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# *p53* and K-*ras* Mutations in Lung Cancers from Former and Never-Smoking Women[1]

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## ABSTRACT

**Somatic *p53* mutations are common in lung cancer. Active cigarette smoking is positively correlated with the total frequency of *p53* mutations and G:C to T:A transversions on the nontranscribed (DNA coding) strand. Mutational hotspots within the *p53* gene, *e.g.*, codon 157, have been identified for tobacco-related lung cancer, whereas these same mutations are found rarely in other cancers. Such data implicate specific *p53* mutations as molecular markers of smoking. Because limited data exist concerning the *p53* mutation frequency and spectra in ex-smokers and nonsmokers, we have analyzed *p53* and K-*ras* mutations in 126 lung cancers from a population-based case-control study of nonsmoking ($n = 117$) or ex-smoking ($n = 9$) women from Missouri with quantitative assessments of exposure to environmental tobacco smoke. Mutations in the *p53* gene were found in lung cancers from lifetime nonsmokers (19%) and ex-smokers (67%; odds ratio, 9.08; 95% confidence interval, 2.06–39.98). All deletions were found in tumors from patients who were either ex-smokers or nonsmokers exposed to passive smoking. The G:C to A:T transitions (11 of 28; 39%) were the most frequent *p53* mutations found and clustered in tumors from lifetime nonsmokers without passive smoke exposure. The incidence of K-*ras* codon 12 or 13 mutations was 11% (14 of 115 analyzed) with no difference between long-term ex-smokers and nonsmokers. These and other results indicate that *p53* mutations occur more commonly in smokers and ex-smokers than in never-smokers. Such comparisons provide additional evidence of genetic damage caused by tobacco smoke during lung carcinogenesis.**

## INTRODUCTION

In the United States, lung cancer accounts for ~28% of the cancer deaths, and smoking is acknowledged as the prominent cause of this disease (1–3). Lung cancer incidence rates for women in the United States continue to climb, whereas for men, a downward trend began several years ago (3). Although the increased risk of lung cancer is less after smoking cessation, it never regains the level of life-long never-smokers, indicating permanent damage in lung tissue from smoking (4). Environmental cigarette smoke from household and workplace exposures also increases the risk of lung cancer in never-smokers (1, 5–9). The number of epidemiological studies published on the effect of passive smoking on the risk of lung cancer has increased from the three by 1982 to 37 by 1997 (reviewed in Ref. 10), and the meta-analysis of these studies indicates an excess risk with a dose-response relationship from both the number of cigarettes and the length of exposure (11). These epidemiological data are consistent with experimental studies on environmental cigarette smoke (12) and with studies indicating that sidestream cigarette smoke contains certain carcinogens at higher concentrations than those formed in the mainstream smoke (1).

Lung carcinogenesis involves a multitude of genetic and epigenetic changes (13–16) including loss of heterozygosity at 3p, 5q, 8p, 9p (*p16/CDKN2*), 9q, 11p, 11q, 13q (*Rb*),[6] 17p (*p53*), and 17q, known and putative sites of tumor suppressor genes (17, 18). The major genetic alterations in lung cancer detected thus far include *p53* gene mutations; inactivation of the Rb pathway including $p16^{INK4}$ and *Rb* mutations; K-*ras* mutations and the disruption of *fragile histidine triad* sequences (recently reviewed in Ref. 15). The *p53* gene on chromosome 17p13.1 codes for the nuclear phosphoprotein p53, a transcription factor involved in the regulation of the cell cycle and apoptosis, and is mutated frequently in human cancers, *e.g.*, >50% of lung cancers carry such a mutation (reviewed in Refs. 19–21). *p53* mutations also have been found in premalignant lesions in lung tissue and indicate an early loss of this tumor suppressor's function in lung carcinogenesis (22–24).

Active cigarette smoking is positively correlated with the total frequency of *p53* mutations and G:C to T:A transversions on the nontranscribed strand of the *p53* gene (for recent reviews on *p53* mutation spectra, see Refs. 25–29). There also are mutational hotspots in the *p53* gene in lung cancer (*e.g.*, codon 157), but these same hotspots are rarely found in other types of cancers, thus suggesting that different mutagens cause different mutations. Perhaps the most convincing evidence linking tobacco carcinogens to direct genetic damage of *p53* coding sequences is the accumulation of BPDE adducts at mutational hotspots within codons 157, 248, and 273 (30) and the induction of *p53* mutations, including codon 248, in human cells exposed to benzo(*a*)pyrene (31). BPDE adducts at these codons also are repaired poorly (32). Among the major hotspots within the *p53* gene, the methylated CpG sites constitute the majority (19, 21, 27, 28). Recently, cytosine methylation at CpG sites was reported to enhance the binding of carcinogens such as benzo(*a*)pyrene to the guanine sites opposite of the 5-methyl-cytosines in the *p53* gene (33, 34).

Although *p53* mutation data are available from >1000 lung cancers, only a small portion of these are *p53* mutations in defined nonsmokers (29). Therefore, we have analyzed *p53* mutations in 117 lung cancers from a population-based case-control study of nonsmoking women from Missouri and nine ex-smokers from the same population (5). *p53* mutations were compared with K-*ras* mutations. IHC

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[6] The abbreviations used are: Rb, retinoblastoma; IHC, immunohistochemistry; GST, glutathione *S*-transferase; ETS, environmental tobacco smoke; BPDE, benzo(*a*)pyrene diolepoxide; SSCP, single-strand conformation polymorphism; WT, wild-type; dNTP, deoxynucleotide triphosphate.

analysis of the p53 protein was carried out as an additional screening method with SSCP, because IHC positivity has been regarded as an indicator of a *p53* mutation. However, it is known that mutation is only one mechanism that increases p53 protein in cells (35). Frequencies of specific mutations in the *p53* gene were compared with the ones in the revised IARC database (29), which is the largest existing *p53* mutation database. Results from epidemiological investigations on the same population have been reported for passive-smoking exposure (5), home radon concentrations (36), dietary practices (37), occupational risk factors (38), and nonneoplastic disease (39). In a separate report (25), we have presented evidence that homozygous deletion of *GSTM1* coding sequences confers a significant risk of lung cancer to lifetime nonsmokers exposed to ETS.

## MATERIALS AND METHODS

**Tumor Series and Study Population.** The study population included 131 Caucasian women from Missouri who were 41–84 years of age at the time of lung cancer diagnosis (Table 1; five cases were excluded from the analysis; see "Reconsideration of Nonamplified Exons"). The study was restricted to Caucasian women because of the low numbers of other ethnic groups and male nonsmokers. Information on active and passive smoking, dietary preferences, inflammatory lung disease, and other risk factors for lung cancer were collected by trained interviewers using a structured questionnaire as described previously (6, 36, 37). Nonsmokers were defined as persons who had neither smoked >100 cigarettes nor used any tobacco products >6 months during their lifetimes (6). Eleven ex-smokers stopped smoking at least 15 years before the study interview, and their smoking histories ranged from 1 to 105 pack-years. Quantitative exposure to ETS, measured in ETS pack-years, was determined by telephone interviews that documented the source (*e.g.,* parent or spouse), intensity, and duration of exposure during childhood and adulthood (5). One ETS pack-year is the exposure, within a confined space such as a room, to ETS produced by an active smoker consuming one pack of 20 cigarettes each day for a year. Passive smoking data were available in 96% (126 of 131) of cases, and for analytic purposes, the population was divided into four groups as follows: 0 pack-years, <21 pack-years, 21–52 pack-years, and >52 pack-years. Measurements of home radon gas concentrations were performed by placing $\alpha$-track detectors in each dwelling that was occupied for at least 1 year by the study subject during the preceding 30 years (36). Home radon measurements were completed in 92% (120 of 131) of these cases, and 83% had <2.5 pCi/l, which is typical for American homes and well below the EPA action level of 4.0 pCi/l. For statistical analyses, the population was divided into quartiles defined by the following breakpoints: 0–0.9, 0.9–1.5, 1.5–2.2, and >2.2 pCi/liter.

**Mutation Analysis of the *p53* Gene.** Complete coding sequences and splice junctions for exons 5–8 were screened for mutations by SSCP analysis (40–43). This newly developed, nonradioactive assay includes analysis at two temperatures, which ensures a high efficiency for mutation detection. DNA was extracted from microdissected, paraffin-embedded tumor sections by a treatment with proteinase K for 3–5 days at 37°C and phenol extraction-ethanol precipitation method. For amplification of p53 exons 5–8, two sets of intronic primers (44), the second set internal to the first one for each exon, were used in two consecutive amplification reactions. One hundred-$\mu$l reactions contained either Dynazyme (Finnzymes, Espoo, Finland) with the recommended buffer or AmpliTaq (Perkin-Elmer, Branchburg, NJ) with a 10× buffer containing 500 mM Tris (pH 9) and 30 mM $MgCl_2$ with 20–30 pmol of each primer and 300 $\mu$M of dNTPs (Pharmacia Biotech). Two control reactions without a template were included in each set of amplifications to rule out contamination. Amplified products were gel-purified on a 4% NuSieve 3:1 agarose gel (FMC Bioproducts, Rockland, ME). Purified samples were analyzed by SSCP using a temperature-controlled electrophoresis system (PhastSystem; Pharmacia Biotech, Piscataway, NJ), PhastGel Homogenous 20% polyacrylamide gels, and native buffer strips. Gels were stained with PhastGel DNA Silver Staining kit (Pharmacia Biotech). Amplified DNA using artificially mutated nested primers for positive controls and confirmed WT lymphocyte DNA for negative controls were included in each SSCP run.

As the second step, all of the repeat and similar positive cases by PCR-SSCP, as well as those showing high positivity by *p53* IHC, were sequenced manually. Direct radioactive dideoxy-sequencing on both the coding and noncoding strands, using the same amplified samples as for SSCP, was done using a Sequenase kit from Amersham. To maximize the labeling, $^{35}$S-labeled dNTP was selected according to the first nucleotide after the primer in each case. After denaturation at 100°C for 3 min, the sequencing reactions were carried out at 42°C. In *p53* mutation analysis, at least two independently amplified DNA samples with identical results were required for any sample to be labeled "mutated."

**Analysis of *p53* Mutation Database.** Lung cancer mutations were graciously provided by Drs. P. Hainaut and G. P. Pfeifer from the recently revised IARC database (29). In this version of the database, the lung cancer mutation entries have been edited to remove duplicate entries and 107 mutations from an anomalous report. In addition, the demographic information and smoking histories have been verified and corrected by manual review of the original publications. Before compiling data shown in Figs. 1 and 2, we excluded mutations from lung cancers associated with occupational exposures (*e.g.,* chromate, asbestos, silicates, and mustard gas) or ionizing radiation (*e.g.,* uranium mining, cancer therapy, and A-bomb survivors).

**Reconsideration of Nonamplified Exons.** There were some samples where, despite repeated amplifications from several tissue sections, one or more exons of *p53* did not amplify. In each case, some exons of *p53* amplified, and we also were able to amplify K-*ras* in these cases, which indicates DNA of sufficient quality for PCR amplifications. Thus, among the 131 cases, 13 miss the mutational data for one exon of *p53,* two cases for two exons, and three cases for three exons. One case with one "missing exon" contained a mutation in another exon. It is possible that in these cases there was a deletion of part of the *p53* gene, a mutation, or a deletion at the site of the PCR primer. Calculations of *p53* mutation frequencies are based on 117 cases of lifetime nonsmokers (104 cases with four exons and 13 cases with three exons of *p53* analyzed) and nine cases of ex-smokers (four exons were analyzed in all cases).

***p53* Immunohistochemistry.** As described previously (45), DO7 monoclonal antibody raised against human WT *p53,* which recognizes both WT and mutated p53 in IHC, was used as the primary antibody in immunohistochemistry. Each case was stained at least twice, and the coded slides were read by three pathologists for a consensus of the extent of positivity.

**Mutation Analysis for K-*ras*.** The sequence area around codons 12 and 13 of K-*ras* was amplified. Amplified strands were gel-purified and sequenced using direct dideoxy-sequencing as described above. PCR amplification was performed in a two-step amplification using external and nested primer sets: external forward (5′-GTACTGGTGGAGTATTTGAT-3′) and external reverse (5′-TGCACCTAGGACTCATGAAAATGGTCAGAG-3′); and nested forward (5′-ACATGTTCTAATATAGTCAC-3′) and nested reverse (5′-CTATTGTTGGATCATATTCG-3′).

The initial PCR amplification was done in a 100-$\mu$l reaction mixture, with 100–300 ng of genomic DNA as template. The nested PCR was done using a 300-$\mu$l reaction mixture. PCR products were generated by using primers at a final concentration of 20 pmol/100 $\mu$l. Amplification was performed with Taq polymerase (2.5 units/100-$\mu$l reaction mixture; Perkin-Elmer, Branchburg, NJ) in a Perkin-Elmer DNA thermal cycler using 35 PCR cycles preceded by a "hot start"

Table 1 *Association of p53 mutation status with patient characteristics*

| | Patients | Mutations | |
|---|---|---|---|
| | *n* | *p53* | K-*ras* |
| Patients | 126[a] | 28 (22%) | 14 (11%) |
| Age range (mean) | 41–84 (71.2) | 66.8 | 75 |
| Smoking history | | | |
| Never-smokers | 117 (93%) | 22 (19%) | 13 (11%) |
| Ex-smokers | 9 (7%)[b] | 6 (67%) | 1 (11%) |
| Histology | | | |
| Adenocarcinoma | 92 (73%) | 23 (25%) | 11 (12%) |
| Broncho-alveolar | 8 (6%) | 0 | 1 (13%) |
| Squamous cell carcinoma | 2 (2%) | 0 | 0 |
| Small cell lung carcinoma | 3 (2%) | 2 (67%) | 0 |
| Other/mixed | 21 (17%) | 3 (14%) | 2 (10%) |

[a] Five of the 131 cases were excluded from the analysis because only two or fewer of the *p53* exons 5–8 could be amplified (see "Reconsideration of Nonamplified Exons" in "Materials and Methods").

[b] Two of the 11 cases were excluded from the analysis because only two or fewer of the *p53* exons 5–8 could not be amplified (see "Reconsideration of Nonamplified Exons" in "Materials and Methods").

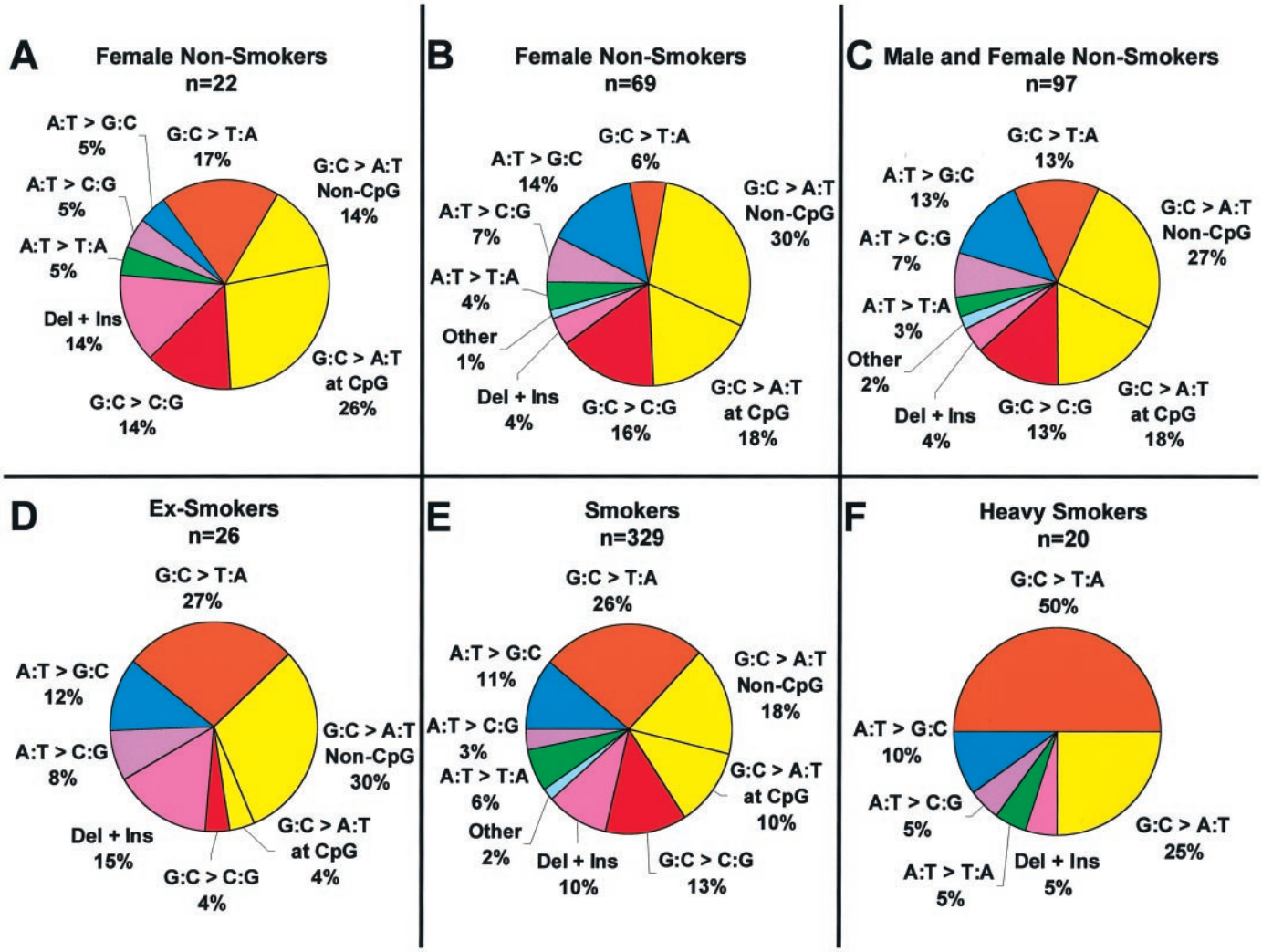


Fig. 1. *p53* mutation spectra in lung cancers from smokers, ex-smokers, and nonsmokers. Because of numerical rounding by Microsoft Excel, percentages in figures may differ from those calculated in the text. *A*, lifetime nonsmokers in this study. *B*, female nonsmokers from the revised IARC database (29). *C*, male and female nonsmokers from the revised IARC database: 69 females, 22 males, and six of unknown gender (29). *D*, ex-smokers; combined data from this study with those in the revised IARC p53 mutation database (29): 12 females, nine males, and five of unknown gender. *E*, smokers from the revised IARC *p53* mutation database (29): 64 females, 186 males, and 79 of unknown gender. *F*, heavy smokers from the revised IARC database (29): three males and 17 of unknown gender.

of 95°C for 5 min. Each PCR cycle consisted of 1 min at 94°C, 1 min at 50°C, and 30 s at 72°C. The PCR buffer consisted of final concentrations of 50 mM potassium chloride, 10 mM Tris-HCl (pH 8.3 at room temperature), 15 mM magnesium chloride, and 7.5 mM ammonium sulfate; each dNTP final concentration was 200 $\mu$mol/liter (800 $\mu$mol/l for all four dNTPs). The final PCR amplified product was 182-bp fragments encompassing nucleotides 42–204 of exon 1 of the K-*ras* gene. The amplified PCR products were electrophoresed in agarose (NuSieve & SeaKem, FMC Corp., Rockland, ME) containing ethidium bromide, run at 150 V for 45 min, and visualized under UV.

Direct sequencing was performed using 5 $\mu$l of the amplified product with $^{35}$S-labeled nucleotide. The sequencing method was similar to the one described for *p53*, except nested primers for K-*ras* were used. Sequences were confirmed by sequencing both strands of the DNA and a positive result by new amplification.

**Statistical Analysis.** Mutational spectra were compared using the Monte Carlo method with the Adams and Skopek algorithm as described by Piegorsch and Bailer (40) using a computer program created by Cariello *et al.* (46).

Maximum likelihood procedures were used in computing odds ratios and 95% confidence intervals. Multivariate logistic regression models were used to adjust for potentially confounding variables: age, *GSTM1* status, vegetable consumption, and domestic radon exposure.

Wald confidence intervals were computed on the bases of the estimated parameter $\beta$, and its SE (47):

$$Exp\ \{\beta_1 \pm 1.96 \times \mathrm{SE}\ (\beta_1)\}$$

## RESULTS

To our knowledge, this is the largest series of *p53* mutations in lung cancers from never-smoking women enrolled in a population-based study of passive smoking. To better understand our results, we have compared them with the revised IARC-hosted database of *p53* mutations in lung cancer (29), which has information of smoking status in 451 cases of the 1346 lung cancers with p53 mutations. The database contains 64 mutations in lung cancers from female smokers and 97 mutations in lung cancers from nonsmokers, of which 69 are female nonsmokers.

***p53* Mutations in Lifetime Nonsmokers.** Twenty-two of the mutations were in lung cancers from 117 lifetime nonsmokers (19%), where three exons (13 cases) or all four exons (104 cases) could be amplified for analysis. The percentage of G:C to A:T mutations of all found mutations was 41% (9 of 22) in lifetime nonsmokers (Fig. 1). Six of 22 (27%) of the mutations in lifetime nonsmokers were G:C to A:T at the CpG sequences. Such mutations are not as common in smokers, especially male smokers (29). Six of 22 (27%) of the *p53* mutations in the lifetime nonsmokers were at the hotspot codons reported in lung cancer, and only one of the hotspot codon mutations was a G→T transversion (Table 2; Fig. 2). Two mutations were found

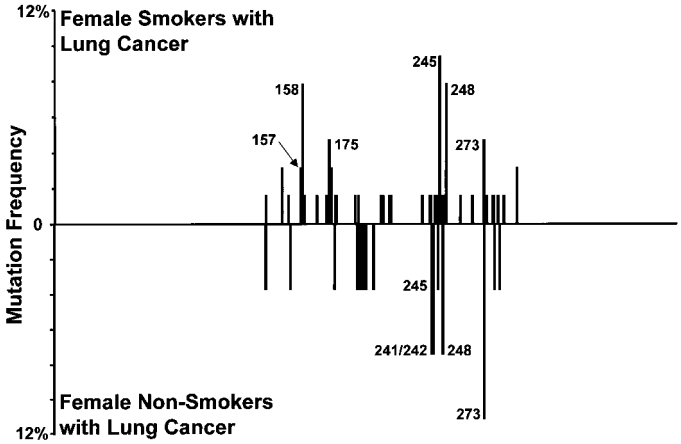


Fig. 2. Distribution of *p53* gene mutations in lung cancers from female nonsmokers ($n = 22$) in this study compared with the reported data from female smokers ($n = 64$) in the revised IARC-based *p53* mutation database (29).

Table 2 *p53 mutations in never-smoker lung cancers*

| ID | *GSTM1* | Passive smoking (pack-years) | *p53* mutations | | |
|---|---|---|---|---|---|
| | | | Exon | Codon | Change |
| R 56 | + | | 5 | 135 | $TGC^{Cys}\rightarrow TTC^{Phe}$ |
| R 53 | + | 0 | 6 | 195 | $ATC^{Ile}\rightarrow AGC^{Ser}$ |
| R 78 | + | 0 | 6 | 198 | $GAA^{Glu}\rightarrow TAA^{STOP}$ |
| R 23 | + | 0 | 7 | 241 | $TCC^{Ser}\rightarrow TTC^{Phe}$ |
| R 82 | − | 0 | 7 | 245 | $GGC^{Gly}\rightarrow GTC^{Val}$ |
| R 116 | NA[a] | 0 | 7 | 248 | $CGG^{Arg}\rightarrow CAG^{Gln}$ |
| R 120 | + | 0 | 7 | 242 | $TGC^{Cys}\rightarrow TAC^{Tyr}$ |
| R 36 | − | 0 | 8 | 273 | $CGT^{Arg}\rightarrow TGT^{Cys}$ |
| R 60 | + | 0 | 8 | 281 | $GAC^{Asp}\rightarrow CAC^{His}$ |
| R 63 | − | 0 | 8 | 273 | $CGT^{Arg}\rightarrow CAT^{His}$ |
| R 76 | + | 0 | 8 | 284 | $ACA^{Thr}\rightarrow ATA^{Ile}$ |
| R 95 | + | <21 | 5 | 179 | $CAT^{His}\rightarrow CTT^{Leu}$ |
| R 115 | + | <21 | 6 | 204 | $GAG^{Glu}\rightarrow TAG^{STOP}$ |
| R 14 | − | 21–52 | 5 | 127–128 | 4-bp deletion |
| R 61 | − | 21–52 | 6 | 193 | $CAT^{His}\rightarrow CGT^{Arg}$ |
| R 128 | − | 21–52 | 8 | 273 | $CGT^{Arg}\rightarrow TGT^{Cys}$ |
| R 32 | NA | >52 | 5 | 151 | 1-bp deletion |
| R 121 | + | >52 | 5 | 161–163 | 7-bp deletion |
| R 30 | − | >52 | 7 | 242 | $TGC^{Cys}\rightarrow TCC^{Ser}$ |
| R 57 | − | >52 | 7 | 248 | $CGG^{Arg}\rightarrow CAG^{Gln}$ |
| R 64 | − | >52 | 7 | 241 | $TCC^{Ser}\rightarrow TGC^{Cys}$ |
| R 127 | + | Unknown | 6 | 196 | $CGA^{Arg}\rightarrow TGA^{STOP}$ |

[a] NA, not applicable.

at codon 241 and again at codon 242. Codon 241 rarely is mutated in lung cancer (seven reported mutations in the revised IARC database; Ref. 29).

***p53* Mutations in Ex-Smokers.** Six p53 mutations were detected in 11 ex-smokers (55%). If the calculation is based on cases with all four exons amplifiable (in one case only one exon, and in one case two exons were amplifiable), the percentage is even higher (6 of 9; 67%). Three of six (50%) *p53* mutations in the ex-smokers were at the hotspot codons in the *p53* gene reported in the literature, and one of these was a G:C to T:A transversion (Table 3). The percentage of hotspot mutations in ex-smokers was higher than the percentage in lung cancers from lifetime nonsmokers in this study (Tables 2 and 3; Fig. 2). The proportion of cases with a *p53* mutation was much higher in lung cancers from ex-smokers than in lung cancers from lifetime nonsmokers (highly statistically significant difference with age adjusted odds ratio of 9.08 and 95% confidence interval of 2.06–39.98). Adjustment for vegetable consumption, *GSTM1* status, or radon exposure did not alter the risk estimate. All four deletions were found in cancers from ex-smokers or those nonsmokers exposed to ETS.

***p53* Immunopositivity and Histology.** Immunopositivity of the p53 protein was detected in 76 of the 131 samples (58%). A statistically positive correlation between IHC positivity and *p53* mutations was not observed. Most of the lung tumors in our study (73%) were adenocarcinomas. Adenocarcinoma is the most common histological subtype in nonsmoking women (48). The percentage of *p53* mutations in adenocarcinomas in our study is 25% (23 of 92).

***p53* Mutations in Relation to Radon Exposure.** Twenty-four of the 28 *p53* mutations were found in lung tumors from the 120 cases with known indoor radon exposure measurements. No statistically significant differences were found among the different radon quartiles (quartile 1, 6 of 30, 20%; quartile 2, 9 of 31, 29%; quartile 3, 4 of 28, 14%; quartile 4, 5 of 28, 18%).

**K-*ras* Mutations.** None of the cancers carried more than one *p53* mutation. However, in five cases, the tumor sample contained a K-*ras* mutation in addition to the *p53* mutation (Table 4). The incidence of K-*ras* codon 12 or 13 mutations among these cases was 11% (14 mutations in 126 successfully analyzed cases) with no difference between long-term ex-smokers (1 of 9; 11%) and lifetime nonsmokers (13 of 117; 11%). Thirteen of the mutations were found at codon 12, and only 1 K-*ras* mutation was found at codon 13 in an ex-smoker (Table 4). Six codon 12 mutations occurred in nonsmokers who had no passive smoking exposure. Only one (7%) of the K-*ras* mutations was a G:C to T:A transversion, which is the most frequent K-*ras* mutation reported in smokers (57%; Ref. 49), but it was found in a tumor sample from a person exposed to passive smoking. The most frequent type of base change was a G:C to A:T transition (7 of 14 mutations).

## DISCUSSION

Our study is consistent with published data indicating a different *p53* mutation spectrum in lung cancers from nonsmokers when compared with lung cancers from smokers (16, 21, 26–28, 50). Differences can be identified in the frequency, type, and distribution of *p53* mutations. A significant finding in this study was the lower frequency of *p53* mutations in lifetime nonsmokers (22 of 117; 19%) when compared with the frequency of ex-smokers (6 of 9; 67%; $P = 0.016$).

Table 3 *p53 mutations in ex-smoker lung cancers*

| ID | *GSTM1* | Smoking | | *p53* mutations | | |
|---|---|---|---|---|---|---|
| | | Ex-smoking (pack-years) | Passive (pack-years) | Exon | Codon | Change |
| R 31 | + | 1.0 | >52 | 7 | 248 | $CGG^{Arg}\rightarrow CAG^{Gln}$ |
| R 52 | + | 1.3 | 21–52 | 6 | 195 | $ATC^{Ile}\rightarrow AGC^{Ser}$ |
| R 5 | + | 2.6 | 21–52 | 7 | 252 | 1-bp deletion |
| R 108 | − | 7.5 | 0 | 7 | 248 | $CGG^{Arg}\rightarrow TGG^{Trp}$ |
| R 8 | − | Unknown | 0 | 5 | 175 | $CGC^{Arg}\rightarrow GGC^{Gly}$ |
| R 42 | − | Unknown | >52 | 8 | 273 | $CGT^{Arg}\rightarrow CTT^{Leu}$ |

Table 4 *K-ras mutations in nonsmoker lung cancers*

| ID | Passive smoking (pack-years) | *p53* mutation | Codon | Change |
|---|---|---|---|---|
| R 1 | 0 | No | 12 | $GGT^{Gly}\rightarrow GCT^{Ala}$ |
| R 12 | 0 | No | 12 | $GGT^{Gly}\rightarrow GGC^{Gly}$ |
| R 26 | 0 | No | 12 | $GGT^{Gly}\rightarrow AGT^{Ser}$ |
| R 36 | 0 | Yes | 12 | $GGT^{Gly}\rightarrow GCT^{Ala}$ |
| R 60 | 0 | Yes | 12 | $GGT^{Gly}\rightarrow GAT^{Asp}$ |
| R 99 | 0 | No | 12 | $GGT^{Gly}\rightarrow GCT^{Ala}$ |
| R 7 | <21 | No | 12 | $GGT^{Gly}\rightarrow AGT^{Ser}$ |
| R 91 | <21 | No | 12 | $GGT^{Gly}\rightarrow GAT^{Asp}$ |
| R 95 | <21 | Yes | 12 | $GGT^{Gly}\rightarrow CGT^{Arg}$ |
| R 113 | <21 | No | 12 | $GGT^{Gly}\rightarrow CGT^{Arg}$ |
| R 33 | 21–52 | No | 12 | $GGT^{Gly}\rightarrow AGT^{Ser}$ |
| R 52 | 21–52[a] | Yes | 13 | $GGC^{Gly}\rightarrow GAC^{Asp}$ |
| R 107 | 21–52 | No | 12 | $GGT^{Gly}\rightarrow GTT^{Val}$ |
| R 121 | >52 | Yes | 12 | $GGT^{Gly}\rightarrow GAT^{Asp}$ |

[a] Ex-smoker.

The *p53* gene mutation frequency that we found in nonsmokers (19%) was similar to previous reports 7 of 35 (20%; Ref. 51); 6 of 23 (26%; Ref. 52); 18 of 69 (26%; Ref. 53); and 9 of 91 (10%; Ref. 54). The frequency of *p53* mutations in ex-smokers in this study (6 of 9; 67%) is similar to that in smokers reported by Greenblatt *et al.* (21) and Hernandez-Boussard and Hainaut (26) but higher than the 29 or 38% found in ex-smokers by Husgafvel-Pursiainen *et al.* (54). More *p53* mutations were found at known lung cancer hotspots in ex-smokers than in lifetime nonsmokers in this study. Because the ex-smokers had stopped smoking >15 years prior to the surgical removal of the lung cancer, our findings indicate the persistence of molecular damage from smoking. This conclusion also is supported by the findings of loss of heterozygosity and microsatellite alterations, which persist in bronchial epithelium years after smoking cessation (12, 55).

In accordance with the revised IARC database information (29), the frequency of G:C to T:A transversions in the *p53* gene of female lifetime nonsmokers reported here is less [4 of 22 (18% of all mutations); 4 of 19 (21%) missense mutations] than in lung cancers from smokers (26% in all smokers and 29% in female smokers; Ref. 29; Fig. 1). Although these mutations have been reported to be more frequent in lung cancers from female smokers (56, 57), such a gender difference was not found in the revised IARC-based *p53* mutation database (Ref. 29; Fig. 1). The number of studies on *p53* mutations in lung cancer from nonsmokers is still small when compared with the number of studies from smokers, but the published studies agree with each other on the key findings. Our current data, along with other studies of nonsmoker lung cancers (51–54, 58–60), are in agreement with the hypothesis that the proportion of G:C to T:A transversions is significantly lower in nonsmokers, in which the majority are G:C to A:T transitions. Huang *et al.* (59) found only one G:C to T:A transversion on the coding strand among the 16 *p53* mutations from nonsmokers, whereas all of the G:C to T:A transversions occurred on the coding strand of *p53* in Takagi *et al.* (53), as well as among our cases. The *p53* mutations among nonsmokers from the revised IARC database (29) include a slightly lower percentage of deletion/insertions than the *p53* mutations from smokers. In this study, *p53* deletions were totally absent in the 11 mutated tumor samples from never-smokers who were without passive smoking exposure. Unfortunately, only a few cases archived in the revised IARC p53 database (29) have information about passive smoking.

The distribution of *p53* gene mutations in our study is different from lung cancers in smokers (Refs. 26 and 29; Fig. 2). Although the total percentage of hotspot mutations in our study (32%) was comparable with that reported for lung cancer in general (33%), several hotspots (codons 157, 158, and 249) remained unchanged in these tumors. Although the total percentage of *p53* mutations at CpG sites did not differ from previous lung cancer reports (26, 27), the percentage in ex-smokers in our study was clearly higher than that in lifetime nonsmokers (Tables 2 and 3). Our findings fit a current model of lung carcinogenesis whereby certain chemical carcinogens, including polycyclic aromatic hydrocarbons, such as BPDE, preferentially bind to specific DNA sequence motifs to generate discrete mutational hotspots in tobacco-associated lung cancers (30). More recent investigations have demonstrated that the hotspots are typically CpG dinucleotides containing 5-methyl cytosine (33, 34, 61).

The *p53* mutation spectra contain some conspicuous differences between nonsmokers and smokers, thus implicating tobacco carcinogens as etiological factors in lung cancer. On the basis of earlier human studies and this one, a *p53* mutation, especially a G:C to T:A transversion, indicates alteration by tobacco carcinogens (29). In experimental models, this mutation is preferentially caused by BPDE, regardless of the gene studied (35). On the other hand, although higher doses of BPDE induce mutations, especially in the G:C bp, the proportion of A:T bp mutations increases when the concentration of BPDE decreases (Ref. 62 and references therein). G:C to A:T at CpG sites are typically spontaneous mutations in animal models, whereas *N*-nitrosamines induce G:C to A:T transitions at non-CpG sites in animals (63–66). Nitrosamines from cigarette smoke or from other environmental sources probably contribute to human lung cancer, because G:C to A:T transitions at non-CpG sites are fairly common in lung cancers from both smokers and nonsmokers (Fig. 1).

A *p53* gene mutation is one of the most frequent genetic events in lung cancer with different prevalences, depending on the tumor histology. Adenocarcinomas are especially common in nonsmokers (48) and also formed a majority among our cases (73%). Our results agree with the reported percentages of *p53* mutations in lung adenocarcinomas, which have been reported to vary from 20% (51, 53) to ~33% (21). Marchetti *et al.* (51) found no difference in *p53* mutation frequency in adenocarcinomas between smokers and nonsmokers.

One hundred and nineteen cases had been analyzed for the *GSTM1* polymorphism in our former study of this population (25), of which, 69 cases were null genotype. Seventeen % of these patients had a *p53* mutation in their tumor sample, whereas 28% of those with *GSTM1* present had a *p53* mutation (Tables 2 and 3). Thus, the *GSTM1* null genotype was associated more with WT *p53* cases than with mutant *p53,* and this study and others (67–70) do not support previous reports indicating the absence of the *GSTM1* gene as a susceptibility factor for *p53* mutations (71, 72).

In smoking-associated adenocarcinoma of the lung, G→T transversions represent the majority (up to 80%) of the K-*ras* mutations (49, 73, 74). In our study, a K-*ras* mutation was found in 11% of the lung cancers, with no difference between lifetime nonsmokers and ex-smokers. Only one of the mutations was a G:C to T:A transversion. Whereas the percentage of *p53* mutations was lower in nonsmokers than ex-smokers, no difference in the rate of K-*ras* mutations was suggested in our tumor series. In the literature, a clear association of K-*ras* mutations with heavy lifetime smoking has been reported (49, 52), and it may be that an induction of a *p53* mutation requires lower cigarette smoke exposure when compared with a smoke-induced K-*ras* mutation. On the other hand, Westra *et al.* (75) found no difference in the frequency of K-*ras* mutations in lung adenocarcinomas from persistent smokers (30%) *versus* former smokers (32%).

In conclusion, *p53* gene mutations are less frequent and the mutation spectra are different in lung cancers of lifetime nonsmokers when compared with smokers. Lung cancers from ex-smokers contain a *p53* mutation spectrum similar to that in smokers, indicating that tobacco carcinogens cause permanent, identifiable damage in lung tissue that can be found decades after smoking cessation. Further studies on molecular markers and the possible long-term effects of past and passive smoking are warranted.

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