TP53 and *KRAS* Mutation Load and Types in Lung Cancers in Relation to Tobacco Smoke: Distinct Patterns in Never, Former, and Current Smokers

Florence Le Calvez,[1] Anush Mukeria,[2] Jay D. Hunt,[1,3] Olaf Kelm,[1] Rayjean J. Hung,[1] Philippe Tanière,[4] Paul Brennan,[1] Paolo Boffetta,[1] David G. Zaridze,[2] and Pierre Hainaut[1]

[1]IARC, Lyon, France; [2]Institute of Carcinogenesis, Cancer Research Center, Moscow, Russia; [3]Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, New Orleans, Louisiana; and [4]Department of Cellular Pathology, The Medical School, University of Birmingham, Egbaston, Birmingham, United Kingdom

Abstract

***TP53* mutations are common in lung cancers of smokers, with high prevalence of G:C-to-T:A transversions generally interpreted as mutagen fingerprints of tobacco smoke. In this study, *TP53* (exons 5-9) and *KRAS* (codon 12) were analyzed in primary lung tumors of never (n = 40), former (n = 27), and current smokers (n = 64; mainly heavy smokers). Expression of p53, cyclooxygenase-2 (Cox-2), and nitrotyrosine (N-Tyr), a marker of protein damage by nitric oxide, were analyzed by immunohistochemistry. *TP53* mutations were detected in 47.5% never, 55.6% former, and 77.4% current smokers. The relative risk for mutation increased with tobacco consumption ($P_{linear\ trend}$ < 0.0001). G:C-to-T:A transversions (P = 0.06, current versus never smokers) and A:T-to-G:C transitions (P = 0.03, former versus never smokers) were consistently associated with smoking. In contrast, G:C-to-A:T transitions were associated with never smoking (P = 0.02). About half of mutations in current smokers fell within a particular domain of p53 protein, suggesting a common structural effect. *KRAS* mutations, detected in 20 of 131 (15.3%) cases, were rare in squamous cell carcinoma compared with adenocarcinoma [relative risk (RR), 0.2; 95% confidence interval (95% CI), 0.07-1] and were more frequent in former smokers than in other categories. No significant differences in Cox-2 expression were found between ever and never smokers. However, high levels of N-Tyr were more common in never than ever smokers (RR, 10; 95% CI, 1.6-50). These results support the notion that lung tumorigenesis proceeds through different molecular mechanisms according to smoking status. In never smokers, accumulation of N-Tyr suggests an etiology involving severe inflammation.** (Cancer Res 2005; 65(12): 5076-83)

Introduction

Lung cancer is the leading cause of cancer death for both women and men worldwide, and about 90% of these cancers are attributable to tobacco smoking (1). Recently, case-control and cohort studies have reported an increased risk of lung cancer in lifetime never smokers exposed to spousal or workplace environmental tobacco smoke (2, 3). Other occupational or environmental exposures may also play a role in this type of cancer (4).

There is evidence that smoking not only affects frequency but also mechanism of lung carcinogenesis. Small cell lung carcinoma (SCLC), a type of cancer with neuroendocrine features, is strongly associated with smoking. Among non–small cell lung carcinomas (NSCLC), squamous cell carcinoma (SCC) is the most common type in smokers, whereas adenocarcinoma is the most prevalent in never smokers (5, 6). Irrespective of histology, common genetic changes in lung cancers include mutations in *TP53*, defects in the $p16^{INK4}$/*RB* pathway, loss of heterozygosity (LOH) at alleles on chromosome 3p encompassing fragile histidine triade gene (*FHIT*), Semaphorin 3B (*SEMA3B*) and *RASSF1A*, aberrant promoter methylation in O^6-methylguanine-DNA methyltransferase (O^6*MGMT*), $p16^{INK4}$, death-associated protein kinase (*DAPK*), tissue inhibitor of metalloproteinase-3 (*TIMP-3*), and *RASSF1A* (7). Mutually exclusive mutations at codon 12 in *KRAS* or in the tyrosine kinase domain of the epidermal growth factor receptor are found in about 30% of adenocarcinomas but are rare in other histologic forms (8–10). *TP53* mutations have been reported more frequent in lung cancers of smokers than never smokers (11). Other alterations regarded as more common in smokers include *KRAS* mutations (codon 12) in adenocarcinomas (12), hypermethylation of $p16^{INK4}$ and *APC* (13), and LOH at 3p and 9p (14).

The diversity of point mutations in *TP53* makes this gene informative to identify tumor- or exposure-specific mutation patterns (11). The IARC *TP53* mutation database (http://www.p53.iarc.fr/index.html) has been developed to identify and compare these mutation patterns (15). This database (R9 update, July 2004) contains 1,648 mutations in primary lung cancers described in over 60 publications, including 738 mutations in patients with information on smoking status (572 in smokers and 166 in subjects described as never smokers). The mutation pattern in smokers shows an excess of transversions at G bases (G to T, 30%), uncommon in never smokers (13%), and in cancers not directly related to tobacco (9%; ref. 16). These transversions often occur at codons 157, 158, 245, 248, 273, experimentally identified as sites of adduct formation by benzo(a)pyrene metabolites, a polycyclic aromatic hydrocarbon (PAH) found in tobacco smoke (17, 18). Mutations at codon 157 are detectable in histologically normal lung tissue adjacent to cancer in smokers as well as in the lungs of smokers without cancer (19). However, studies on mutation patterns in never smokers are limited in statistical power and heterogeneous with respect to ascertainment of past or environmental tobacco exposures. Thus, it has been necessary to combine several studies to detect differences between smokers

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

Requests for reprints: Pierre Hainaut, Molecular Carcinogenesis Group, IARC, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France. Phone: 33-4-72-73-84-62; Fax: 33-4-72-73-83-22; E-mail: hainaut@iarc.fr.

and never smokers. Moreover, factors such as sex, ethnicity, or histologic cancer type may be important confounders (20, 21).

In this study, we have exploited the setting of an epidemiologic study in Russia (22) to analyze *TP53* and *KRAS* mutation patterns in a series of lung cancer patients with well-documented information on exposure to tobacco and other potential risk factors. Tumor specimens were also analyzed by immunohistochemistry for cyclooxygenase 2 (Cox-2) expression (23) and nitrotyrosine (N-Tyr) as markers of tumor inflammation (24, 25). We describe here the differences in mutation patterns in relation with cumulative exposure to direct tobacco smoke.

Materials and Methods

Study population and tumors. The study is based on the setting of a population-based case-control study on lung cancer involving 600 cases and 600 controls from Moscow, Russia. Each participant answered a questionnaire on lifestyle (residence, past health, direct and second-hand exposure to tobacco, diet, alcohol consumption, sexual history, and occupational exposure to selected cancer risk factors; see ref. 22 for detailed description). Informed consents were obtained and the study was approved by relevant Ethical Review Boards. Archived tumor specimens (formalin fixed, paraffin embedded) were available for only a subset of the cases, for which pathology was reviewed by three independent experts. For molecular analyses, we selected cases with confirmed histologic diagnosis of primary lung carcinoma and for whom sufficient material was available for DNA extraction and PCR amplification. In total, 131 lung cancer patients were analyzed, including 40 never smokers (<100 cigarettes smoked in a lifetime), 27 former smokers (smoking cessation for ≥2 years before diagnosis), and 64 current smokers (59 of whom were heavy smokers with a tobacco consumption over 35 pack-years). This represented all never and former smokers with available tumor tissue, as well as a sample of current smokers, selected to be representative of patients with high exposure to direct tobacco smoke.

Mutation analysis. DNA was extracted from selected areas of fresh formalin fixed, paraffin-embedded tumor sections as described elsewhere (26) and was analyzed by denaturing high-performance liquid chromatography (DHPLC) for mutations in exons 5 to 9 of *TP53*, containing over 95% of all mutations described in lung cancers. Briefly, exons 5 to 6, 7, and 8 to 9 were amplified by touchdown PCR as three independent products using primers (see Supplementary Table S4) in a mixture containing 1.5 mmol/L $MgCl_2$ (2 mmol/L for exons 8-9), 200 μmol/L deoxynucleotide triphosphates, 0.4 μmol/L primers, 1.5 units of Platinum Taq DNA polymerase (Life Technologies, Inc., Gaithersburg, MD), 50 mmol/L KCl, and 20 mmol/L Tris-HCl. After denaturation and renaturation, amplicons were injected into a DNASep Column (Transgenomic, Omaha, NE) equilibrated in triethylammonium acetate and DNA was eluted in linear acetonitrile gradients. Throughout the PCR and DHPLC procedures, DNA from cell lines with known mutations in exons 5 to 9 were used as internal standards. For specimens with DHPLC profiles indicating the presence of a mutation, tumor DNA was subjected to a second PCR and bidirectional automated sequencing (ABI PRISM BigDye Terminator v1.1 Cycle sequencing, Applied Biosystems, Foster City, CA). Specimens with matched DHPLC and sequencing results were considered as containing a mutation. Fourteen specimens without mutations in exons 5 to 9 but with positive p53 immunostaining (see below) were further analyzed for mutations in exons 4 and 10 by direct sequencing. *KRAS* mutations at codon 12 were analyzed by mutant-enriched PCR as described elsewhere (27), allowing enrichment of the mutant sequence, and sequenced.

Immunohistochemistry. Deparaffinized tissue sections were labeled using standard protocols with the following antibodies: CM1 (rabbit polyclonal immunoglobulin G anti-human p53, 1/500, Novacastra Laboratories Ltd., Newcastle, United Kingdom), Cox-2 (C-20; goat polyclonal IgG anti-human,1/1000, Santa Cruz Biotechnology, Santa Cruz, CA), and anti-N-Tyr (rabbit polyclonal immunoglobulin G, 1/1,000, Upstate Biotechnology, Lake Placid, NY). Fixed antibodies were detected using biotinylated immunoglobulin G, streptavidine-peroxidase, and diaminobenzidine-based detection (Vector Laboratories, Inc., Burlingame, CA). For Cox-2 and N-Tyr analyses, specimens were scored as "positive" when containing at least 10% of stained cancer cells.

Statistical analysis. Relative risks (RR) and 95% confidence intervals (95% CI) were adjusted for sex, age, and education and calculated by unconditional multivariate logistic regression using the SAS System for Windows (release 8.02), as were pooled t tests for independent samples with equal variances, Satterthwaite t test for samples with unequal variances (when folded F tests, $P < 0.05$), Pearson χ^2 analyses, and Fisher's exact tests.

Results

Clinical and individual characteristics of the patients. A total of 131 patients were included in the study (Table 1). Most never-smoker patients (88%) were women, whereas former and

Table 1. Clinical and individual characteristics of patients and lung tumors studied for *TP53* and *KRAS* mutations

	All subjects (n = 131), n (%)	Never smokers (n = 40), n (%)	Former smokers (n = 27), n (%)	Current smokers (n = 64), n (%)
Gender				
Female	37 (28)	35 (88)	1 (4)	1 (2)
Male	94 (72)	5 (12)	26 (96)	63 (98)
Age at diagnosis (y)				
<50	7 (5)	4 (10)	1 (3)	2 (3)
51-60	47 (36)	11 (28)	8 (30)	29 (45)
61-70	45 (35)	13 (32)	8 (30)	25 (39)
>70	30 (23)	12 (30)	10 (37)	8 (13)
Education				
Basic-middle school	51 (39)	14 (35)	9 (35)	28 (44)
High school-university	79 (61)	26 (65)	17 (65)	36 (56)
Tumor histology				
Adenocarcinoma	47 (36)	24 (60)	8 (30)	15 (23)
SCC	54 (41)	3 (8)	11 (41)	40 (63)
ADC-SCC	16 (12)	5 (12)	4 (15)	7 (11)
SCLC	6 (5)	2 (5)	2 (7)	2 (3)
Others	8 (6)	6 (15)	2 (7)	0 (0)

Table 2. RR of *TP53* and *KRAS* mutation for smoking status, cumulative tobacco consumption, and histology

	TP53 mutated, *n* (%)	*TP53* Wild type, *n* (%)	RR* (95% CI)	*KRAS* mutated, *n* (%)	*KRAS* Wild type, *n* (%)	RR* (95% CI)
Smoking status						
Never smokers (*n* = 40)	19 (47.5)	21 (52.5)	1 (reference)	4 (10)	36 (90)	1 (reference)
Ever smokers (*n* = 91)	63 (70.8)	26 (29.2)	3.2 (0.7-16)	16 (17.6)	75 (82.4)	0.9 (0.1-7.1)
Former smokers (*n* = 27)	15 (55.6)	12 (44.4)	1.7 (0.3-9.2)	8 (29.6)	19 (70.4)	1.6 (0.2-13)
Current smokers† (*n* = 64)	48 (77.4)	14 (22.6)	5.2 (1.0-27)	8 (12.5)	56 (87.5)	0.6 (0.1-4.4)
Total	82 (63.6)	47 (36.4)		20 (15.3)	111 (84.7)	
Tobacco consumption (pack-years)						
0‡ (*n* = 40)	19 (47.5)	21 (52.5)	1 (reference)	4 (10)	36 (90)	1 (reference)
<30 (*n* = 22)	9 (40.9)	13 (59.1)	1.2 (0.2-6.7)	7 (31.8)	15 (68.2)	1.7 (0.2-14)
30-39 (*n* = 16)	11 (73.3)	4 (26.7)	7.6 (1.0-61)	4 (25)	12 (75)	1.3 (0.1-13)
40-49 (*n* = 31)	24 (80)	6 (20)	8.5 (1.3-56)	5 (16.1)	26 (83.9)	0.7 (0.1-6.3)
>50 (*n* = 22)	19 (86.4)	3 (13.6)	13 (1.7-101)	0 (0)	22 (100)	—
Histology						
Adenocarcinoma (*n* = 47)	20 (42.6)	27 (57.4)	1 (reference)	10 (21.3)	37 (78.7)	1 (reference)
SCC (*n* = 54)	40 (76.9)	12 (23.1)	3.0 (1.1- 8.1)	5 (9.2)	49 (90.8)	0.2 (0.07-1.0)
ADC-SCC (*n* = 16)	12 (75)	4 (25)	3.4 (0.9-13)	4 (25)	12 (75)	1.1 (0.2-5.0)
Others (*n* = 14)	10 (71.4)	4 (28.6)	4.3 (1.1-17)	1 (7.1)	13 (92.9)	0.3 (0.03-3.5)

*Multivariate analysis adjusting for (a) sex, age, and education for RR related to smoking status or tobacco consumption and (b) sex, age, education, and smoking status for RR related to histology.

†Two cases were removed for the statistical analysis of *TP53* mutation because no DNA has been amplified.

‡pack-years = number of years of smoking × average number of packs smoked/d.

current smokers were almost exclusively men (96% and 98%, respectively). The predominant tumor histology was adenocarcinoma (60%) in never smokers and SCC in former (41%) and current smokers (63%). Mixed adenosquamous (ADC-SCC) types were observed in 12% of the patients. SCLC were equally distributed among never, former, and current smokers, whereas other types of lung cancers (including poorly differentiated carcinoma) were detected in never and former smokers.

***TP53* and *KRAS* mutations in relation with smoking status and histology.** A total of 129 tumors were successfully analyzed for exons 5 to 9 of *TP53*. Mutations were found in 82 (63.6%), including 19 of 40 never smokers (47.5%), 15 of 27 former smokers (55.6%), and 48 of 64 current smokers (77.4%; Table 2). The RR of having a *TP53* mutation increased with tobacco use (former smokers: RR, 1.7; 95% CI, 0.3-9.2; current smokers: RR, 5.2; 95% CI, 1.0-27), with a linear dose-response (RR, 7.6; 95% CI, 1.0-61 with 30-39 pack-years; RR, 8.5; 95% CI, 1.3-56 with 40-49 pack-years; and RR, 13; 95% CI, 1.7-101 with over 50 pack-years; $P_{\text{linear trend}} < 0.0001$; Table 2). This trend held true when the analysis was limited to current smokers (data not shown). There were, however, differences between histologic types. First, after adjustment for tobacco smoking, *TP53* mutations were more frequent in SCC or other histologic types than in adenocarcinoma (RR for SCC, 3.0; 95% CI, 1.1-8.1; Table 2). Second, smoking did not significantly increase the risk of having a *TP53* mutation in adenocarcinoma (data not shown).

Mutations in *KRAS* (codon 12) were detected in 15% of the subjects (20 of 131) and in only 9.4% of those who had a tobacco consumption over 40 pack-years (5 of 53). *KRAS* mutations were less common in SCC (5 of 54) than in adenocarcinoma (10 of 47; RR: 0.2; 95% CI, 0.07-1.0; Table 2). Of 82 patients with *TP53* mutation, 74 (90.2%) were wild type for *KRAS*; of 20 patients with a *KRAS* mutation, 11 (55%) were wild type for *TP53*. Thus, patients with *TP53* mutations were less likely to have *KRAS* mutations (RR, 0.3; 95% CI: 0.1-0.7). Furthermore, patients with both *KRAS* and *TP53* mutations (9 of 130; see Supplementary Table 8) were diagnosed at an earlier age (median, 58) than patients with either one of the mutations (85 of 130; median, 63; $P = 0.02$, Wilcoxon Mann-Whitney test). Of note, adenocarcinoma of former smokers showed a higher prevalence of *KRAS* mutations (62.5 %, 5 of 8), and a lower prevalence of *TP53* mutations (12.5%, 1 of 8), than adenocarcinoma of never smokers [9.1% (2 of 24) and 45.8% (11 of 24), respectively]. All five *KRAS* mutations were detected in adenocarcinoma of distant-former smokers (cessation of smoking at least 10 years before diagnosis). This group was also more likely to develop adenocarcinoma (87.5%, 7 of 8) than recent-former smokers (cessation between 2 and 9 years before diagnosis). However, these differences were not significant due to small numbers (data not shown; details available in Supplementary Table S8).

Mutation patterns. A total of 91 *TP53* mutations were found in 82 patients, including seven patients with two mutations and one patient with three distinct mutations (Fig. 1; details of the mutations in Supplementary Tables S5, S6, and S7). Missense mutations were more prevalent in current (72.5%) than in never or former smokers (47.6% and 47.3%, respectively). Overall, 10 mutations (11%) were silent base changes or located within introns (other than splice junctions), all of them in never or former smokers. In current smokers, several mutations were detected at codons described as "hotspots" for transversions (codon 157: one mutation, G:C to T:A; codon 158: two mutations, G:C to T:A and G:C to C:G; codon 248: three mutations, one G:C to T:A and two G:C to A:T; codon 273: one mutation, G:C to T:A; refs. 17, 18). Other codons with several mutations in current smokers were codons 205, 215, 220, 236, and 242: 3, 2, 4, 2 and 2 mutations, respectively.

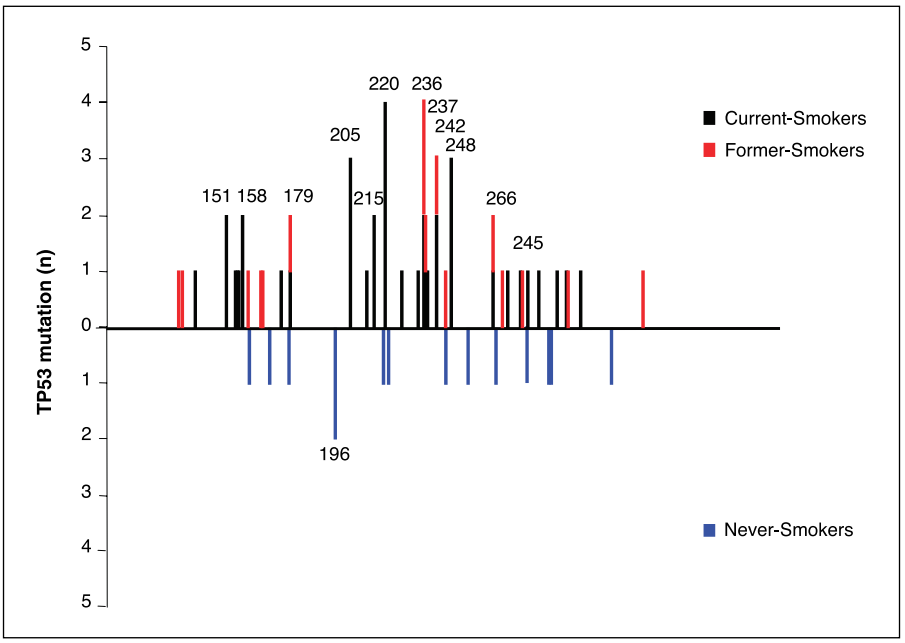


Figure 1. Codon distribution of *TP53* point mutations (exons 5-9) in lung cancer from ever smokers (current in *black*, former in *red*) and never smokers (*blue*).

In former smokers, six mutations were detected at codons also mutated in current smokers [codons 179, 236 (two mutations), 237, 242, and 266]. However, in never smokers, none of the 13 codons listed above was found mutated. Of the 32 missense mutations in SCC of current smokers, 16 fell within β strands and flanking loops that delineate a structural domain at the outer surface of the protein (residues 151, 155-158, and 205, 212, 215, 220; Fig. 2), which were rarely mutated in cancers of former (0 of 9) or never smokers (2 of 10).

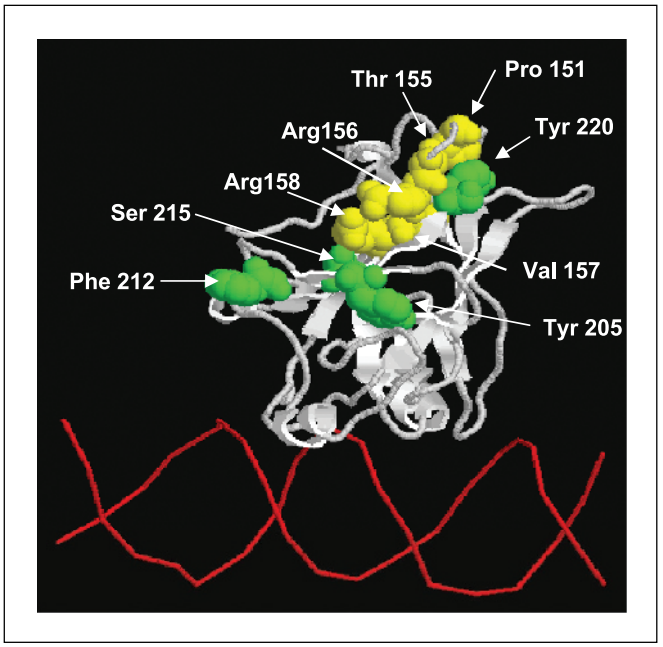


Figure 2. Predicted effect of most frequent *TP53* mutations on the tertiary structure of p53 protein in contact with DNA in SCC of current-smoker patients. Excess of missense mutations (16 of 32) into β strand and flanking loops that delineate a structural domain at the outer surface of the protein, including residues 151, 155 to 158 (*yellow*), and 205, 212, 215, and 220 (*green*).

Patterns of mutations (Fig. 3*A*) show a predominance of transversions in current smokers (64.3%) and of transitions in former (73.7%) and never smokers (64.7%). Only three types of mutations showed consistent differences in relation with tobacco smoke: G:C-to-T:A transversions, A:T-to-G:C transitions (both elevated in ever versus never smokers), and G:C-to-A:T transitions at non-CpG sites (decreased in current versus never smokers). In particular, G:C-to-T:A transversions were common in current smokers (14 of 51, 27.5%), less frequent in former smokers (3 of 19, 15.8%), and rare in never smokers (1 of 21, 4.8%; *P* for difference between never and current smokers = 0.06; Fig. 3*A*). Figure 3*B* shows the variations in prevalence for these three mutation types in relation with cumulative tobacco consumption. Both G:C-to-T:A transversions and A:T-to-G:C transitions increased even at lower levels of tobacco consumption, whereas G:C-to-A:T transitions decreased in a progressive manner with cumulative exposure to tobacco. Figure 3*C* shows the distribution of nucleotide changes in the nontranscribed strand (NTS) for the three mutation types. In ever smokers, there was a strong bias towards G-to-T and A-to-G mutations on the NTS [76.5% (13 of 17) and 71.5% (10 of 14), respectively], suggesting that promutagenic lesions occurring at G or A bases on the transcribed strand may be preferentially repaired. This asymmetry was not observed for G:C-to-A:T transitions, either in ever or in never smokers. The pattern of mutation of *KRAS* at codon 12 showed both transitions (GGT to GAT or AGT) and transversions (GGT to TGT, GTT, or GCT; see Supplementary Table 8). The latter were exclusively found in former and current smokers, although this association was not significant due to small numbers.

Expression of p53 and Cox-2 and detection of nitrotyrosine. Immunohistochemical detection of p53 was informative for 126 tumors. There was a strong association between missense *TP53* mutation and positive staining (RR, 56.5; 95% CI, 15.9-20.1). Overall, 93% (50 of 54) of tumors with *TP53* missense mutation showed p53 protein accumulation. Of cases with mutations other than missense, 80% did not show protein accumulation.

Among 130 informative tumors, 81 expressed Cox-2 protein in at least 10% of cancer cells (62.3%). There was a nonsignificant association between Cox-2 expression and tobacco use (RR, 1.7; 95% CI, 0.4-8.7, ever versus never smokers; Table 3). In particular, of the 20 tumors that expressed Cox-2 in over 50% of the cells, only three were from never smokers. Cox-2 expression was correlated with histology, being less expressed in SCC than adenocarcinoma (RR, 0.3; 95% CI, 0.1-0.9). The presence of N-Tyr as a marker of protein damage by endogenously produced nitrogen species was detected in 30 of 128 informative cases (23.8%) and was significantly associated with never-smoker status. Of 39 tumors of never smokers, 20 (51.3%) were positive for N-Tyr compared with 10 of 89 (11.2%) in ever smokers (RR, 0.1; 95% CI, 0.02-0.6). Of the 17 tumors containing N-Tyr in over 20% of the cells, none was from current smokers. N-Tyr staining was less common in SCC (2 of 53, 3.8%) than adenocarcinoma (16 of 46, 34.8%; RR, 0.1; 95% CI, 0.03-0.8). Thus, accumulation of N-Tyr seems to preferentially occur in tumors of never smokers.

Discussion

TP53 mutation patterns in lung cancers have been the focus of controversy, partially fuelled by the tobacco industry (28–31) but also justified by imprecise assessment of exposure to tobacco in many studies and conflicting interpretation of the effect of potential confounders such as sex and tumor histology. We have taken advantage of the setting of a case-control study to analyze mutation patterns in patients with well characterized smoking status. In this case-control study, estimates of the cumulative risk of lung cancer in smokers were similar to those reported in other, good quality studies.[5] Our results provide conclusive evidence of tobacco-related differences in *TP53* mutation patterns in lung cancers of ever and never smokers. First, *TP53* mutations were strongly associated with tobacco consumption; second, two types of *TP53* mutations were predominant in smokers, G:C-to-T:A transversions and A:T-to-G:C transitions, often at positions identified as experimental sites of adduct formation by metabolites of PAH; third, in SCC of smokers, half of *TP53* missense mutations fell within protein domain rarely mutated in other cancers, suggesting selection of mutants with particular functions; fourth, *KRAS* mutations were predominantly observed in adenocarcinoma of distant-former smokers (smoking cessation $\geq$10 years before diagnosis); fifth, frequent detection of N-Tyr in lung cancers of never smokers suggests a pathogenic role for severe inflammatory stress in these patients.

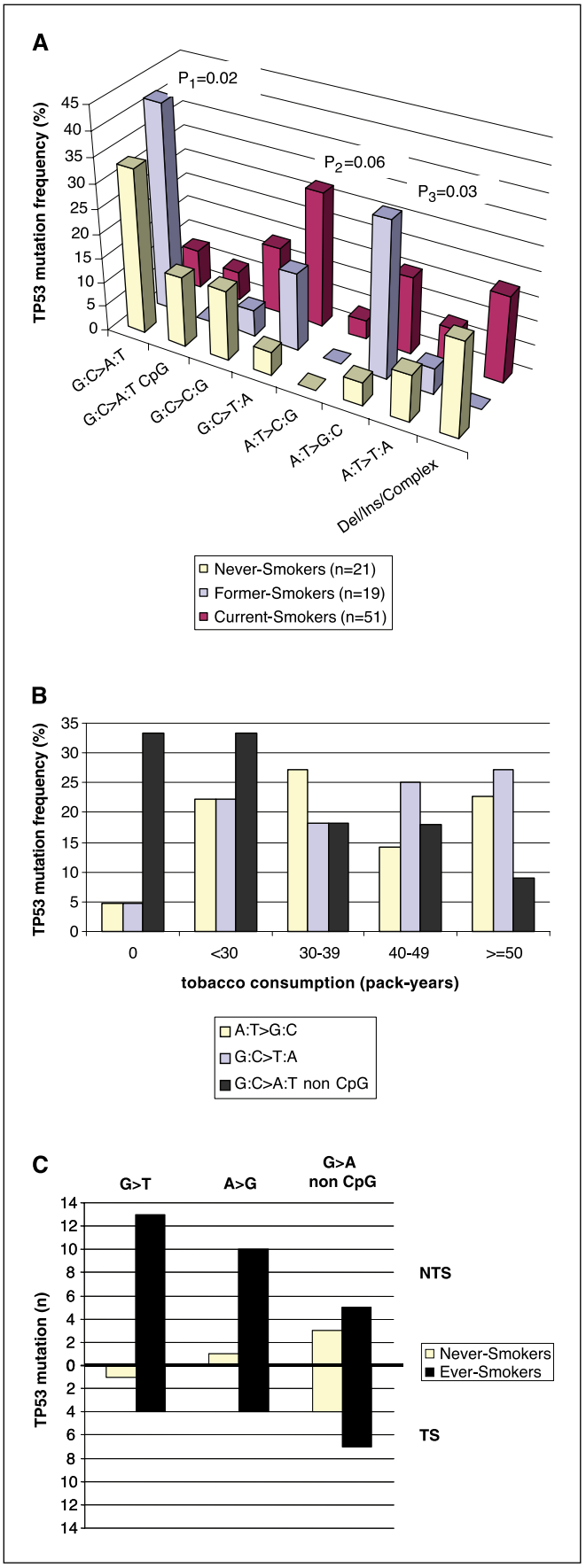
Mutagen fingerprints of tobacco smoke. The risk of *TP53* mutations in lung cancer was strongly associated with heavy tobacco consumption, extending previous reports of a dose-response between cigarette consumption and *TP53* mutations in a smaller series of lung cancer patients (32). The higher prevalence of *TP53* mutation in smokers may reflect increased selection pressure on *TP53* in tissues exposed to smoke. However, if selection was the only factor, mutation patterns should be identical irrespective of smoking status. In contrast, smoking was associated with increased prevalence of G:C-to-T:A transversions and A:T-to-G:C transitions. Both mutation types showed DNA strand bias, with over 70% of G-to-T or A-to-G changes on NTS, consistent with a role for transcription-coupled excision repair in the removal of bulky lesions on transcribed strand (33). The prevalence of G:C-to-T:A transversions observed here was similar to the one in the IARC TP53 mutation database for smokers (27.5% and 30%, respectively) but was much lower for never smokers (4% and 12%, respectively; ref. 28). The higher prevalence in the database possibly reflects misclassification of former smokers as never smokers. The tobacco-related difference in A:T-to-G:C transitions has not been documented before, although strand bias has been noted in database analysis (34). Together, these observations are strong indicators of mutagenesis by tobacco smoke compounds through the formation of bulky adducts.

Studies by Pfeifer et al. have shown that in smokers, G:C-to-T:A transversions often occur at sites of *in vitro* adduct formation by bay region diol epoxides of PAH ("PAH hotspot" codons 157, 158, 237, 245, 248, and 273; ref. 18). This sequence specificity is explained by enhanced adduct formation at guanines adjacent to 5-methylcytosines within CpG repeats present at all these codons except 237 (35). In the present study, one third of G:C-to-T:A transversions in smokers fall at "PAH hotspot" codons. As for A:T-to-G:C transitions, 5 of the 14 mutations in ever smokers occurred at sites of formation of adenine adducts by PAH *in vitro* (codons 236 and 246; ref. 18), whereas five other were detected in exon 6 (codons 220 and 227), in which binding of PAH metabolites was not evaluated. Thus, an important fraction of mutations in smokers bear "signatures" of mutagenesis by PAH metabolites, confirming previous analyses using the IARC *TP53* database. However, smoke compounds other than PAH may also contribute to either G:C-to-T:A or A:T-to-G:C transitions. For example G:C-to-T:A transversions may result from free radical attack generating 8-oxodeoxyguanosine (36), pyridyloxobutyl adducts formed by metabolites of *N*-nitrosamines such as nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) or *N*′-nitrosonornicotine (37), or exocyclic, 3, N^4-ethenodeoxycytidine adducts formed by reactive metabolites of carcinogens or products of lipid peroxidation (38). In the case of A:T-to-G:C transitions, possible mechanisms include formation of 1,N^6-ethenodeoxyadenine that preferentially induces A-to-G transitions in mammalian cells *in vitro* (39).

Histologic types of cancer in relation with exposure to tobacco. In this study, SCC represented 63% of cancers in current smokers, 41% in former smokers, and 8% in never smokers, and adenocarcinoma was the more frequent cancer in never smokers (60%). This distribution reflects the strong association of heavy smoking with SCC, which showed a higher risk to contain *TP53* mutation than adenocarcinoma (RR, 3; 95% CI, 1.1-8.1). Nevertheless, the association between smoking and G:C to T:A was observed even when considering adenocarcinoma separately (8 % of G:C-to-T:A transversions in never smokers versus 34% in ever smokers). Thus, a molecular signature of mutagenesis by smoke compounds is present in the two main types of NSCLC. SCLC were poorly represented in the present series (6 of 131, 5%), precluding further analysis on mutation patterns in this cancer type.

Patterns of mutations in *KRAS* also showed differences in relation with tobacco smoke. Of a total of 20 mutations, 14 were transitions and 6 transversions. All transversions (including five G:C to T:A) were found in ever smokers. *KRAS* mutations were significantly more common in adenocarcinoma (21.3%) than SCC (9.2%) and more likely to occur in tumors of distant-former smokers (7 of 18, 38%) than in tumors of never (4 of 40,10%), recent-former (1 of 9, 11%), or current smokers (8 of 64, 12.5%). Irrespective of histologic type, tumors with *KRAS* mutations were more likely to have wild-type *TP53* and vice versa, in particular in distant-former smokers (five cases with wild-type *TP53* and two cases with silent *TP53*

[5] P. Brennan et al., submitted for publication.



mutations). In contrast, in recent-former and current smokers, half (5 of 9) of tumors with *KRAS* mutation also carried *TP53* mutation. These results suggest that lung tumorigenesis occurs through different pathways in current and in distant-former smokers, with a predominance of *TP53* mutations in current and recent-former smokers, and mutations of *KRAS* and retention of wild-type *TP53* in adenocarcinoma of distant-former smokers. Thus, smoke-induced mutation of *KRAS* may favor the survival of cells that retain a high risk of progression towards malignancy even many years after smoking cessation.

Despite similar mutation types, *TP53* mutations occurred at different codons in SCC and adenocarcinoma. In SCC of current smokers, 50% (16 of 32) of missense mutations were at codons encoding residues in β strands S4 (codons 156, 157, and 158), S6 (codon 205), S7 (codon 215), and in flanking loops (codons 151, 155, and 220; ref. 40). These mutations were not found in adenocarcinoma and represented only 7.2% of all mutations in cancers other than lung (IARC *TP53* mutation database). Several of these residues form contacts between them (e.g., Tyr^{220} with Pro^{151} and Thr^{155}; Ser^{215} with Arg^{158}), stabilizing a protein domain made of short loops exposed at the surface opposite to DNA-binding surface. Functional assays in yeasts indicate that these mutants have lost transactivation properties due to disruption of wild-type p53 conformation (41–43). With one exception (codon 156, arginine to proline), these mutants accumulated in a large proportion of cancer cells. These observations suggest that smoke exerts combined effects that shape *TP53* mutation patterns in SCC. First, smoke compounds induce mutations in a sequence-specific manner. Second, some of the resulting mutants specifically contribute to progression of the metaplasia-dysplasia-carcinoma sequence leading to invasive SCC under continuous exposure to tobacco smoke. This hypothesis implies that common mutants in SCC of smokers may have "gain-of-function" effects through mechanism(s) that remain to be identified.

Mutation patterns in never smokers. We found a higher prevalence of *TP53* mutations in lifetime never smokers (19 of 40, 47%) than in previous reports of similar scope [Husgafvel-Pursiainen et al. (11): 9 of 91, 10%; Vahakangas et al. (44): 22 of 117, 19%; Gealy et al. (45): 6 of 23, 26%; Takagi et al. (46): 18 of 69, 26%; Marchetti et al. (47): 7 of 35, 20%]. This difference may reflect some intrinsic differences between populations under study. The patients in this study were all residents from Moscow and were representative of a population highly exposed to occupational and environmental lung mutagens. The association between *TP53* mutations and environmental and occupational exposures is addressed in a companion, detailed analysis.[6]

Patterns of *TP53* mutations in never smokers showed a high proportion of G:C-to-A:T transitions (10 of 21, 47.6%), especially at

Figure 3. *A, TP53* mutations pattern in tumors from never, former, and current smokers. Ninety-one mutations are represented in 82 patients, including seven patients with two distinct mutations and one patient with three distinct mutations. For some patients, several paraffin-embedded tissues were available. Thus, multiple mutations found per patient were detected either in the same tumor sample or in different tumor samples with various pathologies. Nonadjusted *P*: P_1, G:C > A:T (non-CpG sites) transitions in current smokers compared with never smokers; P_2, G:C > T:A transversions in current smokers compared with never smokers; P_3, A:T > G:C transitions in former smokers compared with never smokers. *B,* proportion of three mutation types, G:C > T:A, A:T > G:C, and G:C > A:T at non-CpG sites, in relation with cumulative exposure to tobacco (in pack-years). *C,* distribution of nucleotide changes G > T, A > G, and G > A at non-CpG sites in the NTS and transcribed strand.

Table 3. RR of Cox-2 and N-Tyr for smoking status, histology, and *TP53* mutation status

	Cox-2$^-$, *n* (%)	Cox2$^+$, *n* (%)	RR* (95% CI)	N-Tyr$^-$, *n* (%)	N-Tyr$^+$, *n* (%)	RR* (95% CI)
Smoking status						
Never smokers (*n* = 40)	17 (43.6)	22 (56.4)	1 (reference)	19 (48.7)	20 (51.3)	1 (reference)
Ever smokers (*n* = 91)	32 (35.2)	59 (64.8)	1.7 (0.4-8.7)	79 (88.8)	10 (11.2)	0.1 (0.02-0.6)
Former smokers (*n* = 27)	12 (44.4)	15 (55.6)	1.3 (0.3-7.3)	22 (84.6)	4 (15.4)	0.2 (0.02-1.2)
Current smokers (*n* = 64)	20 (31.3)	44 (68.8)	2.1 (0.4-11)	57 (90.5)	6 (9.5)	0.08 (0.01-0.5)
Histology						
Adenocarcinoma (*n* = 47)	12 (25.5)	35 (74.5)	1 (reference)	30 (65.2)	16 (34.8)	1 (reference)
SCC (*n* = 54)	23 (42.6)	31 (57.4)	0.3 (0.1-0.9)	51 (96.2)	2 (3.8)	0.1 (0.03-0.8)
TP53 mutation status						
TP53 wild type (*n* = 47)	16 (34.0)	31 (66.0)	1 (reference)	30 (66.7)	15 (33.3)	1 (reference)
TP53 mutated (*n* = 82)	32 (39.5)	49 (60.5)	0.7 (0.3-1.6)	66 (81.5)	15 (18.5)	0.8 (0.3-2.0)

*Multivariable analysis adjusted for age, sex and education (additional adjustment for smoking status in the analysis of histology and *TP53* mutation).

non CpG sites (7 of 21, 33%). This was also the case in former smokers (8 of 19, 42.1%), in particular in those with low tobacco consumption (Fig. 3*A* and *B*). In contrast, only two mutations at "PAH hotspots" were found in never smokers (one G:C-to-T:A transversion and one A:T-to-G:C transition, both in the same patient with a history of moderate exposure to secondary smoke). Thus, the pattern of *TP53* mutations in never smokers was different from the one of current smokers. A high proportion of G:C-to-A:T transitions was also observed in never smokers in the IARC *TP53* database (47%) as well in studies by Vahakangas et al. (44), Gealy et al. (45), and Husgafvel-Pursiainen et al. (11). However, at least half of these transitions were at CpG sites in contrast with non-CpG sites in the present study. G:C to A:T may occur through formation of promutagenic O^6-aklyl adducts induced by tobacco-specific nitrosamines such as NNK, present at significant levels in second-hand tobacco smoke (48). There is experimental evidence that presence of endogenous 5-methylcytosine in *TP53* protects neighboring guanine from O^6-alkylation by NNK (49), explaining the preferential occurrence of these adducts at non-CpG sites. G:C-to-A:T transitions at non-CpG sites in never or former smokers might thus represent a DNA fingerprint for NNK in subjects exposed to secondary smoke.

We found a significant association between never-smoking status and accumulation of N-Tyr in tumor cells. N-Tyr is a stable product of nitration of tyrosine residues and is a biomarker of protein damage by peroxinitrite and other reactive nitrogen species (50). Increased levels of N-Tyr have been reported in plasma proteins of smokers as a marker of ongoing damage by nitrogen species from tobacco (24). In addition, intracellular accumulation of N-Tyr is common in severe forms of inflammatory airway diseases such as chronic obstructive pulmonary disease and asthma and may result from enhanced expression of the NO-generating nitric oxide synthase NOS_2 (51, 52). In inflammatory colorectal cancers, there is a correlation between NOS_2 expression and prevalence of G:C-to-A:T transitions at CpG sites in *TP53*, a type of mutation that occurs through a process enhanced by nitrogen radicals (53). However, we did not observe any correlation between N-Tyr in cancer and a particular *TP53* mutation type. This suggests that in the respiratory airway, severe inflammation contributes to cancer by other mechanisms than *TP53* mutation. Inflammatory airway diseases have multiple endogenous or exogenous causes, such as exposure to inorganic fibers or to particulate materials. It remains to be determined if N-Tyr accumulation in tumors of certain never smokers may correlate with exposure to specific environmental risk factors.

[6] Hunt et al., submitted for publication.

Acknowledgments

Received 2/17/2005; revised 3/24/2005; accepted 4/6/2005.

Grant support: European Commission INCO-Copernicus programme IC15-CT96-0313, IARC Special Training Awards (F. Le Calvez, O. Kelm, and R. Hung), and National Institute of Environmental Health Sciences tenure of a Research Scientist Development Award K01 ES00358 (J.D. Hunt).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Ghyslaine Martel-Planche, Catherine Boillot, Nicole Lyandrat, Sandra Tierrie, and Christine Carreira for excellent technical help; Joelle Fevotte and Andrea 't Mannetje for the coordination of the occupational exposure assessment; industrial hygienists Shterengorz Rafail and Ivanov Nikolay for their expert assessment; and Noemie Travier and Gilles Ferro for data management.

References

1. IARC. IARC Monographs on the evaluation of the carcinogenic risk of chemicals to humans. Tobacco smoke and involuntary smoking. Vol. 83. Lyon (France): IARC. 2004.
2. Dockery DW, Trichopoulos D. Risk of lung cancer from environmental exposures to tobacco smoke. Cancer Causes Control 1997;8:333–45.
3. Boffetta P, Agudo A, Ahrens W, et al. Multicenter case-control study of exposure to environmental tobacco smoke and lung cancer in Europe. J Natl Cancer Inst 1998;90:1440–50.
4. Boffetta P, Nyberg F. Contribution of environmental factors to cancer risk. Br Med Bull 2003;68:71–94.
5. Jedrychowski W, Becher H, Wahrendorf J, Basa-Cierpialek Z, Gomola K. Effect of tobacco smoking on various histological types of lung cancer. J Cancer Res Clin Oncol 1992;118:276–82.
6. Koo LC, Ho JH. Worldwide epidemiological patterns of lung cancer in nonsmokers. Int J Epidemiol 1990;19 Suppl 1:S14–23.
7. Yokota J, Kohno T. Molecular footprints of human lung cancer progression. Cancer Sci 2004;95:197–204.
8. Cooper CA, Carby FA, Bubb VJ, Lamb D, Kerr KM, Wyllie AH. The pattern of K-*ras* mutation in pulmonary

adenocarcinoma defines a new pathway of tumour development in the human lung. J Pathol 1997;181: 401–4.
9. Shigematsu H, Lin L, Takahashi T, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. J Natl Cancer Inst 2005;97:339–46.
10. Rodenhuis S, Slebos RJ. Clinical significance of ras oncogene activation in human lung cancer. Cancer Res 1992;52:2665–9S.
11. Husgafvel-Pursiainen K, Boffetta P, Kannio A, et al. p53 mutations and exposure to environmental tobacco smoke in a multicenter study on lung cancer. Cancer Res 2000;60:2906–11.
12. Husgafvel-Pursiainen K, Hackman P, Ridanpaa M, et al. K-*ras* mutations in human adenocarcinoma of the lung: association with smoking and occupational exposure to asbestos. Int J Cancer 1993;53: 250–6.
13. Toyooka S, Maruyama R, Toyooka KO, et al. Smoke exposure, histologic type and geography-related differences in the methylation profiles of non-small cell lung cancer. Int J Cancer 2003;103:153–60.
14. Wistuba II, Lam S, Behrens C, et al. Molecular damage in the bronchial epithelium of current and former smokers. J Natl Cancer Inst 1997;89: 1366–73.
15. Olivier M, Eeles R, Hollstein M, Khan MA, Harris CC, Hainaut P. The IARC TP53 database: new online mutation analysis and recommendations to users. Hum Mutat 2002;19:607–14.
16. Pfeifer GP, Denissenko MF, Olivier M, Tretyakova N, Hecht SS, Hainaut P. Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers. Oncogene 2002;21:7435–51.
17. Denissenko MF, Pao A, Tang M, Pfeifer GP. Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in P53. Science 1996; 274:430–2.
18. Smith LE, Denissenko MF, Bennett WP, et al. Targeting of lung cancer mutational hotspots by polycyclic aromatic hydrocarbons. J Natl Cancer Inst 2000;92:803–11.
19. Hussain SP, Amstad P, Raja K, et al. Mutability of p53 hotspot codons to benzo(a)pyrene diol epoxide (BPDE) and the frequency of p53 mutations in nontumorous human lung. Cancer Res 2001;61:6350–5.
20. Guinee DG Jr, Travis WD, Trivers GE, et al. Gender comparisons in human lung cancer: analysis of p53 mutations, anti-p53 serum antibodies and C-erbB-2 expression. Carcinogenesis 1995;16:993–1002.
21. Toyooka S, Tsuda T, Gazdar AF. The TP53 gene, tobacco exposure, and lung cancer. Hum Mutat 2003; 21:229–39.
22. Mannetje A, Fevotte J, Fletcher T, et al. Assessing exposure misclassification by expert assessment in multicenter occupational studies. Epidemiology 2003; 14:585–92.
23. Castelao JE, Bart RD III, DiPerna CA, Sievers EM, Bremner RM. Lung cancer and cyclooxygenase-2. Ann Thorac Surg 2003;76:1327–35.
24. Pignatelli B, Li CQ, Boffetta P, et al. Nitrated and oxidized plasma proteins in smokers and lung cancer patients. Cancer Res 2001;61:778–84.
25. Ohshima H, Friesen M, Brouet I, Bartsch H. Nitrotyrosine as a new marker for endogenous nitrosation and nitration of proteins. Food Chem Toxicol 1990;28: 647–52.
26. Taniere P, Martel-Planche G, Maurici D, et al. Molecular and clinical differences between adenocarcinomas of the esophagus and of the gastric cardia. Am J Pathol 2001;158:33–40.
27. Anker P, Lefort F, Vasioukhin V, et al. K-*ras* mutations are found in DNA extracted from the plasma of patients with colorectal cancer. Gastroenterology 1997;112: 1114–20.
28. Pfeifer GP, Denissenko MF, Olivier M, Tretyakova N, Hecht SS, Hainaut P. Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers. Oncogene 2002;21:7435–51.
29. Paschke T. Analysis of different versions of the IARC p53 database with respect to G- ->T transversion mutation frequencies and mutation hotspots in lung cancer of smokers and non-smokers. Mutagenesis 2000;15:457–8.
30. Rodin SN, Rodin AS. Human lung cancer and p53: the interplay between mutagenesis and selection. Proc Natl Acad Sci U S A 2000;97:12244–9.
31. Rodin SN, Rodin AS. On the origin of p53 G:C - -> T:A transversions in lung cancers. Mutat Res 2002;508: 1–19.
32. Kondo K, Tsuzuki H, Sasa M, Sumitomo M, Uyama T, Monden Y. A dose-response relationship between the frequency of p53 mutations and tobacco consumption in lung cancer patients. J Surg Oncol 1996;61:20–6.
33. Chen RH, Maher VM, Brouwer J, van de PP, McCormick JJ. Preferential repair and strand-specific repair of benzoapyrene diol epoxide adducts in the HPRT gene of diploid human fibroblasts. Proc Natl Acad Sci U S A 1992;89:5413–7.
34. Hainaut P, Pfeifer GP. Patterns of p53 G- ->T transversions in lung cancers reflect the primary mutagenic signature of DNA-damage by tobacco smoke. Carcinogenesis 2001;22:367–74.
35. Yoon JH, Smith LE, Feng Z, Tang M, Lee CS, Pfeifer GP. Methylated CpG dinucleotides are the preferential targets for G-to-T transversion mutations induced by benzo[a]pyrene diol epoxide in mammalian cells: similarities with the p53 mutation spectrum in smoking-associated lung cancers. Cancer Res 2001;61: 7110–7.
36. Henderson PT, Delaney JC, Gu F, Tannenbaum SR, Essigmann JM. Oxidation of 7,8-dihydro-8-oxoguanine affords lesions that are potent sources of replication errors *in vivo*. Biochemistry 2002;41:914–21.
37. Hecht SS. Biochemistry, biology, and carcinogenicity of tobacco-specific *N*-nitrosamines. Chem Res Toxicol 1998;11:559–603.
38. Moriya M, Zhang W, Johnson F, Grollman AP. Mutagenic potency of exocyclic DNA adducts: marked differences between *Escherichia coli* and simian kidney cells. Proc Natl Acad Sci U S A 1994;91:11899–903.
39. Pandya GA, Moriya M. 1,N^6-ethenodeoxyadenosine, a DNA adduct highly mutagenic in mammalian cells. Biochemistry 1996;35:11487–92.
40. Cho Y, Gorina S, Jeffrey PD, Pavletich NP. Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science 1994; 265:346–55.
41. Campomenosi P, Monti P, Aprile A, et al. p53 mutants can often transactivate promoters containing a p21 but not Bax or PIG3 responsive elements. Oncogene 2001; 20:3573–9.
42. Di Como CJ, Prives C. Human tumor-derived p53 proteins exhibit binding site selectivity and temperature sensitivity for transactivation in a yeast-based assay. Oncogene 1998;16:2527–39.
43. Shiraishi K, Kato S, Han SY, et al. Isolation of temperature-sensitive p53 mutations from a comprehensive missense mutation library. J Biol Chem 2004; 279:348–55.
44. Vahakangas KH, Bennett WP, Castren K, et al. p53 and K-ras mutations in lung cancers from former and never-smoking women. Cancer Res 2001;61:4350–6.
45. Gealy R, Zhang L, Siegfried JM, Luketich JD, Keohavong P. Comparison of mutations in the p53 and K-*ras* genes in lung carcinomas from smoking and nonsmoking women. Cancer Epidemiol Biomarkers Prev 1999;8:297–302.
46. Takagi Y, Osada H, Kuroishi T, et al. p53 mutations in non-small-cell lung cancers occurring in individuals without a past history of active smoking. Br J Cancer 1998;77:1568–72.
47. Marchetti A, Pellegrini S, Sozzi G, et al. Genetic analysis of lung tumours of non-smoking subjects: p53 gene mutations are constantly associated with loss of heterozygosity at the FHIT locus. Br J Cancer 1998;78: 73–8.
48. U.S. Environmental Protection Agency. Respiratory health effects of passive smoking: lung cancer and other disorders. Vol. EPA/600/6-90/006F. Washington (DC): U.S. Environmental Protection Agency, 1993; 1993.
49. Ziegel R, Shallop A, Upadhyaya P, Jones R, Tretyakova N. Endogenous 5-methylcytosine protects neighboring guanines from N7 and O^6-methylation and O^6-pyridyloxobutylation by the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Biochemistry 2004; 43:540–9.
50. van d V, Eiserich JP, Cross CE. Nitric oxide: a pro-inflammatory mediator in lung disease? Respir Res 2000; 1:67–72.
51. Ichinose M, Sugiura H, Yamagata S, Koarai A, Shirato K. Increase in reactive nitrogen species production in chronic obstructive pulmonary disease airways. Am J Respir Crit Care Med 2000;162:701–6.
52. Di Stefano A, Caramori G, Ricciardolo FL, Capelli A, Adcock IM, Donner CF. Cellular and molecular mechanisms in chronic obstructive pulmonary disease: an overview. Clin Exp Allergy 2004;34:1156–67.
53. Ambs S, Bennett WP, Merriam WG, et al. Relationship between p53 mutations and inducible nitric oxide synthase expression in human colorectal cancer. J Natl Cancer Inst 1999;91:86–8.

TP53 and *KRAS* Mutation Load and Types in Lung Cancers in Relation to Tobacco Smoke: Distinct Patterns in Never, Former, and Current Smokers

Florence Le Calvez, Anush Mukeria, Jay D. Hunt, et al.

Cancer Res 2005;65:5076-5083.