Detection of Mutant p53 Protein in Workers Occupationally Exposed to Benzidine

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Abstract: Detection of Mutant p53 Protein in Workers Occupationally Exposed to Benzidine: Cui-Qin Xiang, et al., Shanghai Municipal Center for Disease Control & Prevention, China—To investigate the expression of mutant p53 protein in workers occupationally exposed to benzidine, we detected mutant p53 protein by immuno-PCR assay in the serum of 331 benzidine-exposed healthy workers, while we classified exfoliated urothelial cells in urine samples with Papanicolaou’s grading (PG). The Papanicolaou’s grading classified exfoliated urothelial cells of the subjects from grade I (normal cells) to grade III (suspicious malignant cells). The subjects were also divided into high, medium and low exposure groups according to the exposure intensity index. The results revealed that mutant p53 protein in the medium and high exposure groups were significantly higher than the in low exposure group (p<0.05), and in PG II and III were significantly higher than in the PG I (p<0.05). There was no significant difference among Papanicolaou’s gradings strata in the low exposure group on the incidence and quantity of mutant p53 protein. In the medium and high exposure groups, the incidence and/or quantity of mutant p53 protein in the stratum of PG II and/or III were significantly higher than that of PG I (p<0.05). Detection of mutant p53 protein in conjunction with benzidine exposure level and Papanicolaou’s gradings of exfoliated urothelial cells could provide more information to help us elevate surveillance efficiency and diagnose bladder cancer in the early period.

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Key words: Benzidine, Exposure intensity index, mutant p53 protein, Exfoliated urothelial cells, Papanicolaou’s grading

Benzidine, a human carcinogen of the bladder was listed as a sufficient carcinogen by the International Agency for Research on Cancer in 1987¹, was once widely used in the dyestuff industries in China. It was officially banned for all industrial applications by the Chinese government in 1976. From then on, benzidine-exposed workers received annual medical examinations. We have carried out medical surveillance for occupational bladder cancer in benzidine-exposed workers in Shanghai continuously for 20 yr using Papanicolaou’s grading, but the efficiency of surveillance is very poor due to the low sensitivity of Papanicolaou’s grading². The mutant p53 protein is detected in many types of neoplasm. Therefore, in order to elevate the efficiency of clinical surveillance and early diagnosis of bladder cancer, we investigated mutant p53 protein in the serum of benzidine-exposed workers and analyzed the results in combination with Papanicolaou’s grade of exfoliated urothelial cells.

Subjects and Methods

Subjects

The study population was 331 benzidine-exposed workers who participated in the medical surveillance in 1999. All of them were members of the benzidine-exposed cohort of the Shanghai dyestuff industry, which was established and has been followed-up since 1983. This cohort initially registered about 1,000 workers from seven factories. Up to 1999, 79 subjects had died of various malignant tumors, and among them 26 cases were of bladder cancer. Industrial hygiene data available for the cohort indicated that benzidine was the only arylamine to which the study subjects were exposed occupationally³. Job titles held by each worker were classified according to different exposure levels to benzidine, based on the classification criteria developed by Bi et al. for this cohort⁴. These criteria were developed based upon knowledge of the operations in the facilities and available industrial hygiene data. Exposure level 1 included
A gene probe (PNC-p53) was constructed according to (1) Plasmid DNA extraction, purification and digestion. An exposure intensity index was estimated as the product of the benzidine exposure level multiplied by the exposure duration in that job. The study subjects were divided into 3 groups according to the exposure intensity index. Subject whose exposure intensity index ≤5 were placed in the low exposure group; Subjects whose exposure intensity index was between 5 and 20 were assigned to the medium exposure group; and Subject whose exposure intensity index >20 were assigned to the high exposure group.

Collection of blood and urine samples
Blood samples of all subjects were collected only after their informed consent was obtained. Blood was immediately frozen at −80°C after collection and was sent to the laboratory later on dry ice. Urine samples were collected from the subjects at the occupational medical clinic between 09:00 AM and 11:00 AM. All samples were the second or later urine of the day. The sample of entire urine was collected from each person.

**Immuno-PCR assays**
Mutant p53 protein in serum was detected using an immuno-PCR assay. The method of detecting mutant p53 protein in serum is described as follows. The molecule of chlorophyll was linked with the molecule of plasmid DNA in a covalent bond to form the plasmid DNA-chlorophyll compound, through connecting to the acidamide chain with the free amidogen (−NH₂) in the emplastic end of bacillus subtilis DB403 plasmid DNA. The head end of the chlorophyll molecule combined with the plasmid DNA in covalence was linked to the mutant p53 protein monoclonal antibody to become the gene probe of mutant p53 protein-chlorophyll-plasmid DNA (PNC-p53). The probe was reacted with mutant p53 protein in serum and the unlinked probes were then washed off. The plasmid DNA resulting from the combination of the gene probe and mutant p53 protein in serum was amplified under certain conditions, which are induced by the plasmid DNA specific primers. The results were observed under ultraviolet light after electrophoresis and staining with EB.

The immuno-PCR detection procedure was as follows. (1) Plasmid DNA extraction, purification and digestion. A gene probe (PNC-p53) was constructed according to the method described by Wu. (2) First, a flexible polycarbonate microtiter plate (Shisheng Co, Shanghai) was coated with 50 µl of 5-fold dilutions of serum sample, overnight at 4°C. Next, the plate was blocked with PBST (PBS solution, Tween) at 37°C for 30 min and incubated with 50 µl of 500-fold dilution of gene probe at 37°C for 45 min. Then the plate was washed with 20 µl PBST for 3 min and the washing procedure was repeated five times. Finally, the plate was cut down along the edge after it had dried up and was subjected to PCR. (3) The PCR was done using 2 µl of forward primer 5’-GGAAGCCTGATCCACCATGACACTTGAC-3’ and 2 µl of reverse primer 5’-AATTCGATGCCTGAAACCTGGCAGG-3’ (primers were synthesized at the Australian Animal Health Laboratory), 2 µl of deoxyribonucleoside triphosphate, 2 µl of Taq DNA polymerase (DNA polymerase and deoxyribonucleoside triphosphate were from Promega), 10 x buffer 3 µl, ddH₂O 19 µl. The PCR program was a 5-min denaturation step at 93°C, followed by 35 cycles of 93°C for 60 s, 50°C for 60 s and 72°C for 120 s, and a final extension step at 72°C for 10 min. The 265bp PCR amplified product was electrophoresed on a 2% agarose gel containing 0.5 µg/ml etidium bromide at 80 V for 30 min. Then the gels were scanned with a VDS (PHARMACIA) and the DNA band intensity was measured using NIH Image 1.54. (4) Determination of the results. If identical 265bp DNA bands existed in both the samples and the positive controls, it indicated that there was mutant p53 protein in the sample serum.

**Classification of exfoliated cells**
The whole urine sample was centrifuged immediately after it had been collected and supernatants were discarded. The remaining pellet of exfoliated cells was firstly fixed with ethanol and ether, then spread on slides, dried and subsequently stained according to Papanicoloau. According to the method of Papanicoloau and Marshall, the cells are always analyzed and classified by the same observer. The morphological grading of cells was performed under light microscopy according to the criteria provided in a Chinese manual. Papanicoloau’s grading classifies exfoliated urothelial cells from grade I (normal cells) to grade V (malignant cells).

**Statistical analysis**
Statistical procedures for all variables included exploratory data analysis and descriptive statistics. The average scanning integral of PCR amplified bands with non-normal distributions was log-transformed then analyzed. All tests of significance were conducted at alpha=0.05 in two-tailed tests.

The incidence of mutant p53 protein was analyzed via χ² test, and log-transformed average scanning integrals of PCR amplified band were analyzed via the F test. SPSS software and SAS software (SAS Institute Inc., Cary, North Carolina, USA) were used for all the analyses.
Results

Characteristics of subjects

The 331 healthy workers occupationally exposed to benzidine included 229 males (range 41–86 yr, mean age 61.2 yr) and 102 females (range 41–77 yr, mean age 60.5 yr). The mean exposure duration was 4.8 yr (range 0.3–25 yr). The subjects were divided into 3 groups based on the exposure intensity index: 143 in the low exposure group, 129 in the medium exposure group and 59 in the high exposure group.

Gel electrophoresis of PCR products

The same 265bp amplified DNA band as found in positive control existed in samples 6, 7, 8 and 9, while no amplified DNA band was seen in samples 4, 5 and 10. Therefore, samples 6–9 were identified as positive and samples 4, 5 and 10 as negative (Fig.1).

Incidences of mutant p53 protein and average scanning integrals of PCR amplified bands among different exposure groups

Incidences of mutant p53 protein in the low, medium and high exposure groups were 30.1%, 48.1% and 49.2% respectively. The incidence of mutant p53 protein in the medium exposure group was very significantly higher than that in the low exposure group ($p<0.01$). The incidence of mutant p53 protein in the high exposure group was significantly higher than that in the low exposure group ($p<0.05$). The average scanning integrals of PCR amplified bands in the low, medium and high exposure groups were 10,741, 13,706 and 12,942 respectively. The log-transformed average scanning integral of PCR amplified bands in the medium exposure group and the high exposure group were significantly than that in the low exposure group ($p<0.05$) (Table1).

Also listed in Table 1 are the stratified data according to Papanicoloau’s grade for each exposure intensity group: 52 in Papanicoloau’s grading I (PG I), 121 in Papanicoloau’s grading II (PG II) and 158 in Papanicoloau’s grading III (PG III). Trend analysis of the exposure intensity index and stratum of Papanicoloau’s grade was performed. The result indicates that there is no correlation between the exposure intensity index and Papanicoloau’s grade ($\chi^2=0.137$, $p=0.711$).

Table 1. Incidences of mutant p53 protein, average scanning integrals of PCR amplified band and trend of exposure intensity index and Papanicoloau’s gradings among different exposure groups

<table>
<thead>
<tr>
<th>Exposure intensity index</th>
<th>Number</th>
<th>Papanicoloau’s grading</th>
<th>p53 positive number</th>
<th>Incidence (%)</th>
<th>Average integral ($\pm S$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low ($\leq 5$)</td>
<td>143</td>
<td>23 52 68</td>
<td>43</td>
<td>30.1</td>
<td>10,741 ± 9,211</td>
</tr>
<tr>
<td>Medium (5–20)</td>
<td>129</td>
<td>21 47 61</td>
<td>62</td>
<td>48.1**</td>
<td>13,706 ± 10,251*</td>
</tr>
<tr>
<td>High (&gt;20)</td>
<td>59</td>
<td>8 22 29</td>
<td>29</td>
<td>49.2*</td>
<td>12,942 ± 9,406*</td>
</tr>
</tbody>
</table>

*p<0.05; ** p<0.01.
The log-transformed average scanning integrals of PCR amplified bands in the PG II and PG III were significantly higher than that in PG I (\(p<0.05\) and \(p<0.01\) respectively) (Table 2). In the medium exposure group, there was also no significant difference within these strata on the incidence of mutant p53 protein, but the log-transformed average integrals in the strata of PG II and PG III were significantly higher than that of PG I (\(p<0.05\)) (Table 3). In the high exposure group, the incidence of mutant p53 protein in the stratum of PG III was significantly higher than that of PG I (\(p<0.05\)) (Table 4). The p53 gene has been found to be the most important tumor-associated human gene, and the development of human tumors is tightly correlated with its deletion, rearrangement and point mutation\(^{17-21}\). p53 allele deletion and point mutation can be detected in many kinds of tumor tissues and 175, 248, 249, 273 and 282 point mutations are very commonly found\(^{22}\). Frequent inactivation of p53 protein in most tumors indicates that the p53 protein plays a role in the development of human tumors and that the p53 gene is a susceptible target of carcinogens\(^{23, 24}\). The p53 protein is a nucleic phosphoprotein of 53,000 Daltons. Its quantity in serum is minimal and its half-life is about 20 min. Therefore, it is very difficult to detect. The detection of mutant p53 protein in healthy subjects indicates that as the p53 suppressive gene mutates the probability of developing cancer increases\(^{25, 26}\). Many studies on the association between p53 gene mutation and tumors show that an abnormal expression of mutant p53 protein is probably linked to carcinogenesis of several

### Table 2. Incidences of mutant p53 protein and average scanning integrals of PCR amplified band among different Papanicoloau’s gradings

<table>
<thead>
<tr>
<th>Papanicoloau’s grading</th>
<th>Number</th>
<th>p53 positive number</th>
<th>Incidence (%)</th>
<th>Average integral ((\chi \pm S))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG I</td>
<td>52</td>
<td>17</td>
<td>31.7</td>
<td>9,305 ± 8,735</td>
</tr>
<tr>
<td>PG II</td>
<td>121</td>
<td>51</td>
<td>38.4</td>
<td>12,657 ± 19,972*</td>
</tr>
<tr>
<td>PG III</td>
<td>158</td>
<td>74</td>
<td>44.9</td>
<td>13,001 ± 9,733**</td>
</tr>
</tbody>
</table>

\(*p<0.05; **p<0.01.\)

### Table 3. Incidences of mutant p53 protein and average scanning integrals of PCR amplified bands in the medium exposure group

<table>
<thead>
<tr>
<th>Papanicoloau’s grading</th>
<th>Number</th>
<th>P53 positive number</th>
<th>Incidence (%)</th>
<th>Average integral ((\chi \pm S))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG I</td>
<td>21</td>
<td>6</td>
<td>28.6</td>
<td>8,202 ± 7,336</td>
</tr>
<tr>
<td>PG II</td>
<td>47</td>
<td>25</td>
<td>53.2</td>
<td>15,998 ± 11,583**</td>
</tr>
<tr>
<td>PG III</td>
<td>61</td>
<td>31</td>
<td>50.8</td>
<td>13,834 ± 9,415*</td>
</tr>
</tbody>
</table>

\(*p<0.05; **p<0.01.\)

### Table 4. Incidences of mutant p53 protein and average scanning integrals of PCR amplified bands in the high exposure group

<table>
<thead>
<tr>
<th>Papanicoloau’s grading</th>
<th>Number</th>
<th>P53 positive number</th>
<th>Incidence (%)</th>
<th>Average integral ((\chi \pm S))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG I</td>
<td>8</td>
<td>2</td>
<td>25.0</td>
<td>9,941 ± 10,126</td>
</tr>
<tr>
<td>PG II</td>
<td>22</td>
<td>9</td>
<td>40.9</td>
<td>12,249 ± 7,610*</td>
</tr>
<tr>
<td>PG III</td>
<td>29</td>
<td>18</td>
<td>62.1*</td>
<td>14,296 ± 10,457*</td>
</tr>
</tbody>
</table>

\(*p<0.05; **p<0.01.\)
types of human cell. Therefore, it is very important for early diagnosis of tumors to detect minimal mutant p53 protein in the human body as early as possible.

This study detected mutant p53 protein in the serum of benzidine exposed workers with immuno-PCR assays. The results indicate that incidences of mutant p53 protein and average scanning integrals of PCR amplified bands escalate with benzidine exposure level. In addition, the incidences of mutant p53 protein and average scanning integrals of PCR amplified bands in the medium and high exposure levels were significantly higher than that in the low exposure level. The difference of incidences of mutant p53 protein and average scanning integrals between the medium and high exposure level groups was minimal. The result seems to show that both the incidence of mutant p53 protein and the quantity of mutant p53 protein in serum dramatically escalated with an increase of exposure level just within the exposure intensity index range of ≤20 of the benzidine exposure level. Once the exposure level exceeded that threshold, the incidence of mutant p53 protein and the quantity of mutant p53 protein did not significantly escalate with the exposure level anymore. Among the different levels of exposure, there were no differences in Papanicolaou’s grading ratios, but significant differences in the incidence and the quantity of mutant p53 protein did exist. This suggests the susceptibility to benzidine-exposure of mutant p53 protein was greater than the pathological change of exfoliated urothelial cells.

The results indicate that the incidence of mutant p53 protein in serum was enhanced by increase of Papanicolaou grade, but no significant differences were present. Regarding the quantity of mutant p53 protein, it was significantly higher in the groups of PG II and PG III than in PG I. That is, when the exfoliated urothelial cells of the benzidine-exposed workers changed from PG I (normal cells) to PG II (proliferative cells) and PG III (suspicious malignant cells), the quantity of mutant p53 protein increased significantly. Therefore workers with higher benzidine exposure levels in combination with the detection of mutant p53 protein in serum should come under the scope of intensive surveillance. It appears that the detection of mutant p53 protein in conjunction with benzidine exposure level and Papanicolaou’s gradings of exfoliated urothelial cells might provide more information to help us elevate surveillance efficiency and diagnose bladder cancer in the early period.

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