

Quantitative Detection of p53 Mutations in Plasma DNA from Tobacco Smokers

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Abstract

In lung tumors, the p53 tumor suppressor gene is commonly mutated with a characteristic mutation spectrum. The amount of and alterations in plasma DNA, such as mutations in p53, were associated with several cancers. Few studies used quantitative methods of high sensitivity. Previously, we observed p53 mutations in the noncancerous tissue that differed from those in lung tumors using the highly sensitive p53 mutation load assay. Based on our observation of an increased p53 mutation load in nontumorous lung tissue in smokers, we hypothesized that plasma DNA may contain mutant p53 indicative of tobacco smoke exposure and will be an effective biomarker of lung cancer or smoking exposure. We modified the p53 mutation load assay to detect mutations at p53 codons 248 and 249, common mutations in lung cancer, in plasma DNA samples with a sensitivity of 1:5,000. The assay was applied to a set of lung cancer cases ($n = 39$), hospital controls ($n = 21$), and population controls ($n = 20$) from a larger study. Controls were selected to consist of equal numbers of both ever and never smokers. The p53 mutation load (mutated p53 copies per total number of p53 copies) was associated with smoking ($P = 0.06$), but not with lung cancer ($P = 0.59$). Most of the individuals with p53 mutations observed in plasma DNA were ever smokers and the p53 mutation load was higher in those who smoked for longer durations ($P = 0.04$). In summary, we were able to detect p53 mutations in plasma DNA from healthy individuals and our data suggest that p53 mutations in plasma DNA may be a marker of carcinogen exposure from tobacco smoke. (Cancer Res 2006; 66(16): 8309-17)

Introduction

Inactivation of the p53 tumor suppressor gene is a critical and early event in lung carcinogenesis (1). The p53 protein functions to induce growth arrest, DNA repair, or apoptosis in response to cellular stress, including DNA damage (2–4). Mutations in p53 are present in >90% of small cell lung cancers and >50% of non-small

cell lung cancers (5–8), suggesting that inactivation of p53 effector pathways often occurs as a consequence of mutation in p53.

The mutation spectrum of p53 seems distinct for particular cancers and exposures, including both the type and the position of mutation (9). For example, dietary aflatoxin B₁ exposure in combination with chronic hepatitis B viral infection was correlated with G:C to T:A transversions that lead to serine substitutions at residue 249 of p53 in hepatocellular carcinoma (10, 11) and exposure to cigarette smoke was correlated with G:C to T:A transversions in lung carcinomas (1, 12–15). Mutations at codons 157, 158, 248, 249, and 273 are more frequently detected in lung tumors than mutations at other positions and are considered “hotspots” for p53 mutation (14, 16, 17).

Circulating extracellular dsDNA fragments, or serum/plasma DNA, has emerged as a potential biomarker of cancer. Several studies showed that concentration of DNA from serum/plasma was significantly higher from patients with a variety of different cancers when compared with healthy controls (18–21). Moreover, DNA alterations in serum/plasma DNA were associated with cancers (21–25), and in several reports, the DNA alterations identified in the serum or plasma of cancer patients were identical to those found in the primary tumor (19, 26–28). In studies of liver cancer, mutations in p53 at Ser²⁴⁹ from plasma DNA were associated with aflatoxin B₁ exposure in cases with hepatitis B viral infection and were detected before cancer diagnosis (29–31).

Most studies examining the association of tumor-related alterations in plasma DNA with disease were qualitative and used assays of low sensitivity. A highly sensitive assay for the detection of p53 mutations is necessary if the method is to be applied before cancer diagnosis, because the concentration of plasma DNA is low in healthy individuals. The previous studies investigating Ser²⁴⁹ p53 mutations in plasma DNA associated with liver cancer had some of the highest sensitivities for detecting mutations (1:250–1:5,000; refs. 29–32). A recent report observed that amounts of Ser²⁴⁹ p53 mutations in plasma were associated with liver cancer (32). We describe the application of a highly sensitive assay (1:5,000) developed to determine the p53 mutation load at codons 248 and 249 using DNA obtained from participants' plasma in a case-control study of lung cancer to determine if the p53 mutation load in plasma DNA was associated with lung cancer or cigarette smoking.

Materials and Methods

Study subjects. Samples for the application of the mutation load assay were selected from within a larger ongoing case-control study from the greater Baltimore area (33) and institutional review board approval was

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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obtained from all participating institutions. Informed consent was obtained from all participants. Fresh-frozen surgical lung cancer specimens and macroscopically normal (nontumorous) lung tissues were obtained at the time of surgery from patients with lung cancer. Blood specimens were processed immediately after collection for isolation of serum or plasma and stored at -70°C .

A target study was planned to consist of 40 lung cancer cases, 20 hospital controls, and 20 population controls for the application of the mutation load assay. Due to the possibility that the DNA concentrations obtained from some samples may be too low for analysis by mutation load assay, additional cases and controls were selected before DNA isolation, based on availability of at least 5 mL plasma. Forty-five lung cancer cases were selected for analysis where frozen tissue was available for DNA sequencing and p53 mutation load analysis. Twenty-eight hospital controls and 27 population controls were frequency matched to cases based on race, age, and gender using interviewer screening data. To examine possible smoking associations, all nonsmoking cases with tissue were included in the study group, and hospital and population controls were selected to consist of equal numbers of ever and never smokers. Complete questionnaire data were unavailable for four cancer patients because patients died before completion of interviews. All experimental procedures were done with coded samples lacking identifiers.

Never smokers smoked <100 cigarettes in their lifetime. Race was classified by self-report.

DNA extraction. DNA was isolated from plasma using the QIAamp spin column procedure with QIAamp DNA Blood Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions with the following modifications: EB buffer (Qiagen) was used as final elution buffer and incubation time was increased to 5 minutes before DNA elution. Pilot experiments showed that AE buffer, the suggested kit elution buffer, inhibited PCR amplification in our assays (data not shown). Plasma (3-4.2 mL) was used for DNA extraction with 600 μL plasma applied to each column. DNA was stored at -20°C until applying to the mutation load assay. Tumor DNA was extracted from fresh microdissected tumor tissue with QIAamp DNA Micro (Qiagen). DNA from nontumorous lung tissue was extracted by Puregene (Gentra System, Minneapolis, MN). Extracted normal or tumor tissue DNA was quantified by spectrophotometer.

Quantification of p53 copies in blood DNA. The concentration of p53 DNA from plasma was measured using a real-time quantitative PCR Taqman assay with the following primers and probe: 5'-CCCTGTGCAGCTGTGGTTG-3', 5'-ATGGCCATGGTGCGGAC-3', 5'-FAM-CCACACCCCGCCCGCA-TAMRA-3', and Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA). A calibration curve composed of a 10-fold serial dilution of known copies of PAT153p53II plasmid, which contains wild-type p53 gene, was used to determine absolute copies of p53 in plasma DNA. Taqman assays were done in triplicate for each sample by ABI 7700 Sequence Detection System (Applied Biosystems). To ensure that similar DNA amounts were used for each plasma sample, experiments were done using 50,000 total p53 copies. DNA isolation failed for three lung cancer cases. Thirty-nine lung cancer cases, 20 hospital controls, and 21 population controls had concentrations of plasma DNA high enough for the application of 50,000 p53 copies to the mutation load assay for codons 248 and 249 in duplicate.

Analysis of p53 mutations by mutation load assay. A highly sensitive assay to estimate the p53 mutation load in nontumorous tissue DNA, the mutation load assay, was described previously (34). The most frequent mutations in lung cancer observed in the IARC p53 mutation database are at positions 273, 249, and 248 (<http://www-p53.iarc.fr/>; ref. 35). A limitation of the mutation load assay is the requirement for a restriction enzyme site for enrichment of mutated DNA sequence (34). Therefore, only mutations at codons 248 and 249 could be examined using the mutation load assay. The standard mutation load assay was optimized for plasma DNA and the slot blot procedure for codons 248 and 249 was developed based on the procedure outlined for codon 157 of p53 (34). In brief, the mutation load assay procedure involves an enrichment of mutated p53 fragments with use of specific restriction enzyme digestion, followed by two consecutive PCR amplifications, enzyme digestions, and a quantification of

p53 mutated fragments with use of an internal control and slot blot hybridization.

Development and testing of the p53 mutation load assay was done using a defined number of mutant p53 copies plus 100 ng genomic DNA wild-type for p53 (Fig. 1). Fifty thousand copies of p53 DNA from plasma DNA, determined by real-time PCR as described above, was used for the application of the mutation load assay. The nontumorous tissue DNA

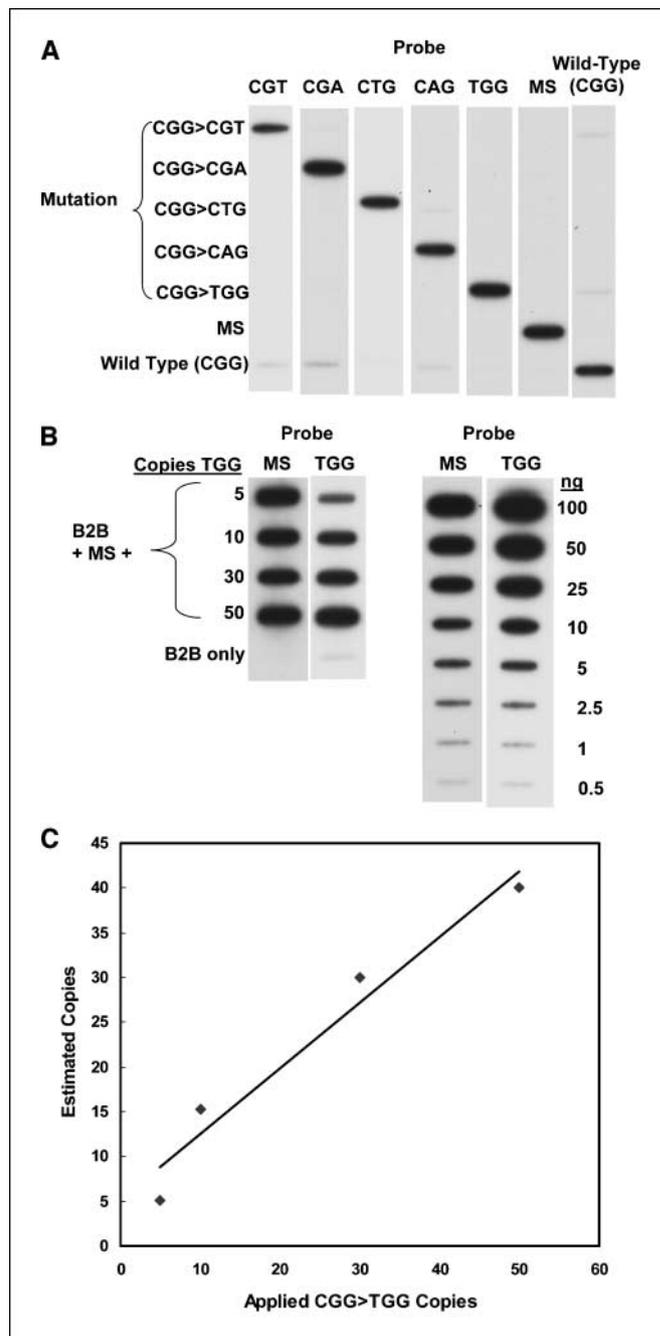


Figure 1. Sequence specificity of the mutation load assay. *A*, selected 248 mutant, MS, and wild-type p53 sequences (10 ng) were applied to membranes. Membranes were probed for 248 mutations, MS, or wild-type p53 sequences as described in Materials and Methods. *B*, test samples with 5, 10, 30, or 50 copies of CGG>TGG codon 248 mutated p53 sequences + 100 ng Beas2B (B2B) cell line DNA + 30 copies of MS or Beas2B alone were assayed using the mutation load assay. Membranes were probed for MS or CGG>TGG mutations. *C*, CGG>TGG p53 mutant copies were estimated using the control ladder in (*B*) and band intensities as outlined in Materials and Methods.

(100 µg; $\sim 3.2 \times 10^7$ p53 copies) was applied to mutation load assay for nontumorous tissue DNA analysis.

DNA was predigested with specific restriction enzymes, either *Msp*I (codon 248) or *Hae*III (codon 249; Roche Diagnostic, Mannheim, Germany), overnight using the reaction conditions specified by the manufacturer. Predigested DNA was purified by QIAquick PCR purification kit (Qiagen) and eluted into 50 µL EB buffer. After purification, PCR amplification was done with the addition of 30 copies of an internal control [mutant standard (MS)] sequence (36) and description provided previously (34). PCR amplification was done in $1 \times$ Pfu buffer (Stratagene, La Jolla, CA) using 0.4 µmol/L sense and antisense primers, 1 µL Pfu DNA polymerase (2.5 units/µL; Stratagene), and 0.5 µL Perfect Match enhancer (Stratagene) in a total of 100 µL reaction volume. Primers for PCR amplification were sense 5'-CCAGTGTGATGATGGTGAGG-3' and antisense 5'-GTTGGCTCTG-ACTGTACCAC-3'. PCR cycling conditions included a 5-minute incubation at 95°C for polymerase activation followed by 40 cycles of 96°C for 45 seconds, 56°C (codon 249) or 60°C (codon 248) for 1 minute, and 72°C for 30 seconds followed by a final extension of 72°C for 5 minutes.

Amplified products were digested overnight with the appropriate restriction enzyme and conditions as indicated above. Digested products were purified using the QIAquick PCR amplification kit into a total volume of 50 µL. Digested amplification product (20 µL) was used for the second PCR amplification using conditions identical to the first PCR amplification but with 20 cycles.

PCR products from the second PCR amplification were purified with the QIAquick PCR purification kit and prepared for application to slot blot as described (34). Slot blot membranes were hybridized with specific ³²P-labeled oligonucleotides for the identification of each 248 (CGG>CTG; CGG>CAG; CGG>TGG) or 249 (AGG>AGT; AGG>ATG) mutation or internal control (MS). Probe sequences were described previously (34). Optimal hybridization and washing conditions were determined to ensure specificity of hybridization for each probe and were as follows (hybridization/washing temperature): all codon 248 mutants: 57°C/63°C; codon 249, AGG>AGT: 60°C/63°C; codon 249, AGG>ATG: 57°C/64°C; MS: 57°C/61.5°C. Specific hybridization of probes was shown (Fig. 1A). Signal intensities were obtained with a Fuji Film FLA-5000 phosphorimager and quantified with Fuji Film Image Gauge software (Fuji Film, Japan).

To determine the p53 mutant copies in plasma DNA samples, negative control intensities from Beas2B (wild-type p53 cell line) were subtracted from the measured intensity for each plasma sample. The amount of internal control and each p53 mutant were determined by generating standard curves using known nanogram amounts applied to each membrane. Standard curves were linear within the ranges examined. Next, the following equation was used to estimate copies for each plasma sample: $C = (MS_{\text{copies}} \times \text{ng Mut}) / (\text{ng MS})$, where C is calculated copies of p53 mutations, MS_{copies} is known copies of MS added into each sample (30 copies), ng Mut is estimated nanogram p53 mutant determined from standard curve, and ng MS is calculated nanogram internal control from standard curve. After calculation, values were normalized to known copies of each mutation that were included in each assay as positive controls. Mutated cell line DNA used as positive controls for p53 codons 248 and 249 were as follows: for codon 248, DNA obtained from these cell lines: NCI-H322 (CGG>CTG), CCRF-CEM (CGG>CAG), and MIAPaCa2 (CGG>TGG); for codon 249, DNA obtained from these cell lines: PLC/PRF/5 (AGG>AGT) and HS700T (AGG>ATG). Wild-type p53 DNA, obtained from Beas2B, a bronchial epithelial cell line with wild-type p53, was used as negative controls in all assays. To estimate p53 copies in cell line DNA, whole human genomic DNA was estimated as 6.6 pg/cell (37).

For normal (nontumorous) tissue DNA, additional processing was done in pre-PCR and post-PCR amplification as described (34).

All positive samples plus 20% randomly selected negative samples were independently duplicated with consistent results (Spearman's correlation coefficient for codon 248: 0.67, $P = 0.0004$; Spearman's correlation coefficient for codon 249: 0.56, $P = 0.002$) and agreement was high among duplicates, considering samples positive or negative for mutation (κ coefficient for codon 248: 0.70, $P = 0.006$; κ coefficient for codon 249: 0.51, $P = 0.03$). All replicates were done on separate days.

Tumor DNA p53 mutation analysis. Tumor DNA was available for sequencing for 38 of the samples. Exons 5 to 8 of p53 were amplified using 1 unit Platinum Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA) with primer sets reported previously (38). Direct sequencing of exons 5 and 7 of p53 from amplified PCR products was done using automated sequencing as described in the previous section. Single-strand conformational polymorphism (SSCP) was done for screening for p53 mutations at exons 6 and 8 using a gel containing 12.5% acrylamide (Gene Gel Excel 12.5/24 kit, Amersham Biosciences, Uppsala, Sweden) and a DNA fragment analyzer (Amersham Biosciences) at 600 V, 25 mA, 15 W, and 10°C for exon 6 and 15°C for exon 8 for 90 minutes. DNA bands were visualized by a DNA Silver Staining kit (Amersham Biosciences). Gels were analyzed by at least two independent readers. Positive samples in SSCP were confirmed to have p53 mutation using direct automated sequencing. All samples positive for p53 mutation by direct sequencing were repeated and mutations were confirmed.

Statistical analysis. All statistical analysis was done using SAS 8.02 (SAS Institute, Cary, NC). Categorical variables were compared using χ^2 tests or Fisher's exact tests when 20% of expected counts were <5. For comparisons of p53 copies, results were similar using parametric and nonparametric tests; therefore, only parametric results (t tests or ANOVA) are shown. Due to the highly skewed nature of the estimated p53 mutation frequency, for comparisons of p53 mutation frequency, nonparametric tests were used (Wilcoxon test for two group comparisons and Kruskal-Wallis tests for more than two comparison groups). Survival analysis was done using PROC LIFETEST in SAS and differences between survival curves were estimated using the Wilcoxon test for equality of survival curves. All P s were two-sided.

Results

Specificity and accuracy of the mutation load assay. The original mutation load assay was developed for use with tissue DNA that was of much higher quality and quantity in comparison with DNA obtained from plasma. The assay procedures were streamlined for use with lower amounts of DNA. Specific hybridization and washing conditions were determined for the detection of the most frequent mutations at codons 248 and 249 (Fig. 1A). To evaluate accuracy of the determination of p53 mutation using the modified mutation load assay, known amounts (5, 10, 30, and 50 copies) of CGG>TGG mutant controls were mixed with 100 ng Beas2B cell line DNA (equivalent to 32,000 copies of wild-type p53) plus 30 copies of MS and applied to the assay. Copies for p53 mutations were determined as described in Materials and Methods. The correlation between the estimated number of 248 mutant copies and copies applied to the assay was high (Pearson's correlation coefficient: 0.98, $P = 0.02$; Fig. 1B and C). Based on this experiment, the estimated lower limit of determination was 1:6,400.

Concentration of circulating p53 DNA in plasma. To quantify the amount of DNA to be applied to the mutation load assay, the p53 copies were determined by real-time PCR amplification, because it was determined that DNA quantification of plasma DNA using a spectrophotometer often inaccurately reflected p53 copies (data not shown). The p53 copies/µL purified from plasma was highly variable in the series of lung cancer cases, hospital controls, and population controls. Therefore, although the means were quite different, these differences were not statistically significant in the study groups (Table 1). Cases, compared with combined controls, were slightly more likely to have sufficient DNA for application of the mutation load assay ($P = 0.06$). No statistical difference was observed for group (cases, hospital controls, and population controls), smoking status, age, gender, or race among those with DNA concentrations in sufficient quantity for application to the mutation load assay and those with lower concentrations of DNA.

Table 1. Characteristics of study population applied to the mutation load assay

	Cases (n = 39), n (%)	Hospital controls (n = 20), n (%)	Population controls (n = 21), n (%)	P
Age, mean ± SD	62.5 ± 9.6	64.4 ± 11.1	65.5 ± 9.9	0.5454*
Race				
African American	10 (26)	4 (20)	6 (29)	0.8114 [†]
Caucasian	29 (74)	16 (80)	15 (71)	
Gender				
Male	22 (56)	9 (45)	12 (57)	0.6622*
Female	17 (44)	11 (55)	9 (43)	
Smoking status				
Never	2 (5)	6 (30)	10 (48)	<0.0001 [‡]
Ever	35 (95)	14 (70)	11 (52)	
Missing	2			
Pack-years of smoking				
Mean ± SD	48.4 ± 31.1	33.4 ± 40.0	17.4 ± 24.6	0.0031*
Missing	3			
Duration of smoking (y)				
Mean ± SD	38.1 ± 16.7	24.8 ± 21.6	14.8 ± 16.6	<0.0001*
Missing	3			
p53 Copies (copy/μL), [§] mean ± SD	760 ± 1,390	2,495 ± 5,707	3,353 ± 10,148	0.9587
Mutation detection in plasma				
No detected p53 mutant DNA	33 (85)	16 (80)	19 (90)	0.6039 [‡]
Detected p53 mutant copies	6 (15)	4 (20)	2 (10)	

*ANOVA test.

[†]χ² test.[‡]Fisher's exact test.[§]Concentration is the number of p53 copies/μL of eluate from column purification.^{||}Kruskal-Wallis test.

Application of the mutation load assay for p53 codons 248 and 249. The characteristics of the study population used for application of the mutation load assay are shown in Table 1. The age, race, and gender distributions were similar between cases and controls. Lung cancer cases were more frequently ever smokers, had higher pack-years of smoking, and smoked for longer durations than both hospital and population controls.

The estimated p53 mutation load in plasma DNA ranged from 3×10^{-5} to 3×10^{-3} mutated p53 copies (Fig. 2A and B). Results from plasma DNA samples 1 and 4 were confirmed using colony hybridization followed by DNA sequencing (Supplementary Fig. S1). Examining duplicated assays, four samples where copies was estimated to be five or less were undetectable on replication. Therefore, we decided that a conservative cutoff should be used for determination of positive samples of having at least 10 detectable copies in one of the replicates (>3 times the detection limit) or 2×10^{-4} p53 mutation load. A p53 mutation load above this limit of determination at codons 248 and 249 was observed in 12 of 80 (15%) samples (Table 2). All of these positive plasma samples were obtained from ever smokers. The median p53 mutation load for all mutations detected at codon 248 (CGG>CAG, $P = 0.60$; CGG>TGG, $P = 0.05$), codon 249 (AGG>ATG, $P = 1.00$; AGG>AGT, $P = 0.74$), and combined p53 mutation load (mutations at all positions) were not statistically different in cases, hospital controls, and population controls ($P = 0.59$). Differences in the levels of CGG>TGG codon 248 mutations were driven by hospital controls—this mutation was not observed in either cases or population controls. The p53 mutation load was higher among ever smokers ($P = 0.05$).

The presence of p53 mutations in plasma DNA was not associated with gender, race, age, case status, or smoking pack-years (Table 3). Plasma DNA samples positive for p53 mutations were more frequent among ever smokers, although this was not statistically significant. The frequency of plasma samples positive for p53 mutations was not statistically different among both current and former smokers ($P = 0.91$). The number of years smoked was higher among those individuals with plasma DNA positive for p53 mutation (Table 3). The association with years of smoking remained if samples where the p53 mutation load was discordant between the replicates (positive/negative) were removed from the analysis ($P = 0.03$) or were considered below the level of determination ($P = 0.03$). Using multivariate logistic regression analysis, years of smoking seemed an independent predictor of the presence of a mutation when adjusted for case status ($P = 0.04$). In this model, lung cancer was not predictive of the presence of a p53 mutation in plasma DNA ($P = 0.46$). The mean follow-up time for lung cancer cases was 46.8 months. Estimated p53 mutation frequency in plasma DNA was not associated with lung cancer survival ($P = 0.09$; Supplementary Fig. S2).

p53 mutation status in tumor tissue DNA. DNA obtained from 38 tumors was sequenced for p53 (exons 5-8) and the distribution of mutation is shown in Table 4. The overall p53 mutation frequency was 34% in these tumors consistent with previous reports for adenocarcinoma and squamous cell carcinomas (SCC) of the lung (5). The majority of mutations in p53 were G:C>T:A mutations consistent with the IARC R10 p53 mutation database (limiting to surgical samples and biopsies, in United States, non-small cell lung cancer, and primary tumors; 46% versus 37%; $P = 0.56$; χ^2 test;

ref. 35). Codon 249 mutations or CGG>CAG mutations at codon 248 of p53 were not detected in any of the tumor samples sequenced. One tumor was positive for CGG>TGG mutation at codon 248 of p53. p53 mutations were not detected in the paired plasma DNA from this individual using the mutation load assay.

p53 Codon 248 and 249 mutation load assay in nontumorous tissue. Nontumorous tissue was available for 24 lung cancer cases for the determination of the p53 mutation load. Amplification was poor or DNA concentration was insufficient for analysis for 10 cases at codon 248 and seven cases at codon 249. The estimated p53 mutation load in nontumorous DNA ranged from 2×10^{-7} to 2×10^{-5} mutated p53 copies per total p53 copies, in a similar range as reported previously (Fig. 2C; ref. 34). It is important to note that much higher amounts of DNA (100 μ g or 3.2×10^6 p53 copies) were used to determine the p53 mutation load in normal tissue. Using a similar cutoff for positive p53 mutation load (10 copies), there were six (43%) cases positive for p53 mutations at codon 248 and 12 (71%) cases positive for p53 mutations at codon 249 from nontumorous tissue DNA (Supplementary Table S1). Due to the low frequency of positive plasma samples, it was difficult to compare these with the p53 mutation

load in normal tissue. Two samples positive for CGG>CAG mutation at codon 248 in plasma were also positive for this mutation in normal tissue by mutation load assay (Supplementary Fig. S3). Among samples with detectable p53 mutations in nontumorous tissue or plasma, the p53 mutation load was higher in plasma DNA than in the nontumorous tissue ($P < 0.0001$; Fig. 2B and C).

Discussion

Many previous studies reported the association of tumor-related DNA alterations in serum/plasma DNA with disease and suggested its potential use for molecular diagnosis and prognosis (21). Based on our observation of an increased p53 mutation load in nontumorous lung tissue in smokers (34), we hypothesized that plasma DNA may contain mutant p53 indicative of tobacco smoke exposure. We developed a highly sensitive assay to estimate the p53 mutation load for several p53 mutations at hotspot codons 248 and 249 in plasma DNA. The p53 mutation load in plasma DNA was determined in a series of ever and never smokers from a group of lung cancer cases and healthy controls, including both population and hospital controls. Mutations of p53 in plasma DNA were not

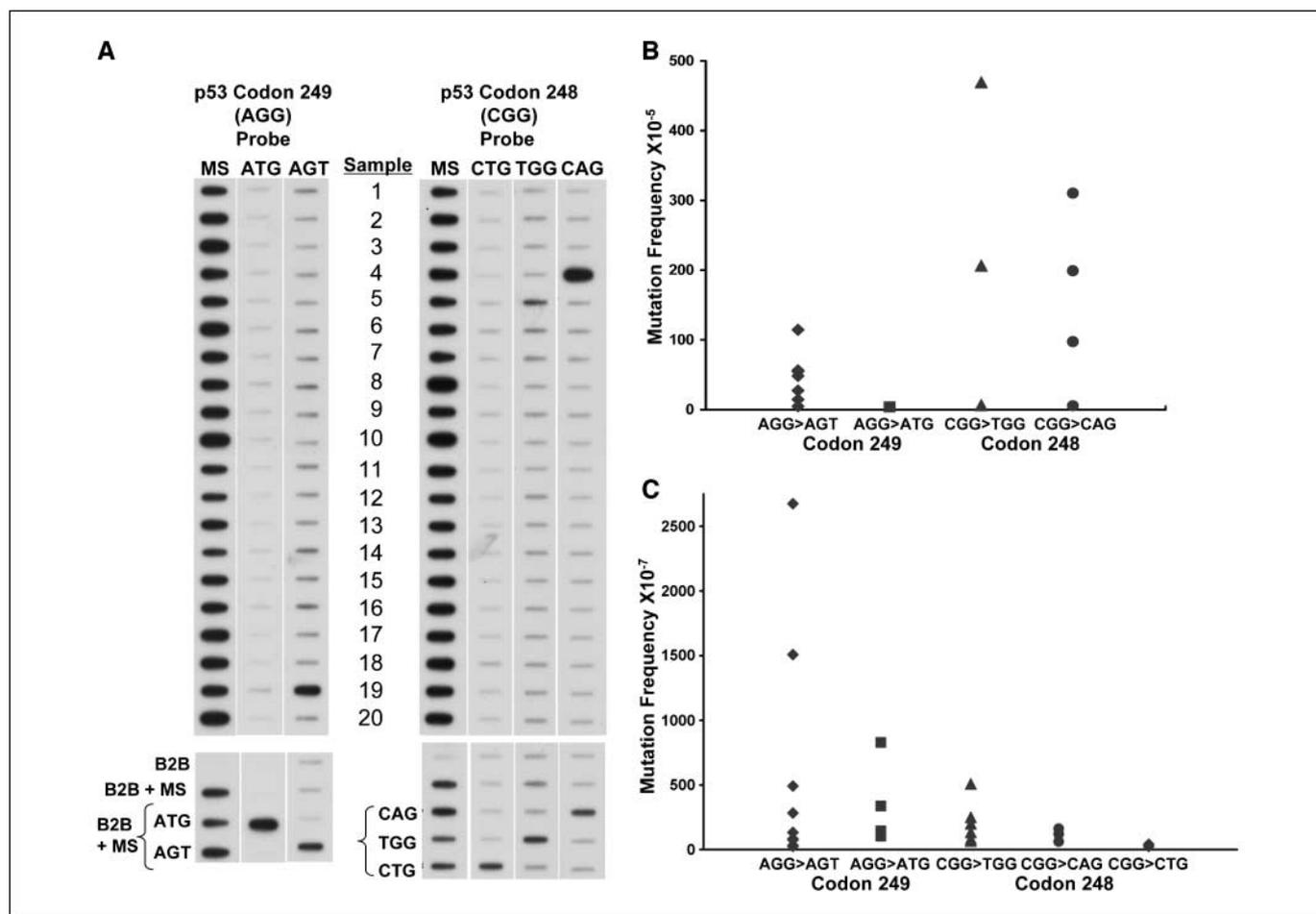


Figure 2. Determination of p53 mutation load in lung cancer cases, hospital controls, and population controls. *A*, representative membrane for 20 samples from the application of the mutation load assay. Samples were probed for p53 codon 248 and 249 mutations. Negative controls, including Beas2B cell line DNA and Beas2B cell line DNA + 30 copies of MS (*B2B* + MS). Positive controls for each mutation included Beas2B + 30 copies of MS + 30 copies of appropriate cell line DNA for codon 249 mutations (ATG or AGT) using HS700T for AGG>ATG and PLC/PRF/5 for AGG>AGT and codon 248 mutations (CAG, TGG, or CTG) using CCRF-CEM for CGG>CAG, MIAPaCa2 for CGG>TGG, and NCI-H322 for CGG>CTG. *B* and *C*, samples assayed for each mutation (*X* axis). Points on graph are samples with mutation load above the limit of detection (3:50,000). Mutation frequency in plasma (*B*) and nontumorous lung tissue (*C*) was estimated as described in Materials and Methods.

Table 2. Characteristics of individuals with a p53 mutation load above level of determination

Sample no.	Group	Smoking status	Gender	Age	Race	Tumor histology	Frequency of p53 mutation ($\times 10^{-5}$), mean (replicate values)			
							Codon 248		Codon 249	
							CGG>TGG	CGG>CAG	AGG>AGT*	AGG>ATG
4	Case	Ever	Female	65	Caucasian	Adenocarcinoma	310 (340, 280)			
19	Case	Ever	Male	59	African American	ND			55 (40, 70)	
26	Case	Ever	Male	61	Caucasian	SCC			42 (0, 84)	
36	Hospital	Ever	Female	51	Caucasian		206 (288, 124)			
38	Case	Ever	Male	74	Caucasian	Adenocarcinoma	199 (74, 324)			
42	Hospital	Ever	Female	38	Caucasian				115 (60, 170)	
50	Hospital	Ever	Male	70	Caucasian		470 (480, 460)			
58	Case	Ever	Male	55	Caucasian	Adenocarcinoma			11 (22, 0)	
61	Hospital	Ever	Female	60	African American				20 (0, 40)	
72	Case	Ever	Female	76	Caucasian	Adenocarcinoma			56 (64, 48)	
73	Population	Ever	Female	67	African American		96 (46, 146)			
76	Population	Ever	Male	79	Caucasian				28 (56, 0)	

Abbreviation: ND, not determined.

*Values of 0 were below the detection limit.

associated with disease, but were associated with years of smoking, suggesting that p53 mutations in plasma DNA may reflect exposure to carcinogens.

The primary strength of the application of the mutation load assay to plasma DNA is the ability to quantify the p53 mutation load with high sensitivity. Many previous studies investigated the presence of tumor-related alterations, such as mutations in p53, in plasma DNA (21, 29–31). However, most of these studies were

primarily qualitative, only examining the presence or absence of a particular alteration. Because assay sensitivity will vary, using nonquantitative methods makes it problematic to compare results from different reports. Moreover, in liver cancer, it was shown that the concentration of Ser²⁴⁹ p53 mutations in plasma was strongly associated with risk of disease (32), suggesting that concentrations of p53 mutations in plasma may be associated with other cancers, including lung cancer, as examined in our study.

Table 3. Distribution of exposures associated with mutations in p53 in plasma DNA

	Mutated p53 DNA fragments		<i>P</i>
	Present (<i>n</i> = 12), <i>n</i> (%)	Absent (<i>n</i> = 68), <i>n</i> (%)	
Case status			
Cases	6 (50)	33 (49)	0.6039*
Hospital controls	4 (33)	16 (23)	
Population controls	2 (17)	19 (28)	
Race			
African American	3 (25)	17 (25)	1.00*
Caucasian	9 (75)	51 (75)	
Gender			
Male	6 (50)	37 (54)	1.00*
Female	6 (50)	31 (46)	
Age, mean \pm SD	62.9 \pm 11.6	63.9 \pm 9.8	0.7504 [†]
Smoking status			
Never	0 (0)	18 (27)	0.0582*
Ever	12 (100)	48 (73)	
Smoking pack-years, mean \pm SD	48.5 \pm 30.2	33.7 \pm 34.7	0.1697 [†]
Smoking duration (y), mean \pm SD	39.2 \pm 15.9	26.3 \pm 20.6	0.0434 [†]

*Fisher's exact test.

†ANOVA test.

Table 4. Summary of sequencing of lung tumors for p53 mutation

Sample no.	p53 Mutation	Exon	Histology
26*	Codon 285 GAG(Glu)>AAG(Lys)	8	SCC
27	Codon 158 CGC(Arg)>CTC(Leu) CpG site	5	Adenocarcinoma
28	Codon 152 1-bp deletion	5	Adenocarcinoma
31	Codon 220 TAT(Tyr)>TGT(Cys)	6	SCC
6	Codon 307 GCA(Ala)>CCA(Pro) + intron 8 (14589) g>t (splice site) 2-bp change	8, intron 8	Adenocarcinoma
32	Codon 277 TGT(Cys)>TTT(Phe)	8	Adenocarcinoma
38*	Codon 286 GAA(Glu)>TAA(Stop)	8	Adenocarcinoma
10	Codon 156 CGC(Arg)>CAC(His) CpG site, codon 248 CGG(His)>TGG(Trp) CpG site	5,7	Adenocarcinoma
11	Codon 245 GGC(Arg)>TTC(Phe) CpG site	7	SCC
13	Codon 157 GTC(Val)>TTC(Phe) CpG site	5	SCC
75	Codon 213 CGA(Arg)>CGG(Arg)	6	Adenocarcinoma
79	Codon 158 CGC(Arg)>CTC(Leu) CpG site	5	SCC
20	Codon 272 GTG(Val)>CTG(Leu)	8	Adenocarcinoma

*Matched plasma DNA samples were positive for p53 mutation load at codon 248 or 249 of p53.

Several previous studies suggested that the levels of DNA in serum/plasma from cancer patients are much higher than those found in healthy controls (19, 21). To detect alterations in serum/plasma DNA before cancer diagnosis, an assay must have high sensitivity. We modified an assay developed previously by our laboratory, with an estimated sensitivity of 10^{-7} on tissue DNA, which was used to estimate the p53 mutation load in nontumorous tissue from lung cancer patients and individuals with chronic inflammatory diseases (34, 39, 40). The estimated limit of determination of our assay for p53 mutations in plasma DNA (1:5,000) was higher than previous reports using RFLP (1:250) and similar to combined RFLP plus short oligonucleotide mass analysis (1:5,000) in liver cancer (29–31). The calculated sensitivity of the mutation load assay for detection of p53 mutations in plasma DNA was limited by the amount of plasma available (3–4.2 mL) or DNA concentration used; if larger volumes of plasma were available, the sensitivity of this assay would be higher.

Using the highly sensitive mutation load assay, p53 mutations were detected in plasma DNA from both hospital and population controls. Therefore, p53 mutations were detected before any cancer diagnosis consistent with observations in liver cancer studies of Ser²⁴⁹ p53 mutations in serum/plasma DNA in controls from regions with high exposure to aflatoxin B₁ (29–31) and the observation in a prospective study that Ser²⁴⁹ mutations in p53 were detected in plasma in 33% of patients before liver cancer diagnosis (31). Interestingly, in the studies of liver cancer, Ser²⁴⁹ mutations in p53 were not detected in serum/plasma of individuals from areas with low aflatoxin B₁ exposure or in European or U.S. populations (29, 31, 32). We report the novel finding of p53 mutations in plasma DNA from a control population of individuals within the United States.

In contrast to aflatoxin B₁-associated liver cancer where Ser²⁴⁹ p53 mutations accounts for ~40% of p53 mutations in liver tumors in areas with high exposure to aflatoxin B₁ (11, 22, 30, 41), in lung cancer, the frequency of 248 and 249 mutations each are only ~4% to 5% of lung cancer mutations (ref. 35; R10 version of IARC database). Therefore, we were able to determine the p53 mutation load in plasma DNA for p53 mutations that were much less prevalent than those associated with liver cancer and for mutations at multiple sites in p53. Our results suggest the applicability of examining p53

mutations in plasma DNA to cancers in addition to liver cancer, where several p53 mutations are common in the mutation spectrum.

The p53 mutation frequency in plasma DNA was similar in lung cancer cases, hospital controls, and population controls and was not associated with lung cancer prognosis. The lack of association of p53 mutation load with prognosis is consistent with several previous reports that indicated that alterations in serum/plasma DNA were not associated with prognosis, stage, or grade of disease (20, 28, 42–45), although some studies suggested that this DNA is higher with advanced disease (18, 46–49).

The p53 mutation load was associated with smoking behavior. In particular, individuals who smoked for longer years had a higher p53 mutational load in plasma DNA. Consequently, our data suggest that the p53 mutational load in plasma DNA more likely reflects carcinogenic exposure than disease status. Consistent with this observation, levels of Ser²⁴⁹ p53 mutations cycled with the seasonal variability of aflatoxin B₁ levels within the diet (30) and these mutations were only detected in geographic regions with high aflatoxin B₁ exposure and hepatitis B viral infection, but not in individuals with only hepatitis B viral infection (29–31). In another study, which examined DNA methylation in plasma DNA, alteration was also higher among smokers (42).

The smoking-associated p53 mutation spectrum in lung tumors is characterized by an increased frequency of G:C to T:A mutations often occurring at codons 157, 158, 245, 248, and 273 (12, 13, 50). Transversions at these positions were experimentally determined to be sites of the formation of DNA adducts after exposure to an active metabolite of benzo[*a*]pyrene, benzo[*a*]pyrene diol epoxide (BPDE; refs. 34, 51). In contrast, the p53 mutations in plasma associated with cigarette smoking at codon 248 were CGG>TGG and CGG>CAG and mutations were frequently observed at codon 249 in the present study. Although G:C>T:A mutations at codon 248 of p53 represent a signature of polycyclic aromatic hydrocarbon (PAH) adduct formation in lung tumors, CGG>TGG (34%) and CGG>CAG (20%) mutations are also commonly observed in smoking-associated lung tumors at codon 248 (IARC p53 mutation database, R10, minus nonsmokers, cell lines and xenografts; ref. 35). The reduced number of G:C>T:A mutations observed in plasma could reflect increased inflammation associated with cigarette smoking. G:C>A:T mutations at CpG sites were associated with increased nitric oxide synthase

expression (52). Interestingly, the frequency of G:C>T:A mutation frequency of p53 differs depending on the location (oral cavity, larynx, or lung) or tumor histologic type (17). Therefore, the mutation pattern more distant from the tumor (such as in plasma) could reflect the levels of oxidative stress instead of direct exposure to PAHs. In addition, because plasma DNA could reflect more recent exposure than mutations in tumors, the reduced frequency of G:C>T:A mutations observed in plasma may reflect the altered composition of cigarettes, including decreasing levels of benzo[*a*]pyrene and increasing levels of tobacco-specific nitrosamines of recent years (17).

All of the plasma mutations at codon 249 were G:C>T:A transversions at the third base. In the IARC p53 mutation database, the most common 249 mutations observed in tumors from smoking-associated lung tumors are AGG>AGT (36%) and AGG>ATG (31%), consistent with the p53 mutation load observed after exposure of Beas2B cells with BPDE (34). Therefore, it was unexpected that none of the mutations were observed at codon 249 at the second position in plasma DNA. Importantly, as observed previously in nontumorous lung tissue, p53 mutations in plasma were detected, which were not selected for in the primary tumor (34).

Our study focused on the detection of p53 mutations at codons 248 and 249 in plasma DNA due to the higher frequency of mutations at these positions in lung cancer (ref. 35; R10 version of IARC database). Given our observed association of p53 mutations at codons 248 and 249 with cigarette smoking, it would be of interest to examine p53 mutations at codon 157 in plasma DNA. Although mutations at codon 157 of p53 are less frequent than mutations at codons 248 and 249, mutations at this position seem to be specific to cigarette smoking-related lung cancer and are rarely detected in other tumors (50). Our preliminary studies suggested that 157 mutations in p53 are rare in plasma DNA (data not shown), causing us to focus on positions 248 and 249.

Total p53 copies in plasma DNA were not associated with lung cancer in contrast to previous studies indicating that higher concentrations of DNA in serum/plasma were associated with disease (18–21, 23, 27, 48, 53). The lack of an association of plasma DNA levels with lung cancer is consistent with a case-control study nested within the European Prospective Investigation of Cancer (EPIC) cohort, where levels of plasma DNA, obtained before lung cancer diagnosis, were not associated with lung cancer (54). Another study observed that although median levels of plasma DNA were higher in cancer patients, plasma DNA levels were not elevated in all cancer patients and that plasma DNA levels had low sensitivity for classification of cancer patients (20). Quantification of p53 copies, using real-time PCR, was used to approximate DNA concentration in our study. It is possible that the plasma DNA concentration differences observed previously in cases compared with healthy individuals may be attributable to repeated DNA sequences. One study observed that repeated DNA sequences are found in serum/plasma DNA at higher levels than coding sequences and suggested that repetitive DNA sequences were preferentially secreted into plasma (55). Therefore, coding DNA levels, such as p53 copies, may be similar in cases and controls. In the EPIC cohort, it was noted that plasma DNA concentrations varied widely from different countries or centers where DNA was isolated (54). A strength of our study is that all participants were from the same geographic area and plasma was isolated by only one center.

The origin of circulating DNA in plasma is unknown at present, but some studies have suggested that it stems in part from tumors, originating from apoptosis, necrosis, or active DNA release from cells (19, 21, 23). One recent study concluded that DNA fragments

found in blood is derived from both apoptotic and necrotic cells (19), whereas another suggested that plasma DNA originates from neoplastic cells that have been engulfed by macrophages (49). In our study, the p53 mutation load was higher in plasma DNA than in nontumorous tissue, suggesting that p53 mutated DNA is enriched in plasma DNA and that perhaps DNA found in plasma is preferentially originating from precancerous or cancer cells. In addition, the p53 mutation load in plasma DNA was not associated with the detection of p53 mutations in lung tumors by DNA sequencing. The detection of mutations in plasma DNA that were not selected in the lung tumors is consistent with our previous demonstration of the detection of different p53 mutations in nontumorous lung tissue than the sequenced mutations in lung tumors (34). Taken together, these results suggest that the p53 mutation in plasma DNA could originate from precancerous cells.

Only one lung tumor was positive for p53 mutation at codon 248 or 249. The matched plasma from this sample was negative for p53 mutations using the mutation load assay. Several reports observed concordance between mutations in serum/plasma DNA and tumors (56–59), whereas other reports noted inconsistency between matched samples (28, 43, 44, 60). The lack of detection of codon 248 p53 mutation in the matched plasma DNA sample could be due to cycling of p53-mutated DNA or presence of p53-mutated DNA only a certain period after exposure. Previously, in several plasma samples from the same individual, obtained longitudinally, p53 mutations were detected in some samples and not others (31), indicating that p53 mutations in plasma DNA may turnover (i.e., p53-mutated fragments are degraded or undetectable at different times). It is also possible that the level of DNA with p53 mutation was below the level of determination of our assay and could be detected if larger plasma volumes were available for analysis. Notably, the original p53 mutation load assay had a higher sensitivity of mutation detection of $(1:10^7)$ due in part to the larger amount of DNA available for assay application (34).

There are several limitations of this study that need to be addressed in future studies. Given that this was a pilot study, the sample size was small for testing differences in the p53 mutation load and survival data was incomplete for some cases. It is possible that differences in the p53 mutation load may be apparent in a larger study sample. Our results show the need for the development of high-throughput assays for the estimation of the p53 mutation load for the application to larger studies. Only 3 to 4.2 mL plasma was available for analysis; larger volumes of plasma would enhance sensitivity of mutation detection. Finally, samples were only available from one time of collection. Because the turnover rate of p53 mutations is unknown, it is possible that some plasma samples were positive for mutations were missed.

In spite of these limitations, by developing a highly sensitive assay, we were able to determine the p53 mutation load from both cases and controls, and observed an association of the p53 mutation load in plasma DNA with smoking. Although the biological role of these mutations is unknown at present, the association of p53 mutation in plasma DNA with smoking described here and the association between aflatoxin B₁ with Ser²⁴⁹ p53 mutations in plasma shown by others (29, 30, 32) suggest that plasma DNA may be an attractive marker for examining carcinogen exposures.

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