Advances in Brief

High Frequency of p53 Mutations in Ultraviolet Radiation-induced Murine Skin Tumors: Evidence for Strand Bias and Tumor Heterogeneity

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Abstract

Exposure to UV radiation has long been associated with the development of skin cancers. To identify the molecular targets in UV carcinogenesis, we analyzed 11 UV-induced murine skin cancers for mutations in the p53 tumor suppressor gene and found a 100% incidence rate. Such a high frequency of p53 mutations is unprecedented and suggests that this gene plays an important role in the development of UV-induced skin cancers. The mutations were predominantly "UV-signature" transitions (C→T and CC→TT) at pyrimidine-rich sequences located on the nontranscribed strand of the gene. In addition, seven tumors harbored multiple mutant alleles of p53, providing strong evidence for tumor heterogeneity at the molecular level.

Introduction

The importance of UV radiation in the pathogenesis of human skin cancers has been investigated from an epidemiological standpoint for several decades (1). Solar UVB radiation (280–320 nm) is absorbed by the skin, producing unique "signature" lesions in DNA that mark the sunlight-associated steps in the development of skin cancers (Ref. 2 and references therein). The two major lesions, cyclobutane dimers and pyrimidine (6–4) pyrimidone photoproducts, result from the formation of covalent links between adjacent pyrimidines and mutations arising at such sites are typically C→T and CC→TT transition events (Ref. 2 and references therein). DNA damage and subsequent mutagenesis may result in genetic alterations, including activation of oncogenes and/or inactivation of tumor suppressor genes, that culminate in the development of cancers (3). Murine skin cancers induced by repeated exposure to UV radiation (4) provide an excellent model system for investigating the molecular mechanisms of UV carcinogenesis since the etiology of these tumors is well defined and other known risk factors are carefully controlled. In previous studies we have shown that codon 61 of the N-ras oncogene is mutated in some of the tumors (5). The relatively low frequency of mutagenesis at the ras locus (20%) suggested that additional genetic alterations, possibly involving tumor suppressor genes such as p53, may play a role in UV carcinogenesis. Mutations in p53 have been detected with a high frequency in many types of cancer (3, 6), and transgenic mice deficient in this gene are predisposed to the development of tumors (7).

Materials and Methods

Tumors and Cell Lines. Cell lines were derived from tumors that were induced in female C3H/HeNCr(MTV−) mice by repeated exposure to UVB radiation (4, 5). Lines 96-1, 97-1, 97-4, 97-5, 98-1, and 98-4 were established from primary skin cancers and only early (<5) in vitro passages were used in the experiments. Tumors 1422, 1463, 1591, 2237, and 2240 were established in culture from the first transplant generation in immunosuppressed, syngeneic mice and these cell lines have been passaged 20 to 25 times in vitro. All cell lines, including control C3H10T1/2 cells, were grown in Dulbecco's modified Eagle's medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT).

DNA Amplification. Exons 4 through 8 of the p53 gene [from the tumor cell lines, C3H10T1/2 cells, and unirradiated C3H/HeNCr(MTV−) mouse epidermis] were individually amplified by PCR (8) using primers that span the intron-exon junctions of the respective exons. Amplicons (PCR products) were verified to be of the expected size by electrophoresis in 1.5% agarose gels.

SSCP Analysis. SSCP analysis (9) was performed using a MDE (mutation detection enhancement) gift from ultrahigh-resolution gel (AT Biochem, Malvern, PA) according to instructions from the manufacturers. In brief, 25 μl amplification reactions were performed 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 sequencing stop solution. The diluted amplicons were heated at 94°C for 2 min, quick cooled on ice, loaded on a 0.25 × mutation detection enhancement gel, and resolved by electrophoresis at 6 W. The entire procedure, including amplification by PCR, was repeated three times.

Nucleotide Sequencing. Amplicons were subcloned into the Smal site of pBluescript II SK− (Stratagene, La Jolla, CA) and XL-1 Blue cells (Stratagene) were transformed with the recombinant plasmids. Eight to 12 representative colonies of transformed cells were picked for each tumor, and each of the inserts was sequenced in both directions with Sequenase version 2.0 (USB, Cleveland, OH). All sequences were amplified and sequenced twice to avoid errors that may have been incorporated by Taq polymerase during the amplification reactions.

Results and Discussion

Frequency of p53 Mutations. SSCP analysis of exons 4 through 8 of the p53 gene in cell lines derived from 11 UV-induced C3H/HeNCr(MTV−) mouse skin tumors indicated the presence of mutations in eight cases. The results shown in Fig. 1 reveal variant bands in exon 5 for the cell lines 98-4, 98-1, and 97-1, and in exon 8 for the cell lines 96-1, 1591, 2237, 2240, and possibly 1422. No variant bands were found for the control C3H10T1/2 and C3H/HeNCr(MTV−) epidermal cells. To preclude overestimation of nucleotide polymorphism, both the SSCP variants and exons 5 and 8 of the apparently normal samples were subcloned into pBluescript and sequenced. By this thorough investigation, all 11 tumor-derived cell lines were found to exhibit p53 mutations (Table 1; Fig. 2) while only wild-type sequences were detected in the control cells. In addition to the mutant sequences, wild-type p53 sequences were also present in tumor cell lines 97-1, 97-4, 97-5, 98-1, 98-4, and 2240.

1 The abbreviations used are: MTV, mammary tumor virus; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.
UV-Induced Mutations of p53. The p53 mutations were predominantly base substitutions at dipyrimidine sequences and no insertions or deletions were observed. Of a total of 28 base changes (identical changes on different alleles in the same cell line were counted only once), 25 (89.3%) were at or opposite dipyrimidine sites (Table 1). Apart from five T→C transitions, all changes at possible UV damage sites were comprised of C→T and CC→TT substitutions typical of UV-induced mutations in mammalian cells (17). In addition, in the case of UV damage repair, the two main lesions are not removed at equal rates, and for any given region of chromatin, (6-4) photoproduct formation (Table 2). The nontranscribed strand accounted for only 35.7% of all TC sites in exons 5 and 8 but contained 80% of all mutations at TC sequences. The strand bias that was observed in this study is in accordance with the principles of preferential DNA repair (14, 15). There is a hierarchy in the repair of DNA whereby transcriptionally active genes are repaired before the rest of the genome (15), and the transcribed strand is processed before the nontranscribed strand (14). This phenomenon, along with the differences in fidelity of DNA replication between the leading and lagging strands (16), has been implicated in the strand specificity of UV-induced mutations in mammalian cells (17). In addition, in the case of UV damage repair, the two main lesions are not removed at equal rates, and for any given region of chromatin, UV-specific mutations in the p53 gene have been detected in human (2, 10, 11) and murine (12) skin tumors. UV mutagenesis. Similar UV-specific mutations in the p53 gene have been observed in human (2, 10, 11) and murine (12) skin tumors.

Thus, the mutations were not artifacts produced during the propagation of the cell lines. This conclusion was further supported when four of the original tumors (97-1, 1422, 1591, and 2237) were examined and found to contain the mutations identified in the corresponding tumor cell lines. The paucity of transitions at the C residue of CG sequences, which usually occur due to spontaneous deamination of 5-methyl cytosine, is also noteworthy since such mutations are common in tumors of internal organs (2, 13).

Preferential Mutagenesis of the Nontranscribed Strand of the p53 Gene. Assignment of mutations at possible UV lesion sites to the transcribed or nontranscribed strands of DNA demonstrated a remarkable strand bias. There was a pronounced tendency for the mutations to originate on the nontranscribed strand (z test, P < 0.0007), with only 24% arising on the transcribed strand (Table 2). Further analysis of our data indicates that the strand specificity was most prominent for mutations at TC sequences (z test, P = 0.0001), the primary sites of (6-4) photoprotein formation (Table 2). The nontranscribed strand accounted for only 35.7% of all TC sites in exons 5 and 8 but contained 80% of all mutations at TC sequences. The strand bias that was observed in this study is in accordance with the principles of preferential DNA repair (14, 15). There is a hierarchy in the repair of DNA whereby transcriptionally active genes are repaired before the rest of the genome (15), and the transcribed strand is processed before the nontranscribed strand (14). This phenomenon, along with the differences in fidelity of DNA replication between the leading and lagging strands (16), has been implicated in the strand specificity of UV-induced mutations in mammalian cells (17). In addition, in the case of UV damage repair, the two main lesions are not removed at equal rates, and for any given region of chromatin, (6-4) photoproducts are repaired more efficiently than pyrimidine dimers (15). While

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**Table 1 p53 mutations in UV-induced murine skin tumors**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Exon</th>
<th>Codon</th>
<th>Nucleotide* change</th>
<th>Amino acid change</th>
<th>Strand*</th>
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<tbody>
<tr>
<td>97-1c</td>
<td>5</td>
<td>140</td>
<td>CCT GTC→CCT TICG</td>
<td>Val→Leu</td>
<td>NA</td>
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<tr>
<td></td>
<td></td>
<td>143</td>
<td>TGC GTC→TGA GTC</td>
<td>Trp→Stop</td>
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<tr>
<td></td>
<td></td>
<td>145</td>
<td>GTC AGC→GTC GGC</td>
<td>Ser→Cys</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>154→155</td>
<td>GTC GCC→GCT TGC</td>
<td>Val Arg→Ala Cys</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>175→176</td>
<td>CAC CAT→CAT CAT</td>
<td>His His→His Tyr</td>
<td>−</td>
</tr>
<tr>
<td>97-5c</td>
<td>5</td>
<td>145</td>
<td>AGC GCC→AGT GCC</td>
<td>Ser→Ser</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>145</td>
<td>AGC GCC→AGT GCC</td>
<td>Ser→Ser</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>163</td>
<td>TCA CAG→TGC CAG</td>
<td>Ser→Ser</td>
<td>NA</td>
</tr>
<tr>
<td>98-1c</td>
<td>5</td>
<td>143</td>
<td>TGC TGC→TGA GTC</td>
<td>Trp→Stop</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>154→155</td>
<td>GTC GCC→GCT TGC</td>
<td>Val Arg→Ala Cys</td>
<td>−</td>
</tr>
<tr>
<td>98-4c</td>
<td>5</td>
<td>143</td>
<td>TGC TGC→TGA GTC</td>
<td>Trp→Stop</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>154→155</td>
<td>GTC GCC→GCT TGC</td>
<td>Val Arg→Ala Cys</td>
<td>−</td>
</tr>
<tr>
<td>96-1</td>
<td>8</td>
<td>270</td>
<td>GTT GCT→GTT IGT</td>
<td>Arg→Cys</td>
<td>−</td>
</tr>
<tr>
<td>97-4c</td>
<td>8</td>
<td>295</td>
<td>CCT GAA→CCT AAA</td>
<td>Gly→Lys</td>
<td>+</td>
</tr>
<tr>
<td>1422</td>
<td>8</td>
<td>275</td>
<td>CCT→CTT</td>
<td>Pro→Leu</td>
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</tr>
<tr>
<td></td>
<td>275</td>
<td>CCT→CTT</td>
<td>Pro→Leu</td>
<td>−</td>
<td></td>
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<tr>
<td></td>
<td>279</td>
<td>GAC GCC→GAC IGC</td>
<td>Arg→Cys</td>
<td>−</td>
<td></td>
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<tr>
<td>1463</td>
<td>8</td>
<td>270</td>
<td>GTT GCT→GTT IGT</td>
<td>Arg→Cys</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>270</td>
<td>GAA GAA→GAA AAA</td>
<td>Glu→Lys</td>
<td>+</td>
<td></td>
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<tr>
<td>1591</td>
<td>8</td>
<td>270</td>
<td>GTT GCT→GTT IGT</td>
<td>Arg→Cys</td>
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<tr>
<td>2237</td>
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<td>270</td>
<td>GTT GCT→GTT IGT</td>
<td>Arg→Cys</td>
<td>−</td>
</tr>
<tr>
<td>2240c</td>
<td>8</td>
<td>267</td>
<td>TTT→TCT</td>
<td>Phe→Ser</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>270</td>
<td>GTT GCT→GTT IGT</td>
<td>Arg→Cys</td>
<td>−</td>
<td></td>
</tr>
</tbody>
</table>

* Sequence of the nontranscribed strand (5'→3') is shown and the affected bases are indicated by underlining. Bases adjacent to the mutation(s) are included to show dipyrimidine sites. Nontranscribed mutations on the same allele are grouped together by brace.

* Strands are indicated as transcribed (+) or nontranscribed (−) for mutations that occur at possible dipyrimidine lesion sites. NA, absence of dipyrimidine sequences at the mutation site.

* These tumors also contained wild-type alleles in addition to mutant alleles.
lines (97-1, 98-1, 98-4, 1463, 2240, 97-5, and 1422) were found to
have demonstrated that different p53 mutants obtained from tumors
of such heterogeneity could provide significant improvement in the
other recent studies (11, 12) along with ours substantiates the role of
preferential repair.

Multiple Alleles of Mutant p53 and Tumor Heterogeneity. Since
primary tumor masses often consist of clones of cells that have dif-
ferent biological properties (18) such as karyotype, surface receptors,
growth rate, and metastatic ability, understanding the molecular basis
of such heterogeneity could provide significant improvement in the
management of cancer metastasis. Previous work by Halevy et al. (19)
has demonstrated that different p53 mutants obtained from tumors
possess distinct biological activities. In our study, seven tumor cell
lines (97-1, 98-1, 98-4, 1463, 2240, 97-5, and 1422) were found to
carry more than one mutant allele of p53. In two of the cases (97-5 and
1422) it is likely that mutant p53 alleles with single base changes were
targets of secondary mutation events, perhaps because of continued
exposure to UV radiation during tumor progression. The mutations in
cell line 97-1 are especially interesting; four different sets of muta-
tions were found in it (Fig. 2a), including one with a stop codon
(mutations at codons 140/143, 145, 154/155, and 175/176 were de-
tected in different subclones). In addition to the p53 mutations, cell
lines 98-1, 98-4, and 97-1 contained identical mutations at codon 61
of the N-ras oncogene (5). Thus different combinations of mutations
in tumor suppressor genes and oncogenes were present, providing a
molecular basis for tumor heterogeneity.

Significance of Silent p53 Mutations in UV Carcinogenesis. All
the mutations detected in cell line 97-5 were synonymous (20), rep-
resenting silent substitutions at codons for serine residues. While such
mutations leave the primary sequence of the protein intact, it is inter-
esting to consider the bias in codon usage in p53. The codons AGC
(position 145) and TCA (position 163), respectively, encode 31.4 and
20.0% of all the serine residues in the p53 gene, but their mutated
counterparts (AGT and TCG) account for only 5.7 and 2.9% of the
amino acid. In all, the substituted codons are 5.5- and 6.9-fold less
favored in the p53 sequence and may lead to a reduction in transla-
tional efficiency. Such an effect, if present, would be most pronounced
for gene sequences containing the double base changes at codons
145/163. The overall codon bias in the mouse (877 genes found in
Genbank 63, compiled by J. Michael Cherry at Massachusetts General
Hospital) follows a similar, albeit less explicit, trend and helps to
explain the significance of rare synonymous mutations in tumors
where a strong selection for transforming base changes must exist.

Concluding Comments. Based on comparative sequencing of the
p53 tumor suppressor gene from a variety of tumor cell lines, we
provide strong evidence for the causal involvement of p53 in the
pathogenesis of UV-induced skin cancers. In a murine model system,
the gene was shown to be mutated, often in a strand-specific manner,
in all 11 tumors examined. Our investigations also furnish direct
evidence of tumor heterogeneity at the molecular level. Future studies
on clonal populations of cells harboring the different p53 alleles
identified in this study could provide new insight into the mechanism
of tumor heterogeneity.

Acknowledgments
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critically reviewing the manuscript and Leigh Anne Roth and Jackie Walker for
excellent technical assistance.

References

Table 2 Strand bias of p53 mutations at different dipyrimidine sites in exons 5 and 8

<table>
<thead>
<tr>
<th>Dipyrnidine</th>
<th>No. of sites</th>
<th>No. of mutations detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>sequences</td>
<td>+ strand*</td>
<td>- strand*</td>
</tr>
<tr>
<td>in exons 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>TC</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>CT</td>
<td>24</td>
<td>15</td>
</tr>
<tr>
<td>CC</td>
<td>18</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td>76</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>+ strand*</td>
<td>- strand*</td>
</tr>
<tr>
<td>in exons 8</td>
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<td></td>
</tr>
<tr>
<td>TT</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>TC</td>
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<td>10</td>
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<td>15</td>
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<td>CC</td>
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<td>31</td>
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<tr>
<td>Total</td>
<td>76</td>
<td>67</td>
</tr>
</tbody>
</table>

* Strand assignments are as in Table 1.
** The mutation at codon 283 may have originated at a TC or a CT site and has been
counted once for each possible site.
*** The mutation at codon 275 was similarly counted once as a CC and once as a CT site.
**** Exact number of mutations detected.

Fig. 2. Mutations in the p53 tumor suppressor gene in UV-induced murine skin cancers. Left, DNA sequence of mutated codons; right, codon numbers. * base substitutions. All
mutations shown in a occur in the tumor cell line 97-1. b, all the mutations detected in
exon 8.

Differences in repair rates may be an important factor in determining
the mutation bias, another possibility is that UV radiation may induce
mutations at specific sites on the p53 gene. For instance, the arginine
to cysteine mutation at codon 270 is common in human (where the
Corresponding codon number is 273) and murine tumors (6). How-
ever, an examination of the UV-induced p53 mutations detected in
other recent studies (11, 12) along with ours substantiates the role of
preferential repair.

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