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K-*ras* Mutation in Sputum of Patients With or Without Lung Cancer

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Abstract K-*ras* mutation appears in about 60% of patients with non-small-cell lung cancer (NSCLC). This frequency and its presence in normal appearing tissues point to the potential of *ras* oncogene mutation to serve as a good biomarker. Using enriched PCR (EPCR), which enables the detection of one mutant allele in the presence of 10,000 normal alleles, we have determined the frequency of mutant *ras* alleles in the sputum samples of patients with or without lung cancer. Samples were collected from 37 patients with NSCLC and from 40 controls who suffered from non-oncological lung diseases, including bronchitis, asthma, and pneumonia. Of the 37 samples obtained from patients with lung cancer, 18 were found to harbor *ras* oncogene mutations (48%). Of the 40 cases that were free of lung cancer, five were found to harbor this mutation (12.5%). The difference between the two frequencies was found to be significant ($P < 0.01$). These findings indicate that (a) K-*ras* oncogene mutation can be identified in routinely obtained sputum samples of patients who may be at risk of developing lung cancer and (b) the higher frequency of these mutations in samples of patients with lung cancer points to the potential use of the *ras* mutation as a biomarker for either exogenous or endogenous exposure to carcinogens. Thus, the ability to examine sputum provides a powerful and convenient source of sampling and may be adapted for future large-scale screening. J. Cell. Biochem. 25S:172–176.

INTRODUCTION

The detection of point mutations in oncogenes and tumor suppressor genes associated with lung cancer is important in understanding the pathogenesis of this tumor type [Hollstein et al., 1991; Westra et al., 1993; Ronai, 1992] and is expected to assist in identifying biomarkers of exposure, diagnosis, and prognosis. Most of the point mutations identified in lung tumors have been in the *ras* oncogene and in the p53 tumor suppressor gene [Hollstein et al., 1991; Mitsudomi et al., 1993; Iman and Harris, 1991]. K-*ras* oncogene mutations were detected in 55–63% of lung tumors, while 52% of the neoplasms revealed p53 gene mutations [Mao et al., 1994b; Mills et al., 1995; Gazzeri et al., 1994]. Although p53 and *ras* mutations are frequent, they represent independent genetic alterations that play different roles in the pathogenesis and in the progression of NSCLC [Mao and Sidransky, 1994]. Whereas p53 mutations were found in both small-cell lung cancer (SCLC) and NSCLC, [Mao et al., 1994b; Gazzen et al., 1994], K-*ras* codon 12 mutations appeared mainly in NSCLC [Suzuki et al., 1990; Sugio et al., 1992]. NSCLC comprises a group of neoplasms that includes, among others, adenocarcinoma, squamous or epidermoid cell carcinoma, and large-cell carcinoma. Several studies have indicated that lung cancer patients whose tumors were found to harbor K-*ras* mutations had poorer survival rates [Rosell et al., 1993]. To provide an understanding of the role K-*ras* mutations may play in the pathogenesis of NSCLC and to examine whether the presence of this mutation could serve as a good biomarker, we recently analyzed lung tissues from normal appearing tissue, tumor tissue, and metastases obtained from patients with NSCLC. As a control, we used lung tissues from patients who lack oncological diseases or lung disorders. For the study, we used EPCR, a highly sensitive PCR-based methodology that selectively amplifies the mutant form even if its incidence is 10^{-4} [Kahn et al., 1991]. K-*ras* codon 12 mutations were found in 4 out of 40 (10%) lung tissues obtained from patients with no lung diseases [Yakubovskaya et al., 1995].

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The same mutation was detected in 9 out of 15 samples of normal appearing lung tissues (60%) obtained from patients with lung cancer, in 15 out of 24 (63%) tumors, and in 8 out of 10 metastases (80%). A less sensitive PCR methodology was able to detect K-*ras* codon 12 mutations in only 1 of the 24 tumors, indicating that the incidence of this mutation in all other cases is very low, regardless of the source of tissue (i.e., "normal appearing," primary, or metastatic) [Yakubovskaya et al., 1995]. The fact that more than 60% of lung tissues taken from patients with lung cancer harbor the K-*ras* codon 12 mutation (as compared with 10% of lung tissues obtained from patients with no lung disorders) points to its potential use as a biomarker to identify patients who have undergone exogenous and/or endogenous exposures to carcinogens and may thus be at higher risk of developing lung cancer.

Support for the potential use of this mutation as a biomarker in lung cancer came from a recent study by Sidransky and colleagues, who have identified K-*ras* mutations in sputum samples derived from patients with lung cancer [Mao et al., 1994b]. Mutant *ras* alleles were detected in the excreta of patients who underwent colonoscopies, yet were free of colorectal diseases at the time of examination. Similarly, the mutant *ras* allele was detected in either first degree relatives of patients with colorectal cancer or in patients who themselves had had colorectal cancer in the past [Tobi et al., 1994]. Mutant *ras* alleles were reported in the pancreatic juice and in the serum of patients with pancreatic cancer [Tada et al., 1993]. With the technology in hand and the finding that *ras* mutations appear to exist in 60% of patients with lung cancer, we have evaluated the frequency of this mutation in the sputum of patients with and without lung cancer.

MATERIALS AND METHODS

Patients

Sputum samples were obtained from patients with NSCLC at the Cancer Research Center of the Russian Academy of Medical Sciences (RAMS) in Moscow. These patients, 37 men aged 41 to 72 years, had mainly squamous cell lung cancer. Diagnoses were confirmed after surgery by histopathologic examination.

Sputum from patients with non-oncological diseases (chronic bronchitis, pneumonia, bronchitis, asthma, heart disease) was obtained in 1994 from hospitals in Moscow. This group included 28 male and 12 female patients. Sputum obtained from patients was frozen within 30 minutes after collection.

DNA Isolation and Enriched PCR Assay

Genomic DNA was isolated using a DNA lysis buffer (10 mM TRIS, 10 mM EDTA, 150 mM NaCl, 2% SDS and 20 μg ml^{-1} Proteinase K, Sigma, St. Louis, MO) followed by extraction with phenol and chloroform and ethanol precipitation. DNA (2 μg) was then subjected to EPCR, as previously described [Kahn et al., 1991], but with the following modifications. In the first amplification reaction, 60 μg of 5′ and 3′ wt primers were employed, whereas 100 μg of the respective 5′ and 3′ primers were used for the second amplification. The primers for the amplification [see Kahn et al., 1991 for details] were gel purified after their synthesis. A second important parameter to enable successful EPCR was to maintain the concentration of the four dNTPs at 0.2 mM. Amplification was performed in a thermocycler (Ericomp, San Diego, CA) using 20 cycles (1′ at 94°C; 1′ at 59°C, 1′ at 72°C) for the first round of amplification and 30 cycles for the second. Ten percent of the amplified material (5 μl) was taken for an intermediate digestion using BstNI restriction enzyme for 2 h at 60°C. Only 1% of the original material (after its digestion to eliminate normal alleles) was used as a template for subsequent amplification; 15 μl of the amplified material was subjected to a restriction enzyme digestion and analysis on 10% PAGE via ethidium bromide staining. The molecular weight markers were generated by digesting SK+ plasmid (Stratagene, La Jolla, CA) with HpaII restriction endonuclease. Each sample was analyzed in three independent reactions, one of which was performed in Moscow and the other two independently in the United States were done.

Statistical Analysis

Standard errors and 95% confidence intervals for individual rates of K-*ras* mutations were computed using the normal approximation to the binomial probability distribution [Fleiss, 1981a]. The statistical significance of the difference between pairs of mutation rates was evaluated using a two-by-two frequency table followed by the chi-square test of association (Fleiss, 1981b]. The differences between groups of mutation rates were tested using the

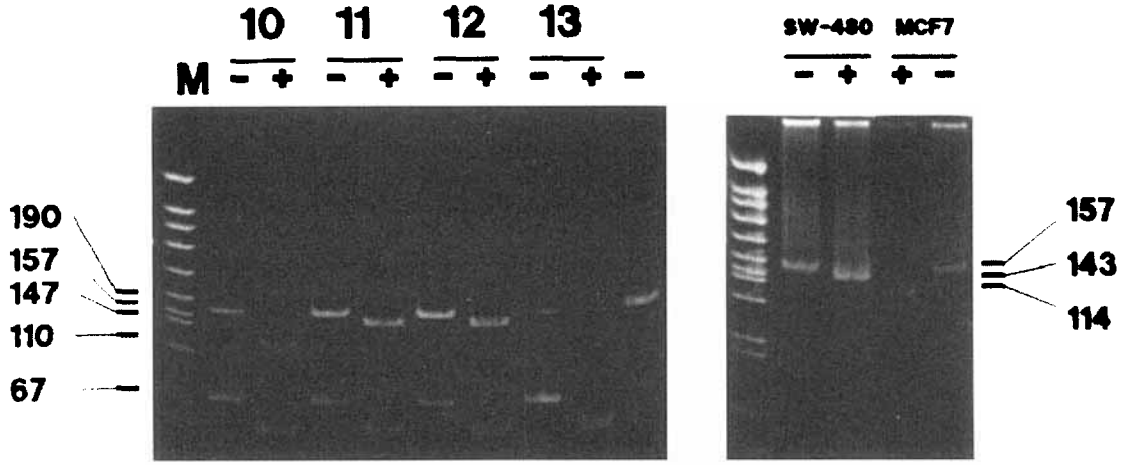

Fig. 1. Enriched PCR Analysis. Genomic DNA was prepared from the sputum samples and was subjected to the Enriched PCR with the modification of temperatures being 94°C, 59°C, and 72°C for denaturation, annealing, and polymerase, respectively. The first round of amplification was performed for only 20 cycles. Five μl of the amplified material were digested using BstNI at 60°C for 1 h. Subsequently, 0.5 μl was used as a template for the second round of amplification. The characteristic 157-bp amplified product of the second round of amplification (− lines) was digested with the restriction endonuclease BstNI (+ lines), and separated on 10% PAGE. The presence of a 143-bp fragment (samples 11, 12 represent sputum of patients with lung cancer) reveals the existence of mutant alleles, whereas a 114-bp fragment represents the normal allele of the K-ras gene. The position of the molecular weight markers is outlined in the left panel, and the respective size of fragments generated in the Enriched PCR is noted in the right panel.

chi-square test for the comparison of proportions from independent samples with one degree of freedom, which assumes that the partitioning of the data into groups was planned a priori (Fleiss, 1981b].

RESULTS

K-*ras* Mutation Identified in the Sputum of Patients With Lung Cancer

Genomic DNA present in the sputum was subjected to an EPCR. Owing to the quality of the DNA in these samples, we increased the amount used as a template for the first round of our reaction to 2 μg. A typical pattern of analysis following an EPCR reaction is shown in Figure 1. The presence of a wild-type band (114 bp) in addition to the mutant band (143 bp) is the result of incomplete digestion of the amplified material obtained after the first round of amplification. Alternatively, although only 1% of the original material was taken to the second round of amplification, the latter could consist of small amounts of genomic DNA that could also serve as a template for amplification, yielding a small portion of normal alleles which would be reflected in the final analysis.

The 37 sputum samples analyzed here were obtained from males who were smokers diagnosed with squamous cell lung cancer; 18 (48%) were found to harbor the K-*ras* codon 12 mutation (Table I). With one exception, all samples revealed the mutant K-*ras* allele only when the sensitive EPCR was used, indicating that although the frequency of *ras* mutation is high (close to 50%), the incidence of the mutation within a test sample is very low. Based on our previous evaluations using dot blot hybridization techniques, we were able to estimate a minimal incidence of one mutant allele among 250 normal alleles, which is reflected in the samples tested here.

TABLE I. K-*ras* Mutation in Sputum of Patients With or Without Lung Cancer†

Sample	Positives n/total	(%)	SE	95% Confidence interval
Sputum of patients without lung cancer	3/28	(12)	5.8	0–22.1
Sputum of patients with lung cancer	18/37	(48)	8.2	32.5–64.7

†When the two patient groups were compared using chi-square analysis; $X^2 - 5.9$ with $p < 0.01$.

K-*ras* Mutation in Sputum Samples From Patients With Non-Oncological Lung Diseases

As a control, we used sputum that was obtained from patients with asthma, chronic bron-

chitis, acute bronchitis, pneumonia, or heart disease. Of the total of 40 cases analyzed, 28 were males (of whom 23 were smokers), and 12 were non-smoking females. EPCR analysis identified 5 of the 40 cases to harbor the mutant K-*ras* allele (12.5%). These mutations were distributed between males (3/28) and females (2/12) (Table I). None of the non-smoking males were found to harbor this mutation. All positives had low incidence of mutant *ras* alleles.

DISCUSSION

The ability to detect mutated genes in excreta of patients prior to the detection of neoplasms by common histopathological procedures provides one of the hallmarks of early diagnosis and potential prevention. Extensive studies that enable the identification of oncogene and tumor suppressor gene mutations are translated into clinically applicable assays that may offer the ability to identify patients at risk of developing certain types of cancer. The present study provides an important advance toward the potential use of the K-*ras* oncogene mutation as a biomarker in lung cancer. Here, we have documented the frequency of this *ras* oncogene mutation in the sputum of patients with lung cancer as compared with controls who suffer from non-oncological disorders. While about 50% of patients with NSCLC harbored the mutant allele, only 12% of the control group was found to contain this mutation. These differences appear to be significant when subjected to statistical analysis ($P < 0.01$). Similar differences were found when the lung tissues of patients without lung cancer were compared with those of patients who have lung cancer (10 and 60%, respectively; $P < 0.001$) [Yakobovskaya et al., 1995]. In all, the present analysis allows one to conclude that the mutant K-*ras* oncogene is present in very low incidence in the sputum samples of patients with lung cancer. The low incidence of mutant *ras* alleles in the sputum of patients without lung cancer is likely to represent mutant cells that are randomly distributed throughout the normal appearing lung tissue.

The presence of mutant *ras* alleles does indicate, however, the occurrence of a genomic event that has yielded this mutation. This could be the result of an exogenous exposure, such as to cigarette smoke, as implicated in numerous studies that associated smoking with a higher incidence of this specific class of mutation [Westra et al., 1993; Husgafvel-Pursiainen et al., 1993] and the respective DNA adducts [Mooney et al., 1995]. The latter could explain the presence of *ras* mutations in 10% of the patients who were free of NSCLC but were heavy smokers. One cannot exclude that such mutations may represent endogenous events as a consequence of impaired repair or detoxification enzyme activities that could be part of predisposition. The presence of mutant *ras* alleles in lung samples could represent a certain degree of exposure, and as such could indicate the existence of additional genomic alterations, which together would put the patient at greater risk of developing lung cancer. Given that about 18% of smokers develop lung cancer and that 60% of lung cancer patients harbor *ras* mutations, one may estimate that about 10% of smokers would harbor mutant *ras* alleles. The latter is in line with the 10–12% of normal lung tissues that were found to harbor *ras* mutations, implying a high accuracy of the analysis performed here.

Mutant *ras* is only one of several potential biomarkers for lung cancer. A high frequency of p53 mutation was reported, as well as mutations in the APC gene, and an instability of microsatellite repeats [Mao et al., 1994a]. An indication was recently demonstrated for the appearance of p53 gene mutations in preneoplastic lesions of sputum samples of patients prior to their diagnosis of lung cancer [Mao et al., 1994b] and in chick cells prior to the development of oral cancer [Boyle et al., 1994]. In all, a reliable protocol of early diagnosis must rely on the combination of a few independent biomarkers, which await the development of technology that will enable their detection in preneoplastic lesions.

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