* $+/-$ mice. Lane 1, normal tissue from unirradiated *p53* $-/-$ mouse. Lanes 2–5, tissues from mouse #38; Lanes 6–8, tissues from mouse #58; Lanes 9–11, tissues from mouse #63; Lanes 12–14, tissues from mouse #66; Lanes 15–16, tissues from mouse #40. (b) SSCP analysis of *p53* exon 5 in UV-induced tumors from *p53* $+/-$ mice. Lane 1, negative control (no DNA); Lanes 2–5, from mouse #38; Lanes 6–8, from mouse #58; Lanes 9–10, from mouse #40; Lanes 11 and 12 from mouse #67; Lanes 13–15, from mouse #63; Lanes 16–18, from mouse #66; Lanes 19–21, from mouse #48; Lane 22, tumor #98.1 with known mutation in exon 5 (positive control). Arrows indicate shifted (mutant) bands

mutations characteristic of those produced by UV radiation. Three mutations resulted in stop codons and four were missense mutations.

To sequence alterations in exons 6–8, pairs of primers were used that amplified only the wild-type allele. One primer was anchored in a region that was deleted in the inactive *p53* allele; the other spanned the intron-exon junctions of each exon examined. Abnormal bands from the SSCP gels were cut out, reamplified, and sequenced. Exons 6–8 were examined only in tumors from the cohort study. As shown in Table 2, of the 13 tumors with mutations, three occurred in exon 5, one was in exon 6, and nine were in exon 8 (69%). All mutations in exon 8 were C→T point mutations at codon 270, which is a mutational hotspot, resulting in an Arg→Cys substitution (Kanjilal *et al.*, 1993; Ananthaswamy *et al.*, 1998). The tumor with an alteration in exon 6 had a silent point mutation in codon 212, as well as a deletion of codon 213. There was no apparent difference in the histological types of tumors exhibiting mutations in *p53* compared to those without detectable mutations (Table 2).

Discussion

These studies demonstrate the extreme sensitivity of *p53*−/− and +/− mice to tumor induction by UV radiation and indicate the critical nature of this gene in protecting against UV carcinogenesis. These results correlate well with the demonstration that *p53*−/− mice exhibit markedly reduced apoptosis (sunburn cell formation) in the epidermis after UV irradiation and +/− mice show an intermediate response between −/− and wild-type mice (Ziegler *et al.*, 1994). Our results differ dramatically from those obtained in studies of chemical carcinogenesis using an initiation-promotion protocol (Kemp *et al.*, 1993), in which tumor progression was enhanced, but the incidence of papillomas actually decreased. This suggests that *p53* may play different roles in the evolution of chemically-induced and UV-induced skin tumors. Although this difference could be due to the disparate genetic backgrounds of the mice used in the two studies, it most likely reflects differences in the biology of the tumors produced during chemical and UV carcinogenesis. Whereas two-stage chemical carcinogenesis protocols induce mainly papillomas, some of which may regress or progress to invasive carcinomas, chronic UV irradiation of most haired, inbred strains of mice induces a variety of carcinomas and sarcomas and only an occasional papilloma (Kripke, 1977). Histological analysis of the tumors produced in this study showed that sarcomas predominated in mice with one or two copies of *p53*, whereas squamous cell carcinoma predominated in *p53*−/− mice. This may indicate that *p53* plays a more important role in regulating epidermal carcinogenesis than mesenchymal tumors. Another possibility is suggested by the finding that in chemically-induced tumors of *p53*+/− mice, the wild-type allele of *p53* was lost (Kemp *et al.*, 1993), whereas in the UV-induced tumors, the wild-type allele of *p53* remained, and *p53* mutations in this allele could be detected in approximately 50% of the tumors. All but one of the mutations were point mutations at

dipyrimidine sites characteristic of UV-induced mutations. Thus, UV-induced mutation in *p53*, rather than loss of the wild-type allele, was a frequent occurrence in UV carcinogenesis in the *p53*+/− mice. This difference could result from the fact that UV radiation generally causes point mutations rather than large deletions in DNA.

Since *p53* plays such an important role in skin cancer development, it is curious that the *p53*−/− mice fail to develop spontaneous skin cancers, even though they develop other types of malignancy with very high frequency. The high rate of cellular proliferation in normal skin would seem to make it an obvious candidate for spontaneous transformation in the absence of *p53* function. The lack of such spontaneous skin cancers in these mice (Donehower *et al.*, 1992; Jacks *et al.*, 1994) suggests that *p53* plays a major role in the response of keratinocytes and other cells of the skin to UV-induced DNA damage and a less prominent or no role in the control of normal proliferation of this tissue. This suggestion is supported by a recent study showing that *p53*+/− mice lacking the *XPC* gene, which is involved in repair of UV-induced DNA damage, are highly sensitive to the acute effects of UV radiation and exhibit increased susceptibility to UV carcinogenesis compared to *XPC*−/− mice with wild-type *p53* (Cheo *et al.*, 1996). However, this study is confounded somewhat by the mixed genetic background of the UV-irradiated mice.

An unexpected finding from this study was the high incidence of ocular tumors in the *p53*−/− mice, most of which were melanomas. Although such tumors occur occasionally in UV-irradiated *p53*+/+ mice, their frequency was significantly increased in the absence of functional *p53*. Another surprising finding was the occurrence of cutaneous melanomas in both *p53*−/− and *p53*+/− mice. Although in humans, cutaneous melanoma is often associated with sunlight exposure, mice rarely develop melanoma in response to UV radiation alone, chemical carcinogens usually being required as initiators or promoters of melanoma development in mouse skin (Donawho and Kripke, 1991). There may have been a higher incidence of cutaneous melanomas in the *p53* deficient mice, but because of the small numbers, the differences were not statistically significant. In this regard, it is interesting to note that patients with Li–Fraumeni syndrome have an excess incidence of cutaneous melanoma, although there are no reports of excess non-melanoma skin cancers (Malkin *et al.*, 1990). Whether this is because non-melanoma skin cancers are generally not reported or these patients do not exhibit the same increased sensitivity to UV carcinogenesis as the *p53*+/− mice is not clear. There is no evidence that ocular melanoma in humans is related to exposure to UV radiation (Kraemer *et al.*, 1987); therefore, one would not expect to find an increased incidence of this tumor in people with Li–Fraumeni syndrome.

In summary, our studies demonstrate the essential role of *p53* in protecting the skin against UV carcinogenesis and strengthen the notion that UV-induced mutations in *p53* found in skin cancers represent molecular lesions that are of functional significance in the evolution of these tumors. In a recent study, we found that application of sunscreens to the skin of mice prior to UV irradiation greatly

decreased the rate of appearance of *p53* mutations in non-tumorous skin (Ananthaswamy *et al.*, 1997). The results reported here further support the concept that *p53* mutations can serve as an early marker of skin cancer development and that the prevention of such mutations is an appropriate indicator of photoprotection.

Materials and methods

Mice

Specific-pathogen-free C57BL/6NCr mice were obtained from the Frederick Cancer Research Facility Animal Production Area. Specific-pathogen-free *p53* knockout mice (TSG-p53) were obtained from GenPharm, Intl. (Mountain View, CA, USA) and backcrossed onto the C57BL/6 background. TSG-p53 mice were derived from those originally produced by Donehower *et al.* (1992). The mice were housed in a pathogen-free barrier facility accredited by the American Association for Accreditation of Laboratory Animal Care, in accordance with current US Department of Agriculture, Department of Health and Human Services, and National Institutes of Health regulations and standards. All procedures were approved by the Institutional Animal Care and Use Committee. Mice were housed under filter-protected cages, and ambient lighting was controlled to provide 12 h light-12 h dark cycles. Autoclaved National Institutes of Health open formula mouse chow and water were provided *ad libitum*.

UV irradiation

UV radiation was provided by a bank of six FS40 sunlamps (National Biological Corp., Twinsburg, OH, USA), which emit a continuous broad spectrum from 250–400 nm, with a peak emission at 313 nm. Approximately 65% of the radiation is emitted within the UV-B (280–320 nm) range (Kripke, 1977); the incident dose-rate averaged 3.75 W/m² UV-B over the course of the experiments. For the cohort study, age-matched mice of the three genotypes were irradiated three times per week for 30 weeks, beginning at 12 weeks of age; all mice had their dorsal fur shaved weekly. All $-/-$ mice, 74% of the $+/-$, and 83% of the $+/+$ mice were males. Since there was no difference in the rate or site of tumor development or histological types of tumors between males and females, the data for males and females were pooled. Mice were individually numbered and observed three times per week for health status; skin lesions were measured weekly with a vernier caliper in two bisecting diameters.

Southern hybridization

Genomic DNA (10 µg) was isolated from the tails of 1-week-old mice and cleaved with *Bam*HI (Boehringer-Mannheim,

Indianapolis, IN, USA). The DNA was subjected to electrophoresis for 18 h at 25 V in 0.8% agarose gel. The DNA was transferred to nitrocellulose paper (0.45 µm pore size) and hybridized with the *p53* exon 2–6 probe supplied by GenPharm Intl. Positive bands were detected by autoradiography.

DNA analysis

Thirty nanograms of genomic DNA extracted from tumor and normal skin were used in each 25 µl of the amplification reaction mixture. Exon 5 of the *p53* gene was amplified by PCR using primers that spanned the intron-exon junctions, as described previously (Kanjilal *et al.*, 1993; Ananthaswamy *et al.*, 1998). For the amplification of exons 6–8, one primer was anchored in exons that were deleted in the targeted allele was used, along with a primer that spanned in the intron-exon junctions of the desired exon. The identity of the PCR products was verified by running them on a 2% agarose gel.

SSCP analysis

SSCP analysis was performed using a mutation detection enhancement (MDE) gel (AT Biochem, Malvern, PA, USA). Briefly, the amplification reaction was performed in a 25 µl volume in the presence of 2.5 µCi of [α -³²P]dCTP, and 1.5 µl aliquots of the products were diluted with 3.5 µl of stop solution. The diluted PCR products were heated at 94°C for 4 min and quick-cooled and loaded onto 0.25 × MDE gel and resolved by electrophoresis at 6 W for 16 h as described previously (Kanjilal *et al.*, 1993). SSCP analyses were repeated three times.

Nucleotide sequencing

The abnormal bands were cut from the SSCP gels, dissolved in 100 µl TE buffer containing 10 mM Tris and 1 mM EDTA (pH 8.0), and heated at 80°C for 30 min. The supernatants were purified by spinning through Microcon-30 tubes (Amicon, Berkeley, CA, USA) and were used as templates for PCR amplification with specific primers. PCR products were sequenced directly in both directions using the Sequenase version 2.0 sequencing kit (USB, Cleveland, OH, USA) according to the manufacturer's instructions. Normal mouse skin DNA and wild-type bands from SSCP gels were also amplified and sequenced along with the mutant bands.

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