Mutations in TP53, but not FGFR3, in urothelial cell carcinoma of the bladder are influenced by smoking: contribution of exogenous versus endogenous carcinogens

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Smoking is a major risk factor for urothelial cell carcinoma of the bladder (UCC). Mutations in the FGFR3 and TP53 genes have been shown to define two distinct pathways in superficial papillary and invasive UCC disease, respectively. We investigated the relationship between smoking and these mutations by means of denaturing high performance liquid chromatography and sequencing for 110 primary UCC of the bladder. This study included 48 current smokers, 31 ex-smokers and 31 non-smokers. Thirty-five of the tumors were grade 1, 37 were grade 2 and 59 grade 3. Smoking was associated with high stage (P = 0.03) and high grade tumors (P = 0.006). Twenty-two of the 110 tumors studied harbored TP53 mutations (20%) and 43 harbored FGFR3 mutations (39%). Odds ratios (OR) were higher for FGFR3 mutations (20%) and 43 harbored TP53 Twenty-two of the 110 tumors studied harbored high stage (P = 0.03) and high grade tumors (P = 0.006). Twenty-two of the 110 tumors studied harbored TP53 mutations (20%) and 43 harbored FGFR3 mutations (39%). Odds ratios (OR) were higher for FGFR3 mutations in current smokers [OR, 2.25; 95% confidence interval (95% CI), 0.65–7.75] and ex-smokers (OR, 1.62; 95% CI, 0.41–6.42) than in non-smokers. Double TP53 mutations and the A:T→G:C base substitution pattern was found only in current smokers. Patients with the FGFR3wild-type/TP53mutated genotype had significantly higher levels of tobacco consumption, as measured in pack-years (P = 0.01). Smoking influenced neither the frequency nor the pattern of FGFR3 mutations. Our results suggest that smoking is associated with invasive and high grade UCCs, at initial presentation, and influenced TP53 or the molecular pathway defined by these mutations. In contrast, FGFR3 mutations are not affected by smoking and probably result from endogenous alterations. These data have potential implications for clinical management and prevention strategies.

Introduction

Bladder cancer is a major human cancer associated with exposure to exogenous carcinogens. Cigarette smoking, occupational exposure to certain chemicals, inflammatory reactions of a parasitic nature (such as schistosomiasis) or other chronic infections and exposure to contaminants in drinking water (such as arsenic) are known to be risk factors for bladder cancer (1–4). Tobacco is considered to be the major external risk factor for bladder cancer [urothelial cell carcinoma of the bladder (UCC)], accounting for the doubling to tripling of risk in individuals who have smoked at some time in their lives (5). About half the cases of bladder cancer in men and one third of those in women are attributed to cigarette smoking (6). Continued smoking following the diagnosis of UCC is a significant clinical problem, as 50% of patients who were smokers at the time of diagnosis continue to smoke (7).

Tobacco smoke is a heterogeneous mixture containing over 60 different substances classified as carcinogens by the IARC. Other carcinogens not evaluated by the IARC are probably also present, but their carcinogenicity is currently unknown. Certain carcinogens present in tobacco damage DNA by creating smoking-related DNA adducts, and may induce specific base changes in cancer-related genes. Aromatic amines, a constituent of cigarette smoke, play a significant role in causing bladder cancer (reviewed in 8). Among the recognized human bladder carcinogens present in cigarette smoke are 4-aminobiphenyl (4-ABP) and 2-naphthylamine. The first of these compounds, 4-ABP, originating primarily from cigarette smoke (6), is a major exogenous factor implicated in the etiology of human bladder cancer (9). Several studies have suggested that differences in carcinogen adduct levels may be closely correlated with differences in bladder cancer risk. DNA adduct levels in white blood cells have been found to be significantly associated with bladder cancer risk. High levels of 4-ABP–DNA adducts have been detected in biopsy samples taken from smokers with bladder cancer. Such adducts have also been found in DNA isolated from exfoliated urothelial cells in the urine of smokers. Indeed, 4-ABP has been shown to be a strong urinary bladder carcinogen in experimental animals (reviewed in 10).

It has been suggested that specific carcinogens leave a characteristic mutational signature in the DNA (8). TP53 is a common target for carcinogenic agents and an excellent candidate for molecular epidemiology studies (11). In lung cancer there is evidence linking G→T transversions at CpG sites in TP53 with smoking (12). In schistosomal bladder cancer there is an excess of C→T transitions at CpG sites that may reflect nitric oxide-induced DNA damage due to the inflammatory reaction caused by the parasite (13). In cyclophosphamide-associated bladder cancer the predominant mutations are G:C→A:T, occurring at non-CpG sites only (14). These studies are consistent with TP53 being a target for

Abbreviations: 4-ABP, 4-aminobiphenyl; CS, current smoker; 95% CI, 95% confidence interval; DHPLC, denaturing high performance liquid chromatography; ES, ex-smoker; mut, mutated; NS, non-smoker; OR, odds ratio; UCC, urothelial cell carcinoma of the bladder; wt, wild-type.

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carcinogens, with a specific TP53 mutation spectrum (15). In UCC, however, although most of the mutations observed are G:C→A:T transitions at CpG and non-CpG sites (16), conflicting results have nonetheless been obtained concerning the influence of smoking on the pattern of TP53 mutations (17–21). Some authors have reported no difference in the mutation spectrum between smokers and non-smokers (19), whereas others found multiple TP53 mutations only in smokers (17). Habuchi et al. (18) found A:T→G:C transitions only in smokers. It was recently demonstrated that G:C→A:T transitions at CpG sites are more frequent in smokers than in non-smokers (20,21). The reason for such discrepancies is not yet clear. A region encompassing codons 280 and 285 may correspond to a unique TP53 mutational hot-spot in bladder cancer (22). A recent study identified codon 273 as a mutational hot-spot associated with smoking (20). However, there is no consensus for the existence of such smoking-associated hot-spots.

Other molecular targets, such as chromosome 9, have also been studied in relation to smoking (23). However, no previous study has addressed the potential impact of smoking on FGFR3 mutations in relation to bladder cancer. FGFR3 belongs to the FGFR (fibroblast growth factor receptor) family, which comprises four structurally related members (FGFR1–FGFR4) (24).

Germline activating point mutations in FGFR3 are responsible for several types of craniosynostosis and dwarfing chondrodysplasia, including thanapophic dysplasia and severe achondroplasia with developmental delay and acanthosis nigricans (reviewed in 25). The same activating somatic point mutations in exons 7, 10 and 15 of the FGFR3 gene are frequently observed in urothelial cell cancers (26). These mutations were found primarily in superficial tumors and were linked to a favorable prognosis (27). Given the high frequency of these FGFR3 mutations, or the mutation spectrum between smokers and non-smokers (19), influence of smoking on the pattern of FGFR3 mutations, or the function of the level of tobacco consumption.

Materials and methods

Study population

We studied 110 patients with primary bladder tumors admitted to the Department of Urology for transurethral resection or radical cystectomy, with no previous treatment. The median age of the patients was 67 years (range 38–93 years).

Patients gave written informed consent and we collected matched tumors and blood samples from each patient. The tumors were graded according to the WHO classification of 1973 (29). Stage was determined according to the TNM classification guidelines (30). Thirty-five tumors were stage PTa, 40 were stage PT1 and 35 were ≥PT2. Fourteen of the tumors were grade 1, 37 were grade 2 and 39 were grade 3.

The smoking history of the subjects was determined by means of personal interviews. The information recorded included the age at which the patient began smoking, time since quitting and tobacco consumption, expressed in pack-years. One pack year = 20 cigarettes per day for 1 year. Current smokers (CS) were defined as patients who had smoked and were smokers at the time of diagnosis. Patients who had stopped smoking <1 year before their diagnosis were considered CS. Forty-eight (44%) patients were defined as current smokers. Patients who had quit smoking at least 1 year before the interview were classified as ex-smokers (ES). Thirty-one (28%) patients were thus defined as ex-smokers. Patients who had smoked <100 cigarettes during their lifetime and had never used any other tobacco-related products were classified as non-smokers (NS): 31 (28%) patients were classified as non-smokers.

DNA extraction

Tumors were snap frozen in liquid nitrogen and stored at −80°C. Venous blood, from matched tumors used as a source of reference DNA, was collected in EDTA-containing tubes and stored at −20°C until DNA extraction. DNA and RNA were extracted simultaneously, by the cesium chloride cushion method, as previously described (31). DNA was extracted from blood with a Qiagen S.A., France.

PCR

For TP53, we screened for mutations in exons 2–11 by DHPLC. PCR was performed in a final volume of 50 µl containing 100 ng genomic DNA, 1× amplification buffer, 1.5 mM MgCl₂, 200 µM each dNTP, 0.15 µM forward primer, 0.15 µM reverse primer and 2.5 U Taq DNA polymerase (HotStarTaq; Qiagen S.A., France).

PCR was carried out as follows: an initial denaturation step (95°C for 15 min) was followed by 40 cycles consisting of denaturation at 94°C for 30 s, primer annealing at 58°C (exons 2–11, but not exon 8) or 59°C (exon 8) for 30 s and extension at 72°C for 30 s. The mixture was then heated at 72°C for 10 min as a final extension step.

For FGFR3 we amplified exons 7, 10 and 15 by PCR in a final volume of 50 µl containing 50 ng genomic DNA, 1× amplification buffer, 1.5 mM MgCl₂, 80 µM each dNTP, 0.3 µM forward primer, 0.3 µM reverse primer and 2.5 U Taq DNA polymerase (HotStarTaq). We used a touchdown PCR technique, with 35 cycles. Reaction mixtures were first heated for 15 min at 95°C. Annealing temperatures were lowered by 0.5°C/cycle from 72 to 62°C over the course of 20 cycles (exon 7), from 71 to 62°C over 18 cycles (exon 10) and from 70 to 61°C over 18 cycles (exon 15), with each annealing step lasting 1 min. For the remaining cycles annealing temperatures were maintained at 62, 62 and 61°C for exons 7, 10 and 15, respectively, with each annealing step lasting 1 min. In each cycle the denaturation step consisted of heating at 94°C for 1 min and the extension step of heating at 72°C for 1.20 min. At the end of the last cycle samples were incubated for an additional 15 min at 72°C.

DHPLC

We carried out DHPLC analysis with a Varian (Prostar) Helix system, using the helix analysis column (the helix DNA² column set). Briefly, the formation of heteroduplexes and homoduplexes was favored by denaturing PCR products, by heating at 95°C for 10 min and allowing the DNA to renature at 62°C (FGFR3) or 88°C (TP53) for 1 h. We applied a 5 µl DNA sample to the column and eluted with a linear acetonitrile gradient as the eluent. The acetonitrile gradient was created by mixing buffer A [100 mM triethylammonium acetate (pH 7.0) and 0.1 mM EDTA] and buffer B [100 mM triethylammonium acetate (pH 7.0), 0.1 mM EDTA and 25% (v/v) acetonitrile]. The flow rate was 0.45 ml/min and we increased the proportion of buffer B by 3.3% per min for 4.5–5.5 min. The melting behavior of the specific DNA was established by repeatedly injecting the sample at temperature steps beginning at 50°C until complete denaturation was reached. A melting curve, in which retention time was plotted against temperature, was produced and the temperature at which 25–50% denaturation was observed was determined (the retention time is 0.75–1 min shorter than that under non-denaturing conditions), using the
universal gradient (Varian). The acetonitrile gradient was then adjusted such that the peaks eluted between 3 and 6.30 min. DNA fragments were monitored by UV absorbency at 260 nm. The primers for TP53 and FGFR3 and specific values for the gradient ranges and mobile phase temperatures used were as previously described (28).

DNA sequencing

PCR products with elution profiles differing from those of the corresponding wild-type DNA were sequenced. Sequencing was performed with a Big Dye Terminator kit, following the Applied Biosystems protocol. Samples were sequenced in an ABI Prism 377 sequencer (Perkin-Elmer Applied Biosystems). If a mutation was identified, matched constitutional DNA samples were also sequenced to confirm the somatic nature of the mutation. The results were confirmed by sequencing on both strands.

Statistical analysis

The relationships between stage and smoking status and between grade and smoking status were assessed by χ² analysis, using contingency tables. Associations between mutations in TP53 and FGFR3, tumor genotypes (according to these mutations) and stage, grade and smoking status were measured by calculating odds ratios (ORs) and 95% confidence intervals (95% CI), by means of logistic regression.

The number of pack-years smoked and the association of this variable with TP53/FGFR3 genotype were evaluated by the Kruskal–Wallis non-parametric ANOVA test and data were expressed as means ± SEM. Values of P < 0.05 were considered significant.

Results

Relationship between smoking and stage and grade of tumors

Smoking was associated with adverse clinico-pathological parameters. UCC tumors from current smokers were of a higher stage (P = 0.03) and higher grade at initial diagnosis than were the tumors of ex-smokers and non-smokers (P = 0.006) (Table I).

Relationship between TP53 and FGFR3 mutations and stage and grade of tumors

TP53 mutations were associated with high stage and high grade tumors, whereas FGFR3 mutations were associated with low stage, low grade tumors (Table II). We further analyzed the relationship between stage or grade and tumor genotype (FGFR3wt/TP53mut and FGFR3mut/TP53wt), rather than TP53 and FGFR3 mutations, to see whether this affected associations. Associations between tumor genotype and stage and grade were stronger than those between mutations and stage and grade (Table III). Tumor genotypes also defined two separate genetic pathways.

Characteristics of TP53 mutations

All the characteristics of TP53 mutations in this cohort are listed in Table IV. Of the 110 tumors studied, 22 harbored TP53 mutations (20%). These mutations were located in exons 4–9; one mutation was located at a splice acceptor site of intron 6. No mutations were found in exons 2, 3, 10 or 11.

Four patients had double TP53 mutations and all of these patients were current smokers. These double mutations were found in patient 41 in exon 5 (codon 175) and exon 8 (codon 306), patient 94 in exon 7 (codon 248) and exon 8 (codon 291), patient 102 in exon 8 (codon 285 and 291) and patient 27 in exon 7 (codons 244 and 245). Fifty percent of TP53 mutations in non-smokers concerned exon 5, whereas this was the case for only 25% of current smokers. In current smokers mutations between codons 241 and 249 accounted for 38% of mutations, whereas such mutations accounted for only 25% of mutations in non-smokers.

Of the transitions (62% of TP53 mutations) detected 13 were G:C→A:T (50%), eight of which occurred at CpG sites (31%), including five in current smokers (31%), two in non-smokers (50%) and one in an ex-smoker (17%). The remaining five G:C→A:T transitions occurred at non-CpG sites (19%), including three in current smokers (19%), one in a non-smoker (25%) and one in an ex-smoker (17%). Three A:T→G:C transitions were detected and all three were found in current smokers.

The transversions (31% of TP53 mutations) observed were five G→T, one C→A, one A→C and one C→G. The C→G

<table>
<thead>
<tr>
<th>Table I. The relationship between stage and grade and smoking status</th>
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<tbody>
<tr>
<td>Histopathology</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Stage</td>
</tr>
<tr>
<td>Ta + T1</td>
</tr>
<tr>
<td>≥T2</td>
</tr>
<tr>
<td>Grade</td>
</tr>
<tr>
<td>G1 + G2</td>
</tr>
<tr>
<td>G3</td>
</tr>
</tbody>
</table>

The P value was calculated in a χ² test. P < 0.05 was considered significant.

<table>
<thead>
<tr>
<th>Table II. The relationship between TP53 and FGFR3 mutations and stage and grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mutations</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>Stage</td>
</tr>
<tr>
<td>Ta + T1</td>
</tr>
<tr>
<td>≥T2</td>
</tr>
<tr>
<td>Grade</td>
</tr>
<tr>
<td>G1 + G2</td>
</tr>
<tr>
<td>G3</td>
</tr>
</tbody>
</table>

The ORs and 95% CI were estimated using logistic regression.

<table>
<thead>
<tr>
<th>Table III. The relationship between tumor genotype and stage and grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGFR3wt/TP53mut</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>Stage</td>
</tr>
<tr>
<td>Ta + T1</td>
</tr>
<tr>
<td>≥T2</td>
</tr>
<tr>
<td>Grade</td>
</tr>
<tr>
<td>G1 + G2</td>
</tr>
<tr>
<td>G3</td>
</tr>
</tbody>
</table>

The ORs and 95% CI were estimated using logistic regression.
transversion was found in intron 6, at a splice acceptor site (patient 30). Two deletions (7% of TP53 mutations) were identified in exon 5 (patients 19 and 20).

No specific TP53 hot-spot was associated with smoking.

Characteristics of FGFR3 mutations

The characteristics of the FGFR3 mutations are listed in Table V. Activating mutations were observed in 43 (39%) of the 110 bladder tumors analyzed, with 68% of these mutations found in exon 7 (codons 248 and 249), 25% in exon 10 (codons 372, 373 and 375) and 7% in exon 15 (codon 652). Seven different mutations were identified, all of which had already been reported in bladder carcinomas: R248C, S249C, G372C, S373C, Y375C, K652E and K652M. The S249C mutation was the most frequent mutation, accounting for 25 (58%) of the 43 mutations found. No specific pattern of mutations was found to be associated with smoking status.

Relationship between TP53 mutations and smoking status

The relationship between TP53 mutations and smoking status is shown in Table VI. OR depended on smoking status, with higher ORs obtained for current smokers (OR, 2.25; 95% CI, 0.65–7.75) and for ex-smokers (OR, 1.62; 95% CI, 0.41–6.42) than for non-smokers. However, despite this trend, no significant association was found between the frequency of TP53 mutations and smoking status.

Relationship between FGFR3 mutations and smoking status

No significant association was found between the frequency of FGFR3 mutations and smoking for current smokers (OR, 0.64; 95% CI, 0.26–1.62) or for ex-smokers (OR, 0.51; 95% CI, 0.18–1.42), with non-smokers used as the reference group (Table VI).

Relationship between tumor genotype, according to TP53 and FGFR3 mutations, and smoking status

When we analyzed the relationship between tumor genotype and smoking, ORs were higher for current smokers (OR, 4.06; 95% CI, 0.26–23.59) than for non-smokers, but no significant association was found (Table VII).

Relationship between tumor genotype and tobacco consumption in pack-years

In non-parametric Kruskal–Wallis ANOVAs comparing tobacco consumption in patients with the different tumor...
The ORs and 95% CI were estimated using logistic regression. Smoking status in epithelial cells. NS, non-smoker; ES, ex-smoker; CS, current smoker.

The relationship between tumor genotype and smoking status according to stage and grade (Table I). These results are consistent with those of Koss et al. (32), who showed that tumors from workers exposed to 4-ABP might be more relevant to high grade, high stage bladder cancer. The issue relating epidemiological studies to the two different forms of UCC has recently been reviewed by Cohen et al. (33).

The frequency of TP53 mutations in the 110 tumors investigated was (20%) consistent with that reported by other authors (21,34). TP53 mutations were associated with high stage and high grade, whereas FGFR3 mutations were associated with low stage and low grade tumors (Table II). Genotyping according to the mutation status of both genes (FGFR3wt/TP53mut) and (FGFR3mut/TP53wt) yielded stronger associations (Table III) and is therefore more useful for defining the two separate molecular pathways previously reported (28).

For TP53, consistent with previous investigations, we found a trend but a non-significant association between the frequency of TP53 mutations in current smokers and that in non-smokers (17–21). The doubling to tripling of bladder cancer risk in smokers with respect to non-smokers identified in epidemiological studies (35) could not be accounted for by TP53 mutations alone. TP53 mutations may be induced by endogenous and exogenous carcinogens and other molecular targets involved in bladder cancer may also be targeted by carcinogens present in tobacco. Indeed, evidence has already been obtained that there is a link between smoking and chromosome 9 alterations in the etiology of bladder cancer (23). Non-smokers may also have been exposed to carcinogens like those present in cigarette smoke, resulting in the generation of TP53 mutations. Some studies have reported exposure to 4-ABP in humans other than through smoking, but the sources of such non-smoking-related exposure remain unclear (36). Moreover, it has been shown that environmental exposure to aromatic amines unrelated to smoking may account for a

### Table V. FGFR3 mutation characteristics and smoking history in primary UCC tumors

<table>
<thead>
<tr>
<th>Exon</th>
<th>Codon</th>
<th>Nucleotide position and base change</th>
<th>Amino acid change</th>
<th>Smoking history</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>248</td>
<td>742C→T Arg248Cys (R248C)</td>
<td>NS: 0</td>
<td>4 (10%)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>249</td>
<td>746C→G Ser249Cys (S249C)</td>
<td>NS: 9</td>
<td>25 (58%)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>372</td>
<td>1114G→T Gly372Cys (G372C)</td>
<td>NS: 0</td>
<td>1 (2%)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>373</td>
<td>1117A→T Ser373Cys (S373C)</td>
<td>NS: 1</td>
<td>2 (5%)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>375</td>
<td>1124A→G Tyr375Cys (Y375C)</td>
<td>NS: 2</td>
<td>9 (21%)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>652</td>
<td>1954A→G Lys652Glu (K652E)</td>
<td>NS: 1</td>
<td>2 (5%)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>652</td>
<td>1955A→T Lys652Met (K652M)</td>
<td>NS: 0</td>
<td>1 (2%)</td>
<td></td>
</tr>
</tbody>
</table>

Codon and mutated nucleotide position are numbered according to the cDNA open reading frame corresponding to the FGFR3b isoform, which is produced in epithelial cells. NS, non-smoker; ES, ex-smoker; CS, current smoker.

### Table VI. The relationship between TP53 and FGFR3 mutations and smoking status

<table>
<thead>
<tr>
<th>TP53wt</th>
<th>TP53mut</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smokers</td>
<td>27</td>
<td>4</td>
</tr>
<tr>
<td>Ex-smokers</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>Current smokers</td>
<td>36</td>
<td>12</td>
</tr>
</tbody>
</table>

### Table VII. The relationship between tumor genotype and smoking status

<table>
<thead>
<tr>
<th>FGFR3wt/TP53wt</th>
<th>FGFR3mut/TP53wt</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smokers</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>Ex-smokers</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Current smokers</td>
<td>16</td>
<td>10</td>
</tr>
</tbody>
</table>

TP53mut genotype had significantly higher levels of tobacco consumption than did patients with the other genotypes \((P = 0.01)\).
significant proportion of cases of bladder cancer in the general population (37).

In our study, however, if tumors were grouped according to genotype for TP53 and FGFR3 mutations, the FGFR3wt/TP53mut genotype was found to be correlated with high levels of tobacco consumption and a statistically significant correlation between the presence of TP53 mutations and total tobacco consumption, expressed in pack-years, was observed (P < 0.01). These results seem to be consistent with previous investigations showing that the frequency of 4-ABP-DNA adducts increases with tobacco consumption (9). A meta-analysis of 43 epidemiological studies concluded that current cigarette smokers have a three times higher risk of urinary tract cancer than non-smokers and that the risk of urinary tract cancer is associated with the number of cigarettes smoked per day (58).

Furthermore, protein and increases in SSCP mobility shifts in the TP53 gene were found in normal urothelium cultured from smokers. For most of the smoker group the number of pack-years smoked was correlated with p53 expression (39). However, no such relationship was reported in other studies (21,40). We studied the association between tobacco consumption, expressed in pack-years, and tumor genotype: FGFR3wt/TP53mut, FGFR3wt/TP53wt and FGFR3mut/TP53wt. When specific subgroups were analyzed, some strong associations were observed (41). The grouping of tumors into FGFR3wt/TP53mut, FGFR3wt/TP53wt and FGFR3mut/TP53wt genotypes is based on the assumption that these three genotypes may correspond to different disease entities (28). Moreover, in our study no special selection criteria were used and primary tumors of all stages and grades were included. Data on tobacco consumption for ex-smokers were also included, because although it has been shown that at least 5 years abstinence ex-smokers and non-smokers are indistinguishable in terms of the levels of carcinogen adducts in the urinary bladder (9), ex-smokers still have a higher risk of urinary tract cancer than non-smokers (42). It has also been suggested that the frequency of TP53 mutations in bladder tumors is dependent on the subject’s smoking history (19).

Data on the effect of smoking on the pattern of TP53 mutations in UCC differ greatly between studies and the situation is less clear than that for lung cancer, in which there is an excess of G→T transversions in smokers (reviewed in 8). It has been suggested that the type of mutations cannot be related to smoking habit, because such mutations occur in both non-smokers and smokers (19). However, Spruck et al. (17) suggested that the carcinogens in cigarette smoke may increase the extent of DNA damage per mutagenic event. In our study TP53 double mutations were found only in current smokers, consistent with this earlier observation. Other studies have also generated results tending to confirm this hypothesis (19,43). Double mutations may be one of several ways in which smoking influences TP53.

In our study A:T→G:C mutations were also limited to current smokers. These results are consistent with those of Habuchi et al. (18), who suggested that these transitions strongly support the involvement in urothelial cancer of a specific mutagen(s) induced by cigarette smoking. One possible explanation is that although 80% of the 4-ABP-DNA adducts at the nucleotide sequence level in human cells treated with N-hydroxy-N-acetyl-4-aminoazobenzene are guanine adducts, 20% are adenine adducts (44). Adducts of aromatic amines, such as 4-ABP, mostly react with DNA at the C-8 of deoxyguanosine, but adducts have also been observed at the N6 of deoxyadenosine (reviewed in 8). However, these mutations might have been induced by other mechanisms or other carcinogens present in tobacco smoke, as some authors have found no relationship between 4-ABP-DNA adducts and TP53 mutations (45) and the mutational signature of the proximate bladder carcinogen N-hydroxy-4-acetylaminobiphenyl is inconsistent with the TP53 mutational spectrum in bladder cancer (46). In this study A:T→G:C TP53 mutations distinguished smokers from non-smokers, suggesting that smoking may have influenced the pattern of TP53 mutations.

Many previous studies have indicated that G→A transitions are the most prevalent type of mutation in bladder cancer, with about half of these transitions occurring at CpG sites (47). In our study, although a large proportion of mutations were transitions (G:C→A:T (50%), including 31% at CpG, the frequency of such mutations was only 31% in smokers, whereas it was 50% in non-smokers. These results contrast with those of previous studies (20,21), in which G→A transitions at CpG occurred preferentially in smokers, possibly reflecting differences in genetic susceptibility and/or background occupational exposure. Indeed, some authors have shown that N-acetyltransferase type II and glutathione S-transferase M1 polymorphism are associated with some TP53 mutation patterns (21,34).

For FGFR3 our data suggest that FGFR3 mutations are less likely to have been induced by smoking, which did not seem to affect the frequency or the spectrum of these mutations. Several lines of evidence support this hypothesis.

First, most of the FGFR3 missense somatic mutations identified in bladder cancer (R248C, S249C, G372C and K652E) were initially described as germinal activating mutations responsible for thanatophoric dysplasia, a lethal form of dwarfism (48) which has not been reported to be linked to smoking.

Second, in the small number of studies carried out to date, FGFR3 mutations have not been detected in the normal bladder epithelium of patients with mutation-containing tumors (26,49). UCCs are characterized by their multifocal nature, and this has led to the development of the concept of ‘field change’, in which the entire urothelium is susceptible to malignant transformation caused by exogenous carcinogens (50). Unlike FGFR3 mutations, deletions of chromosome 9 and/or 9p21 have been reported in hyperplasia and in epithelium of normal appearance adjacent to papillary tumors (51,52). TP53 mutations have also been detected in dysplasia, and even in epithelium with a normal appearance (53).

Third, the frequency of FGFR3 mutations and their patterns have been shown to be similar in studies carried out on different populations in France (26), the UK (54), Japan (49) and The Netherlands (27), which may have different genetic backgrounds and probably do not display the same smoking habits. It seems unlikely that a single carcinogen would be responsible for such a high frequency of mutations. However, the high frequency of FGFR3 mutations in UCC, particularly S249C, remains puzzling. It is currently unclear whether certain carcinogens impose a genotoxic burden on urothelial cells and whether genotoxic agents are produced endogenously. Such a mechanism has been proposed for the pattern of TP53 mutations in shistosomal bladder cancer (13).

Our data do not support the hypothesis that FGFR3 mutations are induced by tobacco carcinogens and suggest instead that these mutations are more likely to be induced by endogenously produced changes, although the possibility that some of these carcinogens induce physiological stresses or genotoxic...
burdens and that susceptible hot-spots in the gene are affected cannot be ruled out.

Conclusion

Our results suggest that TP53 is a target of tobacco carcinogens. Smokers with high levels of tobacco consumption, expressed in pack-years, are more likely to have the FGFR3wt/TP53mut genotype, which is associated with high risk UCC tumors. Smoking seems to affect the pattern of TP53 mutations with no specific hot-spot. In contrast, smoking seems to have no effect on the incidence and pattern of FGFR3 mutations. Future research should focus on elucidating the endogenous processes involved in the induction of FGFR3 mutations. Such research should increase our understanding of the contributions of endogenous and exogenous mechanisms to urothelial carcinogenesis. The observation that smokers have higher stage and grade UCC has important potential implications for curative and preventive medicine.

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