Influence of Cigarette Smoking and Schistosomiasis on p53 Gene Mutation in Urothelial Cancer

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ABSTRACT

The mutation patterns of the p53 tumor suppressor gene have been shown to reflect the specific carcinogen(s) involved, or the epidemiological background in some cancers. To elucidate the impact of cigarette smoking or bilharzial infection on the p53 gene mutation pattern, 61 cases of urothelial cancer from Japan and 7 cases of bladder cancer with schistosomiasis from Egypt were examined for mutations of the p53 gene. In total, p53 gene mutations were detected in 20 Japanese cases (33%) and 6 Egyptian cases (86%). Although the incidence of p53 gene mutation was not significantly influenced by habitual smoking, a different mutation pattern was observed as follows: 4 of 10 mutations in smokers in Japan were A:T to G:C transitions, whereas such mutations were not detected in any of 10 mutations in nonsmokers, or in any of 6 mutations associated with schistosomiasis. Although no specific mutation pattern was detected for the squamous cell carcinomas with schistosomiasis, all 8 base substitutions observed in tumors with squamous cell carcinomas occurred at G:C sites, whereas base substitutions at A:T sites were observed in 33% (6 of 18) of mutations in transitional cell carcinomas. Our results suggest that cigarette smoking may have a significant impact on the mutations of the p53 gene in urothelial cancers. Furthermore, the distinct spectrum of the p53 gene mutation found in tumors with squamous cell carcinomas may reflect their unique etiological backgrounds.

INTRODUCTION

The p53 tumor suppressor gene, which is located on the short arm of chromosome 17, has been demonstrated to encode for a protein involved in the regulation of the cell cycle and growth (1). Mutations within the coding sequence of the p53 tumor suppressor gene are among the most frequent genetic alterations found in a variety of human malignant tumors (2-5). RFLP3 analyses have shown that p53 mutations are frequently associated with the loss of a nonmutated allele on chromosome 17p. In urothelial cancers, analyses of p53 gene mutations by PCR techniques or RFLP analyses of the 17p allele status indicate that alterations of the p53 gene are closely associated with a high grade and/or high stage subset of this type of cancer (5-9).

On the other hand, accumulating evidence suggests that the patterns of p53 gene mutation detected in some kinds of human cancers might reflect endogenous and external mutagenic agents involved in carcinogenesis (2, 10). For example, hepatocellular carcinomas closely associated with aflatoxin B often contain a G:C to T:C transversion at codon 249 of the p53 gene (12, 13). Human squamous cell carcinomas of the skin of cigarette smokers are usually associated with a G:C to T:A transversion at dipyrimidine sites of the p53 gene, thus indicating the causative involvement of UV exposure in this type of mutation (14). In colon cancers, most of the p53 gene mutations were reported to be G:C to A:T transitions, occurring mostly at the CpG (methylcytosine-guanine) dinucleotide (2, 10), which is known to be a frequent spot of "spontaneous" mutations (15). In lung cancers, a G:C to T:A transversion is often observed, and this type of mutation is presumably induced by benzo[a]pyrene which is a cigarette smoke-related carcinogen (16-18).

In urothelial cancers of the bladder, ureter, and renal pelvis, cigarette smoking has been shown to be a major contributing factor to tumorigenesis both in Japan and in western countries (19, 20). About 30 to 50% of bladder cancers may be attributed to cigarette smoking, although the mechanism by which cigarette smoking induces urothelial cancer is unclear. In Egypt, where schistosomiasis is endemic, bladder cancer is the most common form of cancer, and consists mainly of squamous cell carcinomas caused by chronic bilharzial infection (21).

Although epidemiologically linked, the question of whether or not cigarette smoking and chronic schistosomal infection have any impact on mutational events of the p53 gene remains to be answered. There have been few studies thus far investigating the differences in the p53 gene mutation pattern according to the etiological background of each case. In this study, p53 gene mutations were examined in 68 cases of urothelial cancer from 61 Japanese and 7 Egyptian patients to answer the above question. In addition, the relationship between p53 gene mutations and the LOH on chromosome 17p was evaluated to elucidate the role of these genetic alterations in urothelial cancer progression.

MATERIALS AND METHODS

Patients and Samples. Specimens of urothelial cancer were obtained from 61 native Japanese patients and from 7 patients with schistosomiasis in Egypt. In 56 of the Japanese cases, the corresponding normal kidney tissue or peripheral blood was obtained for a sample of normal DNA. Tissues were snap frozen and stored at -70°C until the extraction of the DNA. Each tumor tissue was examined histologically, and only those samples containing predominantly viable cancer cells were used. Histological typing, grading, and staging of the tumors (46 bladder cancers and 15 renal pelvic or ureteral cancers) collected in Japan were assessed according to the WHO (22) and TNM (23) classifications, respectively. The 7 carcinoma tissue samples from Egypt were obtained at the National Cancer Institute in Cairo, and all were diagnosed histologically as invasive (apT2) squamous cell carcinomas associated with schistosomiasis (24). For all Japanese patients, smoking and occupational histories were obtained by the medical staff and reconfirmed in some cases by additional interviews. The 61 Japanese patients consisted of 30 current smokers who smoked more than 5 cigarettes per day, 4 exsmokers who stopped smoking at least 4 years before admission, and 27 who had never smoked. No history of schistosomal infection was found in any of the Japanese cases. Furthermore, no obvious occupational exposure, which has been closely associated with urothelial cancers, was identified. No information on occupational histories and smoking habits was obtained for the Egyptian patients.

SSCP Analysis. High-molecular-weight genomic DNA was extracted by the phenol/chloroform method after protein K digestion from tissue samples and peripheral blood leukocytes. Mutations of the p53 gene from exons 4 to 10 were screened by SSCP analysis of the amplified PCR fragments from the genomic DNA. The SSCP analysis was performed according to the method of Toguchida et al. (4, 25), based on the method of Orita et al. (26), with minor modifications. Eight pairs of oligonucleotide primers were designed to span the...
7 coding exons (exons 4 to 10) of the human p53 gene based on the primer sequence data previously published (4). For exon 5, two overlapping PCR fragments were analyzed by two pairs of primers. The other PCR primer pairs spanned only a single exon. For exon 4, amplified PCR fragments were digested by MspI and were then analyzed. Each 50 μl of PCR mixture contained 200 ng of genomic template DNA, 50 pmol of each primer, 40 μM concentrations each of dATP, dGTP, and dTTP, and 0.2 μl of [α-32P]dTTP (3000 Ci/mmol, 1 mCi/ml), and 2.0 units of Taq polymerase in a buffer containing 1.0 or 1.5 mM MgCl2. Thirty cycles of amplification were carried out in an automatic programmable thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT) at 94°C for 30 s, 55 or 60°C, and 72°C for 1, 1.5, and 2 min, respectively. One-tenth of the volume of each crude amplified sample for SSCP analysis. Each amplified DNA template was purified by an ultrafiltration kit (Suprec-02; Takara Shuzo Co., Kyoto, Japan) or by re-extraction. 

**Direct Sequencing.** Any sample that showed variant bands was sequenced by direct sequencing of the PCR product. For sequencing exons 5 to 8 and the noncoding strand of exon 4, a fragment of the p53 gene from exon 4 to exon 9 (2.9 kilobases in length) was amplified by 32 PCR cycles with oligonucleotide primers described elsewhere (3), and this double-stranded PCR fragment was used as a template. For sequencing exon 10 and the coding strand of exon 4, a single-stranded DNA template was amplified by asymmetric PCR by using the primers for SSCP analysis. Each amplified DNA template was purified by an ultrafiltration kit (Suprec-02; Takara Shuzo Co., Kyoto, Japan) or by recovery from low melting agarose gels by using the phenol/thaw technique. The products were electrophoresed on denaturing 6% polyacrylamide gels with and without 10% glycerol. Electrophoresis was performed at 3 W for 14 to 18 h at room temperature. After being dried, the gels were exposed to X-ray films for 1—2 days at -70°C without intensifying screens.

**SSCP analyses with the use of a CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit (New England Biolabs, Beverly, MA). The products were electrophoresed on denaturing 6% polyacrylamide gels. All putative mutations were confirmed on both coding and noncoding strands in an independent PCR template.** When a mutation was identified, the corresponding normal DNA samples, if available, were also sequenced to confirm that the mutations occurred as somatic events.

**RFLP Analysis.** RFLP analysis was performed on 56 Japanese cases in order to examine for the LOH on chromosome 17p. The analysis was carried out as described previously (9). The following five DNA markers on chromosome 17p were used to detect LOH: YNZ22 (D17S5), YNH37.3 (D17S28), MCT35.1 (D17S31), BHP53 (p53), and HF12–2 (D17S1). Probes YNZ22 and BHP53 were kindly provided by Dr. Y. Nakamura. The original references for all these markers have been cited and recorded in Human Gene Mapping 11 (27). The results of the RFLP analysis in the 48 cases have been published previously (9).

**Statistical Analysis.** All 2 × 2 tables were analyzed by the χ2 test or by the Fisher’s exact probability test. P values less than 0.05 were considered statistically significant.

**RESULTS**

In total, p53 gene mutations were detected in 20 (32%) of 61 Japanese cases and 6 (86%) of 7 Egyptian cases. Table 1 shows the characteristics of the p53 gene mutations together with the relevant histological, epidemiological, and clinical data from each case. Patients 30 and 59 previously received intravesical instillation therapy with pirarubicine (one of the anthracyclines) and intravesical Bacille Calmette-Guerin (BCG) instillation, but all these patients were excluded from further analysis. There were no evident differences in the smoking histories of the patients.

**Table 1: Characteristics of p53 gene mutations in urothelial cancers**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Site</th>
<th>Cell type</th>
<th>Grade/stage</th>
<th>Exon</th>
<th>Codon</th>
<th>Mutation</th>
<th>Amino acid substitution</th>
<th>Smoking history</th>
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<tbody>
<tr>
<td>UT74</td>
<td>B</td>
<td>SCC</td>
<td>3/pT1</td>
<td>4</td>
<td>91</td>
<td>TGG→TGT</td>
<td>Trp→Cys</td>
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<td>CCC</td>
<td>2/pT3</td>
<td>5</td>
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<td>TGG→TGC</td>
<td>Trp→Ser</td>
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<tr>
<td>UT46</td>
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<td>TCC</td>
<td>3/pT1</td>
<td>5</td>
<td>158</td>
<td>CCC→CTC</td>
<td>Pro→Leu</td>
<td>0</td>
</tr>
<tr>
<td>UT8</td>
<td>TCC</td>
<td>2/pT1</td>
<td>5</td>
<td>177</td>
<td>158</td>
<td>TCA→TGC</td>
<td>Ser→stop</td>
<td>0</td>
</tr>
<tr>
<td>UT30</td>
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<td>SCC</td>
<td>3/pT3</td>
<td>5</td>
<td>183</td>
<td>TCA→TGA</td>
<td>Ser→stop</td>
<td>0</td>
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<td>UT70</td>
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<td>SCC</td>
<td>3/pT3</td>
<td>5</td>
<td>183</td>
<td>TCA→TGA</td>
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<td>TCC</td>
<td>3/pT1</td>
<td>5</td>
<td>248</td>
<td>CCG→TGG</td>
<td>Arg→Trp</td>
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<tr>
<td>UT42</td>
<td>TCC</td>
<td>2/pT1</td>
<td>5</td>
<td>255</td>
<td>248</td>
<td>ACC→TTC</td>
<td>Ile→Phe</td>
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</tr>
<tr>
<td>UT58</td>
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<td>TCC</td>
<td>3/pT2</td>
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<td>277</td>
<td>TGG→TTT</td>
<td>Cys→Phe</td>
<td>0</td>
</tr>
<tr>
<td>UT48</td>
<td>B</td>
<td>TCC</td>
<td>3/pT2</td>
<td>8</td>
<td>285</td>
<td>GAG→AAG</td>
<td>Glu→Lys</td>
<td>0</td>
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</table>

**Tumors in smokers from Japan**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Site</th>
<th>Cell type</th>
<th>Grade/stage</th>
<th>Exon</th>
<th>Codon</th>
<th>Mutation</th>
<th>Amino acid substitution</th>
<th>Smoking history</th>
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<td>2/pT2</td>
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<td>273</td>
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<td>Arg→His</td>
<td>4-yr abstinence</td>
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<td>UT59</td>
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<td>TCC</td>
<td>3/pT3</td>
<td>8</td>
<td>277</td>
<td>TGT→TTT</td>
<td>Cys→Phe</td>
<td>4-yr abstinence</td>
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</table>

**Tumors in smokers from Japan**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Site</th>
<th>Cell type</th>
<th>Grade/stage</th>
<th>Exon</th>
<th>Codon</th>
<th>Mutation</th>
<th>Amino acid substitution</th>
<th>Smoking history</th>
</tr>
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<td>UT3</td>
<td>U</td>
<td>TCC</td>
<td>3/pT2</td>
<td>5</td>
<td>136</td>
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<td>Silent</td>
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<td>UT51</td>
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<td>TCC</td>
<td>3/pT4</td>
<td>5</td>
<td>164</td>
<td>AAG→TAG</td>
<td>Lys→stop</td>
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<td>SCC</td>
<td>3/pT3</td>
<td>5</td>
<td>166</td>
<td>TCA→TGC</td>
<td>Ser→stop</td>
<td>20×40–5×10</td>
</tr>
<tr>
<td>UT19</td>
<td>B</td>
<td>SCC</td>
<td>3/pT3</td>
<td>6</td>
<td>192</td>
<td>CAG→TAG</td>
<td>Glu→stop</td>
<td>10×60</td>
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<tr>
<td>UT10</td>
<td>B</td>
<td>TCC</td>
<td>3/pT3</td>
<td>7</td>
<td>246</td>
<td>ATC→ACC</td>
<td>Ile→Thr</td>
<td>10×32–40×10</td>
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<tr>
<td>UT72</td>
<td>P</td>
<td>TCC</td>
<td>3/pT2</td>
<td>7</td>
<td>257</td>
<td>CTC→CGG</td>
<td>Leu→Pro</td>
<td>10×53</td>
</tr>
<tr>
<td>UT50</td>
<td>B</td>
<td>TCC</td>
<td>3/pT2</td>
<td>7</td>
<td>258</td>
<td>GAA→CAA</td>
<td>Glu→Arg</td>
<td>20×50</td>
</tr>
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</table>

**Tumors with schistosomiasis from Egypt**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Site</th>
<th>Cell type</th>
<th>Grade/stage</th>
<th>Exon</th>
<th>Codon</th>
<th>Mutation</th>
<th>Amino acid substitution</th>
<th>Smoking history</th>
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<tbody>
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<td>EBT6</td>
<td>B</td>
<td>SCC</td>
<td>N1</td>
<td>5</td>
<td>146</td>
<td>TGG→TGA</td>
<td>Trp→stop</td>
<td>NI/</td>
</tr>
<tr>
<td>EBT21</td>
<td>B</td>
<td>SCC</td>
<td>N1</td>
<td>5</td>
<td>154</td>
<td>GCC→GTC</td>
<td>Gly→stop</td>
<td>NI</td>
</tr>
<tr>
<td>EBT22</td>
<td>B</td>
<td>SCC</td>
<td>N1</td>
<td>5</td>
<td>157</td>
<td>GTC→TTC</td>
<td>Val→Phe</td>
<td>NI</td>
</tr>
<tr>
<td>EBT25</td>
<td>B</td>
<td>SCC</td>
<td>N1</td>
<td>6</td>
<td>194</td>
<td>5 bp del</td>
<td>Frameshift</td>
<td>NI</td>
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<tr>
<td>EBT12</td>
<td>B</td>
<td>SCC</td>
<td>N1</td>
<td>8</td>
<td>283</td>
<td>GCC→CCC</td>
<td>Arg→Pro</td>
<td>NI</td>
</tr>
<tr>
<td>EBT11</td>
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<td>SCC</td>
<td>N1</td>
<td>10</td>
<td>339</td>
<td>5 bp ins</td>
<td>Frameshift</td>
<td>NI</td>
</tr>
</tbody>
</table>

* Primary site of tumor: B, bladder; U, ureter; P, renal pelvis.
* TCC, transitional cell carcinoma; SCC, squamous cell carcinoma; bp, base pair; del, deletion; ins, insertion; NI, no information.
* According to the WHO (22) and TNM (23) classifications.
* Cigarettes per day × year; →, followed by.
Calmette-Guerin therapy, respectively. In all Japanese cases with p53 gene mutations, no systemic chemotherapy or no radiotherapy was performed previously. Both patients 59 and 86 had stopped smoking 4 years prior to the admission for bladder cancer.

In case 3 (UT3), three somatic mutations were found in exons 5, 6, and 8, and the mutation found in exon 5 was silent. We did not perform cloning on the PCR products to determine the allelic nature of these triple mutations. However, this tumor showed LOH on chromosome 17p, and all variant bands of the three mutations in the SSCP analysis showed much higher intensity than the corresponding normal bands (data not shown). Since the normal faint bands were presumably due to contaminated noncancerous cells as evidenced by LOH analysis, it is most likely that UT3 contained triple tandem mutations. In addition, the intratumoral genetic heterogeneity might have existed in this tumor.

Of the 28 mutations detected, 14 mutations (50%) of the missense type or of the nonsense type were located in highly conserved regions of the p53 gene during the evolution (28). Another five mutations detected outside the conserved regions resulted in a premature termination or a frameshift affecting these conserved regions. Furthermore, all the other point mutations, except for the silent mutation in exon 5 in UT3, resulted in amino acid alterations at codons that are conserved in humans, monkeys, rats, and mice (28).

In order to elucidate the contribution and influence of etiological backgrounds on the p53 mutations, the mutational events were classified according to the pattern of base substitution and the occurrence of frameshifs (Fig. 1). Of the Japanese cases, the incidence of p53 gene mutations was not significantly influenced by habitual smoking: 8 (27%) of 30 current smokers, 2 (50%) of 4 exsmokers, and 10 (37%) of 27 nonsmokers had p53 gene mutations. Significantly, A:T to G:C transitions were observed in 4 (40%) of 10 mutations from smokers, whereas this type of transition was not observed in any of the 10 mutations from nonsmokers (P < 0.05), in either of the 2 mutations from exsmokers, or in any of the 6 mutations associated with schistosomiasis (Figs. 1 and 2). On the other hand, G:C to A:T transitions were detected in 4 (40%) of 10 mutations from nonsmokers, whereas such a mutation was detected in only 1 (10%) of 10 mutations from current smokers. G:C to A:T transitions at the CpG site were observed in two tumors (UT16 and UT46) from nonsmokers and in one tumor (UT86) from an exsmoker.

With respect to the histopathological findings, the incidence of p53 gene mutations increased with tumor grade and stage in bladder cancers in the Japanese cases (Table 2). Although the number of renal pelvic and ureteral cancers examined was small, no obvious difference was found between these cancers and bladder cancers with respect to the incidence and pattern of p53 gene mutations. With respect to the cell type, p53 gene mutations were found in 4 (67%) of 6 Japanese cases with squamous cell carcinomas. Together with the Egyptian cases, 10 (77%) of the 13 urothelial tumors with squamous cell carcinomas contained p53 gene mutations. There appears to be no specific base substitution pattern for squamous cell carcinomas with schistosomiasis. However, all 8 base substitutions observed in tumors with squamous cell carcinomas occurred at C:G sites, whereas base substitutions at A:T sites were detected in 6 (33%) of the 18 mutations in pure transitional cell carcinomas (Table 1).

Of the 56 cases examined by RFLP analysis, 50 cases were constitutionally heterozygous at least one locus on chromosome 17p. Of these informative cases, the LOH on chromosome 17p was detected in 21 cases. The frequency of p53 mutations in tumors with the LOH was significantly higher (57%; 12 of 21) than the frequency in tumors without the LOH (3%; 1 of 29) (P < 0.0001). In 9 cases with the LOH on chromosome 17p, there were no p53 gene mutations detected by SSCP analysis. To exclude the possibility of pseudonegative results from the SSCP analysis, direct sequencing of the coding region from exons 4 to 8 in 7 of these 9 cases was performed, but no mutation was detected (data not shown). The proportion of cases with advanced tumors (apT2) was significantly higher in cases with both the p53 gene mutation and the 17p loss (100% of 12) than in cases with only the 17p loss and no detectable p53 gene mutations (44% of 9; P < 0.01), or in cases with neither the p53 gene mutation nor the 17p loss (10% of 28, P < 0.0001).

DISCUSSION

Since the p53 gene is a frequent target of mutation in various human cancers, the concept that “carcinogens leave fingerprints” can be confirmed by analyzing the p53 gene mutation spectrum in human cancers (10, 11). Convincing examples of the close relationship between each carcinogen and its particular mutation pattern in the p53 gene has been demonstrated in several cancers (2, 10–18).

Considering the above evidence, the A:T to G:C transitions observed in tumors from the current smokers in this study strongly suggest the involvement of a specific mutagen(s) induced by cigarette smoking in urothelial cancer. Although cigarette smoking has been shown to increase the levels of various carcinogens such as aromatic amines excreted in urine (29, 30), it is unclear from this study which mutagen is responsible for the specific A:T to G:C transition observed. Interestingly, in experimental rat bladder cancers induced by dietary N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide, about 70% of the Harvey ras gene mutations were A:T to G:C transitions (31). It is therefore of interest to know whether the same or similar metabolite(s) such as dietary N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide is selectively increased in urothelial cells by cigarette smoking. Since the mutations other than the A:T to G:C transitions might be caused by other mutagens induced by cigarette smoking, a considerable proportion of the p53 gene mutations found in smokers may be attributed to a mutagen(s) induced as a result of cigarette smoking.

In a recent study examining p53 gene mutations of bladder cancers in the United States and Denmark (32), no predominant A:T to G:C transition mutations have been observed in association with cigarette smoking. Interestingly, in small cell lung cancers, A:T to G:C transi-
HAEMATOBIAE EGGs MAY INDUCE BLADDER CANCER (33). FURTHERMORE, CLASSIFICATIONs, RESPECTIVELY.

**REFERENCES**

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