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Advances in Brief

Selective Targeting of *p53* Gene Mutational Hotspots in Human Cancers by Etiologically Defined Carcinogens[1]

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Abstract

In lung and liver cancers, *p53* mutations are mostly G:C to T:A transversions. This type of mutation is known to be induced by benzo(*a*)pyrene and aflatoxin B_1 which are associated with the etiology of lung and liver cancers, respectively. Using a novel assay based on DNA polymerase fingerprint analysis, we identified *p53* nucleotides targeted by these carcinogens. Thirteen of 14 nucleotide residues of the *p53* gene which underwent G:C to T:A mutations in lung cancers were targeted by benzo(*a*)pyrene. Similarly, aflatoxin B_1 formed adducts at a mutational hotspot specific for liver cancer. The same nucleotide (third base of codon 249), which mutates rarely in lung cancers, was not a target for benzo(*a*)pyrene. These *in vitro* observations indicate that *p53* mutational hotspots identified in different tumors are selected targets specifically for the etiologically defined environmental carcinogens.

Introduction

Human cancers commonly display mutations in the *p53* tumor suppressor gene that are scattered over nearly 100 different base pairs. Both sites and types of mutations differ among cancers of different tissue origin (1). Table 1 shows *p53* mutations identified in lung and liver cancers as compared with colon cancers. Most of the mutations in colon cancers were G:C to A:T transitions which predominantly occurred at CpG sites (1). These G:C to A:T mutations may result from the deamination of 5-methyl cytosines found at CpG sites (2). When compared with colon cancers, lung cancers as well as primary liver cancers from China and southern Africa displayed different spectra of mutations dominated by G:C to T:A transversions (Refs. 1 and 3–7, 25, 26; and Footnote 3). The reasons of tissue-specific mutations of the *p53* are unknown. One hypothesis is that *p53* mutational spectra reflect the etiological contributions of factors acting as carcinogens in these tissues (1). Both BP,[4] present in tobacco and other combustion products, and AFB, present in foods contaminated by certain *Aspergillus* species, are carcinogens which induce G:C to T:A transversions (8–13). It is well known that the selectivity of this mutagenic activity is determined primarily at the level of base-specific adduct formation between DNA and the activated forms of these carcinogens (14–16). We therefore studied directly the interaction of activated forms of BP and AFB with the *p53* DNA sequences and identified nucleotide residues which are selectively targeted by these carcinogens.

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[3] T. Soussi and C. Caron de Fromentel, personal communication.

[4] The abbreviations used are: BP, benzo(*a*)pyrene; BPDE, benzo(*a*)pyrene7,8-diol-9,10-epoxide; AFB, aflatoxin B_1; AFBE, aflatoxin8, 9-epoxide; DPFA, DNA polymerase fingerprint analysis.

Materials and Methods

Plasmid pC53SN3 (17) containing a full-length human *p53* complementary DNA insert was treated with activated forms of AFB or BP, namely AFBE and BPDE. Nucleotide residues which formed adducts with the carcinogens were identified by DPFA.

Plasmid DNA was purified by cesium chloride gradient centrifugation (18). Stock solutions of AFBE (6.0 mM in acetone) and BPDE (1.5 mM in dimethyl sulfoxide) were diluted in acetone and dimethyl sulfoxide, respectively, to obtain 1.2 and 0.3 nmol of each carcinogen under 10 μl of working solution. Ten μl of each working solution were reacted with 30 μg of plasmid. After treatment with AFBE or BPDE, plasmid DNA was precipitated in ethanol, resuspended in 10 mM Tris-HCl (pH 7.4): 1 mM EDTA, and stored at −70°C until analysis. The number of adducts per μg of plasmid DNA were determined by high-pressure liquid chromatography analysis following hydrolysis as described (19, 20).

DPFA is based on dideoxynucleic acid sequencing which depends upon base-specific termination of DNA polymerase-catalyzed primer extension reactions. A commercial kit (T_7 sequencing kit; Pharmacia, Uppsala, Sweden) was used without major modification of supplier's instructions. Double-stranded DNA was denatured to single strands by incubation in 0.2 M NaOH for 10 min or by heating at 95°C for 5 min and then subjected to sequencing reaction. Four separate reactions were performed, all containing template DNA, four deoxynucleotides (each including a different chain-terminating dideoxynucleotide), and one of the following primers: p53-F3 (5′-GTTGGCTCTGACTGTACCAC-3′); p53-F10 (5′-TCATCTTCTGTCCCTTCCC-3′); p53-F11 (5′-GTTGATTCCACACCCCCG-3′); p53-F12 (5′-ACTCCCCTGCCCT-CAACAAG-3′); p53-R3 (5′-CTGGAGTCTTCCAGTGTGAT-3′); p53-R10 (5′-AAATATTCTCCATCCAGTGG-3′); p53-R11 (5′-AA-ATTTCCTTCCACTCGG-3′); or p53-R13 (5′-TTCCGTCCC-AGTAGATTACC).

The products of sequencing reaction were separated by denaturing polyacrylamide gel electrophoresis (6 to 10% gels). Sequencing gels were dried and exposed to X-ray films at −70°C for 2 to 4 days.

Results

The plasmid pC53SN3 (17) containing a full-length wild-type *p53* complementary DNA insert was treated with activated forms of AFB and BP to produce 0.3 to 2 adducts per 1,000 bases. pC53SN3 plasmid DNA treated under the same conditions with solvents only was used as control.

DNA Polymerase Fingerprint Analysis. *p53* nucleotide residues specifically targeted by AFB and BP were identified by DPFA. This method is based on the dideoxynucleic acid sequencing which depends upon base-specific termination of DNA polymerase-catalyzed primer extension reactions (21). The analysis of control DNAs by autoradiography shows base-specific terminations at the expected nucleotide residues (Fig. 1). Bulky deoxynucleotide adducts formed by carcinogens such as BP and AFB are no longer recognized by T_7 DNA polymerase (22), which dissociates from the DNA template after encountering an adduct (23), and the synthesis reaction terminates at the nucleotide preceding the adduct (22). As a result, bands

Table 1 *p53 substitution mutations in cancers of the lung, liver, and colon*

Mutation	Lung[a]	Liver[b]	Colon[c]
G:C->T:A	26 (46)[d]	11 (92)	0 (0)
G:C->A:T	13 (23)	0 (0)	31 (76)
G:C->C:G	11 (19)	1 (8)	1 (2)
A:T->T:A	3 (5)	0 (0)	3 (7)
A:T->G:C	2 (3.5)	0 (0)	6 (15)
A:T->C:G	2 (3.5)	0 (0)	0 (0)
Total	57 (100)	12 (100)	41 (100)

[a] Data from Refs. 3 to 7 and Footnote 3.
[b] Data obtained in hepatocellular carcinomas from China and southern Africa (25, 26).
[c] Data from Ref. 1.
[d] Numbers in parentheses, percentage of mutations.

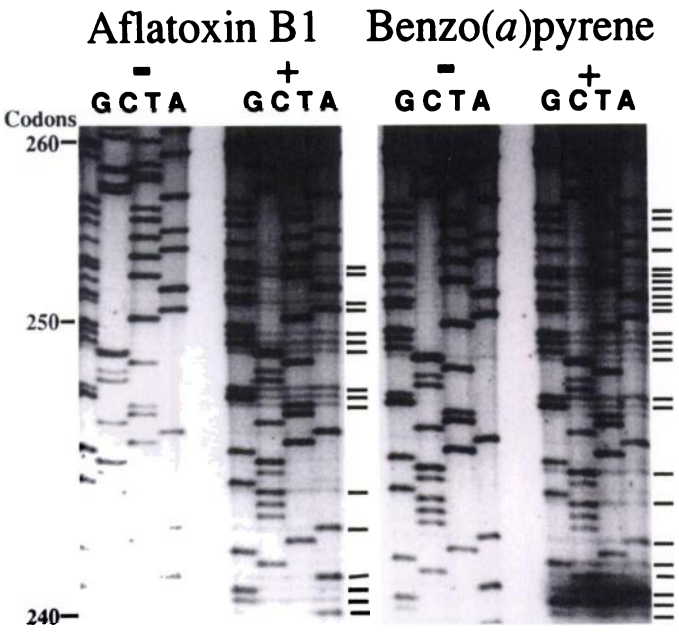


Fig. 1. Fingerprint analysis of the binding of AFB and BP to the *p53* DNA sequences. AFB and BP fingerprints on the transcribed strand between codons 240 and 260 are shown (+). Control DNAs treated with solvents only are also shown for comparison (−). *C, G, T, A* indicate deoxynucleotides in the template DNA. The presence of a band common to four lines (−) indicates the presence of an adduct at the adjacent 5′ nucleotide (*upper band* on the gel) in the template DNA. Note that most of the premature terminations occur at nucleotides preceding guanine residues in the template DNA.

Table 2 *Percentage of p53 nucleotides (exons 5 to 8) targeted by benzo(a)pyrene and aflatoxin B_1*

Base	Benzo(*a*)pyrene (%)	Aflatoxin B_1 (%)
Guanine	87	62
Cytosine	14	6
Thymine	19	2
Adenine	11	3

common to all four lanes (G, C, T, A) reflecting immature chain terminations at the sites of the modified nucleotides in the template DNA were detected (Fig. 1). Single-strand breaks which may occur at the sites of alkaline-labile adducts could also cause premature chain terminations (14–16, 24). In our assay, such a chain termination will also occur at the nucleotide preceding the adduct and provide the same type of information. We compared data generated from heat- and alkaline-denatured plasmids. The sequencing quality obtained with a heat-denatured plasmid was poor, but the pattern of premature chain terminations was similar to that of an alkaline-denatured plasmid (data not shown).

DPFA was repeated several times on different batches of plasmids treated with the same carcinogens, and essentially identical results were obtained. The intensity of adduct-related extra bands detected by this assay was dependent on the adduct:DNA ratio as expected. Similar to previous findings with the BP (16), the sequence selectivity of the binding of AFB and BP did not vary significantly at different adduct:DNA ratios (data not shown).

Analysis of adduct formation with nucleotides of exons 5, 6, 7, and 8 of the *p53* gene (a total of 1086 bases) indicated that 36% and 20% of bases were targeted by BP and AFB, respectively. As shown in Table 2, both BP and AFB targeted preferentially guanine residues. BP also targeted 11 to 19% of non-guanine residues (Table 2). Thus, compared with BP, the binding of AFB to *p53* sequences was restricted to fewer residues and more specific for guanine residues.

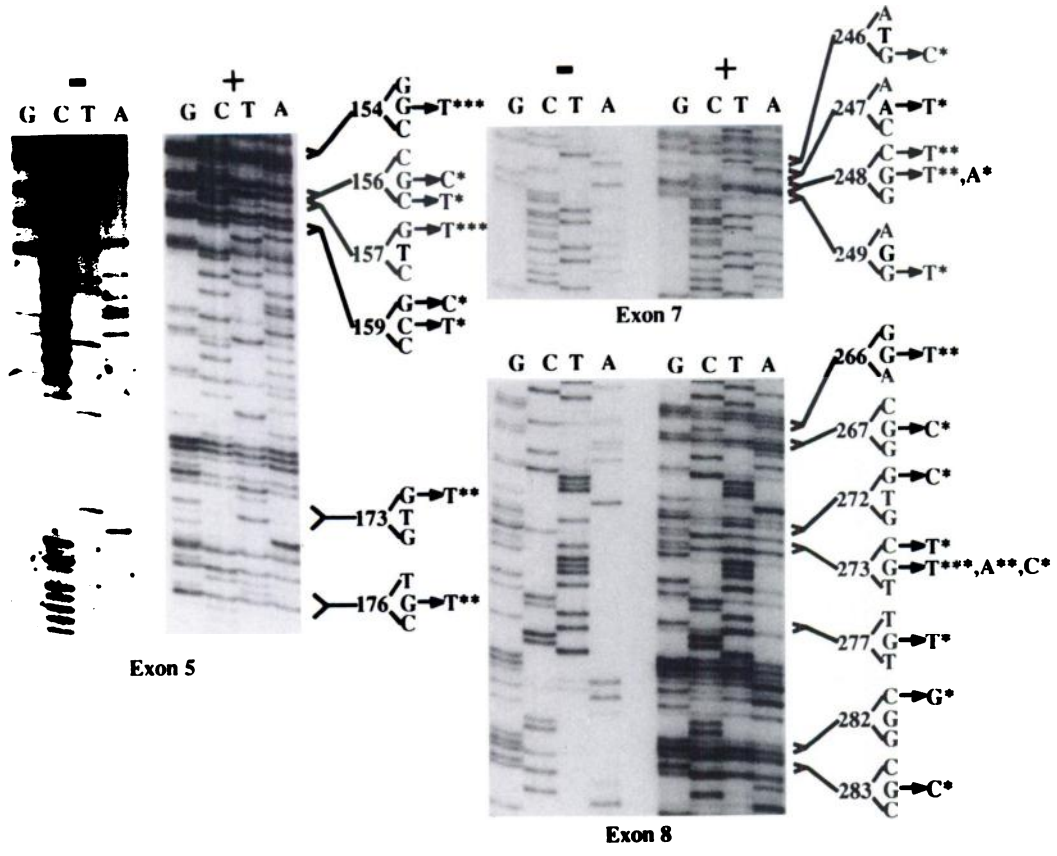
Selective Targeting of *p53* Nucleotides by Chemical Carcinogens. Fig. 2, *Top*, shows fingerprint analysis of BP-DNA adducts in the nontranscribed strand in three regions of the p53 gene (exons 5, 7, and 8) which bear most of the mutational hotspots in lung cancer. With one exception, all of the guanine residues mutated in lung cancers were targeted by BP. Table 3 shows the binding of the BP to base pairs previously found to be mutated in lung cancers. Of the 40 different base pairs involved in 57 mutations identified in these cancers, 32 (80%) were targeted by BP. Twenty-six of 57 mutations were G:C to T:A transversions, and 25 of them (96%) occurred in base pairs modified by BP. Guanine residues at codons 154, 157, 248, and 273 of the *p53* gene are mutational hotspots in lung cancers (Table 3). It is noteworthy that all of these residues were strongly targeted by BP. However, due to the small number of mutations identified (1 to 2 mutations per residue), it is difficult to relate the strength of the BP interaction to the mutation frequency in other residues (Table 3).

Fig. 2, *bottom*, compares the binding of AFB and BP to the region around codon 249 of the *p53* gene. The same carcinogen:plasmid DNA ratio was used for both AFB and BP. The binding of AFB to the codon 249 region of the *p53* gene was stronger than the binding of BP. Both carcinogens targeted the same residues with the exception of one residue. The exception was the last nucleotide of codon 249, which was targeted by AFB only, but not by BP. This guanine residue is a mutational hotspot in liver cancers from patients at high risk of exposure to AFB (25, 26). In contrast, the same residue mutated rarely in lung cancers. As shown in Table 2, only one of 57 mutations thus far identified in lung cancers occurred at this guanine residue.

Discussion

Our studies demonstrate that most of the *p53* nucleotides previously identified as mutational hotspots in lung and liver cancers are preferred targets for the environmental carcinogens BP and AFB. Previous studies have shown that both BP and AFB induce substitution mutations at the target nucleotides, the most frequent being G to T transversions (8–13). In this regard, it is noteworthy that the majority of *p53* mutations in lung and liver cancers are G to T transversions (Table 1). Most of these G to T mutations could, therefore, be induced by the etiologically defined environmental carcinogens BP and AFB.

Many factors are involved in the complex process of mutation in cells exposed to environmental carcinogens. For the AFB and BP, mutational specificity is determined by the sequence specificity of targeting (14–16). The interaction of these muta-



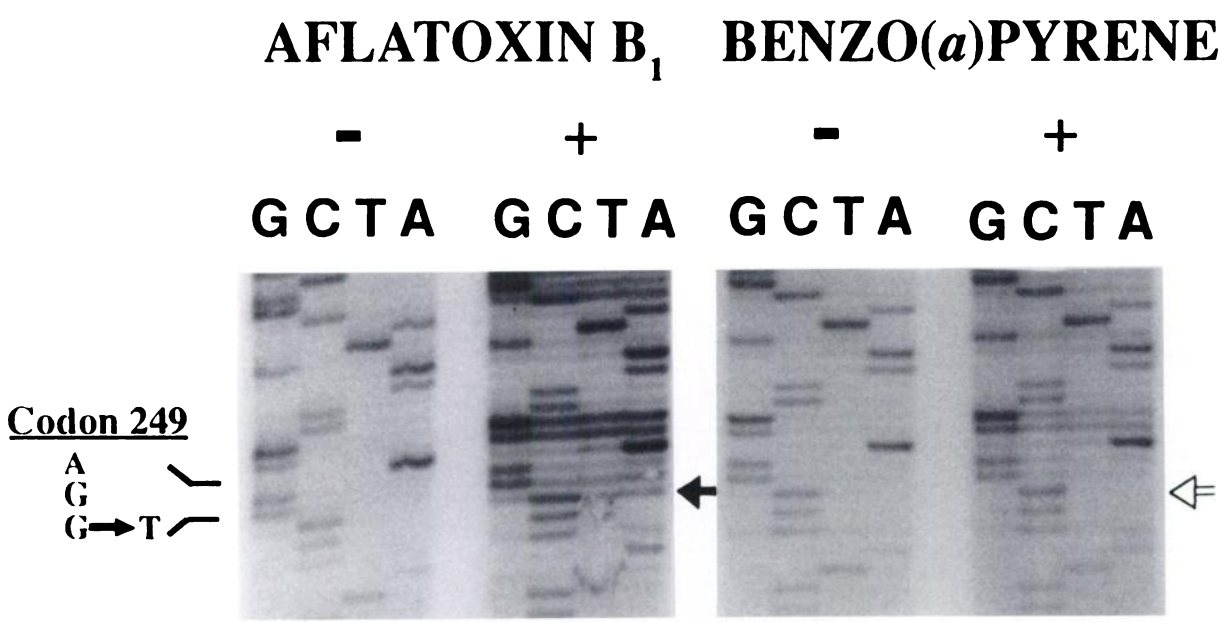


Fig. 2. *Top*, lung cancer-associated mutational hotspots in exons 5, 7, and 8 of the *p53* gene selectively targeted by BP. The nucleic acid sequences of the control (−) and BP-treated (+) *p53* gene (nontranscribed strand) at codons 150 to 180 (exon 5), at codons 243 to 258 (exon 7), and at codons 265 to 285 (exon 8) are shown. *Numbers at the right of (+) lines* indicate *p53* codons which are found to be mutated in lung cancers. Mutated nucleotide residues and types of mutations are also shown. *, Number of mutations as described in Refs. 3–7 and Footnote 3. *Bottom*, differential targeting of codon 249 bases of the *p53* gene by aflatoxin B_1 and benzo(*a*)pyrene. Note that the second G residue is targeted by aflatoxin B_1 but not by benzo(*a*)pyrene.

gens in equimolar amounts with the double-stranded DNA results in adduct formation mostly with guanine residues. However, all guanines are not equally targeted. The secondary structure mostly determined by the relative positions of different nucleotides in a short sequence probably plays a critical role in the specificity of targeting. Our assay allowed the determination of this critical step in the targeting of *p53* sequences by AFB and BP.

Although nearly all guanine residues which are replaced by thymines in lung and liver cancers were targeted by BP or AFB,

Table 3 *Binding of benzo(a)pyrene to p53 gene base pairs mutated in the lung cancers*

Codon/base	BP[a]	*p53* substitution mutations[b] G:C->T:A	Total
132/3	★G:C	1	1
134/3	T:A		1
135/2	★G:C		1
138/2	C:G★		1
143/1	★★G:C	1	1
151/1	★C:G★		1
151/2	C:G★	1	1
154/2	★★G:C	3	3
156/2	★★G:C★		1
156/3	C:G★★		1
157/1	★★G:C	3	3
159/1	★★G:C		1
159/2	C:G★★		1
173/1	★G:C	2	2
176/2	★G:C	2	2
179/3	T:A		1
193/2	A:T		1
194/2	T:A		1
196/1	★★C:G★★		1
198/1	★★G:C	2	2
213/1	★★C:G★		1
229/3	T:A		1
237/3	★★G:C		1
242/2	★★G:C★		1
244/1	★★G:C		1
246/3	★G:C		1
247/2	A:T		1
248/1	★C:G★★		2
248/2	★★G:C★★	2	3
249/3	G:C	1	1
266/2	★G:C	2	2
267/2	★★G:C		1
272/1	★G:C		1
273/1	G:G★★		1
273/2	★★G:C★	3	6
277/2	★G:C	2	2
282/1	★C:G★★		1
283/2	★★G:C		1
286/2	A:T		1
302/3	★★G:C	1	1
Total mutations		26	57
BP targeted		25	49
% targeted		96	86

[a] Binding of BPDE to nucleic acid residues in the base pair determined as described in the legends to Figs. 1 and 2. ★ and ★★, weak and strong binding of BP to the nucleotide next to the star, respectively.

[b] Data compiled from Refs. 3 to 7 and Footnote 3.

not all targeted guanines on the *p53* gene were found to be mutated in these tumors. For example, 87% of guanine residues on exons 5 to 8 of the *p53* gene were targeted by BP (Table 2), but only 12% of them have been shown to be mutated in lung cancers. This finding is not totally unexpected, because selective targeting is necessary but not sufficient in the cellular process of genetic mutation (27). DNAs undergoing transcription (active genes) are preferentially modified by chemical carcinogens (27, 28). Since the *p53* gene is active in normal cells, it is probably more accessible to AFB and BP. In response to preferential targeting, transcriptionally active genes are repaired preferentially (27–30). Thus, damaged *p53* sequences will probably be repaired efficiently in normal cells. However, due to a preferential repair of the transcribed strand of the active genes, the nontranscribed strand is repaired less efficiently (28–30). The strand bias in the repair of the *p53* gene could result by a higher frequency of mutation in the nontranscribed strand. In this regard, it is noteworthy that 36 of 37 G to T mutations of the *p53* gene which were identified in lung and liver cancers occurred at the nontranscribed strand (Table 3, Refs. 25 and 26).

In addition to factors involved in the targeting and repair of *p53* nucleotides, other factors directly related to the biological functions of the *p53* oncoprotein may also contribute to the frequency and nature of mutations detected in tumors. The wild-type *p53* functions as a growth suppressor protein, and most mutant *p53* genes are deficient in growth suppressor activity (1, 17, 31). Thus, *p53* is considered a tumor suppressor gene. As reviewed by Hollstein *et al.* (1), *p53* mutations found in different human tumors are not random and occur on about 90 residues within a 600-base pair region. In addition, silent or double mutations of the *p53* gene are extremely rare. Both data suggest that only those mutations which contribute to the loss of *p53* growth suppressor function will persist and be detectable in tumors.

Furthermore, different mutant *p53* proteins display different biological properties. For example, some *p53* mutants are able to induce wild-type *p53* to a mutant conformation, whereas some others are not (for a review, see Ref. 31). It also appears that some mutants of the *p53* can act as an oncogene (31). In this regard, liver-specific hotspot mutations found at the third base of codon 249 of the *p53* gene deserve further discussion. As shown in Fig. 2, *bottom*, AFB forms an adduct at this guanine residue, whereas BP does not. This finding correlates well with the high frequency of mutations at this residue in aflatoxin-related liver cancers as opposed to the very low frequency of mutation in BP-related lung cancers. AFB also binds other residues in the *p53* gene (for example, G residues at codon 248 as shown in Table 3), and yet codon 249 mutation is detected in up to 50% of liver cancers in Qidong, China (26) and in Mozambique.[5] In contrast, codon 248, which is a hotspot for mutation in other tumors, was not found to be mutated in liver cancers from the same geographical areas (25, 26). Therefore, the unexpectedly high frequency of codon 249 mutation in liver cancers studied could be due to other factors in addition to its being a selected target for AFB. For example, a site-specific repair defect or strong tissue-specific selection for a $249_{Arg \rightarrow Ser}$ mutant form of *p53* protein could be involved. Alternatively, tissue-specific cocarcinogenic factors such as hepatitis B virus could contribute to the strong selection for this mutation in liver cancers.

The direct role of exogenous carcinogens in the induction of mutations in the *p53* gene has not been addressed previously. Our data indicate that most of the mutations identified in lung and liver cancers occur in base pairs selectively targeted by two major carcinogens etiologically associated to these tumors. Since both AFB and BP induce G:C to T:A transversions and produce cancer in animals, it is likely that they also target the *p53* gene and induce mutations leading to the loss of its tumor suppressor functions. Close correlation between mutagen-mediated targeting and tumor-specific mutations in *p53* may indicate a direct role of exogenous carcinogens in the inactivation of this gene in human cancers. Future studies are designed to explore a number of other environmentally important carcinogens with respect to *p53* gene damage. Finally, further investigations will be focused on studying molecular and cellular events that follow selective targeting of the *p53* gene by environmental carcinogens.

[5] M. Ozturk *et al.*, Lancet, in press.

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