Increased Prevalence of K-ras Oncogene Mutations in Lung Adenocarcinoma

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Abstract

Reported estimates of ras mutation prevalence in lung adenocarcinoma of 15–24% may be underestimates because of the insensitivity of the assays used. We have devised a rapid, non-radioactive assay for ras mutations, which detects 1 mutant allele/10⁹ normal alleles and have used it to study DNA isolated from 53 lung tumor samples (including 28 adenocarcinomas) previously analyzed by PCR/allele specific oligonucleotide hybridization, which is less sensitive. We detected mutations in 13 of 28 samples, including 7 not detected by PCR/allele specific oligonucleotide hybridization. We also found ras mutations in 14 of 25 previously unstudied samples (56%). Our results indicate that the prevalence of K-ras codon 12 mutations in lung adenocarcinoma is higher than previously reported; thus, ras mutations may be more clinically useful as molecular markers for lung cancer than has been appreciated.

Introduction

Molecular tumor markers, including mutant ras alleles, may offer clinically useful tools for diagnostic and prognostic purposes in lung cancer. Several studies have reported that 15–24% of human lung adenocarcinomas contain mutations in K-ras codon 12 (1–4) and that ras mutations portend a poor prognosis in lung adenocarcinoma (2, 5, 6). A potential difficulty with these studies, however, lies in the insensitivity of the assays used to detect ras mutations. In the largest series of ras mutations in lung cancer (1), as well as in most other recent studies of ras mutations in human tumors, ras mutations have been detected by PCR/ASO-H, which detects only mutations which are present in at least 10% of the total number of copies of the corresponding ras gene in the DNA sample (2, 7, 8). In tumors in which a small fraction of the malignant cells contain a ras mutation or in studies of DNA isolated from clinical samples containing malignant cells mixed with genetically normal cells, this limited assay sensitivity risks a high rate of false negative results.

To address this concern, we have used a simple, highly sensitive assay for K-ras codon 12 mutations to reexamine DNA samples isolated from lung adenocarcinomas which were previously reported as being ras mutation negative by PCR/ASO-H, as well as to study 25 previously unstudied samples. The detection of significant numbers of mutations which had been undetectable by PCR/ASO-H would indicate that ras mutations are of greater potential clinical utility as tumor markers than suggested by the reported prevalence of 15–24% and might also have an impact on prognostic studies. In addition, demonstration that many lung adenocarcinomas have ras mutations in only a small fraction of tumor cells would have implications for our understanding of the role of ras mutations in the process of lung carcinogenesis.

Materials and Methods

DNA samples isolated from 53 resected lung tumors (28 adenocarcinomas, 12 squamous cell carcinomas, 9 large cell carcinomas, 2 bronchoalveolar carcinomas, and 2 carcinoid tumors), previously analyzed for ras mutations by PCR/ASO-H by Rodenhuis and Slebos (1) at the Netherlands Cancer Institute, were reanalyzed for ras mutation status. The assay used, PCR-PIREMA, was adapted from a method which detected as little as 1 mutant allele in N-ras codon 12 or 61 per 10⁶ normal alleles (9). PCR-PIREMA was performed blinded with regard to tumor histology and ras mutation status according to PCR/ASO-H. DNA isolated (10) from lung adenocarcinoma tissues obtained at the New York University Medical Center was phenol/chloroform extracted and desalted and concentrated to 5–10 µl in Microcon 100 concentrators (Amicon, Inc., Beverly, MA) twice, prior to PCR.

Mutations in K-ras codon 12 were detected as shown in Fig. 1. The most important modification from the method as described previously (9) was the use of greatly decreased concentrations of nucleotides and MgCl₂ in the first and second PCR reactions and lengthened annealing and synthesis times. A gel purification step (9) was also eliminated, which decreases assay sensitivity but simplifies the procedure. The modified protocol detects mutant alleles present at the level of 0.1%, as determined by titration of samples known to contain ras mutations (9). All samples were subjected to the entire PCR-PIREMA process at least twice. Extensive measures were taken to prevent cross-contamination of samples (9). Multiple normal controls and negative controls (no DNA in the PCR reaction) were included in all experiments.

Results

Among the 53 samples tested by PCR-PIREMA were 22 from adenocarcinomas which had been mutation negative by PCR/ASO-H (1). Of these 22, 7 (32%) were mutation positive by PCR-PIREMA (Fig. 2). The 6 adenocarcinomas which had been mutation positive by PCR/ASO-H were all positive for the same mutation by PCR-PIREMA (Table 1). Although DNA from the majority of samples is no longer available for study, by extrapolating from our data to the entire original group of 181 adenocarcinomas, we estimate that the true percentage which would be expected to be mutation-positive would be 24% (the percentage positive by PCR/ASO-H) (1) + 76% (the percentage negative by PCR/ASO-H) × 0.32, or a total of 48%. Of the 23 non-adenocarcinomas, all of which had been mutation negative by PCR/ASO-H, only one was found by PCR-PIREMA to contain a mutation (GTT in one large cell sample).

The K-ras codon 12 mutation status was also determined by PCR-PIREMA for 25 lung adenocarcinoma tissues from the New York University Medical Center. Fourteen samples (56%; 95% confidence interval, 35–76%) contained mutations (5 TGT, 4 GTT, 4 GAT, 1 AGT). The percentage of mutation-positive samples is not statistically different from the percentage in the Dutch group if a true positive percentage of 48% is assumed (2-tailed t test, P = 0.55) but is significantly greater than the 24% originally reported for the Dutch group (P = 0.0026).

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2 To whom requests for reprints should be addressed, at Research Service 151, New York V.A. Hospital, 423 E. 23 St., New York, NY 10010.
3 The abbreviations used are: ASO-H, allele specific oligonucleotide hybridization; PCR-PIREMA, PCR-primer introduced restriction with enrichment for mutant alleles.
Discussion

These data indicate that an assay which detects mutations present in 0.1% of alleles can detect K-ras codon 12 mutations in about 50% of lung adenocarcinomas. Our data demonstrate that the largest previously published report on ras mutations in lung adenocarcinoma (1) contained significant numbers of false negatives and thereby underestimated the prevalence of ras mutations in lung adenocarcinoma.

Because other smaller series using similarly insensitive assays have reported mutation frequencies even less than 24%, they may have contained even higher percentages of false negatives.

Two factors, in conjunction with the use of an insensitive assay, could have contributed to difficulty in detecting mutations in these studies. First, in some cases, only a small fraction of tumor cells may contain the mutation. Since cancer is a clonal disease, one might expect that in mutation-positive cases, the mutation would be contained in all cells of the tumor. In fact, this has not to our knowledge been previously studied in lung adenocarcinoma; furthermore, in the one malignancy of which we are aware where this question has been previously studied in lung adenocarcinoma; furthermore, in the one malignancy of which we are aware where this question has been previously studied in lung adenocarcinoma; furthermore, in the one malignancy of which we are aware where this question has been previously studied in lung adenocarcinoma; furthermore, in the one malignancy of which we are aware where this question has been previously studied in lung adenocarcinoma; furthermore, in the one malignancy of which we are aware where this question has been previously studied in lung adenocarcinoma; furthermore, in the one malignancy of which we are aware where this question has been previously studied in lung adenocarcinoma; furthermore, in the one malignancy of which we are aware where this question has been previously studied in lung adenocarcinoma; furthermore, in the one malignancy of which we are aware where this question has been previously studied in lung adenocarcinoma; furthermore, in the one malignancy of which we are aware where this question has been previously studied in lung adenocarcinoma; furthermore, in the one malignancy of which we are aware where this question has been previously studied in lung adenocarcinoma; furthermore, in the one malignancy of which we are aware where this question has been previously studied in lung adenocarcinoma; furthermore, in the one malignancy of which we are aware where this question has been previously studied in lung adenocarcinoma; furthermore, in the one malignancy of which we are aware where this question has been previously studied in lung adenocarcinoma; furthermore, in the one malignancy of which we are aware where this question has been previously studied in lung adenocarcinoma; furthermore, in the one malignancy of which we are aware where this question has been previously studied in lung adenocarcinoma; furthermore, in the one malignancy of which we are aware where this question has been previously studied in lung adenocarcinoma; furthermore, in the one malignancy of which we are aware where this question has been previously studied in lung adenocarcinoma.
Table 1 Prevalence of K-ras codon 12 mutations in lung tumors by diagnosis and detection method

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of mutants/total (%)</th>
<th>PCR-PIREMA</th>
<th>ASO-H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma</td>
<td></td>
<td>13/28 (46)</td>
<td>6/28 (21)</td>
</tr>
<tr>
<td>Bronchial/uloeolar</td>
<td></td>
<td>0/2 (0)</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td>Cell carcinoma</td>
<td></td>
<td>1/9 (11)</td>
<td>0/9 (0)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td></td>
<td>0/12 (0)</td>
<td>0/12 (0)</td>
</tr>
<tr>
<td>Carcinoid</td>
<td></td>
<td>0/2 (0)</td>
<td>0/2 (0)</td>
</tr>
</tbody>
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K-ras MUTATIONS IN LUNG ADENOCARCINOMA

Fig. 2. PCR-PIREMA analysis of DNA samples from tumors previously analyzed by ASO-H. a, enriched screening (BstNI digestion). Lane 1, undigested PCR product of 192 base pairs. Lane 2, normal control; Lanes 3–5, DNA samples from lung adenocarcinomas. The 192-base pair band in Lanes 4 and 5 indicates the presence of a mutation in those samples. b, verification for TGT mutation by NalII digestion. Lanes 1–4, adenocarcinoma samples; Lane 5, normal control. The digested 157-base pair band in Lanes 1, 3, and 4 indicates the presence of a TGT mutation in those samples. For the 7 samples from adenocarcinomas that are positive for ras mutations by PCR-PIREMA but were mutation negative by ASO-H, the mutant genotypes are: GAT (4 samples); TGT (2 samples); and GTT (1 sample). These are the same mutations which predominated in the original analysis (1).
sis times, optimized assay specificity while maintaining sufficient yield.

Because it requires minimal amounts of starting material, PCR-PIREMA can be easily applied to tissue biopsies or cells in body fluids prior to surgery; e.g., we are using the method to detect mutations in bronchoalveolar lavage fluid from patients undergoing diagnostic bronchoscopy for suspected lung cancer (20). The clinical utility of ras as a biomarker for lung cancer has been suggested by investigators who found ras mutations in stored sputum samples from patients later diagnosed with lung adenocarcinoma (21). The assay used in that study, although sensitive, is labor intensive, necessitating cloning of sputum DNA followed by radioactive ASO-H. In contrast, the assay described here can be easily applied on a large scale in the clinical arena. With the demonstration that mutations in K-ras codon 12 are twice as prevalent as previously appreciated, their potential as clinically useful tumor markers is increased.

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References

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