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Advances in Brief

Mutability of p53 Hotspot Codons to Benzo(a)pyrene Diol Epoxide (BPDE) and the Frequency of p53 Mutations in Nontumorous Human Lung

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Abstract

p53 mutations are common in lung cancer. In smoking-associated lung cancer, the occurrence of G:C to T:A transversions at hotspot codons, *e.g.*, 157, 248, 249, and 273, has been linked to the presence of carcinogenic chemicals in tobacco smoke including polycyclic aromatic hydrocarbons such as benzo(*a*)pyrene (BP). In the present study, we have used a highly sensitive mutation assay to determine the p53 mutation load in nontumorous human lung and to study the mutability of p53 codons 157, 248, 249, and 250 to benzo(*a*)pyrene-diol-epoxide (BPDE), an active metabolite of BP in human bronchial epithelial BEAS-2B cells. We determined the p53 mutational load at codons 157, 248, 249, and 250 in nontumorous peripheral lung tissue either from lung cancer cases among smokers or noncancer controls among smokers and nonsmokers. A 5–25-fold higher frequency of GTC^{val} to TTC^{phe} transversions at codon 157 was found in nontumorous samples (57%) from cancer cases ($n = 14$) when compared with noncancer controls ($n = 8$; $P < 0.01$). Fifty percent (7/14) of the nontumorous samples from lung cancer cases showed a high frequency of codon 249 AGG^{arg} to AGT^{ser} mutations ($P < 0.02$). Four of these seven samples with AGT^{ser} mutations also showed a high frequency of codon 249 AGG^{arg} to ATG^{met} mutations, whereas only one sample showed a codon 250 CCC to ACC transversion. Tumor tissue from these lung cancer cases (38%) contained p53 mutations but were different from the above mutations found in the nontumorous pair. Noncancer control samples from smokers or nonsmokers did not contain any detectable mutations at codons 248, 249, or 250. BEAS-2B bronchial epithelial cells exposed to doses of 0.125, 0.5, and 1.0 μM BPDE, showed G:C to T:A transversions at codon 157 at a frequency of 3.5×10^{-7}, 4.4×10^{-7}, and 8.9×10^{-7}, respectively. No mutations at codon 157 were found in the DMSO-treated controls. These doses of BPDE induced higher frequencies, ranging from 4–12-fold, of G:C to T:A transversions at codon 248, G:C to T:A transversions and G:C to A:T transitions at codon 249, and C:G to T:A transitions at codon 250 when compared with the DMSO-treated controls. These data are consistent with the hypothesis that chemical carcinogens such as BP in cigarette smoke cause G:C to T:A transversions at p53 codons 157, 248, and 249 and that nontumorous lung tissues from smokers with lung cancer carry a high p53 mutational load at these codons.

Introduction

The *p53* tumor suppressor gene is mutated commonly in human cancer (1–3). The frequency, timing, and mutation spectrum of the p53 can provide clues to the etiology and pathogenesis of human cancer (4, 5). Different carcinogens have been found to be associated with different characteristic mutations. For example, exposure to UV light is correlated with transition mutations at dipyrimidine sites (6); dietary aflatoxin B_1 exposure is correlated with G:C to T:A transversions that lead to serine substitutions at residue 249 of p53 in hepatocellular carcinoma (7, 8); and exposure to cigarette smoke is correlated with G:C to T:A transversions in lung carcinomas (9–11). The presence of a characteristic mutation in nontumorous tissue can provide a molecular linkage between exposure to a specific carcinogen and a particular type of cancer. Codons 157, 158, 248, 249, and 273 have been designated as p53 mutational hotspots in lung cancer (10, 12, 13). Earlier reports have also described codon 157 as a hotspot that is frequent in lung cancer associated with smoking. The majority of mutations found at these codons are represented by G:C to T:A transversions. In smoking-associated lung cancer, G:C to T:A transversions have been linked to the presence of BP in cigarette smoke. BPDE,[6] the metabolically activated form of BP, binds to guanosine residues at these hotspot codons (14–16), which are sites of slow nucleotide excision repair (17). Rodin and Rodin (18) argue against linking BPDE with a higher frequency of G:C to T:A transversions at the above codons in lung cancer among smokers. The basis of their argument is the lack of any statistically significant difference in the frequency of G:C to T:A transversions at p53 hotspot codons in lung cancer cases (both smokers and nonsmokers) compared with nonlung cancers, when only G:C to T:A transversions were taken into account. However, the possibility of misclassification of the cases as smokers or nonsmokers may confound this conclusion (19). A recent study by Hainaut and Pfeifer (19) reports a reassessment of the most updated p53 mutational database and the recent data on nonsmokers and has additionally reaffirmed the association between smoking and G:C to T:A transversions in lung cancer.

To link a mutation at a specific site to a particular carcinogen, it is important to evaluate the mutability of that specific site (at the DNA level without any selection process) to the carcinogen in question. In the present study, by using a highly sensitive genotypic mutation assay, we have tested two hypotheses: (*a*) that BPDE induces G:C to T:A transversions at p53 hotspot codons 157, 248, and 249; and (*b*) that nontumorous lung tissue carries a high p53 mutational load in cancer cases.

Materials and Methods

Fresh-frozen surgical peripheral lung samples were collected from 14 lung cancer patients. These cases were all smokers (mean ± SD: 31 ± 10 pack-year) between 41 and 76 years of age (mean ± SD: 60 ± 9 years). The normal controls were obtained from eight organ donors without cancer. Five of eight normal controls were smokers and three were nonsmokers with serum cotinine levels of 331 ± 77 ng/ml and 1 ng/ml, respectively. Smokers among control were between 22 and 45 years of age, whereas nonsmokers were 1–32 years of age.

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[6] The abbreviations used are: PAH, polycyclic aromatic hydrocarbons; BP, benzo(*a*)pyrene; BPDE, benzo(*a*)pyrene-diol-epoxide; WT, wild-type; MS, mutant standard; 4-HNE, 4-hydroxynonenal.

Cell Culture and BPDE Treatment. BEAS-2B, a bronchial epithelial cell line with WT p53 (20) was grown in LHC-9 medium (Biofluid, Rockville, MD). At ~70% of confluency, the cells were treated with 0.125, 0.5, or 1.0 μM doses of BPDE (Midwest Research Institute, NCI Chemical Repository, Kansas City, MO) or with DMSO (Sigma Chemical Co., St. Louis, MO) for 1 h. The medium was then replaced with normal medium, and cells were allowed to grow for 96 h before the extraction of genomic DNA.

Analysis of G:C to T:A Transversion at p53 Codon 157. To analyze p53 codon 157, we have modified a highly sensitive mutation assay originally developed by Ceratti *et al*. (21, 22). The basic principle used in this methodology is the elimination of the WT sequence by specific-restriction enzyme digestion, followed by size fractionation and the amplification of mutated fragments. Cloning and subsequent oligonucleotide plaque-hybridization or slot blot hybridization of amplified DNA fragments are used to measure the frequency of a mutated allele by comparison with an internal control added to each sample at the outset.

Codon 157 contains a portion of the BstUI restriction site (CGCG, spanning the nucleotide residues 13146–13149) and, therefore, is amenable to the above analysis. However, the presence of a second BstUI site (spanning the residue 13151–13154) next to codon 157 does not allow the enrichment of 157-mutated fragments by specific-restriction digestion. Therefore, modifications to the assay were made to alter the second BstUI site during the first amplification cycle using a mutated primer. Briefly, 35 μg of genomic DNA, corresponding to 10^7 copies of p53, were digested with *Sau*3AI restriction enzyme for the enrichment of the p53 sequence containing exon 5. Thirty copies of an 804-bp internal control or MS were added to the digested DNA. A 700-bp to 1 kb fragment population was isolated by size fractionation on 1% agarose gel. This DNA preparation, which contains 855-bp *Sau*3AI fragments containing p53 exon 5 and 804-bp MS, was used as the template for the first round of amplification consisting of 30 cycles with *Pyrococcus furiosus*-DNA polymerase (Stratagene, La Jolla, CA) and mutated antisense primer. The 132 bp-amplified product was then digested with 50 units of BstUI to eliminate codon 157 WT sequences while leaving the mutated sequence intact. After purification, using a Qiagen column (Qiagen, Valencia, CA), the DNA was amplified using a second set of primers that also contains the *Eco*RI cloning site for 15 cycles. The second amplified product was digested with BstUI to remove the remaining WT sequences and then subjected to slot blot analysis. To ascertain the specificity of hybridization and to quantify the mutation frequency, known amounts of authentic mutant, internal control, or MS and WT DNA were loaded on the same membrane. The membranes were then hybridized with ^{32}P-labeled 19-mer oligonucleotides complimentary to a G:C to T:A transversion at codon 157, or to the MS or the WT sequences. The mutation frequency was determined by comparing the phosphorimager signal from the unknown sample with the signals from known amounts of authentic mutant and MS loaded on the same filter. The assay was validated by mixing 5, 10, 20, 50, and 100 copies of p53 sequences, containing codon 157 G:C to T:A mutations, with genomic DNA corresponding to 10^7 copies of p53. A sensitivity of about 10^{-6} was achieved with this assay (Fig. 1*A*).

Analysis of p53 Codons 248, 249, and 250. We have used a highly sensitive mutation assay to determine the p53 mutation load as described earlier in detail (21, 22). Briefly, genomic DNA containing ~3×10^7 copies of p53 was enriched in the mutated p53 sequence by specific restriction digestion with either *Msp*I (codon 248) or *Hae*III (codon 249 and 250), followed by size fractionation. The enriched DNA was then amplified using *P. furiosus* and Taq DNA polymerases (Foster City, CA). The amplified DNA was cloned into λ-gt10, and plaques were hybridized with different ^{32}P-labeled 19-mer oligonucleotide probes that were each specific for a single bp mutation at the *Msp*I (codon 248) or *Hae*III sites (codon 249 and 250), or the WT or MS sequences. For each sample, ~1500 plaques were analyzed on 8–10 membranes. The mutation frequencies were calculated by comparison with the internal control or MS plaques that were produced by a known copy number of MS added in each sample.

Results

BPDE-induced G:C to T:A Transversion at p53 Codon 157. DNA from the BEAS-2B cell line, exposed to 0.125, 0.5, and 1.0 μM doses of BPDE or DMSO control, was analyzed for G:C to T:A transversions at codon 157 by the assay described above. Exposure of cells to 0.125, 0.5, and 1.0 μM BPDE induced G:C to T:A transversions at codon 157 at frequencies of 3.5×10^{-7}, 4.4×10^{-7}, and 8.9×10^{-7}, respectively. No mutations were found in DMSO-treated control samples (Figs. 1, *B* and *C*, and 3).

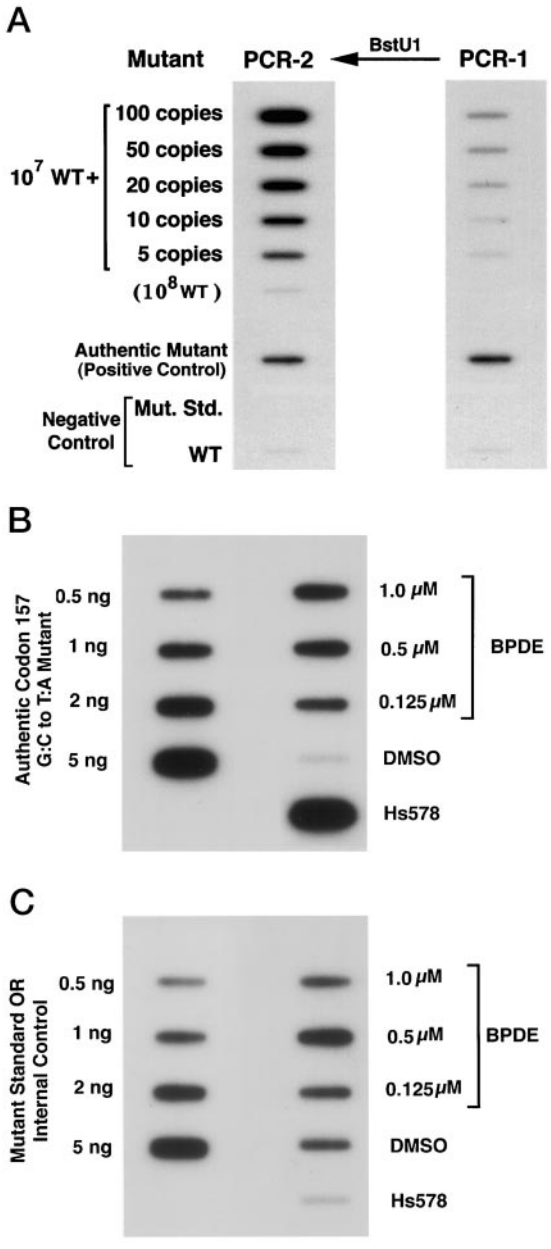

Fig. 1. *A*, slot blot hybridization by codon 157 G:C to T:A mutant probe in a mixing experiment designed for the validation of codon 157 mutation assay. Different copy numbers of authentic codon 157 G:C to T:A mutants were added to 10^7 copies of genomic DNA with the WT p53 sequence and subjected to mutation analysis. A cloned 850-bp DNA fragment containing p53 codon 157 G:C to T:A mutations was used as a positive control, whereas MS (internal control) and WT p53 sequence were used as negative controls when hybridized to G:C to T:A mutant probes. *B* and *C*, slot blot hybridization with either G:C to T:A mutant probe (*B*) or probe for MS (*C*). Amplified DNA from either BPDE treated or DMSO control groups were loaded on the membrane. Known quantities of G:C to T:A mutants (described in *A*) or MS were also loaded on the same membrane for quantitation. DNA from the breast cancer cell line Hs578, which contains G:C to T:A mutations at codon 157, was used as a positive control when hybridized with the G:C to T:A mutant probes, whereas it acted as negative control when hybridized with the MS probe.

BPDE-induced Mutations at Codons 248, 249, and 250. Exposure of 0.5 and 1.0 μM BPDE induced codon 248 CGG^{arg} to CTG^{leu} transversions at frequencies of 26 and 43 $\times$ 10^{-7}, respectively, compared with 2.7×10^{-7} in the control; codon 249 AGG^{arg} to

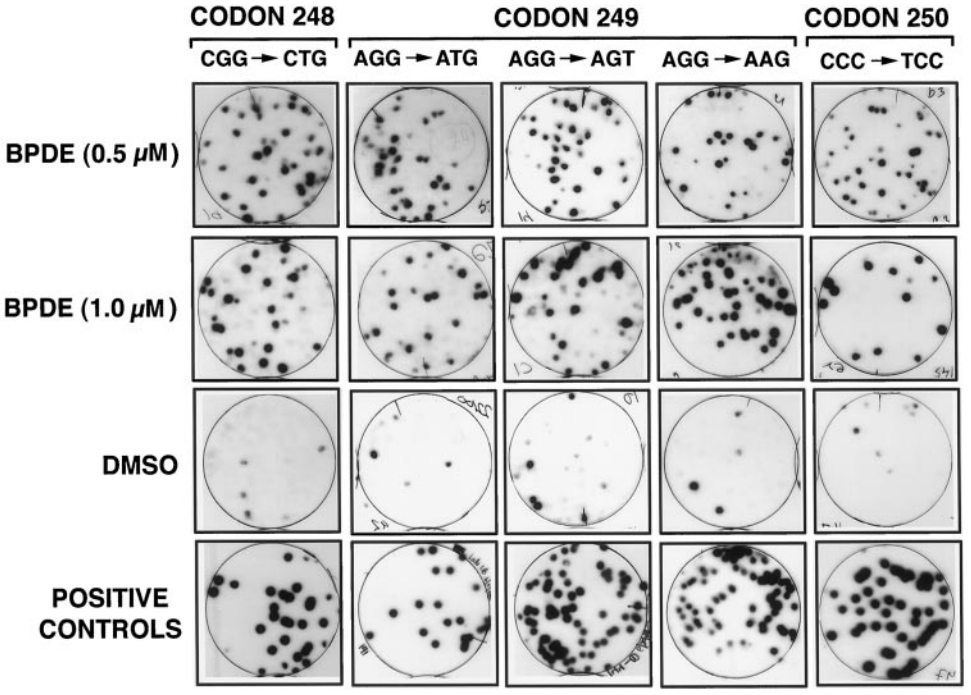

Fig. 2. Identified p53 codon 248, 249, and 250 mutant λ-plaques on representative membranes from different treatment groups. DNA samples from either BPDE-treated or DMSO controls were analyzed for bp changes at the *Msp*I site (codon 248) and *Hae*III site (codons 249 and 250) by *Msp*I and *Hae*III RFLP/PCR. Thirty copies of the MS or internal control were added to 3×10^7 copies of the p53 allele. The RFLP/PCR products were cloned into λ-gt10 and plated on *Escherichia coli* C600Hfl, and plaque screens were analyzed by hybridization with different mutant-specific oligonucleotide probes as well as probes for the WT sequence and MS. With each hybridization and washing, positive control membranes containing the authentic mutants were added to serve as positive controls. Analyses included 1500 plaques on 8–10 LB plates.

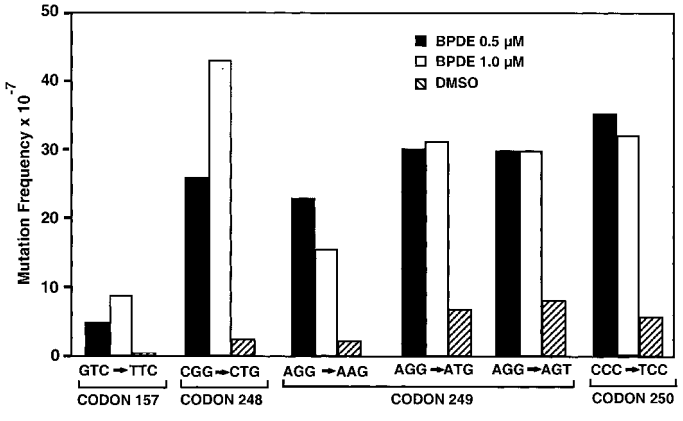

Fig. 3. Absolute p53 mutation frequencies in BPDE-treated and DMSO control BEAS-2B cells. Mutation frequencies were calculated from the percentage of the identified λ-plaques (mean of percentage was taken from 8–10 membranes, each with a total of 150–180 plaques, for each specific oligonucleotide probe) by calibration with the MS contents of the RFLP/PCR products, the initial number of p53 copies, and the number of copies of MS added at the outset of the experiment.

ATG^{met} at frequencies of 30 and 32 $\times$ 10^{-7}, respectively, compared with 6 $\times$ 10^{-7} in the control; codon 249 AGG^{arg} to AGT^{ser} transversions at a frequency of 30 $\times$ 10^{-7} compared with 8.3 $\times$ 10^{-7} in the controls; codon 249 AGG^{arg} to AAG^{lys} transitions at frequencies of 23 and 15.6 $\times$ 10^{-7}, respectively, compared with 2.4 $\times$ 10^{-7} in the controls; and codon 250 CCC^{pro} to TCC^{ser} transitions at frequencies of 35 and 32 $\times$ 10^{-7}, respectively, compared with 7$\times$ 10^{-7} in the controls (Figs. 2 and 3). No other mutations were found at these codons.

p53 Codon 157, 248, 249, and 250 Mutation Load in Nontumorous Tissue from Cancer Cases and Noncancer Controls. DNA from peripheral lung tissue samples, either from cancer cases or noncancer controls, was analyzed for the load of G:C to T:A transversions at codon 157 and mutations at codons 248, 249, and 250 using the assay described above. Eight of 14 (57%) of the nontumorous samples from lung cancer cases showed a 5–25-fold higher frequency of G:C to T:A transversions at codon 157 compared with normal controls ($P < 0.01$; Figs. 4 and 5*B*; Table 1). Fifty percent (7/14) of the nontumorous samples from lung cancer cases showed a high frequency of codon 249 AGG^{arg} to AGT^{ser} transversions

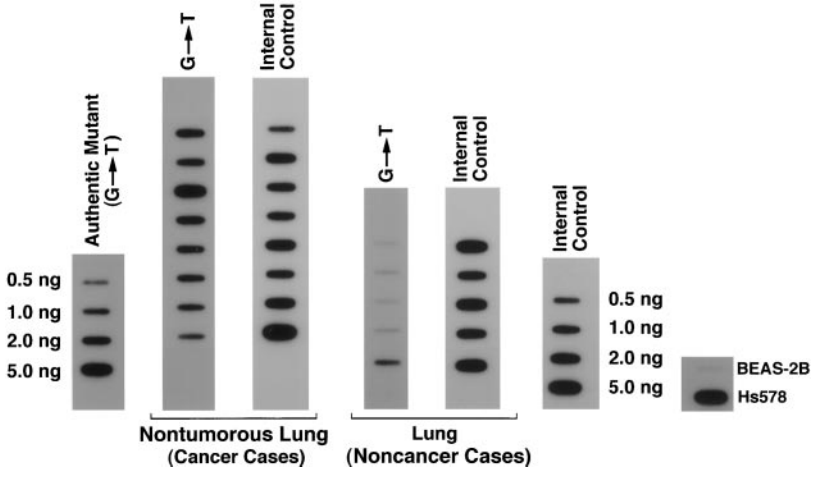

Fig. 4. Slot blot hybridization with ^{32}P-labeled oligonucleotide specific to either codon 157 G:C to T:A transversions or the internal control as described in Fig. 1*A*. Amplified DNA from different nontumorous lung tissue samples either from cancer cases or noncancer controls was loaded on the membranes. After hybridization with the mutant probe, membrane was stripped and hybridized with the probe for internal control. DNA from BEAS-2B and Hs578 cell lines were used as negative and positive controls, respectively.

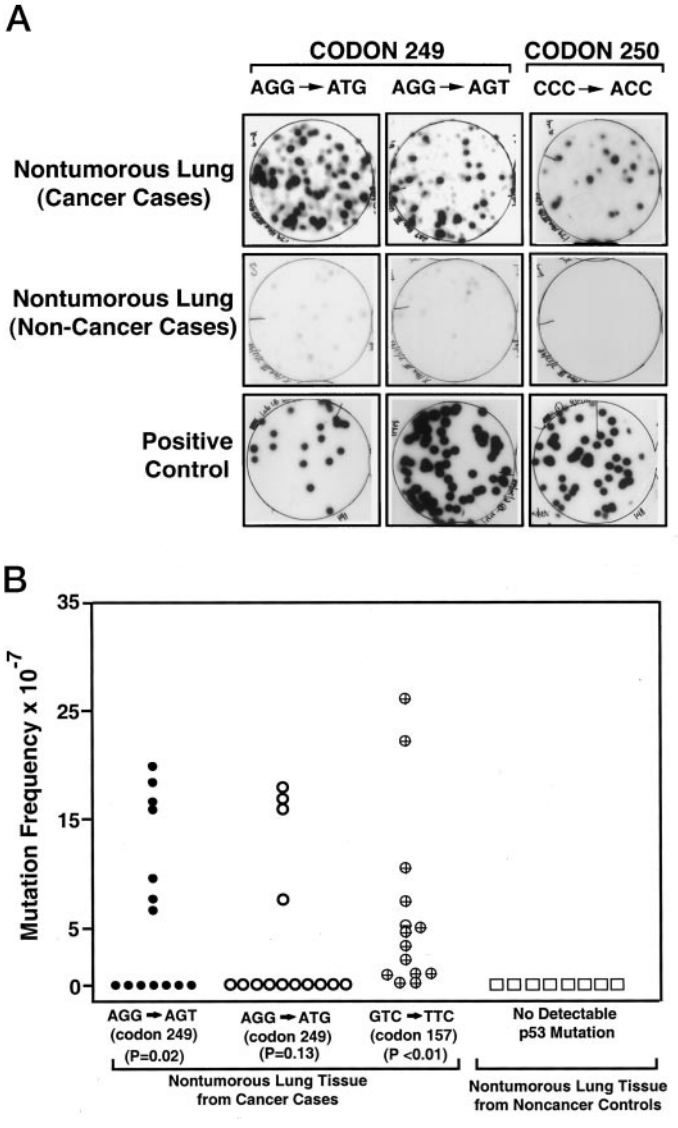

Fig. 5. *A*, identified p53 codons 249 and 250 mutant λ-plaques on representative membranes from nontumorous tissue either from cancer cases or noncancerous controls. Analysis was performed as described in Fig. 2. *B*, absolute mutation frequencies of G:C to T:A transversions at codons 157, 249, and 250. Mutation frequencies were calculated as described in Fig. 3.

($P < 0.02$). Four of these seven samples with AGT^{ser} mutations also showed a high frequency of codon 249 AGG^{arg} to ATG^{met} mutations, whereas only one sample showed codon 250 CCC^{pro} to ACC^{thr} transversions. Noncancer control samples from smokers and nonsmokers did not contain any detectable mutations at codons 248, 249, or 250 (Fig. 5, *A* and *B*).

Discussion

p53 mutations are common in lung cancer with a prevalence of 33% in adenocarcinomas, 65% in squamous cell carcinoma, and 70% in small cell lung cancer [reviewed in Ref. (4)]. In tobacco-associated lung cancer, the spectrum of p53 mutations is dominated by G:C to T:A transversions with a DNA-coding strand bias and a reduced frequency of G:C to A:T transitions (10, 12, 19). This prevalence of G:C to T:A transversions is consistent with the presence of different PAH in cigarette smoke that form bulky DNA adducts and with the preferential repair of the transcribed DNA strand (23). p53 mutations can also be caused by tobacco-specific *N*-nitrosamines (24, 25). Compared with other types of cancer, an unusual series of mutational hotspot codons, *i.e.*, 157, 179, 245, 248, 249, 273, and 282 has been identified in smoking-associated lung cancer (10, 12). BP, a PAH found in cigarette-smoke, has been linked to G:C to T:A transversions at these hotspots. BPDE has been shown to selectively target deoxyguanine at codons 157, 248, and 273 (14), which are sites of slow nucleotide excision repair (17).

Whereas codons 248 and 273 are mutational hotspots in other types of cancer, codon 157 has been described as a mutational hotspot only in lung cancer (10, 12, 13, 19). The high frequency of a specific mutation in tumors may represent both the mutability of a particular codon as well as the selective growth advantage that the mutated cell may attain because of this mutation (5, 18). A carcinogen can induce a number of different mutations in a gene with a preferential induction of a particular mutation at a specific site, which may provide a selective growth advantage (21). To additionally investigate the possible involvement of BP, we have, for the first time, directly evaluated the mutability of codon 157 to BPDE by using a highly sensitive genotypic assay. Exposure of BEAS-2B cells to different doses of BPDE produced G:C to T:A transversions at codon 157 in p53. Our results are consistent with the hypothesis that BPDE induces G:C to T:A transversions at codon 157 of p53. We also evaluated the mutability of codons 248, 249, and 250 to BPDE in BEAS-2B cells. In addition to the high frequency of G:C to T:A transversions at codons 248 and 249, we also observed a high frequency of G:C to A:T and C:G to T:A transitions at codons 249 and 250, respectively. The induction of G:C to A:T transitions, in addition to G:C to T:A transversions, has been reported in BP-induced mouse skin tumors (26). In other studies, BPDE-induced hypoxanthine phosphoribosyltransferase mutation spectra in Chinese hamster V-79 cells varied with the use of different dosages (27). An early study has shown that BP induces G:C to T:A and C:G to A:T transversions at p53 codon 248 in the hepatocellular carcinoma cell line, HepG2 (28). However, no mutations were observed at codon 249 in the above study, which is different from our results. There could be several reasons for the absence of codon 249 mutations in the above study by Cherpillod and Amstad (28), including: (*a*) the sensitivity of our assay with the cloning of mutated fragments and oligonucleotide hybridization is 10 times higher than the slot blot analysis used in their assay; (*b*) they used BP instead of BPDE, the active metabolite of BP; and (*c*) hepatocellular carcinoma cells were used instead of bronchial epithelial cells. Our results indicate that BPDE preferentially induces G:C to T:A transversions at the p53 mutational hotspots found in human lung cancer.

The detection of specific mutations in cancer-related genes in nontumorous tissue can provide insight into the association between exposure to a particular carcinogen and cancer. Recently, p53 and K-ras mutations have been reported in malignant and nonmalignant bronchial biopsies from patients with non-small cell lung cancer (29). We additionally extended our study by analyzing nontumorous lung tissue from lung cancer cases who were smokers for the p53 hotspot mutation by using the highly sensitive mutation assay. The presence of a high frequency of G:C to T:A transversions at codons 157 and 249 in nontumorous lung tissue in smoking-associated lung cancer in our study is consistent with the hypothesis that bulky carcinogens including BP induce G:C to T:A transversions at the hotspot codons.

Cigarette smoking is also associated with an increased oxidative stress including lipid peroxidation (30, 31). An increased level of 4-HNE-adducts at codon 249 of p53 was found when genomic DNA was treated with 4-HNE.[7] Furthermore, we have earlier reported that

[7] M. S. Tang, personal communication.

Table 1 *p53 mutation frequencies in nontumorous peripheral lung tissue in lung cancer cases and normal controls*

Samples	Mut. freq.[a] $\times 10^{-7}$ codon 157 (GTC to TTC)	Mut. freq. $\times 10^{-7}$ codon 249 (AGG to AGT)	Mut. freq. $\times 10^{-7}$ codon 249 (AGG to ATG)	Mut. freq. $\times 10^{-7}$ codon 250 (CCC to ACC)	Sex/age	Smoking status (pack-yr)	Cotinine (ng/ml)
Normal controls							
CC	0.4	ND	ND	ND	M/41	N/A	308
L	0	ND	ND	ND	F/45	N/A	245
W	0	ND	ND	ND	M/44	N/A	414
Z	0.1	ND	ND	ND	F/36	N/A	260
B	0.1	ND	ND	ND	M/22	N/A	431
E	0.2	ND	ND	ND	F/1	N/A	1
F	0.5	ND	ND	ND	M/31	N/A	1
X	0.6	ND	ND	ND	M/32	N/A	1
Nontumorous lung from cancer patients							
257	22.4	18.6	0	ND	M/53	20	N/A
267	5.4	6.8	0	6.8	M/53	53	N/A
227	26.2	16.8	16	ND	M/69	36	N/A
179	10.7	20	18.1	ND	M/65	42	N/A
221	4.8	16	17.1	ND	M/76	45	N/A
231	7.6	9.7	7.8	ND	F/57	40	N/A
255	3.6	7.9	0	ND	M/71	25	N/A
261	0.9	0	0	ND	F/68	30	N/A
279	5.2	0	0	ND	F/57	14	N/A
303	2.3	0	0	ND	M/61	28	N/A
309	0.2	0	0	ND	M/74	31	N/A
317	1.0	0	0	ND	M/64	20	N/A
319	0.2	0	0	ND	M/49	37	N/A
323	1.0	0	0	ND	M/41	23	N/A

[a] Mut. freq., mutation frequency; ND, not detected; N/A, not available.

exposure of the TK-6 lymphoblastoid cell line to 4-HNE induced G:C to T:A transversions at codon 249 of p53 (32). However, we did not find any of the above mutations at codon 248, which also is one of the hotspots in lung as well as in other types of cancer and a target for BPDE adduct formation. This could either be attributable to the absence of 248 mutations or their presence below the limit of detection.

In the present study, one possibility for the occurrence of a lower frequency of codon 157 mutations in BPDE-treated cells, compared with the mutation frequencies at codons 248, 249, and 250 and the presence of comparable frequency of codon 157 mutation to codon 249 mutation in nontumorous tissue in lung cancer cases, could be attributable to the possible clonal expansion, to some degree, of the cells containing codon 157 mutations in the lung tissue. On the contrary, one explanation for the presence of a low frequency of C:G to A:T mutation at codon 250 in nontumorous lung tissue is that this particular mutation does not seem to provide any growth advantage to the mutant cells.

In this study, we selected lung cancer cases that did not contain any mutations in codons 157, 248, 249, and 250 in their tumors (33). However, 38% of the tumor samples in cancer cases showed either G:C to T:A transversions at codons 154, 179, and 273 or 1 bp deletion at codons 273 and 300 (33). This argues against any possible contamination of nontumorous tissue samples with tumor cells. Furthermore, these results suggest that, although codon 249 and 157 mutations can occur early in the process, these may not always be selected during tumorigenesis. This observation contradicts the recent hypothesis by Rodin and Rodin (18) that the high frequency of G:C to T:A transversions at hotspot codons in lung cancer is solely attributable to the selection of these mutations.

We also examined the possibility of measuring the p53 mutational load among normal individuals (without any precancerous or cancerous condition) who were either smokers ($n = 5$) or nonsmokers ($n = 3$). We did not observe any mutation at codons 157, 248, or 249 in normal, noncancer controls, either among smokers or nonsmokers.

This nondetectability could be attributable to the small sample size and the young age of donors in our sample set (average 37 years) or the presence of these mutations below the limit of detection by the assay at early age. Future studies with a larger sample set of individuals with a wide range of ages will be needed to investigate the p53 mutation load in smoking *versus* nonsmoking normal individuals.

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References

1. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. p53 mutations in human cancers. Science (Wash. DC), *253:* 49–53, 1991.
2. Hainaut, P., Hernandez, T., Robinson, A., Rodriguez-Tome, P., Flores, T., Hollstein, M., Harris, C. C., and Montesano, R. IARC Database of p53 gene mutations in human tumors and cell lines: updated compilation, revised formats and new visualization tools. Nucleic Acids Res. *26:* 205–213, 1998.
3. Hainaut, P., and Hollstein, M. p53 and human cancer: the first ten thousand mutations. Adv. Cancer Res., *77:* 81–137, 2000.
4. 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.
5. Hussain, S. P., and Harris, C. C. Molecular epidemiology of human cancer: contribution of mutation spectra studies of tumor suppressor genes. Cancer Res., *58:* 4023–4037, 1998.
6. 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.
7. Hsu, I. C., Metcalf, R. A., Sun, T., Welsh, J. A., Wang, N. J., and Harris, C. C. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. Nature (Lond.), *350:* 427–428, 1991.
8. Bressac, B., Kew, M., Wands, J., and Ozturk, M. Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. Nature (Lond.), *350:* 429–431, 1991.
9. Takeshima, Y., Seyama, T., Bennett, W. P., Akiyama, M., Tokuoka, S., Inai, K., Mabuchi, K., Land, C. E., and Harris, C. C. p53 mutations in lung cancers from non-smoking atomic-bomb survivors. Lancet, *342:* 1520–1521, 1993.
10. Hernandez-Boussard, T. M., and Hainaut, P. A specific spectrum of p53 mutations in lung cancer from smokers: review of mutations compiled in the IARC p53 database (Published erratum in Environ. Health Perspect., *106:* A421, 1998). Environ. Health Perspect., *106:* 385–391, 1998.
11. Suzuki, H., Takahashi, T., Kuroishi, T., Suyama, M., Ariyoshi, Y., and Ueda, R. p53 mutations in non-small cell lung cancer in Japan: association between mutations and smoking. Cancer Res., *52:* 734–736, 1992.
12. Bennett, W. P., Hussain, S. P., Vahakangas, K. H., Khan, M. A., Shields, P. G., and Harris, C. C. Molecular epidemiology of human cancer risk: gene-environment interactions and p53 mutation spectrum in human lung cancer. J. Pathol., *187:* 8–18, 1999.
13. Pfeifer, G. P., Denissenko, M. F., and Tang, M. p53 mutations, benzo[a]pyrene and lung cancer: a reply. Mutagenesis, *13:* 537–538, 1998.
14. Denissenko, M. F., Pao, A., Tang, M., and Pfeifer, G. P. Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in P53. Science (Wash. DC), *274:* 430–432, 1996.

15. Denissenko, M. F., Chen, J. X., Tang, M. S., and Pfeifer, G. P. Cytosine methylation determines hot spots of DNA damage in the human P53 gene. Proc. Natl. Acad. Sci. USA, *94:* 3893–3898, 1997.
16. Weisenberger, D. J., and Romano, L. J. Cytosine methylation in a CpG sequence leads to enhanced reactivity with Benzo[a]pyrene diol epoxide that correlates with a conformational change. J. Biol. Chem., *274:* 23948–23955, 1999.
17. Denissenko, M. F., Pao, A., Pfeifer, G. P., and Tang, M. Slow repair of bulky DNA adducts along the nontranscribed strand of the human p53 gene may explain the strand bias of transversion mutations in cancers. Oncogene, *16:* 1241–1247, 1998.
18. Rodin, S. N., and Rodin, A. S. Human lung cancer and p53: the interplay between mutagenesis and selection. Proc. Natl. Acad. Sci. USA, *97:* 12244–12249, 2000.
19. Hainaut, P., and Pfeifer, G. P. Patterns of p53 G–>T transversions in lung cancers reflect the primary mutagenic signature of DNA-damage by tobacco smoke. Carcinogenesis (Lond.), *22:* 367–374, 2001.
20. Reddel, R. R., Ke, Y., Gerwin, B. I., McMenamin, M. G., Lechner, J. F., Su, R. T., Brash, D. E., Park, J. B., Rhim, J. S., and Harris, C. C. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. Cancer Res., *48:* 1904–1909, 1988.
21. Aguilar, F., Hussain, S. P., and Cerutti, P. Aflatoxin B1 induces the transversion of G–>T in codon 249 of the p53 tumor suppressor gene in human hepatocytes. Proc. Natl. Acad. Sci. USA, *90:* 8586–8590, 1993.
22. Hussain, S. P., Amstad, P., Raja, K., Ambs, S., Nagashima, M., Bennett, W. P., Shields, P. G., Ham, A. J., Swenberg, J. A., Marrogi, A. J., and Harris, C. C. Increased p53 mutation load in noncancerous colon tissue from ulcerative colitis: a cancer-prone chronic inflammatory disease. Cancer Res., *60:* 3333–3337, 2000.
23. Smith, L. E., Denissenko, M. F., Bennett, W. P., Li, H., Amin, S., Tang, M., and Pfeifer, G. P. Targeting of lung cancer mutational hotspots by polycyclic aromatic hydrocarbons. J. Natl. Cancer Inst., *92:* 803–811, 2000.
24. Ronai, Z. A., Gradia, S., Peterson, L. A., and Hecht, S. S. G to A transitions and G to T transversions in codon 12 of the Ki-ras oncogene isolated from mouse lung tumors induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and related DNA methylating and pyridyloxobutylating agents. Carcinogenesis (Lond.), *14:* 2419–2422, 1993.
25. Hecht, S. S. Metabolically activated carcinogens and mutations in the p53 tumor suppressor gene in lung cancer [editorial]. J. Natl. Cancer Inst., *92:* 782–783, 2000.
26. Wei, S. J., Chang, R. L., Merkler, K. A., Gwynne, M., Cui, X. X., Murthy, B., Huang, M. T., Xie, J. G., Lu, Y. P., Lou, Y. R., Jerina, D. M., and Conney, A. H. Dose-dependent mutation profile in the c-Ha-ras proto-oncogene of skin tumors in mice initiated with benzo[a]pyrene. Carcinogenesis (Lond.), *20:* 1689–1696, 1999.
27. Wei, S. J., Chang, R. L., Bhachech, N., Cui, X. X., Merkler, K. A., Wong, C. Q., Hennig, E., Yagi, H., Jerina, D. M., and Conney, A. H. Dose-dependent differences in the profile of mutations induced by (+)-7R,8S-dihydroxy-9S,10R-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene in the coding region of the hypoxanthine (guanine) phosphoribosyltransferase gene in Chinese hamster V-79 cells. Cancer Res., *53:* 3294–3301, 1993.
28. Cherpillod, P., and Amstad, P. A. Benzo[a]pyrene-induced mutagenesis of p53 hotspot codons 248 and 249 in human hepatocytes. Mol. Carcinog., *13:* 15–20, 1995.
29. Lang, S. M., Stratakis, D. F., Freudling, A., Ebelt, K., Oduncu, F., Hautmann, H., and Huber, R. M. Detection of K-ras and p53 mutations in bronchoscopically obtained malignant and non-malignant tissue from patients with non-small cell lung cancer. Eur. J. Med. Res., *5:* 341–346, 2000.
30. Rajpurkar, A., Dhabuwala, C. B., Jiang, Y., and Li, H. Chronic cigarette smoking induces an oxidantantioxidant imbalance in the testis. J. Environ. Pathol. Toxicol. Oncol., *19:* 369–373, 2000.
31. Panda, K., Chattopadhyay, R., Chattopadhyay, D. J., and Chatterjee, I. B. Vitamin C prevents cigarette smoke-induced oxidative damage *in vivo*. Free Radic. Biol. Med., *29:* 115–124, 2000.
32. Hussain, S. P., Raja, K., Amstad, P. A., Sawyer, M., Trudel, L. J., Wogan, G. N., Hofseth, L. J., Shields, P. G., Billiar, T. R., Trautwein, C., Hohler, T., Galle, P. R., Phillips, D. H., Markin, R., Marrogi, A. J., and Harris, C. C. Increased p53 mutation load in nontumorous human liver of wilson disease and hemochromatosis: oxyradical overload diseases. Proc. Natl. Acad. Sci. USA, *97:* 12770–12775, 2000.
33. Skaug, V., Ryberg, D., Kure, E. H., Arab, M. O., Stangeland, L., Myking, A. O., and Haugen, A. p53 mutations in defined structural and functional domains are related to poor clinical outcome in non-small cell lung cancer patients. Clin. Cancer Res., *6:* 1031–1037, 2000.