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Advances in Brief

Carcinogen-specific Mutational Pattern in the *p53* Gene in Ultraviolet B Radiation-induced Squamous Cell Carcinomas of Mouse Skin[1]

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Abstract

We have examined 35 epidermal tumors induced in mice of four different strains by chronic exposure to ultraviolet B radiation for the presence of aberrations in the *p53* tumor suppressor gene. Polymerase chain reaction products from *p53* exons 5 to 8 were screened by single-strand conformation polymorphism analysis and sequencing. Base substitutions were found in seven tumors (20%). All mutations occurred at dipyrimidine sequences; most frequent were C→T single base and CC→TT tandem transitions suggesting the involvement of UV radiation in the genesis of the mutations. Three base substitutions were located at codon 148, and all dipyrimidine-derived mutations occurred at sites where the sequence is present in the nontranscribed DNA strand, indicating some site and strand specificity of the ultraviolet B-induced *p53* mutations.

Introduction

Mutations of the *p53* gene are frequently observed in human and animal tumors (1). There are several lines of evidence in support of the hypothesis that wild-type *p53* acts as a tumor suppressor gene which may lose its growth-restricting function by allelic deletion and/or introduction of point mutations in one of the evolutionarily highly conserved regions of the gene (for a review, see Ref. 2). Mutations are scattered around the gene, and base substitutions have been demonstrated in over 90 different codons (1). In a limited but increasing number of cancers the observed types of *p53* base substitutions correspond to the types of mutations predicted to be induced by certain carcinogens which are suspect risk factors for these cancers. One of the best examples stems from the analysis of human SCCs[3] of the skin where UV light is presumed to represent the major etiological risk factor. In a considerable number of these tumors tandem CC→TT base substitutions have been detected in the *p53* gene, a type of mutation which is only known to be induced by UV (3). Moreover, all mutations detected in the human SCCs occurred at dipyrimidine sites and were mostly C→T transitions (3, 4), both of which would be predicted for UV-induced mutations (5, 6). The present study was undertaken to verify the results obtained with the human SCCs by using a well-defined experimental system where UV light is the only known cancer risk factor.

Materials and Methods

The details of the experimental procedure used for tumor induction by chronic UVB radiation in mice of the indicated strains have been reported elsewhere (7, 8). In brief, SKH-1 hr mice were exposed twice per week to gradually increasing doses from 1 kJ/cm² to 6 kJ/cm² starting at Wk 4 of age and lasting until sacrifice of the animals. Papillomas were isolated between 22 and 26 wk and carcinomas between 32 and 36 wk after the start of treatment. Chronic irradiation of shaved SENCAR, BALB/c, and C3H/He mice was carried out 3 times/wk with 2.88 kJ/cm² from FS40 sun lamps. Exposures were continued until sacrifice of the animals. Tumors (SCCs) were isolated from SENCAR and BALB/c mice between 18 and 24 wk and from C3H/He mice between 26 and 36 wk after start of treatment. Two of the tumors from SENCAR mice were induced by single UVB radiation (8.64 J/cm²). Tumors were carefully removed; one portion of each tumor was processed for routine histology. The other portion was lyophilized and kept stored at −20°C prior to isolation of DNA and PCR.

PCR conditions were as recently described (9). In brief, for PCR amplification of the evolutionarily highly conserved regions of the mouse *p53* gene, the following primers were used.

Exon 5: MA1, 5′-TCC AGT ACT CTC CTC CCC TCA AT-3′
MA14, 5′-AGA GCA AGA ATA AGT CAG AAG CC-3′
MA13, 5′-AGG GCT TAC CAT CAC CAT-3′

Exon 6: MD1, 5′-AGC CCT CAA CAC CGC CTG TGG-3′
MD11, 5′-AAT TAC AGA CCT CGG GTG GCT-3′

Exon 7: MB1, 5′-GCC GGC TCT GAG TAT ACC ACC AT-3′·
MB14, 5′-GGA AAC AGA GGA GGA GAC TTC AT-3′
MB15, 5′-GGT AGA TAG GGT AGG AAC-3′

Exon 8: MB2, 5′-TCT TAC TGC CTT GTG CTG GTC CT-3′
MB12, 5′-CAG GTG GGC AGC GCT GTG GAA GG-3′
MB13, 5′-CCT GCG TAC CTC TCT TTG-3′

Genomic DNA (200 ng) was incubated in a total volume of 20 μl with 0.5 μM of each primer, 200 μM of each deoxyribonucleoside triphosphate, *Taq* polymerase buffer, and 0.04 units of *Taq* polymerase (both from Stehelin, Basel, Switzerland) and amplified for 35 cycles. Aliquots of the initial amplification reactions were subsequently reamplified for 15 rounds in a "hot mix" containing unlabeled and 5′-^{32}P-labeled primers in a 9:1 ratio. With the exception of exon 6, nested 3′-primers were used for these reamplifications (MA13 for exon 5, MB15 for exon 7, and MB13 for exon 8). For SSCP analysis, amplification products of the second PCR reaction (being 174 to 230 base pairs long) were diluted 6-fold in a sequencing stop solution, heat denatured at 95°C for 5 min, chilled on ice, and immediately loaded onto a 0.02-cm-thick 6% nondenaturating polyacrylamide gel containing 0.5× TBE buffer and 5% glycerol. Electrophoresis was carried out at 30 W of constant power for 3 to 3.5 h at room temperature under cooling with a fan.

Genomic DNA from those tumors with *p53* SSCP band shifts was reamplified in a volume of 100 μl using the same primers as for the initial amplification. The PCR products were extracted with chloroform-isoamyl alcohol and purified with the Magic Prep system (Promega, Madison, WI). The fragments were sequenced in both directions using the same primers as for SSCP analysis, except for exon 5 where

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[3] The abbreviations used are: SCC, squamous cell carcinoma; UVB, ultraviolet B; PCR, polymerase chain reaction; SCCP, single-strand conformation polymorphism; TBE, Tris-borate/EDTA.

another internal 3′-primer [5′-AGA TGG AGG CTG CCA GTC CTA AC-3′ (MA12)] was used. Sequencing was performed with 5′-^{32}P-labeled primers using the Sequenase II kit (US Biochemicals, Cleveland, OH).

Results and Discussion

Epidermal tumors were induced by chronic UVB radiation in mice of 4 different strains (SKH-1 hr, SENCAR, BALB/c, and C3H/He). The histological classification of the tumors is given in Table 1. Eight papillomas, all from SKH-1 hairless mice, and 27 SCCs, mostly of a poorly differentiated type, were examined for aberrations in one of the 4 evolutionarily highly conserved regions (exons 5 to 8) of the *p53* gene by PCR-SSCP and subsequent sequencing. In total, PCR products from 7 different tumors showed SSCP band shifts (see Fig. 1). For characterization of the *p53* changes that led to the observed mobility shifts in the SSCP analysis, we have subsequently sequenced the amplified DNA fragments. Base substitutions were detected in exons 5, 7, and 8 of the *p53* gene. In all 7 cases single or double base substitutions were detected (Fig. 2). The mutational spectrum observed in the 7 mutated tumors, and the predicted amino acid changes resulting from the base substitutions are presented in Table 1.

In SKH-1 hr mice, *p53* mutations were present in 22% (2 of 9) of the SCCs, while no mutations were detected in the 8 papillomas from mice of this strain. Similarly, 50% (3 of 6) of the SCCs from BALB/c mice and 29% (2 of 7) of those from C3H/He mice showed *p53* base substitutions, while no aberrations of the gene were detected in the 5 SCCs that were investigated in SENCAR mice. The apparent differences in the mutation frequency of SCCs between the various strains, however, were not statistically significant.

One tumor (No. 26) harbored two independent mutations, both in exon 8. One of these, a CC→TT double base substitution, which occurred at a dipyrimidine site and involved codons 279 and 280, is clearly linked to UV radiation. The second mutation, which involved codon 270, is a C→T transition at a T-CpG sequence. Since the mutated C is located at the 3′-end of a TC pair, this base substitution could also be induced by UV radiation. An alternative explanation, however, would be that it resulted from spontaneous deamination of 5-methylcytosine present at this position (10). It is interesting to note that both mutations in Tumor 26 are probably located on the same *p53* allele. We conclude this from the results of the SSCP analysis of DNA from this particular tumor which demonstrates the presence of signals for both the mutated and the wild-type allele (Fig. 1).

Only single codons were affected by base substitutions in the remaining 6 mutated tumors, and the mutations were in all cases located at dipyrimidine sequences (Table 1). Three of these latter tumors showed C→T transitions, one a C→A transversion. Two tumors harbored tandem base substitutions, one a CC→TT and the other a CC→AT change. The predicted amino acid changes of the various mutations are given in Table 1.

The predominant lesions induced by UVB radiation in DNA of pro- and eukaryotic cells are cyclobutane pyrimidine dimers and pyrimidine-pyrimidone (6-4) photoproducts (11, 12). Both types of photoproducts represent noncoding DNA lesions

Table 1 p53 *mutations in UVB-induced mouse skin tumors*

			p53 aberration				
Case	Strain	Tumor type	Exon	Codon[a]	Sequence[b]	Base change	Amino acid substitution
1	SKH-1 hr	SCC					
3	SKH-1 hr	SCC					
5	SKH-1 hr	SCC					
7	SKH-1 hr	PAP[c]					
8	SKH-1 hr	PAP					
9	SKH-1 hr	SCC (PD)					
10	SKH-1 hr	SCC (PD)					
11	SKH-1 hr	SCC	8	275	tgcc C tggg	C to T	Pro to Leu
12	SKH-1 hr	PAP					
13	SKH-1 hr	PAP					
14	SKH-1 hr	PAP					
15	SKH-1 hr	PAP					
16	SKH-1 hr	SCC (PD)					
17	SKH-1 hr	SCC (PD)					
18	SKH-1 hr	SCC	8	275	tgc CC tggg	CC to AT	Pro to Ile
19	SKH-1 hr	PAP					
20	SKH-1 hr	PAP					
21	SENCAR	SCC (PD)					
22	SENCAR	SCC (PD)					
23	SENCAR	SCC					
24	SENCAR	SCC (PD)					
25	SENCAR	SCC (PD)					
26	BALB/c	SCC (PD)	8	270	gtt C gtgtt	C to T	Arg to Cys
			8	279/280	gaccg CC gtaca	CC to TT	Arg Arg to Arg Cys
27	BALB/c	SCC (PD)					
28	BALB/c	SCC (PD)	8	275	tgcc C tggg	C to T	Pro to Ser
30	BALB/c	SCC (PD)	5	148	aca CC tcca	CC to TT	Pro to Phe
31	BALB/c	SCC (PD)					
32	BALB/c	SCC (PD)					
33	C3H/He	SCC (PD)					
34	C3H/He	SCC (PD)					
35	C3H/He	SCC	5	148	aca C ctcca	C to T	Pro to Ser
36	C3H/He	SCC (PD)					
37	C3H/He	SCC (PD)					
38	C3H/He	SCC (PD)	7	238	agct C ctgc	C to A	Ser to Tyr
39	C3H/He	SCC (PD)					

[a] Codon numbers were deduced from Bienz *et al.* (21).
[b] Sequence of the nontranscribed strand in 5′ to 3′ direction.
[c] PAP, papilloma; SCC (PD), SCC (poorly differentiated).

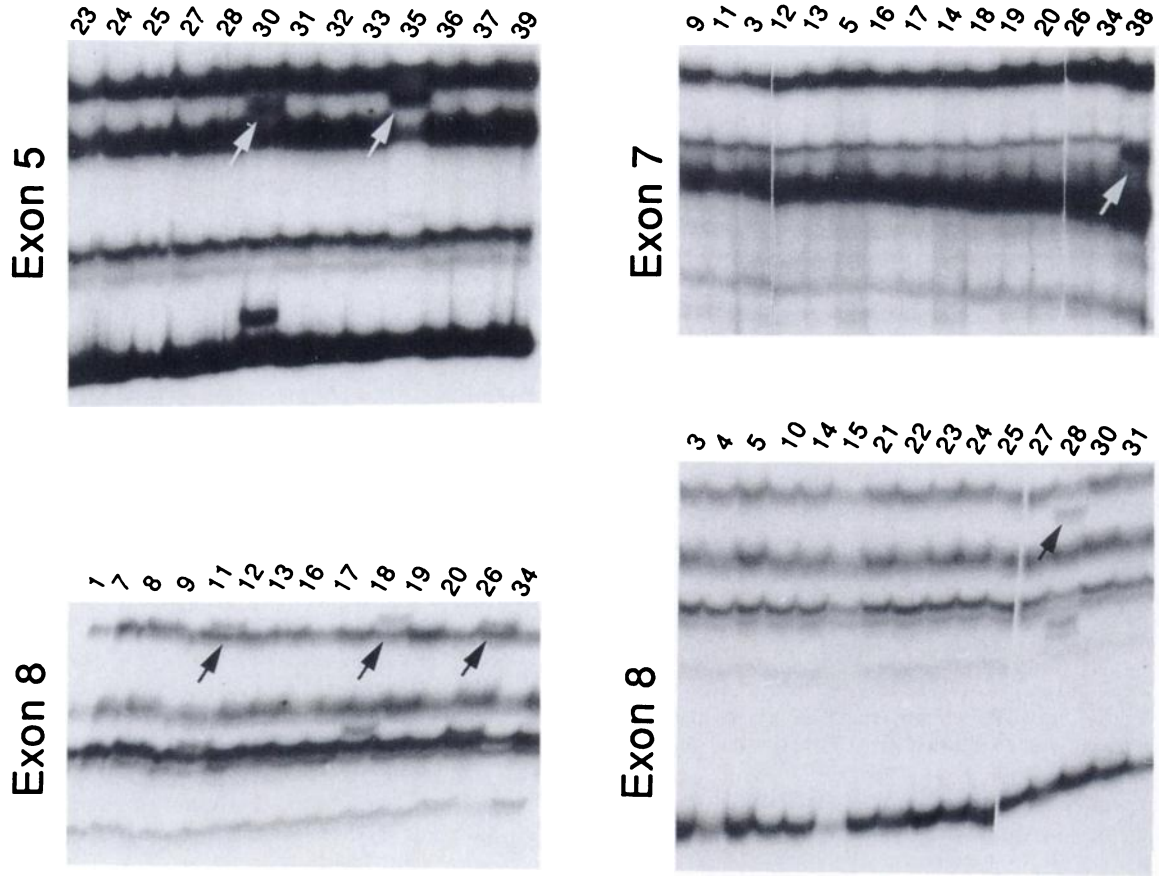

Fig. 1. SSCP analysis of *p53* aberrations in UVB radiation-induced mouse squamous cell carcinomas. PCR products from the 7 mutated samples, defined by a variant migration and denoted by an *arrow*, are shown together with representative samples that exhibited unchanged banding patterns. Tumor identification numbers are given on *top* of each *lane*; exon numbers are noted at the *left* of each *panel*.

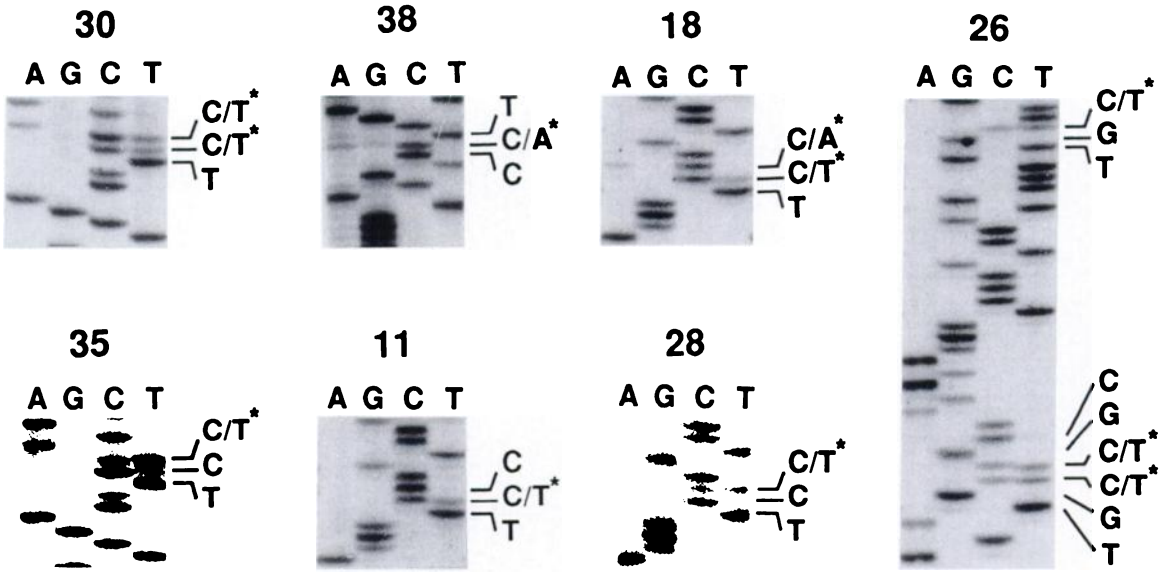

Fig. 2. Identification of *p53* base substitutions in UVB radiation-induced mouse squamous cell carcinomas. The sequences correspond to the 5′-3′ direction of the nontranscribed strand when read from *top* to *bottom*. Base substitutions are indicated by *asterisks*.

which are potentially mutagenic and result in very characteristic mutational patterns (5, 6). C→T transitions at dipyrimidine sites are the most frequent alterations. In the majority of cases, single base substitutions are observed which occur at the 3′-C of CC and TC sequences, while CC→TT double base substitutions (5 to 15% of all mutations) are somewhat less frequent. Typically, about 5 to 15% of mutations are C→A transversions (3, 5, 6). In our present study, all the base substitutions occurred at dipyrimidine sites, and 82% (9 of 11) of these were C→T transitions including two CC→TT double mutations, strongly suggesting that these mutations were directly induced by absorption of UV radiation by the target DNA. A similar frequency distribution of *p53* base substitutions has been recently described in the study of Brash *et al.* (3) for SCCs of the human

skin where UV light is assumed to be the major etiological risk factor. Mutations at T were not detected in the human (3) and mouse (this study) SCCs, although UVB radiation is known to induce cyclobutane thymidine dimers in mouse skin (8, 13–15). This is, however, to be expected since DNA polymerase preferentially inserts adenine opposite to noncoding lesions which leads to the correct sequence in those instances where thymidine is affected but generates C→T substitutions in those cases where cytosine is affected (16).

All SCCs with *p53* mutations showed both mutated and wild-type-specific signals during PCR-SSCP analysis and sequencing, suggesting that the mutations were in all cases heterozygous. We have tried to carefully separate the tumors from the normal surrounding tissue and to eliminate contaminating dermal tissue during isolation of the DNA. The possibility that some DNA from normal tissue was present during PCR amplification, however, cannot be entirely ruled out. Mutation of one *p53* allele coupled with loss of the second one is a frequent phenomenon in many human cancers, suggesting that these mutations may act recessively (for a review, see Ref. 2). Other data demonstrate that *p53* may have suppressor or promoter functions depending on its conformation, whereby the mutated form may function in a dominant negative manner (17). The fact that no loss of heterozygosity was observed in the UVB-induced mouse SCCs may indicate that the mutated alleles act in a dominant negative mode in these tumors. All dipyrimidine sites to which mutations were targeted in the mouse SCCs of this study were exclusively located on the nontranscribed strand of the *p53* gene, suggesting strand bias in the formation or repair of UV photoproducts. In fact, preferential removal of UV-induced pyrimidine dimers from the transcribed DNA strand has been demonstrated in bacteria and in mammalian cells resulting in strand specificity of UV-induced mutations (6, 18). In our study, 476 base pairs in exons 5 to 8 of the *p53* gene were examined for aberrations. The total numbers of CC plus TC pairs within this sequence on the transcribed and nontranscribed strands amount to 66 and 71, respectively. If one assumes identical probabilities for the occurrence of mutations at each of the CC and TC gene loci, the observed bias for mutations at the nontranscribed strand is highly significant ($P = 0.015$ using Fisher's exact test). It has to be considered, however, that the observed frequency distribution of *p53* mutants (mutated tumors) does not necessarily reflect the frequency distribution of *p53* mutations, since the magnitudes of the mutation-mediated selection pressure during clonal expansion of the mutated tumor cells may well differ between different mutational loci. In fact, there is some evidence of a nonrandom distribution of mutational sites within the *p53* gene in the UVB-induced SCCs of this study. Of the 7 mutated tumors, 3 harbored base substitutions at codon 275 and 2 at codon 148 of the gene. Mouse codons 275 and 148 correspond to codons 278 and 151, respectively, of human *p53*. Base substitutions in either of these 2 codons were also detected in 2 of the 14 mutated human SCCs studied by Brash *et al.* (3) and in a variety of human internal malignancies (1).

Mutations of *p53* were not detected in any of the 8 UVB-induced papillomas while present in 26% (7 of 27) of the squamous cell carcinomas. Although this difference in the mutation frequency between both tumor types was not statistically significant, it is noteworthy that similar findings have been reported in two comparable studies, where papillomas and carcinomas were induced in mice by sequential treatment with 7,12-dimethylbenz(*a*)anthracene and a phorbol ester (19, 20). Therefore, mutations of the *p53* gene do not seem to occur during initiation of skin carcinogenesis in mice but may rather represent a genetic change associated with the progression from papillomas to carcinomas.

The results of the present investigation clearly demonstrate that the *p53* gene is a mutational target during UVB radiation-induced mouse skin carcinogenesis. Additional studies with larger numbers of tumors will be required to confirm the apparent absence of *p53* mutations in the early preneoplastic papillomas and the site and strand specificity of the mutations in the SCCs.

References

1. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. *p53* mutations in human cancers. Science (Washington DC), *253:* 49–53, 1991.
2. Levine, A. J., Momand, J., and Finlay, C. A. The *p53* tumor suppressor gene. Nature (Lond.), *351:* 453–456, 1991.
3. Brash, D. E., Rudolph, J. A., Simon, J. A., Lin, A., McKenna, G. J., Baden, H. P., Halperin, A. J., and Ponten, J. A role for sunlight in skin cancer: UV-induced *p53* mutations in squamous cell carcinoma. Proc. Natl. Acad. Sci. USA, *88:* 10124–10128, 1991.
4. Pierceall, W. P., Mukhopadhyay, T., Goldberg, L. H., and Ananthaswamy, H. N. Mutations in the *p53* tumor suppressor gene in human cutaneous squamous cell carcinoma. Mol. Carcinog., *4:* 445–449, 1991.
5. Drobetsky, E. A., Grosovsky, A. J., and Glickman, B. W. The specificity of UV-induced mutations at an endogenous locus in mammalian cells. Proc. Natl. Acad. Sci. USA, *84:* 9103–9107, 1987.
6. Vrieling, H., van Rooijen, M. L., Groen, N. A., Zdzienicka, M. Z., Simons, W. I. M., Lohman, P. H. M., and van Zeeland, A. A. DNA strand specificity for UV-induced mutations in mammalian cells. Mol. Cell. Biol., *9:* 1277–1283, 1989.
7. Das, M., Bickers, D. R., Santella, R. M., and Mukhtar, H. Altered patterns of cutaneous xenobiotic metabolism in UVB-induced squamous cell carcinoma in SKH-1 hairless mice. J. Invest. Dermatol., *84:* 532–536, 1985.
8. Strickland, P. T. Photocarcinogenesis and persistent hyperplasia in UV-irradiated SENCAR mouse skin. Environ. Health Perspect., *68:* 131–134, 1986.
9. Kress, S., König, J., Schweizer, J., Löhrke, H., Bauer-Hofmann, R., and Schwarz, M. *p53* mutations are absent from carcinogen-induced mouse liver tumors but occur in cell lines established from these tumors. Mol. Carcinog., in press, 1992.
10. Rideout, W. M., III, Coetzee, G. A., Olumi, A. F., and Jones, P. 5-Methylcytosine as an endogenous mutagen in the human LDL receptor and *p53* genes. Science (Washington DC), *249:* 1288–1290, 1990.
11. Setlow, R. B., and Carrier, W. L. Pyrimidine dimers in ultraviolet-irradiated DNAs. J. Mol. Biol., *17:* 237–254, 1966.
12. Brash, D. E., and Haseltine, W. A. UV-induced mutation hotspots occur at damage hotspots. Nature (Lond.), *298:* 189–192, 1982.
13. Ley, R. D., Sedita, B. A., and Grube, D. D. Absence of photoreactivation of pyrimidine dimers in the epidermis of hairless mice following exposures to ultraviolet light. Photochem. Photobiol., *27:* 483–485, 1978.
14. Cooke, A., and Johnson, B. E. Dose response, wavelength dependence, and rate of excision of ultraviolet radiation-induced pyrimidine dimers in mouse skin DNA. Biochim. Biophys. Acta, *517:* 24–30, 1978.
15. Vink, A. A., Berg, R. J. W., de Gruijl, F. R., Roza, L., and Baan, R. A. Induction, repair, and accumulation of thymine dimers in the skin of UV-B-irradiated hairless mice. Carcinogenesis (Lond.), *12:* 861–864, 1991.
16. Topal, M. D. DNA repair, oncogenes, and carcinogenesis. Carcinogenesis (Lond.), *9:* 691–696, 1988.
17. Herskowitz, I. Functional inactivation of genes by dominant negative mutations. Nature (Lond.), *329:* 219–222, 1987.
18. Mellon, I., Spivac, G., and Hanawalt, P. C. Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene. Cell, *51:* 241–249, 1987.
19. Burns, P. A., Kemp, C. J., Gannon, J. V., Lane, D. P., Bremner, R., and Balmain, A. Loss of heterozygosity and mutational alterations of the *p53* gene in skin tumours of interspecific hybrid mice. Oncogene, *6:* 2363–2369, 1991.
20. Ruggeri, B., Caamano, J., Goodrow, T., DiRado, M., Bianchi, A., Tono, D., Conti, C. J., and Klein-Szanto, A. J. P. Alterations of the *p53* tumor suppressor gene during mouse skin tumor progression. Cancer Res., *51:* 6615–6621, 1991.
21. Bienz, B., Zakut-Houri, R., Givol, D., and Oren, M. Analysis of the gene coding for the murine cellular tumour antigen p53. EMBO J., *3:* 2179–2183, 1984.

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