Relationship between TP53 tumour suppressor gene mutations and smoking-related bulky DNA adducts in a lung cancer study population from Hungary

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Lung cancer rate in Hungary is one of the highest in the world among men and also very high among women, for reasons not clearly understood yet. The aim of the study was to explore characteristics of DNA damage and TP53 gene mutations in lung cancer from Hungary. Tissue samples from 104 lung resections for lung cancer patients, both men and women, operated on for non-small cell lung cancer, specifically, primary squamous cell carcinoma or adenocarcinoma were studied. Of the cases, 37% smoked up to the surgery, 24% stopped smoking within 1 year before the surgery, 26% stopped smoking more than a year before the surgery and 13% never smoked. TP53 mutations were detected by denaturant gradient gel electrophoresis, automated capillary electrophoresis single-strand conformation polymorphism and sequencing. Bulky DNA adduct levels were determined by 32P-post-labelling in non-tumorous lung tissue. In total, 45% (47/104) of the cases carried TP53 mutation. The prevalence of TP53 mutations was statistically significantly associated with duration of smoking, tumour histology and gender. Smokers had approximately twice as high bulky adduct level as the combined group of former- and never-smokers (10.9 ± 6.5 versus 5.5 ± 3.4 adducts/106 nucleotides). The common base change G → T transversion (8/43; 19%) was detected exclusively in smokers. For the first time, we demonstrate that most carriers of G → T transversions had also a high level of bulky DNA adducts in their non-tumourous lung tissue. Our study provides evidence for a high burden of molecular alterations occurring concurrently in the lung of lung cancer patients.

Materials and methods

Study population

The sample set consisted of 104 lung cancer cases, 62 males (60%) and 42 females (40%), who underwent lung resection for primary lung cancer. This series of cases with adenocarcinoma (n = 67) and squamous cell carcinoma (SCC; n = 37), the two major histological types of lung cancer, were selected from a larger Hungarian study population comprising various histological types of lung cancer. In the sample set, among men, 55% (34/62) presented with adenocarcinoma and 45% (28/62) with SCC. Among women, 79% (33/42) of the cases had adenocarcinoma. After the selection for histology, there was no additional selection for smoking status. The sample set partially overlaps with the study population of a previously published work (17). The samples of lung tumour and histologically non-tumourous peripheral lung tissue were obtained with informed consent of the patients and the research was approved by the local and national ethics committee in Hungary. Information on smoking history was obtained from the patients by self-reporting. Three major smoking categories were defined as never-smokers (10.9 years (4), but the risks associated with other aetiological factors are not known to be different in Hungary from those that exist in many other European countries (6–8).

The tumour suppressor gene TP53 is one of the most studied genes in cancer research. The protein product of TP53 participates in cell cycle control, apoptosis and DNA repair and is central in cellular response to different kinds of stress (9). In non-stress situations, the p53 protein is expressed in almost all tissues at low level and degrades rapidly. DNA damage, activation of oncogenes and also non-genotoxic stresses, e.g. heat and hypoxia, can lead to activation and stabilization of the p53 by post-translational modifications (9). TP53 gene mutations are common in human tumours and several types of environmental cancer have been shown to contain mutations associated with exposures (10,11). According to the International Agency for Research on Cancer (IARC) database, in average, 39% of lung cancers carry TP53 mutations (12). In many cancers, the prevalence of TP53 mutations tend to be higher in smokers than in non-smokers (13–15) and a special mutation spectrum attributed to tobacco smoking has been reported (16).

In the framework of a multi-end point molecular epidemiological study among Hungarian lung cancer patients, we explored molecular DNA alterations showing association with cigarette smoking. We investigated the frequency and the type of TP53 gene mutations and analysed the results in relation to smoking characteristics and gender in the two major histological types of lung cancer. In parallel, we determined the levels of bulky DNA adducts in non-tumorous tissue specimens from the same patients, to search for association between TP53 mutations and tobacco smoke-induced primary DNA damage.

Introduction

Lung cancer is among the leading causes of cancer death in the developed countries (1). In Hungary, incidence of lung cancer is very high, it is 138/100 000 among men and 73/100 000 among women according to statistics from 2007 (2–4). The primary risk factor for lung cancer is smoking (5). Besides smoking, aetiological factors for lung cancer include exposure to other lung carcinogens, such as asbestos and radon. In Hungary, cigarette consumption is high, with a prevalence of ~30% of regular daily smokers in the population at age of 15+ years (4), but the risks associated with other aetiological factors are not known to be different in Hungary from those that exist in many other European countries (6–8).
follows: (i) smokers, that included current smokers who smoked up to surgery, and those patients who stopped smoking within 1 year before surgery, (ii) former-smokers who gave up smoking >1 year before surgery and (iii) never-smokers. The smoking categories were set up on the basis of previous studies (17,18). Out of the 104 lung cancer patients, 37% (38/104) were current smokers, 24% (25/104) quit smoking within 1 year before the surgery, 26% (27/104) had given up smoking >1 year before the surgery and 13% (14/104) had never smoked. Heavy smoking was common among both men and women. Almost all the ever-smoking men consumed 20 or more cigarettes per day (93%, 51/55), and most of them had smoked longer than for 20 years (85%, 46/54). More than half of the smoking women (66%, 21/32) had similarly smoked at least one pack per day and had a long smoking history (>20 years of smoking). Women were in majority among the never-smokers (9 out of 14). Demographic characteristics and data on smoking history of the present study population are given in Table I.

DNA isolation

Samples of macroscopically non-tumorous peripheral lung tissue (100–500 mg) and tumour tissue (20–500 mg) were obtained from the resected lobes of the patients and frozen within 2 h of dissection. The tissue samples were stored at −80°C prior to DNA isolation. DNA was isolated by a phenol–chloroform–isoamyl alcohol extraction procedure as described previously (17).

TP53 mutation detection

TP53 mutations were analysed in exons 5–9 and 11. The TP53 gene sequences were amplified from the DNA samples by polymerase chain reaction (PCR). Denaturant gradient gel electrophoresis (DGGE) and automated capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) were applied to screen for TP53 mutations according to Holmila and Husgafvel-Pursiainen (19). In short, in DGGE analysis, the PCR products were run in perpendicular acrylamide gels containing denaturing gradient in 1× Tris-acetate-EDTA buffer at 60°C (in DCode Universal Mutation Detection System apparatus; Bio-Rad, Hercules, CA) followed by ethidium bromide staining and detection by ultraviolet. For CE-SSCP, the diluted PCR products were denatured at high temperature and then plunged into ice directly. The CE-SSCP analysis was performed using ABI PRISM 310 capillary sequencer (Applied Biosystems or with ABI PRISM 3100 Avant capillary sequencer using a separation media of 5% GeneScan Polymer (Applied Biosystems, Foster City, CA) in 1× running buffer containing ethylenediaminetetraacetic acid (Applied Biosystems) and 10% glycerol.

The locations and types of the mutations detected by DGGE or CE-SSCP were determined by direct sequencing (ABI Prism 310 capillary sequencer). As reported before (19), DNA was amplified by PCR reaction and the PCR products were purified with QIAquick PCR purification Kit (Qiagen, Hilden, Germany). The sequencing reaction was prepared with BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems). Polymorphisms were ascertained by sequencing the DNA derived from both tumour and non-tumorous tissues. All mutation analyses were performed at the laboratories of the Finnish Institute of Occupational Health (Helsinki, Finland).

Bulky DNA adducts were determined in non-tumorous peripheral lung DNA by the 32P-post-labelling method essentially as described before (17). Briefly, DNA (4 µg) was digested overnight with micrococcal nuclease (Sigma, St Louis, MO) and spleen phosphodiesterase (ICN and MP Biomedicals). Adduct enrichment was made with nuclease P1 (Sigma) digestion of the normal mononucleotides. Radiolabelling occurred with 50 µCi carrier-free [γ-32P]ATP (end-labeling grade; ICN Biomedicals, Inc., Aurora, OH and MP Biomedicals, LLC, Irvine, CA) and 6 U of T4 polynucleotide kinase (USB Corporation, Cleveland, OH and Fermentas, Vilnius, Lithuania). Multidirectional thin-layer chromatography of the radiolabelled DNA digests was performed on 10 × 10 cm polyethyleneimine/ cellulose sheets (Macherey-Nagel, Düren, Germany) as detailed earlier (17). Radioactivity patterns were detected by Cerenkov counting and electronic autoradiography (InstantImager; Packard Instrument Co., Inc., Meriden, CT). Background radioactivity of the blank area, corrected for the size of the adduct areas was subtracted from the radioactivity of the adduct areas. DNA adduct levels were calculated by normalization for an in vitro-modified benzo[a]pyrene diol-epoxide-DNA standard (110 adducts/106 nucleotides) as described earlier (17). Two to four replicate analyses were performed with each human DNA sample in separate assays.

Results

Tumour histology and smoking habits

The histological type of the tumour showed close association with smoking habits. All SCCs were among ever-smokers. The proportion of SCC against adenocarcinoma rose with the duration of smoking. Among those who had smoked for 35 years or less, adenocarcinoma predominated, but the difference disappeared over a longer duration of smoking. All never-smokers, mostly women, presented with adenocarcinoma (Table I).

Table I. Demographic characteristics and details of smoking for the lung cancer cases studied

<table>
<thead>
<tr>
<th>Patient feature</th>
<th>Smokers a  (n = 63), n (%)</th>
<th>Former-smokers b  (n = 27), n (%)</th>
<th>Never-smokers  (n = 14), n (%)</th>
<th>All cases  (n = 104), n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (no. of cases)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>37 (59)</td>
<td>20 (74)</td>
<td>5 (36)</td>
<td>62 (60)</td>
</tr>
<tr>
<td>Female</td>
<td>26 (41)</td>
<td>7 (26)</td>
<td>9 (64)</td>
<td>42 (40)</td>
</tr>
<tr>
<td>Tumour histology (no. of cases)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>38 (60)</td>
<td>15 (56)</td>
<td>14 (100)</td>
<td>67 (64)</td>
</tr>
<tr>
<td>SCC</td>
<td>25 (40)</td>
<td>12 (44)</td>
<td>–</td>
<td>37 (36)</td>
</tr>
<tr>
<td>Age at diagnosis (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age range</td>
<td>35–78</td>
<td>45–79</td>
<td>48–76</td>
<td>35–79</td>
</tr>
<tr>
<td>Mean age</td>
<td>56 ± 9</td>
<td>61 ± 3</td>
<td>59 ± 9</td>
<td>58 ± 9</td>
</tr>
<tr>
<td>Median age</td>
<td>57</td>
<td>60</td>
<td>58</td>
<td>58</td>
</tr>
<tr>
<td>Daily smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4–25 cigarettes/day (no. of cases)</td>
<td>49 (78)</td>
<td>15 (56)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>26–60 cigarettes/day (no. of cases)</td>
<td>12 (19)</td>
<td>11 (41)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ND a</td>
<td>2 (3)</td>
<td>1 (4)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mean consumption (cigarettes/day)</td>
<td>23 ± 10</td>
<td>28 ± 15</td>
<td>–</td>
<td>24 ± 12</td>
</tr>
<tr>
<td>Median consumption (cigarettes/day)</td>
<td>20</td>
<td>23</td>
<td>–</td>
<td>20</td>
</tr>
<tr>
<td>Duration of smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5–20 yrs (no. of cases)</td>
<td>12 (19)</td>
<td>9 (33)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>21–35 yrs (no. of cases)</td>
<td>25 (40)</td>
<td>10 (37)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>36–60 yrs (no. of cases)</td>
<td>25 (40)</td>
<td>6 (22)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ND a</td>
<td>1 (2)</td>
<td>2 (7)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mean yrs of smoking</td>
<td>33 ± 12</td>
<td>28 ± 9</td>
<td>32 ± 11</td>
<td></td>
</tr>
<tr>
<td>Median yrs of smoking</td>
<td>33</td>
<td>30</td>
<td>–</td>
<td>30</td>
</tr>
</tbody>
</table>

aSmokers included current smokers and those who quit smoking within 1 year before surgery.

bFormer-smokers were defined as those, who gave up smoking >1 year before the surgery.

ND, not defined.
**TP53 mutations versus histology, gender and smoking characteristics**

Forty-five percent of the subjects (47/104) carried a TP53 mutation, and altogether, 51 TP53 mutations were detected; 44 cases had a single mutation, two cases carried a double mutation and one case three different mutations (complete TP53 mutation data are presented in the Supplementary Table I, available at Mutagenesis Online). The mutation frequency was significantly higher in SCC (70%, 26/37) than in adenocarcinoma (31%, 21/67) ($P = 0.0002$; Table II). About half of the smokers (i.e. those who smoked up to surgery and short-term quitters) carried a mutation, and they tended to carry more mutations (49%, 31/63) than former-smokers (33%, 9/27) ($P = 0.25$; Table II).

Overall, men exhibited statistically significantly higher mutation frequency than women (55 versus 31%), ($P = 0.045$; Table II). When stratified according to histological type and ever-/never-smoking status, there was no statistically significant difference between the genders. However, the difference between ever-smoker males (38%, 11/29) and ever-smoker females (13%, 3/24) was close to borderline significance ($P = 0.059$) in the adenocarcinoma group, whereas in SCC group, the mutation frequency was fairly similar among male and female ever-smokers. Self-reported never-smokers, all with adenocarcinoma, were also found to carry mutations, with an overall frequency of 50% (total 7/14; males 3/5 and females 4/9) (Table II).

The frequency of mutations increased with the duration of smoking. Among those who had smoked up to 20 years, mutations were found in 14.3% (3/21) of the cases, whereas among those who had smoked longer than for 20 years, more than half carried a TP53 mutation (36/66, 54.5%) ($P = 0.002$) (Figure 1).

**Codon distribution and types of TP53 mutations**

Sequence identification was successful for 43 of the 51 mutations. In adenocarcinoma, mutations were more distributed along the gene, whereas in SCC, 86% (26/37) of the mutation were found in codons situated in exons 5 and 8. The most commonly mutated TP53 codon was codon 175 (three mutations), followed by codons 146, 157, 248 and 266, each with two mutations. The codons where the mutations had occurred in never-smokers were different from those mutated in smokers, with the exception of one.

The most common sequence change detected was G → A transition (8/43 plus one intronic G → A transition found in a former smoker, 21%), followed by G → T transversion (8/43, 19%), frameshift mutation (7/43, 16%) and G → C transversion (7/43, 16%) (Figure 2). G → A transitions were more commonly seen among former-smokers (5 out of 10) and never-smokers (2 out of 6) than among smokers (2 out of 27 mutations) (Figure 2A). G → T transversions were found exclusively in smokers, as were the majority of G → C transversions (Figure 2A).

Almost all frameshift mutations were seen in ever-smokers. They were more frequent at a long smoking history with at least 35 years of smoking (4/12, 33%) as compared to fewer years of smoking (2/23, 9%). Among never-smokers, missence mutations dominated.

**Mutations versus smoking-related DNA adducts**

We analysed smoking-related bulky DNA adducts in non-tumorous lung tissue in relation to TP53 mutation in the tumour tissue from the same patients.

Smokers, including those who smoked up to surgery and short-term quitters, had approximately twice as high bulky DNA adduct level as the merged group of former-smokers and never-smokers, 10.9 ± 6.5 versus 5.5 ± 3.4 adducts/10⁸ nucleotides (mean ± standard deviation). The difference between the combined groups was statistically significant ($P < 0.0001$). Among smokers, bulky DNA adduct levels were the same in mutation carriers and non-carriers. However, among former-smokers and never-smokers, the mutation carriers tended to have higher level of DNA adducts in comparison to those with the wild-type TP53 gene ($P ≥ 0.1$) (Table III).

Furthermore, we investigated the different types of TP53 mutations that had occurred in the tumour by the levels of bulky DNA adducts measured in the non-tumorous lung tissue. The carriers of a G → T transversion were those found with a high burden of adducts in their lungs (Figure 2B). Five out of eight (62.5%) cases with a G → T transversion exhibited >12 adducts/10⁸ nucleotides, in one case, adducts were at the medium level and in only two cases (2/8, 25%) adducts were in the lowest range (<6 adducts/10⁸ nucleotides) (Figure 2B).

**Discussion**

The present study provides evidence for a high burden of smoking-related molecular alterations occurring concurrently in the lungs of lung cancer patients. We observed frequent TP53 mutations and a simultaneous high level of lung DNA adducts, predominantly detected in G → T mutation carriers, among lung cancer patients who smoked. We investigated a series of 104 lung SCC and adenocarcinoma cases from Hungary, in which both male and female cases were adequately represented (with 60 and 40%, respectively). The majority in

<table>
<thead>
<tr>
<th>Table II. Lung cancer cases with and without TP53 gene mutation by gender, tumour histology and smoking status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
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<tr>
<td></td>
</tr>
<tr>
<td>TP53 mutation positive</td>
</tr>
<tr>
<td>TP53 mutation negative</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

*Smokers include current smokers and those who quit smoking within 1 year before surgery.
†Former-smokers were defined as those, who gave up smoking >1 year before the surgery.
‡Difference between females and males.
§Difference between adenocarcinoma and SCC.
NS, not significant, difference between smokers and never-smokers.
NS, not significant, difference between smokers and never-smokers.
both genders, >90% of men and 66% of women had smoked >20 cigarettes per day on average and had a long history of smoking with a median of 30 years of smoking, reflecting heavy smoking habits of Hungarian smokers (4).

In the current series of lung cancers, 45% of all cases carried a TP53 mutation; 55% of men and 31% of women were positive for mutation. The overall mutation frequency was relatively high as compared to that detected in many lung cancer populations (20–24); however, some studies have also reported higher frequencies (25–28). We found that smokers (the combined group of those who smoked up to surgery and short-term quitters) representing 59% of the male and 41% of the female cases carried more mutations than the former-smokers. The frequency of mutations increased with the increasing duration of smoking, in line with earlier findings (27,29). A high mutation load in heavy smokers with long duration of smoking is in accordance with the epidemiological evidence of associating heavy and persistent smoking with high risk of lung cancer (30). Among never-smokers, half of cases carried a mutation, a frequency higher than that found in many studies (31) but not exceptional (27).

One of the most common base changes detected in the present set of lung tumours was G → T transversion that was found exclusively in smokers. According to the IARC TP53 mutation database, the mutation spectrum characteristic to lung cancer contains ~30% G → T transversions, detectable on many of the frequently mutated codons (12). A similar mutation profile has been found in non-tumorous lung tissue from smoking lung cancer patients (32). This specificity is compatible with the reported presence of polycyclic aromatic hydrocarbon (PAH)-related bulky DNA adducts in lung tissue from smokers (33), as well as with the evidence of association between DNA adducts and types of mutations resulting from exposure to benzo[a]pyrene (BaP) and other PAH compounds (16,34,35). For the first time, we were able to demonstrate that a majority of the lung cancer cases exhibiting a G → T transversion were, indeed, those with high levels of smoking-related bulky adducts in non-tumorous lung tissue. Among former- and never-smokers, higher levels of bulky DNA adducts were detected in non-tumorous lung tissue from TP53 mutation carriers as compared to non-carriers (P = 0.076). The present findings strongly support the postulation that the specific mutation spectrum of smokers originates from continued exposure to a complex mixture of carcinogenic and mutagenic PAHs (34–37). Other factors, such as selective DNA repair, are likely to participate to some extent in the

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**Fig. 1.** Occurrence of TP53 mutation by the duration of smoking. Percentages of TP53 mutations or wild type are indicated: M, mutated; WT, wild type.

**Fig. 2.** Distribution of base changes in the exons according to smoking status (A) and bulky DNA adducts level as detected from paired non-tumorous lung tissues (B). Smokers include current smokers and those who quit smoking within 1 year before surgery.
process (34,36–39). It is also likely that other tobacco smoke carcinogens also contribute to the mutation profile (34,40,41).

Besides G → T transversions, also G → A transitions, frameshifts and G → C transversions were commonly found in this study population. G → C transversions were detected in smokers, almost exclusively, whereas G → A transitions were more prevalent among former- and never-smokers. Previous studies have also associated G → A transitions with never smoking (16,22,27). Frameshift mutations were frequently seen in smokers, who had smoked >35 years; none was detected in smokers who had smoked for 20 or fewer years.

The codon distribution of the mutations followed overall the general pattern found in lung cancer (31). The most commonly mutated codon in our study was codon 157, one of the mutation hot spots in human cancer, followed by codons 146, 248 and 266. Codon 157 is frequently mutated in lung cancer patients with and without TP53 mutation (12). In our study, G → T transversions was the most common type of mutation, followed by G → A transitions and frameshifts (116,22,27). The most frequent frameshift mutations were observed in smokers, who had smoked >35 years; none was detected in smokers who had smoked for 20 or fewer years.

The prevalence of TP53 mutations in SCC was particularly high (70%) in our study, similar to that observed by Le Calvez et al. (27) recently. The mutation frequency reported for SCC in the IARC database is clearly lower [48.7%; (12)] than the present one; this may again reflect the effect of heavy smoking in the present lung cancer series. On the other hand, the mutation frequency detected in adenocarcinoma was very close to that reported in the database (12), with 30.6% in the database Version R13 versus 31% in our series. In our study, adenocarcinomas from male patients carried more mutations than adenocarcinomas from female patients. The overall codon distribution of the mutations followed the pattern reported in database for lung cancer (12).

In summary, we investigated a series of non-small cell lung cancer, SCC or adenocarcinoma histological type in a Hungarian study population. We found statistically significant association between TP53 mutations and duration of smoking, gender and histology. We explored that smoking-related bulky DNA adducts were at a high level in the non-tumorous lung tissue in those cases who carried a G → T transversion, a signature mutation of tobacco smoking. Collectively, our findings underline the impact of the mutagenic and carcino-TP53 mutations and DNA adducts in lung cancer

Table III. Levels of bulky DNA adducts in non-tumorous lung tissue of lung cancer patients with and without TP53 mutation in the tumour

<table>
<thead>
<tr>
<th>Smoking status</th>
<th>Bulky DNA adducts/10^8 nucleotides, mean ± SD (n)</th>
<th>P-value^a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TP53 mutation carriers</td>
<td>TP53 mutation negative cases</td>
</tr>
<tr>
<td>Smoker^b</td>
<td>10.9 ± 6.4 (31)</td>
<td>10.9 ± 6.7 (32)</td>
</tr>
<tr>
<td>Former-smoker^c</td>
<td>7.6 ± 4.2 (9)</td>
<td>5.0 ± 2.7 (18)</td>
</tr>
<tr>
<td>Never-smoker^c</td>
<td>5.7 ± 3.5 (7)*</td>
<td>4.0 ± 3.1 (7)**</td>
</tr>
</tbody>
</table>

^aDifference between TP53 mutation positive and mutation negative cases.
^bSmokers included current smokers and those who quit smoking within 1 year before surgery.
^cFormer-smokers were defined as those, who gave up smoking ≥1 year before the surgery.

*Difference between TP53 mutation carrier smokers and never-smokers, P = 0.065.
**Difference between TP53 mutation negative smokers and never-smokers, P = 0.0008.

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TP53 mutations and DNA adducts in lung cancer

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