Analysis of p53 and K-Ras Gene Mutations in Patients with Chronic Bronchitis Using Laser Capture Microdissection Microscope and Mutation Detection

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Background Although p53 and K-ras gene mutations have been frequently identified in lung cancer patients, it is obscure whether these mutations are detected in epithelial cells from the patients with chronic bronchitis.

Methods The morning sputum samples were taken from 46 female patients with chronic bronchitis who exposed to smoky coal emissions containing polycyclic aromatic hydrocarbons (PAHs). Epithelial cells were isolated from sputum using laser capture microdissection microscope. Analysis of p53 mutations was carried out using polymerase chain reaction (PCR) and single-stranded conformational polymorphism (SSCP), and K-ras mutation was detected using PCR and denaturing gradient gel electrophoresis (DGGE).

Results Mutations in p53 and K-ras genes were identified in 5 of 46 patients (10.9%). One patient had both a p53 mutation and a K-ras mutation. Three patients had only a p53 mutation, and one patient had only a K-ras mutation.

Conclusion p53 and K-ras mutations are present in epithelial cells from the sputum of patients with chronic bronchitis who were exposed to smoky coal emission but had no evidence of lung cancer, suggesting that these mutations may be associated with exposure to PAHs present in smoky coal emissions.

Key Words: p53 mutation, K-ras mutation, laser capture microdissection microscope, chronic bronchitis

Mutations in the p53 tumor suppressor gene and K-ras oncogene have been frequently identified in lung tumors, sputum and bronchoalveolar lavage (BAL) samples of lung cancer patients¹⁶. They have been also found in fluid samples of patients prior to presenting clinical symptoms of lung cancer, suggesting they may provide useful markers for early detection of lung cancer. However, these mutations are detectable by only sensitive methods because of the small fraction of epithelial cells among all exfoliated cells in sputum samples. Several sensitive methods have been developed, most of them for detecting mutations at codon 12 of the K-ras gene where most K-ras mutations occur⁷. The detection of p53 mutations in sputum samples has been even more complicated because they occur at many codons throughout the p53 gene⁷. These methods for detecting K-ras and p53 mutations are usually laborious and time-consuming. In our previous studies, a method was established which combines sputum cytology and laser capture microdissection techniques to separate tumor cells from sputum of lung cancer patients for analysis of p53 and K-ras mutations⁷. This method has shown an advantage over other available methods in the detection of low fraction mutations in the exons of the p53 gene and K-ras gene. In the present study, we applied this method to identify p53 and K-ras mutations in epithelial cells taken from the sputum of female patients with chronic bronchitis in Xuan Wei County, Yunnan Province, China. These patients were exposed to smoky coal emissions containing polycyclic aromatic hydrocarbons (PAHs). We hypothesized that these gene mutations may occur in these patients.

Methods

Patients and Sputum Samples
Forty six female patients with chronic bronchitis from Xuan Wei County, Yunnan Province, China participated in this study. All the patients provide informed consent and received clinical examination. No evidence of lung cancer was found. This study was conducted according to the recommendations of the World Medical Association Helsinki Declaration (1989)⁸. The protocol of the study accorded with the requirements for protection of human subject certification by the U.S. Environmental Protection Agency (EPA). The morning sputum samples from each patient was stored in 40 ml Saccomanno’s solution (Lerner Laboratories, Pittsburgh, PA, USA) and kept at −4°C.
The samples were centrifuged at 600 g for 10 min and the cell pellets were resuspended in fresh Saccamanno’s solution. Cytological examination was performed to determine whether the sputum samples were derived from the lower respiratory tract and to confirm the presence of bronchial epithelial cells.

**Isolation of Epithelial Cells from Sputum**

Each sample was centrifuged and the cells were resuspended in 1 ml of phosphate buffered saline (PBS) and transferred to a Cyto-Tek specimen chamber. After centrifugation with a Cyto-Tek centrifuge (VWR, Bridgeport, NJ, USA), the cells were stained with eosin and hematoxylin and were cytologically examined. Approximately 150, morphologically benign, epithelial cells were captured on a “cap” with a laser capture microdissection microscope.

**Analysis of p53 and K-ras Mutations**

The captured cell samples were lysed with 15 μl of lysis solution containing protease K and heated at 95°C for 5 min to inactive the protease K. Analysis of p53 and K-ras gene mutations was conducted as previously described. Briefly, for K-ras mutation analysis, an aliquot of each cell lysate was used for polymerase chain reaction (PCR) in a 25 μl reaction mixture containing reagents and [α-32p]dATP (NEN, Boston, MA, USA). The PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) and mutant sequences were isolated and further characterized for mutation. For p53 mutation analysis, the equivalent 20 cells from each cell lysate were used to amplify each of the three fragments corresponding to exons 5–6, 7 and 8 separately in a 25 μl reaction mixture containing reagents and [α-32p]dATP and primers. The PCR products were analyzed by single-stranded conformational polymorphism (SSCP). Brains appearing in each autoradiogram corresponding to a mutant(s) allele and those corresponding to the wild type allele in each cell sample were scanned and the intensity of each band was measured. Mutant alleles appearing on the gel were isolated, further amplified and characterized by sequencing using a sequencer.

**Results**

As shown in Table 1, p53 and K-ras mutations were identified in epithelial cells taken from sputum of 5 patients with chronic bronchitis. The mutation frequency among 46 patients was 10.9%. Patient 1 had mutations both in p53 gene and K-ras gene. In this patient p53 mutation occurred in exon 7 codon 258, from GAA to TAA (Glu to stop), whereas K-ras mutation occurred in codon 12, from GGT to GTT (Gly to Val). Patient 2 had a p53 mutation occurring in exon 7 codon 248, from CGG to CTG (Arg to Leu). In patient 3, one p53 mutation was identified which occurred in exon 5 codon 136, from CAA to CAG (Gln to Gln). Similarly, patient 4 had a p 53 mutation occurring in exon 5 codon 156, from CGG to CCC (Arg to Pro). In patient 5, a K-ras mutation was identified which occurred in codon 13, from GGC to GCC (Gly to Ala).

<table>
<thead>
<tr>
<th>Patients</th>
<th>p53 Mutations</th>
<th>Amino acid changes</th>
<th>K-ras Mutations</th>
<th>Amino acid changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E7 cod 258, GAA to TAA</td>
<td>Glu to stop</td>
<td>Cod 12, GGT to GTT</td>
<td>Gly to Val</td>
</tr>
<tr>
<td>2</td>
<td>E7 cod 248, CGG to CTG</td>
<td>Arg to Leu</td>
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<tr>
<td>3</td>
<td>E5 cod 136, CAA to CAG</td>
<td>Gln to Gln</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>E5 cod 156, CGG to CCC</td>
<td>Arg to Pro</td>
<td>Cod 13, GGC to GCC</td>
<td>Gly to Ala</td>
</tr>
</tbody>
</table>

Epithelial cells were taken from sputum using laser capture microdissection microscope. Analysis of p53 and K-ras mutations was carried out using PCR+SSCP or PCR+DGGE respectively as described in Methods. PCR : polymerase chain reaction, SSCP : single-stranded conformational polymorphism, DGGE : denaturing gradient gel electrophoresis.

**Discussion**

These results demonstrate that the approach of epithelial cell isolation by combining sputum cytocentrifugation and laser capture microdissection and PCR+DGGE or PCR+SSCP analysis provides a simple and sensitive screening tool for K-ras and particularly for p53 mutations that occur at many codons of the p53 gene in sputum samples of patients with chronic bronchitis. Furthermore, the results show that p53 and K-ras mutations were present in epithelial cells isolated from sputum of these patients who were exposed to smoky coal emissions containing PHAs.

It is unknown whether the presence of p53 and K-ras mutations in morphologically benign bronchial epithelial cells in sputum was associated with an increase risk of lung cancer development in these patients with chronic bronchitis. K-ras mutations have been identified in the sputum of lung cancer patient and detection of these mutations in sputum correlated with the presence of identical mutations in matched lung tumor, implying that detection of these mutations in sputum may be a useful biomarker for lung cancer. K-ras mutations have also been detected in the sputum of patients without lung cancer but who had evidence of bronchitis, asthma and pneumonia. Taken together, these evidences indicate that K-ras mutation may be useful marker for lung cancer detection.

The p53 gene has been in mutated form at high frequencies and in many human tumors. In lung cancer,
p53 mutations have been identified in 40–50% of the cases. It has been shown that p53 mutations in lung cancer are different from those in other cancers and that an excess of G to T transversion is characteristic for these tumors. p53 mutations were also found in the dysplastic bronchial epithelium of patients without lung cancer.

In the present study, although the patients did not smoke, they were exposed to smoky coal emissions which contained a high proportion of PAHs for a long time. Evidences have suggested an association between exposure to smoky coal emissions and increased lung cancer risk.

In conclusion, by using laser capture microdissection microscope and mutation analysis, we have shown that p53 and K-ras mutations are present in non-malignant bronchial epithelial cells from the sputum of patients with chronic bronchitis who were exposed to smoky coal emission but had no evidence of lung cancer. The results suggest that these mutations may be associated with exposure to PAHs present in smoky coal emissions.

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References


