Detection of Oncogene Mutations in Sputum Precedes Diagnosis of Lung Cancer

Li Mao, Ralph H. Hruban, Jay O. Boyle, Melvyn Tockman, and David Sidransky

Materials and Methods

Patients. We identified 15 patients from the JHLP trial who went on to develop adenocarcinoma or large cell carcinoma of the lung. These patients were chosen simply on the availability of paraffin-embedded tissue following surgical resection and a negative sputum cytology prior to definitive diagnosis. Tumors not containing K-ras mutations were sequenced for p53 mutations to identify tumor-specific markers (11, 12).

 stored, nebulized sputum samples from the JHLP archives containing 2% Carnobax/50% alcohol (Sacconamino’s fixative) preservative solution were located (details on collection of samples in Refs. 5-7). From each sample, 5 ml were removed (approximately 50,000-500,000 cells), spun at 1000 x g for 5 min., and rehydrated in 5 ml of normal saline. Cells were then resuspended 1000 x g, resuspended in 1 ml of 1% sodium dodecyl sulfate/proteinase K (5 mg/ml), and then incubated at 60°C for 6 h. DNA was extracted twice with saturated phenol-chloroform and ethanol precipitated. Dried DNA was resuspended in 50 μl, and 2 μl were used for each PCR reaction performed in a separate PCR-dedicated room to eliminate the possibility of contamination.

Molecular Analysis of Sputum. Sputum DNA was amplified by PCR with primers for K-ras and p53 that contained EcoRI sites to facilitate cloning. Following 35 cycles of amplification, products were cleaved with EcoRI and ligated to Lambda Zap II (Stratagene, La Jolla, CA) (11, 12). XLI-blue cells infected with bacteriophage were plated on L-Agar at a density of 500-3000 plaques/plate, transferred to nylon membranes, and hybridized with oligonucleotides specific for wild type or mutant K-ras and p53. The oligonucleotides used for hybridizations were labeled with [γ-32P]ATP and hybridized according to the method of Sidransky et al. (11, 12). Oligonucleotides used for detection included

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT ras</td>
<td>5'-GGAGCTGGTGCGCTAGGCCAATGGG-3'</td>
</tr>
<tr>
<td>Val12 mutant</td>
<td>5'-GGAGCTGGTGCGCTAGGCCAATGGG-3'</td>
</tr>
<tr>
<td>Asp13 mutant</td>
<td>5'-GGAGCTGGTGCGCTAGGCCAATGGG-3'</td>
</tr>
<tr>
<td>Ser12 mutant</td>
<td>5'-GGAGCTGGTGCGCTAGGCCAATGGG-3'</td>
</tr>
<tr>
<td>Cys12 mutant</td>
<td>5'-GGAGCTGGTGCGCTAGGCCAATGGG-3'</td>
</tr>
<tr>
<td>WT p53</td>
<td>5'-ATGGGGGCAATGGGGAACCCG-3'</td>
</tr>
<tr>
<td>His127 mutant</td>
<td>5'-CTGGAGGCGATCCCTGGGAACCCG-3'</td>
</tr>
<tr>
<td>Gly131 mutant</td>
<td>5'-CTGGAGGCGATCCCTGGGAACCCG-3'</td>
</tr>
</tbody>
</table>

Results

We sought to develop a method for the early detection of lung cancer that could augment current cytological detection based on the emerging molecular biology of this neoplasm. Adenocarcinomas were chosen because these tumors have a higher incidence of K-ras mutations (30%) than other lung tumors (13, 14). Moreover, we looked for p53 gene mutations because these are the most common genetic alterations found in these tumors and a variety of other cancers (15-17). Sequence analysis of the PCR products from the two target genes in 15 patients identified 10 primary tumors which contained

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3 The abbreviations used are: JHLP, Johns Hopkins Lung Project; PCR, polymerase chain reaction; WT, wild type.
either a K-ras or a p53 gene mutation (Table 1). The K-ras mutations were those commonly associated with adenocarcinoma of the lung and occurred predominantly at codon 12 (13, 14). The two p53 mutations occurred at codons 273 and 281, and both were described previously in lung cancers (15–17). Many tumor samples were small (<1 cm) and DNA was insufficient for extensive sequence analysis of p53 (exons 5–8) in all tumors.

Following identification of tumors with oncogene mutations, we obtained all of the available corresponding sputum samples from these affected patients. None of the patients who we analyzed ever had a positive sputum cytology in the JHLP study. Most samples were read as minimally or modestly dysplastic but were not diagnostic for cancer and were not followed by any clinical intervention. Therefore, all but one of the patients (L4 evaluated because of cough) were diagnosed by chest X-ray. Those sputum samples that were available (generally one to two samples prior to clinical diagnosis) were analyzed by a PCR-based assay able to detect 1 mutant-containing cell among an excess background of 10,000 normal cells (11, 12). This assay is based on the amplification of sputum DNA, followed by cloning into a phage vector and transfer to nylon membranes. A mutant-specific oligomer is then hybridized to each of the filters to identify specific point mutations in either the K-ras or the p53 gene present in sputum.

Using this assay, we detected neoplastic cells in previous “negative” cytological sputum samples from 8 of the 10 patients who had tumors containing oncogene mutations. Positive sputum samples harboring a clonal population of cancer cells were obtained from 1 to 13 months prior to clinical diagnosis (Table 1).

Detection of one cancer cell among 160 normal cells (1/320 = 1/160; each normal cell contributes 2 WT alleles) was detected in sputum from patient L4 13 months prior to his clinical diagnosis (Table 1). The tumor from this patient contained >95% positive cells despite the presence of mutations in their primary tumors (Table 1). Furthermore, we were not able to trace the first emergence of a clonal population of cancer cells in positive patients by our sputum assay because of missing samples prior to diagnosis. However, in 6 of the 10 patients, sputum samples were available >24 months prior to diagnosis and were negative. This suggests 13–24 months as a limit for molecular diagnosis by our assay. As a further control, prior to clinical presentation, 6 of 8 patients who initially tested positive were found to test negative in sputum samples obtained following complete surgical resection of their tumor.

Discussion

We detected rare cancer cells in the sputum of 8 of 10 patients with oncogene mutations in their primary tumor prior to clinical diagnosis.

In two patients, both of whom had only a single sputum sample available, we were not able to detect a clonal population of mutant-containing cells despite the presence of mutations in their primary tumors (Table 1). Furthermore, we were not able to trace the first emergence of a clonal population of cancer cells in positive patients by our sputum assay because of missing samples prior to diagnosis. However, in 6 of the 10 patients, sputum samples were available >24 months prior to diagnosis and were negative. This suggests 13–24 months as a limit for molecular diagnosis by our assay. As a further control, prior to clinical presentation, 6 of 8 patients who initially tested positive were found to test negative in sputum samples obtained following complete surgical resection of their tumor.

**Discussion**

We detected rare cancer cells in the sputum of 8 of 10 patients with oncogene mutations in their primary tumor prior to clinical diagnosis.

**Table 1 Lung cancer patients with gene mutation analysis of sputum**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (yr)/sex</th>
<th>Tumor location</th>
<th>Tumor type/stage</th>
<th>Tumor mutations</th>
<th>Pre-Dx&lt;sup&gt;a&lt;/sup&gt; (mo.)</th>
<th>Diagnosis (M/T)</th>
<th>Postoperation</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>65/M</td>
<td>RUL</td>
<td>A/T1N0M0</td>
<td>K-ras-12Ser</td>
<td>3</td>
<td>1/270</td>
<td>Negative</td>
</tr>
<tr>
<td>L2</td>
<td>57/M</td>
<td>LUL</td>
<td>A/T1N0M0</td>
<td>K-ras-12Asp</td>
<td>1</td>
<td>1/300</td>
<td>N/A</td>
</tr>
<tr>
<td>L3</td>
<td>63/M</td>
<td>LUL</td>
<td>A/T1N0M0</td>
<td>K-ras-12Val</td>
<td>4</td>
<td>1/100</td>
<td>Negative</td>
</tr>
<tr>
<td>L4</td>
<td>51/M</td>
<td>LUL</td>
<td>A/T1N0M0</td>
<td>p53-273His</td>
<td>13</td>
<td>1/320</td>
<td>N/A</td>
</tr>
<tr>
<td>L5</td>
<td>67/M</td>
<td>LUL</td>
<td>A/T1N0M0</td>
<td>K-ras-12Cys</td>
<td>1</td>
<td>1/1200</td>
<td>Negative</td>
</tr>
<tr>
<td>L6</td>
<td>67/M</td>
<td>RUL</td>
<td>A/T1N0M0</td>
<td>p53-281Gly</td>
<td>Negative</td>
<td>0</td>
<td>Negative</td>
</tr>
<tr>
<td>L7</td>
<td>70/M</td>
<td>RUL</td>
<td>A/T1N0M0</td>
<td>K-ras-12Cys</td>
<td>1</td>
<td>1/220</td>
<td>Negative</td>
</tr>
<tr>
<td>L8</td>
<td>59/M</td>
<td>RUL</td>
<td>A/T1N0M0</td>
<td>K-ras-12Cys</td>
<td>1</td>
<td>1/170</td>
<td>Negative</td>
</tr>
<tr>
<td>L9</td>
<td>48/M</td>
<td>RUL</td>
<td>L/T1N0M0</td>
<td>K-ras-12Cys</td>
<td>Negative</td>
<td>0</td>
<td>Negative</td>
</tr>
<tr>
<td>L10</td>
<td>63/M</td>
<td>RUL</td>
<td>A/T1N0M0</td>
<td>K-ras-12Cys</td>
<td>1</td>
<td>1/330</td>
<td>Negative</td>
</tr>
<tr>
<td>L11</td>
<td>63/M</td>
<td>RUL</td>
<td>A/T1N0M0</td>
<td>None</td>
<td>None</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>L12</td>
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<td>LUL</td>
<td>A/T1N0M0</td>
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<tr>
<td>L13</td>
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<td>N/D</td>
</tr>
<tr>
<td>L14</td>
<td>61/F</td>
<td>RUL</td>
<td>A/T1N0M0</td>
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<td>N/D</td>
</tr>
<tr>
<td>L15</td>
<td>62/M</td>
<td>LUL</td>
<td>A/T2N0M0</td>
<td>None</td>
<td>None</td>
<td>N/D</td>
<td>N/D</td>
</tr>
</tbody>
</table>

<sup>a</sup> Staging system is according to the ASCC (35), 1986. All sputum samples for patients L1 to L10 were reamplified and recloned, and the assay was repeated for verification. Control patients without cancer were negative by this assay.  
<sup>b</sup> Pre-Dx, months prior to actual clinical diagnosis; M/T, number of mutant alleles/total clones with target gene insert; RUL, right upper lobe; LUL, left upper lobe; RLL, right lower lobe; LLL, left lower lobe; A, adenocarcinoma; L, large cell carcinoma; N/A, not available; ND, not done.

Fig. 1. Gene mutations in sputum. Many clones with a PCR insert from the p53 gene hybridized to an oligomer probe specific for the codon 273 Arg-His mutation in the patient’s (L4) tumor (T). A fewer number of clones hybridized to the same specific probe in the patients sputum (S). There were no hybridizing clones to this probe in a control sputum from a patient (L15) without a p53 gene mutation in his primary lung cancer (C).

Fig. 2. Confirmation of detected mutations. Sequencing gels of pooled clones for p53 show the WT sequence in a normal (N) control and the 273 CGT-CAT (Arg→His) mutant sequence (arrow) in the tumor (T) with a residual WT band. One positive plaque (clone) depicted in Fig. 1 (S) was picked and sequenced revealing the same mutant band in sputum (S) as that found in the primary tumor. In each panel, the lanes represent A, C, G, and T terminations from left to right.
Those patients whose tumors did not contain oncogene mutations and control patients without cancer were also negative by our assay (data not shown). There did not appear to be any correlation between the site of the tumor and the ability to detect mutations in sputum samples by this assay. “False negative” samples may be due to inappropriate collection, poor cellular content, or the variable presence of tumor cells in sputum. Information gained from cytological diagnosis in sputum through routine light microscopy has previously suggested that examination of two or three samples may improve the diagnostic yield significantly (18, 19), and this likely pertains to molecular diagnosis as well. The percentage of cells identified here is significantly lower than those previously identified in urine (11) and stool (12) using a similar approach. This may be in part due to a large dilution effect from the inflammatory cells present in sputum samples (18, 19) or additionally, from the larger number of normal epithelial cells derived from the large surface area of the bronchopulmonary system.

Our stringent selection bias (negative sputum cytology and surgical resection) provided a group of patients with small lesions and a good outcome. All of these patients were eventually diagnosed by a positive radiograph and only six died because of their disease. Thus, patients with mutations detected in sputum 1–4 months prior to diagnosis still had small tumors amenable to surgical resection and a very favorable survival rate at 5 years (60%). In the JHLP trial, those patients diagnosed by sputum cytology generally had a better survival rate than those diagnosed by chest X-ray. Thus, our molecular screening approach may prove useful as an adjunct to cytological analysis and allow identification of patients who are candidates for surgical resection.

Patient L4 with a large T3 lesion had the longest interval (13 months) from sputum detection to diagnosis. He also had a negative computed tomography scan-guided transthoracic biopsy 6 months prior to definite diagnosis because a small lesion was noted on chest X-ray. This patient thus had two opportunities for routine cytological detection prior to clinical diagnosis. He died of metastatic disease 4 months following surgical resection. This particular case serves to illustrate the limitations of routine cytology and the ability to augment morphological analysis through the use of probes for specific gene mutations.

We chose a laboratory detection method that necessitates cloning because it is quantitative and allows a precise estimation of the number of cancer cells present. Other PCR-based techniques (20–22) or tests using ligation in detection or amplification (23) could also be chosen since they can detect one mutant copy among $10^5$ normal copies and are more amenable to automation. Furthermore, we targeted K-ras because of alterations previously found in adenocarcinomas, although these mutations are not found in other lung tumors. Although p53 mutations are found in the majority of epithelial cancers, they usually occur as late events (24, 25). Recent evidence suggested that p53 mutations may occur somewhat earlier in lung carcinogenesis (26–28) and our early detection of a p53 mutation in the sputum of patient L4 supports this. However, because p53 mutations are so varied, a specific oligomer has to be synthesized for identification of each mutation. Despite these limitations our findings suggest that K-ras and p53 may be suitable targets for early detection strategies. As other gene alterations involved in lung cancer progression are identified, it will be possible to incorporate new markers into this novel molecular approach.

The sensitivity of this type of molecular approach raises the very real possibility of identifying cancer before a tumor mass can be seen by radiological methods. Patients thus identified could be candidates for chemoprevention strategies (29, 30). Repeated molecular diagnosis could serve as important intermediate endpoints in such chemoprevention approaches (31). In the absence of visible tumors, improved bronchoscopic assay detecting intrinsic alterations in autofluorescence between normal and neoplastic cells could be used (32). Following visualization of “affected” mucosa, the laser bronchoscope could be used therapeutically to eliminate the cancer clone and prevent the development of a lung tumor. Novel biological approaches including various types of gene therapy could also be considered (33, 34).

We have demonstrated the ability to detect a clonal population of mutation-containing cells among an excess background of normal cells in sputum cytology samples. Due to the unique nature of these archival samples, we were able to follow the development of clinical lesions after sputum collection, indicating that these gene mutations may be detected with significant lead time prior to clinical diagnosis. Furthermore, those patients identified were amenable to surgical resection and possible cure. Because so many patients die of lung cancer each year, these results have significant implications for the use of emerging molecular techniques as adjuncts to cancer screening. Furthermore, the ability to detect gene mutations more than 1 year prior to clinical diagnosis holds promise for screening of patients at high risk for this deadly disease.

Acknowledgments

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References


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