High Frequency of K-ras Codon 12 Mutations in Bronchoalveolar Lavage Fluid of Patients at High Risk for Second Primary Lung Cancer

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ABSTRACT

A high frequency of K-ras mutations may indicate preneoplastic changes in the bronchial epithelium as a result of genotoxic injury. With the use of sensitive detection techniques, we report a higher prevalence of K-ras mutations in bronchoalveolar lavage than has been reported previously for lung cancer. A PCR/ligase chain reaction technique was used to determine K-ras codon 12 mutations in a group of 52 bronchoalveolar lavage specimens from patients at risk of a second lung cancer. Of the specimens examined, 84% contained at least one mutation in K-ras codon 12, corroborated by an allele-specific hybridization method. These results suggest that point mutations in K-ras codon 12 are widespread in the bronchial epithelium. Based on these preliminary findings, further evaluation of this efficient sensitive assay to monitor K-ras status should be conducted in larger clinical cohorts where clinical outcomes will ultimately be available. Such a trial will define the utility of K-ras codon 12 mutation status as a marker of lung cancer.

INTRODUCTION

As the leading cause of cancer deaths in the United States, lung cancer is predicted to account for 159,000 deaths in 1996 from 177,000 expected new cases (1). To reduce this high rate of mortality, new approaches to lung cancer early detection are needed. Recent research into molecular changes which occur in lung cancer development has revealed a number of potential targets, including the ras and p53 oncogenes, for early detection of lung cancer (2, 3). To advance these targets toward clinical application, we conducted a pilot analysis in BAL fluid, since the BAL procedure samples the site of lung carcinogenesis (4).

With more than 80% of the activating mutations of the K-ras oncogene at codon 12 or 13 in human non-small cell lung cancer and in colorectal cancer, this genetic lesion is a logical candidate to evaluate for population-based screening applications (5). Rapid economical techniques are needed to determine the utility of K-ras mutations for early cancer detection in premalignant clinical specimens. Only a small, variable percentage of bronchial epithelial cells in a typical clinical specimen will have a relevant K-ras codon 12 mutation. Therefore, assay sensitivity is critical.

Earlier methods for detecting ras mutations include PCR with single-strand conformation polymorphism analysis (6) and PCR amplification using mismatched primers to create a "designed RFLP" (7). These and similar methods have a sensitivity limit of 3–25% mutant alleles in a normal DNA background. Recently, techniques such as PCR-PIREMA have been reported with an apparent sensitivity of less than 1% (8), which is comparable to the PCR/LCR assay used in this report. The PCR-PIREMA technique was used to detect ras mutations in 14 of 25 (56%) previously unstudied lung adenocarcinoma samples (9). The PCR-PIREMA technique can detect K-ras oncogene mutations identical to the corresponding resected tumor in the BAL fluid of lung cancer patients (10). In addition, an allele-specific oligonucleotide hybridization technique has been used to show K-ras mutations in sputum specimens that corresponded to the mutations in tumors subsequently removed from patients with lung cancer (11). A high frequency of K-ras mutation was seen in normal-appearing lung tissue and sputum of patients with lung cancer in another recent report using an enriched PCR method (12). These findings support the utility of BAL fluid for screening for evidence of ras mutations. The refinement of these and other high sensitivity PCR-based techniques for K-ras mutation detection will make prospective
screening trials in high-risk populations possible so that the clinical consequences of the mutations can be reliably defined.

MATERIALS AND METHODS

Collection and Processing of BAL Specimens. BAL specimens in normal saline-containing protease inhibitors were obtained from stage I, resected, non-small cell lung cancer patients using a previously described procedure (13). The specimens were collected at clinical centers of the LCEDWG and shipped on ice to the Biomarkers and Prevention Research Branch. Cell pellets of approximately $1 \times 10^6$ cells were aliquoted from each specimen and frozen at $-80^\circ C$ for extraction of DNA. DNA was extracted from control cell lines and from BAL samples using standard procedures (14). DNA pellets were resuspended in sterile water and quantitated by $A_{260}$.

PCR for K-ras Codon 12. The region surrounding codon 12 of K-ras was amplified to increase the number of target sequences for the LCR assay. Primers previously reported by Gumerlock et al. (15) were used to generate a PCR product of 115 bp. The samples were amplified for 35 cycles of $94^\circ C$ for 1 min, $55^\circ C$ for 1 min, and $72^\circ C$ for 30 s. Each 50-μl reaction contained the following: 20 μM Tris-Cl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM each deoxynucleotide triphosphate, 25 pmol RS53 primer, 25 pmol RS54 primer, 2.5 units Taq DNA polymerase, and 100 ng purified human template DNA (15). Each set of PCRs performed using the BAL samples included three positive mutant controls (GGT → AGT, GGT → TGT, and GGT → CGT) and one negative control (no DNA template).

To ensure that the PCR amplification was successful, wild-type LCR primers were used to analyze all PCR samples that did not produce mutant results. Each sample produced a signal for wild-type LCR K-ras product, ensuring that lack of mutant signal meant that the sample contained only the wild-type sequence, not an unsuccessful PCR.

LCR for K-ras Codon 12 Mutations. A multiplexed LCR was performed to determine the first base of codon 12 of the K-ras gene, as previously described (16). In the LCR method an oligonucleotide product can be produced only if a pair of DNA primers bind at the site of a specific single-base mutation. The ligated product of the LCR reaction then serves as a target sequence in the next round of amplification. This results in exponential production of product through multiple cycles of LCR (17). The new assay utilizes several modifications of the originally described LCR conditions (16, 18). Following amplification, 1 μl of each PCR product (including positive and negative controls) was added to a master LCR mixture containing six mutant primers and two 32P-labeled invariable primers. Similar experiments were performed using identical conditions with the substitution of two wild-type primers for the set of six diagnostic primers to confirm the presence of wild-type PCR product. The primers were designed so that the lengths of the sense and antisense LCR products for detection of the Gly → AGT, wild-type codon, Ser → AGT, Cys → TGT, and Arg → CGT mutant codons range from 47 to 59 bp. Products in this size range were differentiated using denaturing 10% polyacrylamide gels. All specimens for analysis were encoded so that the operator was blind to the source of the specimens.

RESULTS

The K-ras LCR assay was used to determine mutations present in pulmonary epithelial cells from the BAL fluid of previously resected non-small cell lung cancer patients. These patients have an extraordinary risk of developing a new primary lung cancer at a cumulative frequency of 1–3% per year (19).

This PCR/LCR assay identified a higher incidence of K-ras first-base codon 12 mutations than has been reported previously. The combined data for 42 specimens presented in this report and 10 previously reported specimens (16) show that 44 of 52 (84%) of the specimens have either a single GGT → TGT or both GGT → TGT and GGT → AGT mutations in K-ras codon 12 (Table 1). Of the 52 specimens, 34 (65%) have GGT → TGT mutations and 10 (19%) have both GGT → TGT and GGT → AGT mutations. Although the GGT → AGT mutation was not present in any of the BAL specimens, the mutant control cell line H157 produced the expected result. The assay was validated by oligonucleotide screening, cloning, and DNA sequencing as reported previously (16).

Fig. 1 shows representative results of applying the PCR/LCR method to a set of BAL specimens. Of the 18 specimens shown, 4 have both GGT → AGT and GGT → TGT mutations (specimens 1, 3, 11, and 20). Thirteen specimens contain GGT → TGT mutations only. The only GGT → AGT mutations that were found were with the cases in combination with GGT → TGT mutations.

From the aggregate analysis, 19 of 34 BAL samples that produced GGT → TGT mutations on the initial PCR/LCR analysis were reexamined in an independent blinded analysis. Each sample again produced the GGT → TGT mutation. Two DNA specimens which displayed a GGT → TGT mutation on initial analysis showed both a GGT → TGT and GGT → AGT mutation on repeat analysis, indicating possible heterogeneity within these samples. Six of the 10 samples that produced both GGT → AGT and GGT → TGT mutations were amplified in a separate PCR reaction, and a blinded LCR analysis was repeated on the independent PCRs. All six of the double mutant samples were confirmed. Repeat analysis of all of the specimens that showed no ras mutations confirmed the absence of mutations.

DISCUSSION

Using a recently reported high-sensitivity assay, we have found a higher than expected frequency of codon 12 ras mutations (84%) in bronchial lavage specimens from individuals at very high risk of developing cancer (16). In the initial methods article, we outlined the extensive validation analysis for this LCR assay, documenting a sensitivity for mutational detection consistently better than 1 cell in 100. Previous studies have shown that K-ras mutations found in sputum or BAL specimens

<table>
<thead>
<tr>
<th>Mutation</th>
<th>No. mutated</th>
<th>%</th>
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<tbody>
<tr>
<td>TGT only</td>
<td>24</td>
<td>65</td>
</tr>
<tr>
<td>TGT and AGT</td>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td>CGT</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>44</td>
<td>84</td>
</tr>
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Table 1 First-base K-ras codon 12 mutations in 52 BAL specimens
A high proportion (39%) of atypical alveolar hyperplasias were recently reported to contain K-ras codon 12 mutations (23). The relationship of alveolar hyperplasia to the eventual development of lung cancer has been anecdotally proposed but is currently not established. The high frequency of K-ras mutations found in that study supports the concept of lung cancer arising from a background of field cancerization. Both the significance as well as the timing of the occurrence of K-ras mutations in the progression of lung cancer is still uncertain (24, 25).

As suggested by Sidransky (3), certain mutations we observe may reflect initiation events that do not become clonally expanded. The clinical literature already reflects that ras mutations can be found in pancreatitis (26) as well as the colonic lavages of individuals with only a family history of colon cancer but no evidence of cancer (27). In a population of patients with bronchitis, asthma, pneumonia, or heart failure, but without lung cancer, 10% had ras mutations as determined by an enriched PCR technique (12), yet these patients have increased risk for lung cancer.

In addition to determining the significance of a positive ras mutation assay, another issue fundamental to the development of molecular diagnostics for early cancer detection is the possibility of false-negative results. This is a particularly important issue for high-sensitivity, PCR-based assays. We have previously outlined the sampling problems inherent in the use of highly sensitive ras assays (16). Molecular early cancer detection approaches will often entail identifying mutations in specimens when only a small number of the available cells in the clinical specimen contain mutations. The number of informative bronchial epithelial cells recovered during a BAL procedure may be less than 1% of the cells comprising the clinical specimen. A clinical assay will be valid only if the mutated epithelial cells are recovered in the BAL specimen. Specimen handling procedures are critical to consider because the distribution of "informative" cells is likely not to be homogeneous in small clinical specimens used for early cancer detection. In the origi-

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Fig. 1  K-ras codon 12 mutational analysis results for BAL specimens and mutant control DNA. Lanes 1–18. LCR bands for 18 BAL specimens, as numbered above the lanes (samples 2 and 12 were omitted due to lack of clinical specimen). Lanes 19 and 25 are PCR-negative controls, as marked above the lane. Lanes 20–22, results for mutant control DNA from cell lines A549, Calu1, and H157, representing GGT → AGT, GGT → TGT, and GGT → CGT mutations, respectively. The results of HeLa DNA reaction with mutant or wild-type primers are shown in Lanes 23 and 24.
inal design of the LCEDWG trial, we attempted to standardize the specimen-handling issues (13) but these conditions were not optimized for a PCR-based assay. Further research delineating the specimen-handling procedures to minimize these potentially confounding issues is essential if these assays are to be used for clinical management decisions.

Based on this pilot experience, a clinical study of ras mutations analyzed with a high-sensitivity ras assay such as the PCR/LCR technique should be conducted in a large cohort whose eventual cancer outcome will be known so that the clinical utility of determining ras mutation status can be elucidated. The molecular diagnostic questions to evaluate include whether the cysteine (TGT) and serine (AGT) K-ras codon 12 amino acid substitutions lead to a difference in clinical progression to cancer compared to the glycine (GGT) wild-type codon, as well as the clinical status of the subjects who have both GGT → TGT and GGT → AGT mutations compared to those with GGT → TGT. In addition, this assay method is being extended to permit analysis of other potential codon 12 and codon 13 mutations so that a more complete understanding of the contribution of K-ras mutations to lung carcinogenesis can be established.

ACKNOWLEDGMENTS

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REFERENCES

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