Increased Prevalence of K-ras Oncogene Mutations in Lung Adenocarcinoma

Nancy E. Mills, Charles L. Fishman, William N. Rom, Neil Dubin, and Daniel R. Jacobson

Department of Medicine, Divisions of Hematology [N. E. M., D. R. J.], Oncology [N. E. M.], Pulmonary and Critical Care Medicine [C. L. F., W. N. R.], and Environmental Medicine [N. D.] and Kaplan Comprehensive Cancer Center [D. R. J.], New York University Medical Center, New York, New York 10016

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/10^9 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 10^6 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 MgCl_2 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).
K-ras MUTATIONS IN LUNG ADENOCARCINOMA

Fig. 1. PCR-PREMA protocol. First PCR reactions contained 10 μM concentrations of each nucleotide, 1.2 mm MgCl₂, and fully mismatched primers flanking K-ras exon 1 (Step 1). Aliquots of the first PCR products were used in second PCR reactions containing 4 μM concentrations of each nucleotide and 0.6 mm MgCl₂, using a 5'-mismatched primer, to introduce a new BstNI restriction site into PCR products derived from alleles containing the normal sequence at positions 1 and 2 of codon 12 (Step 2). The first and second PCR reactions were cycled at 94°C for 1 min, 55°C (for the first PCR) or 40°C (for the second PCR) for 2 min, and 74°C for 3 min. PCR products from Step 2 were digested with BstNI (Step 3). Step 2 and 3 were then repeated (Step 4) except on samples in which mutations would be strongly visible after a single round of enrichment. The digests were then amplified under "standard" PCR conditions (200 μM each nucleotide, 1.5 mm MgCl₂, 55°C annealing) using the same primers as in Step 2 followed by repeat BstNI digestion (Step 5a); these products were electrophoresed on 2.5% agarose gels and stained with ethidium bromide, with a digestion-resistant 192-base pair (bp) band indicating the presence of a K-ras codon 12 mutation. To identify the specific base substitution present, Step 4 PCR products were amplified with different mismatched 5'-primers which introduced new restriction sites into the PCR product, dependent upon both the specific mutations and the introduced substitution(s) (Step 5b, verification). PCR conditions for verification were 200 μM each nucleotide, 1.5 mm MgCl₂, 50°C for 30 s, 50°C for 1 min, 74°C for 1 min for 40 cycles. These PCR products were digested with the appropriate restriction enzymes and electrophoresed as above; the presence of a digested PCR product indicates the presence of a specific mutation. The PCR primers and restriction endonucleases used are as described previously (9), except that since the screening step now uses BstNI rather than Rsal (Step 2), the fourth last base of all 5'-primers has been changed from C to A to eliminate an unnecessary mismatch: mismatched base.

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 examined (acute myelogenous leukemia), ras mutations are generally present in only a fraction of the malignant cells, and this fraction varies widely from case to case (9, 11–14). Second, clinical tissue samples obtained during surgical resection of lung masses generally contain both tumor cells and genetically normal cells; DNA isolated from such specimens will be derived from both populations. Thus, the samples from the Netherlands Cancer Institute included samples containing as few as 25% malignant cells (15). Similarly, one smaller study included some samples containing as few as 20% malignant cells (2); the latter group acknowledged the consequent likelihood of some false negatives. Both the presence of ras mutations in a minority of the malignant cells and the mixture of normal with malignant cells...
in clinical samples could lead to purified DNA samples in which the percentage of mutant alleles is less than the detection threshold of the assay used, i.e., 10%. Many genetic changes have been identified in lung cancer (16), but little is known about the chronology of their development. Some genetic changes may represent early activation events, while others are likely to occur later, as progression events related to invasion and metastasis. Limited evidence has been interpreted as suggesting that in some human tumor types, including lung cancer, ras mutations may fall into the former category (17). On the other hand, if significant numbers of cases of lung adenocarcinoma have ras mutations in only a small percentage of the cancer cells (as implied by the detection of these mutations by PCR-PIREMA but not by PCR/ASO-H), then this would suggest that the ras mutation was not an early, initiating event, but was more likely to represent a secondary event in a subclone of the tumor, as has been argued for acute myelogenous leukemia (18). This concept is consistent with the association of ras mutations with increased tumor growth and invasiveness, as suggested by the poorer prognosis of mutation-positive than mutation-negative cases treated by surgical resection (2, 5, 6).

The PCR-PIREMA assay is rapid, nonradioactive, and readily adaptable to processing large numbers of clinical samples and can also be used to detect all activating mutations in K- and N-ras codons 12, 13, and 61 (9). The protocol modifications reported here (decreased nucleotide and MgCl₂ concentrations in the PCR reactions) are critical to the assay specificity. When PCR-PIREMA is performed under “standard” PCR conditions (i.e., 200 μM each nucleotide and 1.5-2.0 mM MgCl₂), the normal controls frequently fail to digest completely on enriched screening (Fig. 1, Step 4) even when a large excess of the screening enzyme is used (9). We reasoned that this difficulty might arise from the high misincorporation rate of Taq polymerase, of 1 error/10⁴ bases under “standard” PCR conditions (19). The enrichment process would enrich not only for PCR products derived from authentic mutant alleles but also for PCR-introduced errors in codon 12, which would change the PCR product to BstNI resistant. Standard PCR conditions maximize polymerization speed and yield, but at the expense of polymerase fidelity (19). We reasoned that this difficulty might arise from the high misincorporation rate of Taq polymerase, of 1 error/10⁴ bases under “standard” PCR conditions (19). The enrichment process would enrich not only for PCR products derived from authentic mutant alleles but also for PCR-introduced errors in codon 12, which would change the PCR product to BstNI resistant. Standard PCR conditions maximize polymerization speed and yield, but at the expense of polymerase fidelity, as we have interpreted the detection of these mutations by PCR-PIREMA but not by PCR/ASO-H, then this would suggest that the ras mutation was not an early, initiating event, but was more likely to represent a secondary event in a subclone of the
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.

Acknowledgments

We thank Drs. Sjoerd Rodenhuis and Bert Top of the Netherlands Cancer Institute for supplying DNA samples and Dr. Rodenhuis for his careful review and helpful comments on the manuscript. We thank Drs. Jaishree Jagirdar, John Scholes, and Michael Ittman for assistance in procuring pathological samples and Dr. Joel Buxbaum for support.

References

Increased Prevalence of K-ras Oncogene Mutations in Lung Adenocarcinoma


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/55/7/1444

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.