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Frequent mutation of the p53 gene in human esophageal cancer

(tumor suppressor gene/DNA sequence analysis/nitrosamines)

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ABSTRACT **Sequence alterations in the p53 gene have been detected in human tumors of the brain, breast, lung, and colon, and it has been proposed that p53 mutations spanning a major portion of the coding region inactivate the tumor suppressor function of this gene. To our knowledge, neither transforming mutations in oncogenes nor mutations in tumor suppressor genes have been reported in human esophageal tumors. We examined four human esophageal carcinoma cell lines and 14 human esophageal squamous cell carcinomas by polymerase chain reaction amplification and direct sequencing for the presence of p53 mutations in exons 5, 6, 7, 8, and 9. Two cell lines and five of the tumor specimens contained a mutated allele (one frameshift and six missense mutations). All missense mutations detected occurred at G·C base pairs in codons at or adjacent to mutations previously reported in other cancers. The identification of aberrant p53 gene alleles in one-third of the tumors we tested suggests that mutations at this locus are common genetic events in the pathogenesis of squamous cell carcinomas of the esophagus.**

Esophageal cancer is the sixth most common cancer worldwide in males. In some regions of Asia and Africa, it is the leading cause of cancer deaths (1). The uneven geographical distribution of this disease, even within areas of ethnic homogeneity, suggests that certain local dietary and cultural practices are important risk factors in high-incidence regions of certain countries (2). In other parts of the world, epidemiological studies have shown that tobacco and alcohol, particularly in combination, are the prevailing risk factors (3).

Carcinogens with significant mutagenic activity are components of various substances associated with this risk (tobacco, opium resin, and N-nitrosamines). Transforming point mutations in the *ras* protooncogene family have been observed in esophageal tumors induced in rats by *N*-nitrosomethylbenzylamine treatment (4), and the activated forms of these oncogenes have been frequently associated with many human cancers (5); however, they have been conspicuously absent in human esophageal squamous cell carcinomas (ESC) tested to date. *ras* mutations are often found in malignancies of the lower digestive tract, but they have not been detected in neoplasms of the stomach or esophagus (6, 7). Discrete DNA changes at loci other than *ras* may alter the function of genes critical for growth control or differentiation of squamous cells of the upper gastrointestinal tract.

It is becoming apparent that lesions in the p53 tumor suppressor gene also occur in several types of human cancers (8–10) and possibly with considerable frequency as is the case for the *ras* oncogenes. The p53 gene may be a broad target for DNA damage in carcinogenesis, because recent work suggests that proper function of the gene product in controlling growth is compromised by mutations throughout a significant region of the coding sequence [i.e., exons 5–9 (9–11)], whereas transforming *ras* mutations are restricted to discrete codons (12, 13). As none of the p53 mutations in exons 5–9 discovered in human neoplasms were detected in normal tissue of the cancer patients for which both tumor and uninvolved tissue were analyzed (refs. 8–11 and 14; M.C.H. and C.C.H., unpublished observations), these sequence alterations not present in these individuals in the germ line presumably arose during the development of the tumor (15).

The effectiveness as mutagens of tobacco and certain N-nitrosamines, two exposures linked to elevated risk of ESC, suggested to us that one relevant site of DNA damage in this cancer could be the p53 gene locus. As, to our knowledge, there have been no investigations reported on the presence of p53 lesions in ESC, we initially analyzed p53 sequences in tumor cell lines (16) established in our laboratory from surgical specimens of ESC (United States cancer patients). The discovery of a mutant p53 allele in two of the cell lines reported below indicated that this locus could have been an important genetic step in the *in vivo* neoplastic transformation of these cells; this we subsequently explored by screening a series of 14 primary ESC. Direct analysis of these primary tumors showed that the mutations occur *in vivo* and suggested a first approximation of the extent of p53 involvement in human esophageal cancer in patients residing in France, an area where the most common risk factors of esophageal cancer, alcohol and tobacco, are thought to be at work.

MATERIALS AND METHODS

Cell Lines and Tumor Samples. The four cell lines used in this study were established in culture from primary ESC obtained from the Departments of Pathology at the University of Maryland, the University of Alabama, and the Washington Veterans Administration Hospital. The growth characteristics, keratin expression, and karyotypic features of these tumor cell lines have been described (16, 17). The primary tumor tissues were from esophageal cancer patients at Edouard Herriot Hospital (Lyon, France). Upon surgical removal, samples were frozen in liquid nitrogen and stored at −70°C. The tumors ranged from well-differentiated to poorly differentiated squamous cell carcinomas.

Preparation of DNA. Cells or tissues were placed in lysis buffer (Applied Biosystems) and incubated at 55°C for 1–3 hr. After phenol and chloroform extractions, DNA was precipitated in ethanol and resuspended in sterile TE buffer (10 mM Tris, pH 8.0/1 mM EDTA) for storage. Prior to the polymerase chain reaction (PCR), an aliquot of the DNA stock was reprecipitated in ethanol and resuspended in sterile water. DNA stocks were kept physically separate from areas where PCR reaction products were handled. Separate pipet-

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Abbreviations: ESC, esophageal squamous cell carcinoma(s); PCR, polymerase chain reaction.

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ting devices and laboratory materials were set aside to be used exclusively for working with DNA stocks.

Normal cells from tumor samples 11 and 13 were obtained by cutting 20-μm-thick sections of frozen tissue embedded in optimal cutting temperature (O.C.T.) compound (Miles) at −10°C with a microtome and collecting cells from an artery within the tumor mass (sample 11) or stromal cells (sample 13) with the aid of an 18-gauge needle and a dissecting microscope. Cells were incubated at 42°C in TE buffer containing proteinase K for 20 min, and 2 μl of this solution was added directly to the PCR mixture as described below.

PCR and Primers. Amplifications were performed in 100-μl volumes with 500 ng of genomic DNA and 1 μM primers, in 50 mM Tris/pH 9.0/3 mM $MgCl_2$/1 mM dATP/1 mM dCTP/1 mM dGTP/1 mM dTTP (Pharmacia) containing 2.5 units of *Thermus aquaticus* (*Taq*) polymerase (Perkin–Elmer/Cetus) for 35 cycles of 94°C denaturation (30 sec), 60°C annealing (1 min), and 78°C extension (30 sec) in an automated Perkin–Elmer/Cetus thermal cycler (18). Initial denaturation was at 98°C for 8 min before addition of polymerase. Primers for PCR and sequencing were synthesized with an Applied Biosystems DNA synthesizer (model 380B) or purchased from Operon Technologies (Alameda, CA). Primer sequences are as follows: no. 232, ACGTGAATTCCTTGCCACAGGTCTCCCCAA; no. 233, TGCACCTAGGAGGGGTCAGAGGCAAGCAGA; no. 236, ACGTGAATTCTGTTCACTTGTGCCCTGACT; no. 237, ACTGGCCTCATCTTGGGCCT; no. 238, TGTGCAGGGTGGCAAGTGGC; no. 239, GCCTCTGATTCCTCACTGAT; no. 240, TTAACCCCTCCTCCCAGAGA; no. 270, TGCACCTAGGAGCAATCAGTGAGGAATCAG; no. 271, CAGCCCTGTCGTCTCTCCAG; no. 314, ACGTGAATTCTTGGGAGTAGATGGAGCCTGG; no. 800, CTGGAAACTTTCCACTTGAT; no. 802, GATTTCCTTACTGCC.

To control for DNA contamination of PCR reactions, all experiments included one or more reaction tubes in which no DNA was added. Pipetting devices and PCR reagents were kept strictly separated from any PCR products. All mutations were confirmed by a complete repeat of the experimental procedure: amplification of stock genomic DNA, fragment purification, and sequencing of the DNA strand complementary to that sequenced in the initial experiment.

Dideoxy Sequencing of Amplified DNA. PCR-amplified DNA was purified on 4% agarose gels. Excised gel slices containing the DNA fragment were incubated in elution buffer (0.5 M sodium acetate/1 mM EDTA) at room temperature overnight, and the DNA was subsequently precipitated with ethanol. This material was sequenced directly without any intermediate cloning steps. Amplified template DNA was annealed with 25 nmol of primer and sequenced by the dideoxy chain-termination method (19) with Sequenase kit reagents (United States Biochemical). Radioactive label incorporation was achieved by a 2-min 42°C preincubation of the sequencing reaction containing the α-^{35}S-labeled deoxynucleotide (New England Nuclear) corresponding to the first base of the nascent chain as the only deoxynucleotide. Reactions were electrophoresed on 8% polyacrylamide/urea gels for 1–3 hr. Dried gels were exposed to Kodak X-AR 5 film at room temperature for 1–3 days.

RESULTS

Genomic DNA was purified from four human esophageal carcinoma cell lines and 14 frozen ESC specimens. Coding regions of the p53 gene amplified from this material by PCR was sequenced directly. The sequences amplified for analysis of mutations encompassed exons 5, 6, 7, 8, and 9, as most p53 somatic mutations in human tumors localized thus far fall within this area (9, 10, 14). No preselection of tumors on the basis of karyotype, histological features, or patient prognosis was made. The histopathological diagnosis of these surgical specimens was squamous cell carcinoma in all cases. Two of the 14 patients received chemotherapy and radiotherapy prior to surgery; however, p53 mutations were not detected in the tumors of these two patients. Tumors ranged from 2 to 10 cm in length, and each specimen was trimmed of surrounding grossly normal tissue prior to DNA extraction.

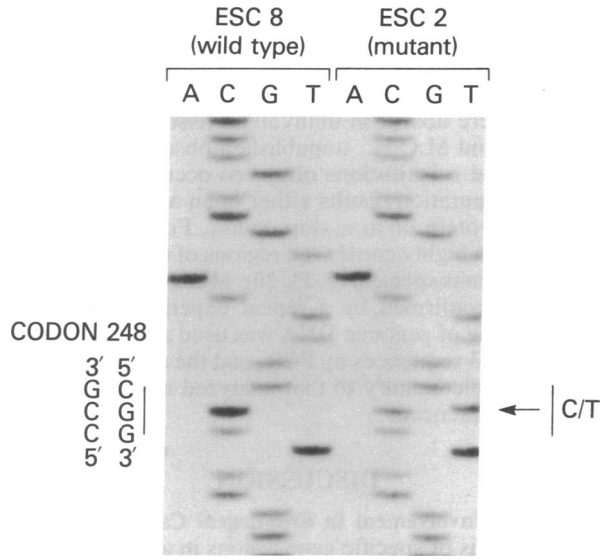

FIG. 1. Examples of p53 gene sequence analysis in ESC. DNA was isolated from squamous cell carcinoma no. 8 (wild type) and no. 2 (mutated) and subjected to amplification of exon 7; the gel-purified PCR product was sequenced directly with oligonucleotide no. 238 antisense primer as described in *Materials and Methods*. The nucleotide substitution in tumor no. 2 shown in this sequencing gel is indicated by the arrow. Both wild-type ($^{-CGG-}_{-GCC-}$) and mutant ($^{-CAG-}_{-GTC-}$) alleles are present in this tumor sample.

A point mutation in the p53 gene was found in two of the four cell lines tested and in 5 of the 14 (36%) primary tumor tissues (Fig. 1 and Table 1). Only the altered sequence was found in the two cell lines and in tumor number 13, indicating a homozygous or hemizygous state of the altered p53 locus. In the remaining tumors with a mutant p53 allele, the wild-type sequence was also present and yielded a signal approximately equal in intensity to the mutant, indicating either that the normal allele was retained in the tumor or that the tumor tissue sample contained a significant number of nontumor cells. A small sample of frozen tissue was still available from two tumors (no. 11 and no. 13) in which a p53 mutation was observed. This was used to prepare 20-μm-thick cryostat sections from which normal cells of an artery (no. 11) or stroma (no. 13) adjacent to tumor cells were isolated for analysis. The PCR-amplified DNA from these preparations revealed only the normal allele upon sequencing, indicating

Table 1. p53 gene mutations in human esophageal cancer

Sample	Exon	Codon	Nucleotide	Amino acid change
Cell line (2/4)*				
HCE-4	7	245	GGC → GTC	Gly → Val
HCE-6	8	278	CCT → TCT	Pro → Ser
Tumor (5/14)*				
No. 2	7	248	CGG → CAG	Arg → Gln
No. 3	8	278	CCT → CTT	Pro → Leu
No. 6	8	263	Frameshift	
No. 11	6	192	CAG → TAG	Stop
No. 13	5	154	GGC → GTC	Gly → Val

*Number of positive samples/number of samples tested.

that the base substitutions found in the tumor cells are somatic mutations. This is in keeping with other instances reported to date where normal cells could be obtained from patients with tumors harboring mutant forms of p53: the mutations were absent in uninvolved tissue (refs. 8–11 and 14; C.C.H. and M.C.H., unpublished observations).

All six base substitutions observed occurred at G·C base pairs. Each mutation results either in an amino acid change in the p53 protein or in a stop codon. Four are located at codons within highly conserved regions of the p53 gene (85% homology across species) (9, 11, 20). Mutants detected in this study were confirmed by a repeat experiment in which a second sample of genomic DNA was used as a PCR template to amplify p53 sequences by PCR, and the strand of the PCR product complementary to that analyzed in the first experiment was sequenced.

DISCUSSION

p53 Gene Involvement in Esophageal Cancer in Humans. Direct analysis of specific gene lesions in an extensive array of human tumors has indicated that cancer is the result of an accumulation of discrete genetic changes in the cell genome occurring over an extended period of time (5, 15, 21–23).

Many carcinogens have the potential to generate the mutations in protooncogenes and tumor suppressor genes that have been detected in human tumor samples. There has been no evidence, however, for mutation of these genes in carcinomas of the esophagus, although overexpression and amplification of growth factor/receptor sequences are frequently associated with this cancer (24). While carcinogens can induce gene amplification in experiments *in vitro* (25), a hallmark of carcinogens, including those associated with a high risk of esophageal cancer, is their capacity to induce point mutations (26–29). The presence of a mutation in the p53 gene in 5 of the 14 ESC examined here suggests the possibility that this locus is a relevant site of gene damage by mutagenic carcinogens implicated in the etiology of this disease.

Our results showing that mutant alleles are present in one-third of the ESC tested may be a conservative estimate of p53 lesion involvement in this cancer population. We deduce from hospital pathology records that the tumors analyzed in this study were composed predominantly of neoplastic cells, but should the fraction of nontumor cells exceed that of tumor cells in some samples, a mutant allele would be difficult to detect against a background of wild-type sequence. Also, as is the case for reports by others on p53 mutations in tumors, we cannot exclude the presence of a mutation occurring in a region of the gene not sequenced that could interfere with the synthesis or function of a biologically active p53 gene product. Assuming that indeed not all ESC harbor modified p53 alleles, as is apparently true of colorectal carcinomas and lung cancers, we speculate that genetic events at other loci can confer comparable phenotypic changes to the cell. This is in keeping with the notion that there are multiple molecular pathways leading to malignancy.

Mutation Spectra in p53 Sequences. Basic research in genetics has revealed that spontaneously arising mutations are not random and that mutagens generate specific, characteristic changes in a given DNA sequence, both with respect to the position and the nature of the alteration (30–32). Mutation spectra (reviewed in ref. 33) may prove quite useful in toxicology and molecular epidemiology to address the issue of whether genetic anomalies in human tumors arise spontaneously during the course of biological function or are induced by exposure to environmental mutagens (34). Two emerging features of p53 gene lesions in human cancer, an apparently broad sequence target area for detrimental mutations and the frequency of p53 gene anomalies in many types of human malignancies (9, 10, 14), eventually may be exploited to yield mutational spectra that would suggest the source of DNA damage in specific cancer groups.

The number of mutants identified in this study alone is too small to suggest a mutation profile. The missense mutations we detected occurred exclusively at G·C base pairs, where both G → A and G → T substitutions were generated, whereas in colon tumors, mutations, predominantly transitions, were found both at G·C and A·T pairs (9), and seven of the eight G → A transitions occurred at CpG sites (Table 2). This dinucleotide is a hot spot for spontaneous mutations, which is attributed to deamination of methylcytosine (35, 36). Only one of the four G → A p53 mutations that we observed in esophageal tumors was at a CpG dinucleotide.

A G → A transition is also the anticipated base substitution following DNA alkylation at deoxyguanosine and mispairing of O^6-methyldeoxyguanosine with thymine (37). Levels of O^6-methyldeoxyguanosine ranging from 10 to 10^3 fmol per mg of DNA have been detected in esophageal tissue of individuals residing in France (38, 39), although the nature of exposure responsible for the presence of the premutagenic lesion in this population is not known. In the *lacI* gene of *Escherichia coli*, 99% of mutations recovered following treatment with the esophageal carcinogen *N*-nitroso-*N*-methyl-N^{α}-acetoxybenzylamine were G → A transitions distributed in a nonrandom fashion, primarily at $A(G)_nA$ sequence motifs (40). It will be of interest to examine the spectrum of mutations in conjunction with measurement of tissue O^6-methyldeoxyguanosine levels in esophageal tumors of patients from a high incidence area such as Linxian, China, where methylating N-nitrosamines have been detected in food samples (41).

In the patient group studied here, tobacco is possibly the major source of carcinogen exposure. Tobacco smoke contains a variety of mutagens, but it is not clear whether a characteristic pattern of mutations will be observed with complex mixtures. Methylating nitrosamines that contribute to the carcinogenicity of tobacco smoke are present in relatively significant amounts and induce primarily G → A transitions (42). The mutational spectrum at the mammalian adenine phosphoribosyltransferase locus induced by the principal mutagenic metabolite of benzo[a]pyrene, another component of tobacco smoke, shows predominantly G → T substitutions, and most mutations are clustered at runs of guanines (43). Since the position of frequent mutations in a defined sequence is the highly discriminating feature of mutational specificity, laboratory studies to generate mutational spectra of chemicals at p53 gene sequences, hampered at present by technical difficulties, could be particularly valuable.

Identification of Cancer Genes That Participate with p53 in Esophageal Carcinogenesis. The identification of p53 gene mutation as one event in the natural history of esophageal cancer may assist in defining other molecular steps in the disease pathway. In cancers of the colon and lung, both *ras* and p53 mutations are characteristic lesions (5, 9, 14), and transfection experiments have shown that introduction of both a *ras* oncogene and p53 sequences is sufficient to transform primary rat cells (44–47). Oncogenes other than *ras* may cooperate functionally with the p53 gene to render basal

Table 2. Analysis of p53 gene missense mutations in human tumors of the digestive tract

Cancer site	No. of mutations detected					
	Total	At A·T	At G·C	G → A	G → T	G → C
Esophagus	6	0	6	4	2	0
Colon*	13	4†	9	8	0	1

*Data from refs. 8 and 9.
†Three mutations were A·T → G·C and one was A·T → T·A.

epithelial cells of the esophagus malignant, as *ras* mutations are apparently not contributing in most cases to neoplastic transformation of these cells in humans. The development of techniques for culturing primary normal human esophageal cells *in vitro* has provided an appropriate experimental model for analysis of complementary activity of oncogenes in human esophageal cancer, and recent success in immortalization of these cells by introducing a plasmid encoding the simian virus 40 large tumor antigen is promising (48). One candidate that may cooperate with a mutant p53 gene in esophageal tumors is the epidermal growth factor receptor. Structural and functional anomalies involving the locus coding for this protein are typical features of esophageal carcinomas. Numerous studies have reported on the presence of altered receptor number, affinity, gene copy number, or mRNA level in human ESC or cell lines (6, 49–52).

The chromosome region encompassing the *HST1* and *INT2* genes coding for fibroblast growth factor-like proteins is also intriguing. Although there is no consistently detectable RNA message from either gene in esophageal tissues, whether normal or tumor, this region of chromosome 11 is amplified in almost one-half of ESC that have been tested (refs. 53–55; M.H., unpublished observations), and a homogeneously staining region at 11q12 indicative of amplified sequences was found in several human esophageal carcinoma cell lines (17).

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1. Muir, C. S. (1988) in *Accomplishments in Cancer Research 1988 Prize Year*, General Motors Cancer Research Foundation, eds. Fortner, J. G. & Rhoads, J. E. (Lippincott, Philadelphia), pp. 126–144.
2. Day, N. E. & Munoz, N. (1982) in *Cancer Epidemiology and Prevention*, eds. Schottenfeld, D. & Fraumeni, J. F. (Saunders, Philadelphia), pp. 596–623.
3. Yu, M. C., Garabrant, D. H., Peters, J. M. & Mack, T. M. (1988) *Cancer Res.* **48,** 3843–3848.
4. Wang, Y., You, M., Reynolds, S. H., Stoner, G. D. & Anderson, M. W. (1990) *Cancer Res.* **50,** 1591–1595.
5. Bos, J. L. (1989) *Cancer Res.* **49,** 4682–4689.
6. Hollstein, M. C., Smits, A. M., Galiana, C., Yamasaki, H., Bos, J. L., Mandard, A., Partensky, C. & Montesano, R. (1988) *Cancer Res.* **48,** 5119–5123.
7. Jiang, W., Kahn, S. M., Guillem, J. G., Lu, S. H. & Weinstein, I. B. (1989) *Oncogene* **4,** 923–928.
8. Baker, S. J., Fearon, E. R., Nigro, J. M., Hamilton, S. R., Preisinger, A. C., Jessup, J. M., vanTuinen, P., Ledbetter, D. H., Barker, D. F. & Nakamura, Y. (1989) *Science* **244,** 217–221.
9. Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Cleary, K., Bigner, S. H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins, F. S., Weston, A., Modali, R., Harris, C. C. & Vogelstein, B. (1989) *Nature (London)* **342,** 705–708.
10. Takahashi, T., Nau, M. M., Chiba, I., Birrer, M. J., Rosenberg, R. K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A. F. & Minna, J. D. (1989) *Science* **246,** 491–494.
11. Lane, D. P. & Benchimol, S. (1990) *Genes Dev.* **4,** 1–8.
12. Barbacid, M. (1987) *Annu. Rev. Biochem.* **56,** 779–827.
13. Bos, J. L. (1988) *Mutat. Res.* **195,** 255–271.
14. Iggo, R., Gatter, K., Bartek, J., Lane, D. & Harris, A. L. (1990) *Lancet* **ii,** 675–679.
15. Fearon, E. R. & Vogelstein, B. (1990) *Cell* **61,** 759–767.
16. Banks-Schlegel, S. P. & Quintero, J. (1986) *Cancer Res.* **46,** 250–258.
17. Whang-Peng, J., Banks-Schlegel, S. P. & Lee, E. C. (1990) *Cancer Genet. Cytogenet.* **45,** 101–120.
18. Mullis, K. B. & Faloona, F. A. (1987) *Methods Enzymol.* **155,** 335–350.
19. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
20. Soussi, T., Cardon de Fromentel, C., Mechali, M., May, P. & Kress, M. (1987) *Oncogene* **1,** 71–78.
21. Cairns, J. (1978) in *Cancer, Science and Society* (Freeman, San Francisco), pp. 69–88.
22. Bishop, J. M. (1987) *Science* **235,** 305–311.
23. Weinstein, I. B. (1988) *Cancer Res.* **48,** 4135–4143.
24. Tahara, E. (1990) *J. Cancer Res. Clin. Oncol.* **116,** 121–131.
25. Lavi, S. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 6144–6148.
26. Miller, E. C. & Miller, J. A. (1966) *Pharmacol. Rev.* **18,** 805–838.
27. McCann, J., Choi, E., Yamasaki, E. & Ames, B. N. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 5135–5139.
28. International Agency for Research on Cancer (1987) *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Overall Evaluations of Carcinogenicity: An Updating of IARC Monographs Volumes 1 to 42* (IARC, Lyon, France).
29. Lawley, P. D. (1989) *Mutat. Res.* **213,** 3–25.
30. Benzer, S. & Freese, E. (1958) *Proc. Natl. Acad. Sci. USA* **44,** 112–119.
31. Miller, J. H. (1983) *Annu. Rev. Genet.* **17,** 215–238.
32. Horsfall, M. J., Gordon, A. J., Burns, P. A., Zielenska, M., van der Vliet, G. M. & Glickman, B. W. (1990) *Environ. Mol. Mutagen.* **15,** 107–122.
33. Thilly, W. G. (1990) *Annu. Rev. Pharmacol. Toxicol.* **30,** 369–385.
34. Ames, B. N. (1983) *Science* **221,** 1256–1264.
35. Coulondre, C., Miller, J. H., Farabaugh, P. J. & Gilbert, W. (1978) *Nature (London)* **274,** 775–780.
36. Lindahl, T. (1982) *Annu. Rev. Biochem.* **51,** 61–87.
37. Mitra, G., Pauly, G. T., Kumar, R., Pei, G. K., Hughes, S. H., Moschel, R. C. & Barbacid, M. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 8650–8654.
38. Montesano, R., Hall, J., Hollstein, M., Mironov, N. & Wild, C. P. (1990) in *DNA Damage and Repair in Human Tissues*, eds. Sutherland, B. M. & Woodhead, A. D. (Plenum, New York), pp. 437–452.
39. Umbenhauer, D., Wild, C. P., Montesano, R., Saffhill, R., Boyle, J. M., Huh, N., Kirstein, U., Thomale, J., Rajewsky, M. F. & Lu, S. H. (1985) *Int. J. Cancer* **36,** 661–665.
40. Horsfall, M. J. & Glickman, B. W. (1988) *Carcinogenesis* **9,** 1529–1532.
41. Singer, G. M., Chuan, J., Roman, J., Min-Hsin, L. & Lijinsky, W. (1986) *Carcinogenesis* **7,** 733–736.
42. Hecht, S. S. & Hoffmann, D. (1988) *Carcinogenesis* **9,** 875–884.
43. Mazur, M. & Glickman, B. W. (1988) *Somat. Cell Mol. Genet.* **14,** 393–400.
44. Parada, L. F., Land, H., Weinberg, R. A., Wolf, D. & Rotter, V. (1984) *Nature (London)* **312,** 649–651.
45. Eliyahu, D., Raz, A., Gruss, P., Givol, D. & Oren, M. (1984) *Nature (London)* **312,** 646–649.
46. Hinds, P., Finlay, C. & Levine, A. J. (1989) *J. Virol.* **63,** 739–746.
47. Jenkins, J. R., Rudge, K. & Currie, G. A. (1984) *Nature (London)* **312,** 651–654.
48. Light, B., Stoner, G. D., Gerwin, B. I., Terada, M. & Harris, C. C. (1990) *Proc. Am. Assoc. Cancer Res.* **31,** 136 (abstr.).
49. Hunts, J., Ueda, M., Ozawa, S., Abe, O., Pastan, I. & Shimizu, N. (1985) *Jpn. J. Cancer Res.* **76,** 663–666.
50. Yamamoto, T., Kamata, N., Kawano, H., Shimizu, S., Kuroki, T., Toyoshima, K., Rikimaru, K., Nomura, N., Ishizaki, R., Pastan, I., Gamou, S. & Shimizu, N. (1986) *Cancer Res.* **46,** 414–416.
51. Ozawa, S., Ueda, M., Ando, N., Abe, O. & Shimizu, N. (1987) *Int. J. Cancer* **39,** 333–337.
52. Lu, S. H., Hsieh, L. L., Luo, F. C. & Weinstein, I. B. (1988) *Int. J. Cancer* **42,** 502–505.
53. Tsuda, T., Tahara, E., Kajiyama, G., Sakamoto, H., Terada, M. & Sugimura, T. (1989) *Cancer Res.* **49,** 5505–5508.
54. Tsuda, T., Nakatani, H., Matsumura, T., Yoshida, K., Tahara, E., Nishihira, T., Sakamoto, H., Yoshida, T., Terada, M. & Sugimura, T. (1988) *Jpn. J. Cancer Res.* **79,** 584–588.
55. Tsutsumi, M., Sakamoto, H., Yoshida, T., Kakizoe, T., Koiso, K., Sugimura, T. & Terada, M. (1988) *Jpn. J. Cancer Res.* **79,** 428–432.