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p53 Gene Deficiency Does Not Enhance Instability of Mouse Minisatellites in Somatic Cells of Normal Tissues

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The effect of p53-deficiency on somatic mutation of minisatellites in normal tissues was examined using p53-deficient (−/−) mice. In total, 248 mice consisting of three different genotypes, +/+, +/− and −/−, were obtained and DNA from their embryos was probed with two minisatellites, Pc-1 and Pc-2. The somatic mutation was detected by Southern blot hybridization as the presence of a third nonparental band reflecting mosaicism in tissues. Mutation frequency of Pc-1 for (+/+) and (+/−) was 1.3% and 1.4%, respectively, which is consistent with previous studies. On the other hand, none of the mice lacking the p53 gene (−/−) exhibited mutation. The Pc-2 probe did not show any somatic mutation in the three groups. These results suggest that the p53 deficiency does not enhance the genomic instability of the minisatellite loci in normal somatic cells.

Key words: Genomic instability — p53-Deficient mouse — Minisatellite — *In vivo* mutation

The p53 tumor suppressor gene has received much attention because it is frequently mutated in cancers of humans and experimental animals.[1–4] Half or more of all human tumors examined have detectable p53 genetic lesions.[5] The p53 protein appears to mediate an important cell cycle checkpoint in progression from G1 to S phase, since the progression of cells expressing high levels of this protein is often blocked.[6,7] Loss of the G1-S checkpoint can lead to genomic instability, inappropriate survival of genetically damaged cells, and the evolution of cells to malignancy.[8] Alterations of the p53 gene were found in the late stage of carcinogenesis in most cases and enhanced malignant progression.[1,2] This suggests that such p53 deficiency influences a late stage, when other genetic changes already exist in cells. On the other hand, genetic studies of esophageal cancer show that the p53 gene mutation may be the step in the pathway of carcinogenesis.[9,10] This raises the possibility that loss of the p53 gene function induces genomic instability even at the early stage, i.e., in normal or non-cancerous somatic cells, as well as in cancer cells.

We previously isolated from the mouse genome two hypervariable minisatellites, designated as Pc-1 and Pc-2, located on chromosomes 4 and 6, respectively.[11–13] They consist of tandem repeats of short sequences and show extensive allelic variation due to differences in the number of repeats. The variation presumably arises through unequal meiotic or mitotic exchanges between tandem arrays.[14] These length-change mutations can be detected not only in germline cells but also in somatic cells by standard Southern blot hybridization.[14] Indeed, we established that mutations at the Pc-1 and Pc-2 minisatellite loci frequently occur after exposure of carcinogenic agents[15,16] and during metastatic processes,[17] using mouse fibrosarcoma cell lines. These results indicate that the minisatellite probes can monitor somatic mutations reflecting genomic instability.

Recently, p53-deficient mice have been developed by gene targeting techniques.[7,18] Combined use of this mouse and the minisatellites allows us to investigate whether the absence of the p53 gene affects genomic instability in normal cells *in vivo*. Here we describe the somatic mutation frequencies of the Pc-1 and Pc-2 minisatellite loci in p53-deficient and normal mice.

MATERIALS AND METHODS

Mice and mating procedure The p53-deficient mouse was established previously.[18] The mouse genome is composed of the C57BL/6(B6) and CBA genomes. Heterozygotes for the p53 gene were mated with B6 and the progeny heterozygous for p53 (+/−) were obtained. The mice (F1) were further mated with MSM and N2 heterozygotes were produced. MSM is an inbred strain derived from the Japanese wild mouse, *Mus. m. molossinus*.[19] The two types of mice, F1 males and N2 females, both heterozygous for the p53 mutation, were chosen as parental stocks for crossing. Their progeny were classified into three types in respect of the p53 gene: homozygotes for the mutation (−/−), heterozygotes (+/−), and wild-type (+/+). That determination was carried out using a three-primer set (Fig. 1). One primer (primer 2) was located in exon 3 of the p53 gene,[20] a second primer (primer 1) in a region 5′ to exon 1, and the remaining one (primer 3) in the inserted neo gene. Their sequences were AATTGACAAGTTATGCATCCATACAGTAC-

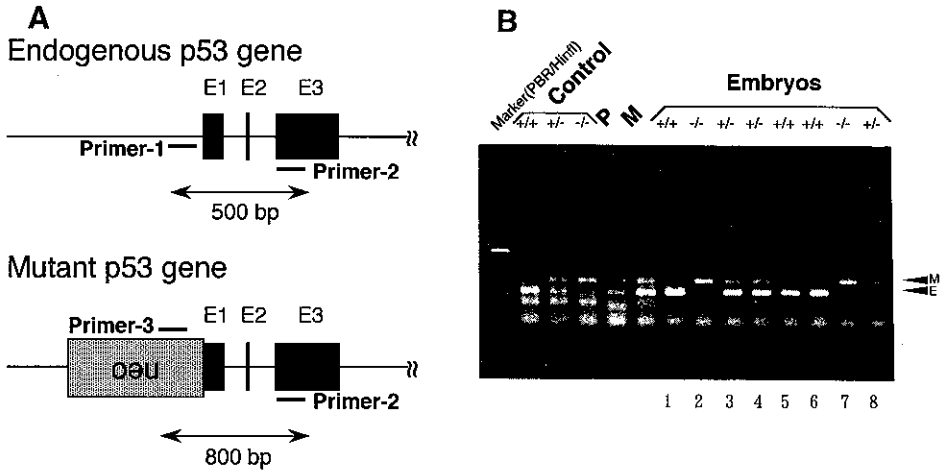

Fig. 1. p53 Geno-typing of embryos obtained. Maps in the left-hand panel display the locations of the three primers used: Primer 1 is located in a region 5′ to exon 1, primer 2 is in exon 3 of the p53 gene, and primer 3 is in the inserted neo gene. The pair of primer 1 and primer 2 amplified a p53-gene fragment of 500 bp. Primer 2 and primer 3 gave an 800 bp fragment containing a part of the p53 and the neo gene. The right-hand panel shows examples. The mice giving only a 500 bp band (marked by arrowhead E) are wild-type (+/+): lanes 1, 5 and 6; the mice showing an 800 bp band (arrowhead M) are mutant (−/−): lanes 2 and 7; and the mice with both bands are heterozygotic (+/−): lanes 3, 4 and 8.

A, ACTCCTCAACATCCTGGGGCAGCAACAGAT, and GAACCTGCGTGCAATCCATCTTGTTCAATG, respectively. Primer 1 and primer 2 amplified a region of the endogenous p53 gene to produce a fragment of 500 bp. Primer 2 and primer 3 gave an 800 bp fragment comprising a part of the neo and p53 genes. Therefore, the 500 bp band and the 800 bp band indicate the presence of the normal allele and mutant allele, respectively.

Genomic DNA of embryos of day 15 or day 16 was extracted by standard protocols[21] and subjected to polymerase chain reaction (PCR).[22] The reaction was carried out in 10 μl containing 50 ng of DNA, 40 ng of each primer, 10 m*M* Tris-HCl, pH 8.3, 50 m*M* potassium chloride, 1.5 m*M* magnesium chloride, 0.01% gelatin, and 0.5 unit of Taq polymerase (Takara, Kyoto), overlaid with 20 μl of mineral oil. Thermal cycling was carried out as followed: denaturing at 94°C for 1 min, annealing at 52°C for 1 min, polymerization at 72°C for 1 min, for 35 cycles. Aliquots of 5 μl of product were separated by electrophoresis on 1% agarose gel. The gel was stained with ethidium bromide to visualize bands.

Southern blotting and hybridization Transfer of DNA to a nylon membrane (Nytran, S&S, Dassel) was performed by a standard protocol.[11, 21] Pc-1 and Pc-2 probes, consisting of tandem repeats of GGGCA and GGCAGGA sequences, respectively, were labeled using a random priming protocol.[21] Hybridization was performed in 4× SSC (1×SSC: 0.15 *M* sodium chloride, 1.5 m*M* sodium citrate, pH7.6) and 1% SDS at 65°C. Final washes employed 0.1×SSC and 0.1% SDS at 65°C.

RESULTS

Somatic mutation of Pc-1 in p53 knock-out mice Mice hemizygous for the p53 mutation (+/−) were mated with each other and a total of 248 progeny were obtained. DNA was prepared from total embryos and p53 genotype was determined by PCR analysis (Fig. 1). The pair of primer 1 and primer 2 can amplify a region of the p53 gene to produce a 500 bp fragment, detection of which indicates the presence of the normal p53 allele. A second set of primer 3 and primer 2 provides an 800 bp fragment comprising a part of the p53 gene and the neo gene, which shows the existence of the disrupted p53 allele. Therefore, which band pattern is given in a DNA sample, a 500-bp band, a 800-bp band or both, determines one of the three p53 genotypes. The embryos were typed as follows: 62, homozygous for p53 mutation (−/−); 123, heterozygous (+/−); 63, wild type of the p53 gene (+/+).

Each of the DNA samples was digested with *Hinf* I, and the digests were separated by 1.3% agarose gel electrophoresis and hybridized with the ^{32}P-labeled Pc-1 probe. Fig. 2 shows one such experiment. The left panel displays DNA fingerprints that were obtained after washing under a less stringent condition. The three parental strains exhibited patterns with bands of different sizes,

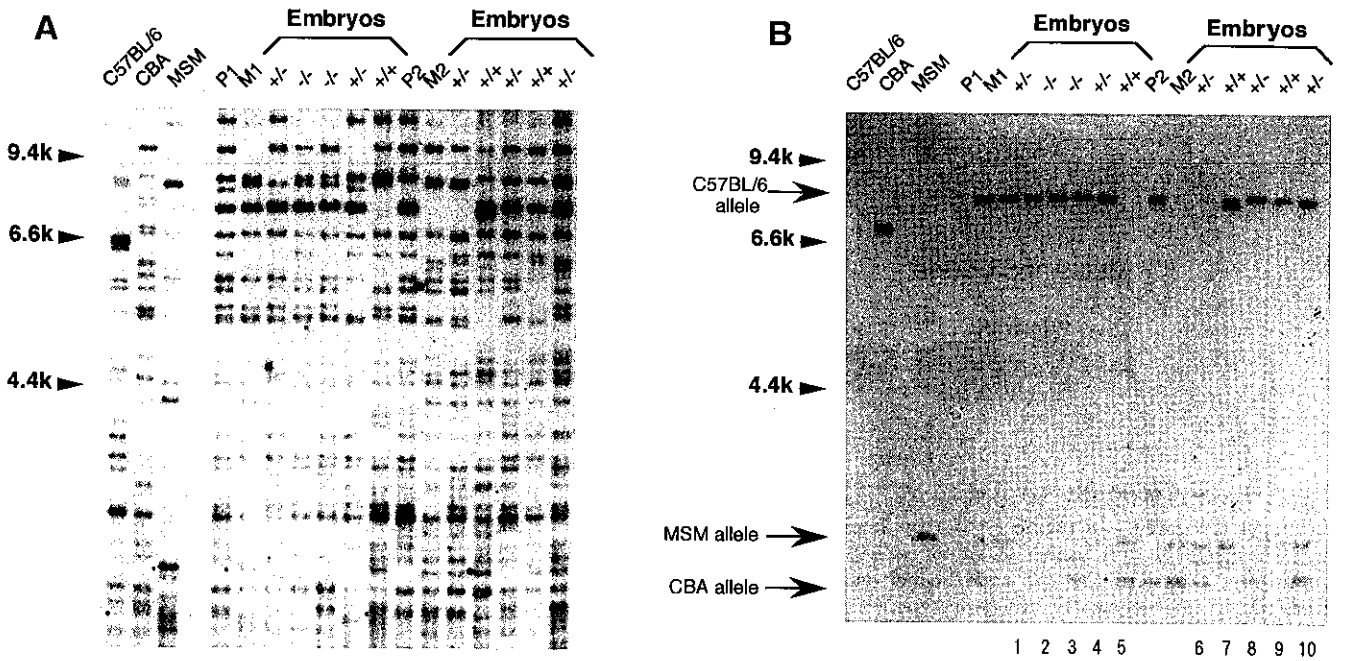

Fig. 2. Mutations in the Pc-1 locus. DNA was isolated from embryos and digested with *Hinf* I. The digests were subjected to Southern hybridization using the Pc-1 probe. The filter was washed in 1×SSC at 60°C (left). This condition provides DNA fingerprint patterns. The filter was washed further in 0.1×SSC at 65°C (right). This reveals the Pc-1 locus. Arrows indicate the positions of the parental C57BL/6, CBA and MSM alleles. The positions of B6 allele in embryos are different from that of B6 control. This is due to the germline mutation in the parental stock. Lane 7 shows an additional band reflecting somatic mutation.

and embryos showed a variety of patterns consisting of some of the above bands. Washing of this filter under a stringent condition revealed the Pc-1 locus (right panel). The identification of Pc-1-specific bands was done by comparison of the patterns obtained from less stringent and stringent washing. The locus was basically detected as two bands depending on the source; the C57BL/6(B6) allele appeared at about 8.0 kb and the CBA allele at about 2.1 kb. The band sizes varied to some extent due to recombination between repeats. The band at around 2.4 kb that hybridized with Pc-1 was derived from the MSM genome, but was not allelic to the bands of 8.0 kb and 2.1 kb. This unexpected finding was based on the following considerations. First, there were several individual mice exhibiting the three bands. An example is seen in lane 10 of Fig. 2B; the mouse has two bands of 8.0 kb and 2.1 kb, indicating that the Pc-1 locus is occupied by the B6 and CBA alleles. This was confirmed by PCR analysis using the polymorphic D4Mit9 marker located 2 cM distal to the Pc-1 locus.[23] The additional band of 2.4 kb seen in this lane must have derived from a region of the MSM genome different from the Pc-1 locus. Second, the 2.4 kb band and D4Mit9 showed different segregation patterns (data not shown). The Pc-1 homologous sequence of the 2.4 kb band in the MSM genome was thus designated as Pc-1m. The Pc-1m locus also showed extensive allelic variations in germline cells (data not shown).

The Pc-1 locus undergoes somatic mutations. Mutations of this type occur during very early development and are detected by the presence of a third nonparental band in a blot pattern of somatic tissues, resulting from mosaicism in cells.[12, 13] Suppose that a zygotic cell comprises A and B alleles and a descendant undergoes a somatic mutation from B to B′; then DNA from the embryo consisting of such mosaic cells shows three bands, A, B and B′. Three samples showed a nonparental band in the B6 allele. These nonparental bands were confirmed by analysis using two other enzymes. Their digestion changed the sizes of both parental (B) and nonparental bands (B′) and the size changes were apparently the same (Fig. 3). Besides, the band-signal ratios between the mutated band (B′) and its parental band (B) were almost the same, implying that the origin of the two bands is the same. The mutated band was either larger or smaller than the parental band, and the band-signal ranged from 0.1 to 1 relative to that of the parental band. Bands with signals weaker than 0.1 may have been missed. The nonparental band was not detected either in

the CBA allele or at the Pc-1m locus. This may result from weak signals. The mutation frequencies for the three different genotypes of the p53 gene are summarized in Table I. The frequencies for (+/+) and (+/−) were 1.3% and 1.4%, respectively, which is consistent with previous studies.[12, 13] None of the mice lacking the p53 gene (−/−) exhibited mutation, suggesting that the p53 deficiency does not enhance the genomic instability of the Pc-1 locus in normal somatic cells.

Somatic mutation of Pc-2 The filters used for the Pc-1 probe were washed and rehybridized with the Pc-2 probe (Fig. 4). The left panel shows DNA fingerprints obtained by washing under a less stringent condition. Some bands are similar to those detected by Pc-1. The right panel exhibits bands at the Pc-2 locus. The C57BL/6 and CBA DNA provided bands of 5.6 kb and 4.6 kb, respectively, whereas the MSM genome was not hybridized with Pc-2.

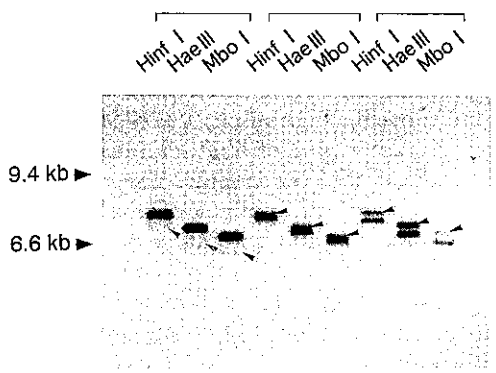

Fig. 3. Somatic mutations in three different enzyme digests. DNA samples showing somatic mutation in the *Hinf* I digests were digested with *Hae* III and *Mbo* I, and the three digests were subjected to hybridization with Pc-1. All three digests gave a nonparental band with the same intensity (marked by arrowheads).

Table I. Summary of Somatic Mutations for Pc-1 and Pc-2

Genotype of p53[a]	No of mice scored		No of alleles scored		Somatic mutation		Rate[c] (%)	
	Pc-1	Pc-2[b]	Pc-1	Pc-2	Pc-1	Pc-2	Pc-1	Pc-2
+/+	62	54	79	68	1	0	1.3	0
+/−	123	102	145	128	2	0	1.4	0
−/−	63	43	76	52	0	0	0	0

a) +/+, wild type for the p53 gene; +/−, heterozygote; −/−, homozygous mutant.
b) Pc-2 assay was not done for some of the mice used for Pc-1.
c) The mutation rate is expressed as %/mouse.

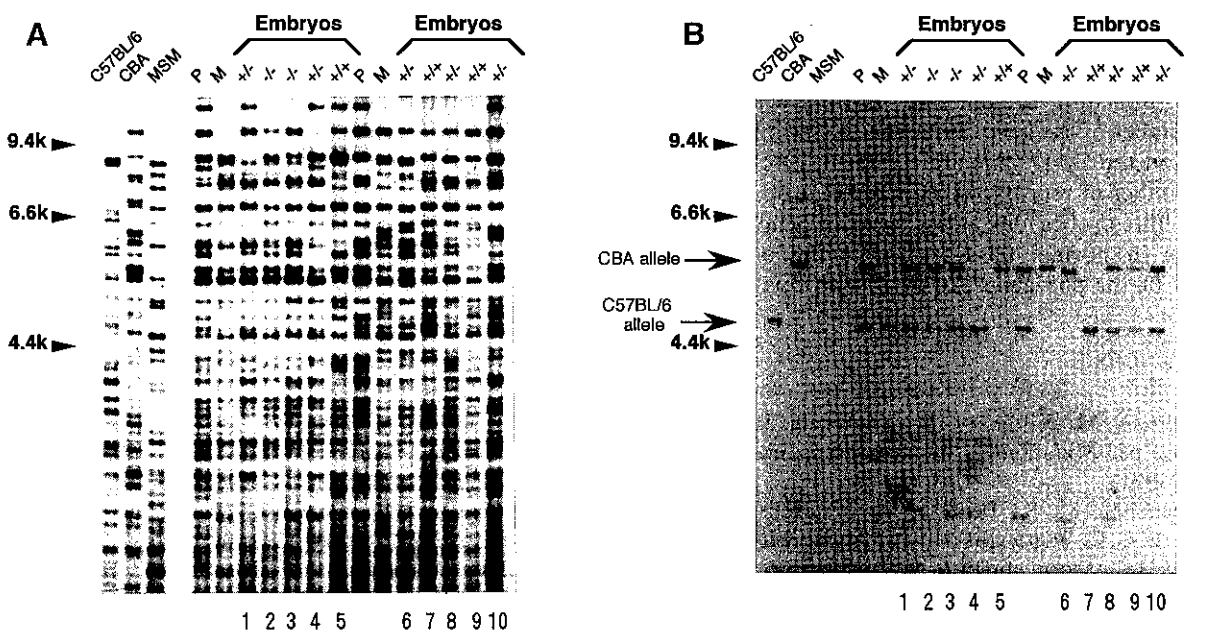

Fig. 4. Variations at the Pc-2 locus. The filters used for the Pc-1 probe were washed thoroughly to remove the probe, then rehybridized with Pc-2. The left panel shows DNA fingerprints and the right exhibits bands at the Pc-2 locus. Hybridization and washing conditions were the same as with Pc-1. Bands marked by arrows indicate alleles of the parental C57BL/6 and CBA alleles. MSM has no hybridizing sequence at the Pc-2 locus.

None of the mice in the three different genotypes underwent somatic mutation (Table I). This is consistent with the hypothesis that p53 deficiency does not enhance genomic instability of the minisatellite loci in cells of normal tissues.

DISCUSSION

In the present study, we examined the *in vivo* effect of p53 deficiency on minisatellite mutation, one type of genomic instability, using p53-deficient mice. The mutation in normal somatic cells is detected by Southern blot hybridization as extra bands of minisatellites different from pre-existing bands. The frequency of mutations detected in normal embryos (wild type and heterozygote) was approximately 1.3%, consistent with previous results,[12, 13] and the frequency was not increased in embryos of p53-deficient mice. The results imply that absence of the p53 gene does not increase the somatic mutation frequency of the hypervariable minisatellites, Pc-1 and Pc-2, in cells of normal tissues.

We previously reported that mutations of the hypervariable minisatellites accumulated in cell clones obtained after exposure of chemical carcinogens.[15, 16] Also, mutations occurred during the metastatic process of mouse fibrosarcoma cell lines.[17] These findings suggested that minisatellite mutation due to rearrangement of repeats is an indicator of genomic instability related to malignant progression of tumor cells. On the other hand, it was reported that the mutation or loss of both p53 alleles allows gene amplification of the CAD gene to occur in mammalian cells.[24, 25] The gene amplification is another indicator of genomic instability affecting the copy number of the gene. Since the alterations in minisatellites and the CAD gene are the result of change of the copy number of repeats, we inferred that our assay using minisatellite probes may assess levels of genomic instability even in normal tissues, if it is influenced by the absence of the p53 gene. Analysis with this assay would provide insight into possible relationships between cell cycle control and the ability to undergo chromosomal rearrangements in an early stage of carcinogenesis. However, we failed to find a positive relationship.

An inherited cancer predisposition, Li-Fraumeni syndrome, has been linked to a variety of germline p53 mutations.[26] Half of the individuals in Li-Fraumeni families developed cancer by the age of 30, in contrast to a 1% incidence in the general population.[27] p53-Deficient mice are presumed to be a useful model for Li-Fraumeni syndrome. Donehower *et al.* demonstrated that mice homozygous for a null p53 allele were developmentally normal but showed a high susceptibility to the early onset of cancer. Actually 74% of homozygotes had developed tumors by the age of six months. The most frequently observed tumor type was malignant lymphoma, although soft tissue sarcomas and osteosarcomas were also observed.[17, 28]

Tumor susceptibility of the homozygotes may be ascribed to genomic instability that increases the chance of both activation of oncogenes and impairment of tumor suppressor and mutator genes. If this is so, we would expect that length-change mutations of hypervariable minisatellites would accumulate in p53-deficient mice, since their loci are prone to rearrangement due to recombination.[11–17] Our results, however, demonstrated no accumulation of such mutations in cells of normal tissues, suggesting that the absence of the p53 gene does not affect recombination of the minisatellites. This may be interpreted as indicating that the tumor progression accompanying minisatellite instability does not always proceed through pathways involving mutation of p53. Otherwise, it is possible that the absence of p53 can be functionally bypassed in normal cells. This is probably compatible with the finding that mutation of the p53 gene is rare in the early stage of tumor development but quite common in the late stage, where other genetic alterations have already occurred.[1–4]

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REFERENCES

1) Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C. C. p53 Mutations in human cancers. *Science,* **253,** 49–53 (1991).
2) Greenblatt, M. S., Bennett, W. P., Hollstein, M. and Harris, C. C. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res.,* **54,** 4855–4878 (1994).
3) Kress, S., Sutter, C., Strickland, P. T., Mukhtar, H., Schweizer, J. and Schwarz, M. Carcinogen-specific mutational pattern in the p53 gene in ultraviolet B radiation-induced squamous cell carcinomas of mouse skin. *Cancer Res.,* **52,** 6400–6403 (1992).
4) Makino, H., Ishizaki, Y., Tsujimoto, A., Nakamura, T., Onda, M., Sugimura, T. and Nagao, M. Rat p53 gene mutations in primary Zymbal gland tumors induced by 2-amino-3-methylimidazo[4,5-*f*]quinoline, a food mutagen. *Proc. Natl. Acad. Sci. USA,* **89,** 4850–4854 (1992).
5) Chang, F., Syrjanen, S., Kurvinen, K. and Syrjanen, K. The p53 tumor suppressor gene as a common cellular target in human carcinogenesis. *Am. J. Gastroenterol.,* **88,** 174–186 (1993).
6) Michalovitz, D., Halevy, O. and Oren, M. Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. *Cell,* **62,** 671–680

(1990).
7) Hartwell, L. H. and Kastan, M. B. Cell cycle control and cancer. *Science,* **266,** 1821–1828 (1994).
8) Wang, L. D., Hong, J. Y., Qiu, S. L., Gao, H. and Yang, C. S. Accumulation of p53 protein in human esophageal precancerous lesions: a possible early biomarker for carcinogenesis. *Cancer Res.,* **53,** 1783–1787 (1993).
9) Gao, H., Wang, L. D., Zhou, Q., Hong, J. Y., Huang, T. Y. and Yang, C. S. p53 Tumor suppressor gene mutation in early esophageal precancerous lesions and carcinoma among high-risk populations in Henan, China. *Cancer Res.,* **54,** 4342–4346 (1994).
10) Mitani, K., Takahashi, Y. and Kominami, R. A GGCAGG motif in minisatellites affecting their germline instability. *J. Biol. Chem.,* **265,** 15203–15210 (1990).
11) Suzuki, S., Mitani, K., Kuwabara, K., Takahashi, Y., Niwa, O. and Kominami, R. Two hypervariable minisatellites; chromosomal location and simultaneous mutation. *J. Biochem.,* **114,** 292–296 (1993).
12) Kelly, R., Bulfield, G., Collick, A., Gibbs, M. and Jeffreys, A. J. Characterization of a highly unstable mouse minisatellite locus: evidence for somatic mutation during early development. *Genomics,* **5,** 844–856 (1989).
13) Jeffreys, A. J., Wilson, V. and Thein, S. L. Hypervariable minisatellite regions in human DNA. *Nature,* **314,** 67–73 (1985).
14) Suzuki, S., Takada, T., Sugawara, Y., Muto, T. and Kominami, R. Quercetin induces recombinational mutations in cultured cells as detected by DNA fingerprinting. *Jpn. J. Cancer Res.,* **82,** 1061–1064 (1991).
15) Kitazawa, T., Kominami, R., Tanaka, R., Wakabayashi, K. and Nagao, M. 2-Hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (N-OH-Pphip) induces recombinational mutations in mammalian cell lines as detected by DNA fingerprinting. *Mol. Carcinog.,* **9,** 67–70 (1994).
16) Takada, T., Suzuki, S., Sugawara, Y., Kominami, R., Arakawa, M., Niwa, O. and Yokoro, K. Somatic mutation during metastasis of a mouse fibrosarcoma line detected by DNA fingerprint analysis. *Jpn. J. Cancer Res.,* **83,** 165–170 (1992).
17) Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery Jr, C. A., Butel, J. S. and Bradley, A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature,* **356,** 215–221 (1992).
18) Tsukada, T., Tomooka, Y., Takai, S., Ueda, Y., Nishikawa, S., Yagi, T., Tokunaga, T., Takeda, N., Suda, Y., Abe, S., Matsuo, I., Ikawa, Y. and Aizawa, S. Enhanced proliferative potential in culture of cells from p53-deficient mice. *Oncogene,* **8,** 3313–3322 (1993).
19) Bonhomme, F. and Guenet, J.-L. "Genetic Variants and Strains of the Laboratory Mouse," ed. M. F. Lyon and A. G. Searle, p. 658 (1989). Oxford University Press, New York.
20) Bienz, B., Zakut-Houri, R., Givol, D. and Oren, M. Analysis of the gene coding for the murine cellular tumor antigen p53. *EMBO J.,* **3,** 2179–2183 (1984).
21) Sambrook, J., Fritsch, E. F. and Maniatis, T. "Molecular Cloning: a Laboratory Manual," 2nd Ed. (1989). Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
22) Saiki, R. K., Gelfand, D. H., Stoffel, S., Sharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Ehrrich, H. A. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science,* **239,** 487–491 (1988).
23) Dietrich, W., Miller, J. C., Steen, R. G., Merchant, M., Darmon, D., Nahf, R., Gross, A., Joyce, D. C., Wessel, M., Dredge, R. D., Merquis, A., Stein, L. D., Goodman, N., Page, D. C. and Lander, E. A comprehensive genetic map of the mouse genome. *Nat. Genet.,* **7,** 220–245 (1994).
24) Yin, Y., Tanisky, M. A., Bischoff, F. Z., Strong, L. C. and Wahl, G. M. Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. *Cell,* **70,** 937–948 (1992).
25) Livingstone, L. R., White, A., Sprouse, J., Livanos, E., Jacks, T. and Tlsty, T. D. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. *Cell,* **70,** 923–935 (1992).
26) Srivastava, S., Zou, Z., Pirollo, K., Blattner, W. and Chang, E. Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. *Nature,* **348,** 747–749 (1990).
27) Malkin, D. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasmas. *Science,* **250,** 1233–1238 (1990).
28) Harvey, M., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., Bradley, A. and Donehower, L. A. Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. *Nat. Genet.,* **5,** 225–229 (1993).