Detection of K-*ras* Mutations of Bronchoalveolar Lavage Fluid Cells Aids the Diagnosis of Lung Cancer in Small Pulmonary Lesions[1]

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ABSTRACT

An increased prevalence of K-*ras* oncogene mutation in lung adenocarcinoma has been shown by PCR-primer-introduced restriction with enrichment for mutation alleles (PCR-PIREMA) experiments. In the present study we investigated whether this method is useful for the diagnosis of lung cancer in small pulmonary lesions, which are difficult to diagnose cytologically as lung cancer by bronchoscopic examination. We examined bronchoalveolar lavage fluid (BALF) cells from 33 patients with single nodular pulmonary lesions of less than 2 cm in diameter (measured on chest computed tomography scans) for K-*ras* (codon 12) mutation, by PCR-PIREMA. Transbronchial fiberscopic examinations had not revealed lung cancer cytologically in any of the patients. The final diagnoses for the 33 lesions were 20 adenocarcinomas, 5 cases of focal fibrosis, 5 cases of pneumonia, 1 case of tuberculosis, 1 hamartoma, and 1 case of lymph node swelling. BALF cell lysates were amplified and digested with a restriction enzyme to detect the K-*ras* oncogene. Only the normal K-*ras* was observed after the first amplification and digestion for each of the 33 patients. Three amplifications and digestions were performed for each sample. We detected mutation of K-*ras* in BALF cells from 15 (75%) of 20 lung cancer patients and in cells from only 4 (31%) of 13 patients with nonmalignant lesions. The detection rate of the K-*ras* mutation in lung cancer was significantly greater than that in nonmalignant lesions ($P = 0.012$). Our results indicate that the detection of the codon 12 K-*ras* mutation in BALF cells by PCR-PIREMA aids the diagnosis of lung cancer in patients with small pulmonary lesions with negative cytological findings.

INTRODUCTION

Lung cancer is the leading cause of cancer deaths in Japan. To improve the prognosis of lung cancer patients, many oncologists have been trying to develop tests that will facilitate the earlier diagnosis and treatment of lung cancer and thereby decrease the mortality from this disease. Most early-stage lung cancers show no symptoms and are detected as an abnormal shadow on a chest roentgenogram or a chest CT[3] scan. Lung cancer appears as small nodules in the peripheral lung, and pathological or cytological diagnosis is essential for the diagnosis of lung cancer. Patients suspected of having lung cancer often undergo fiberscopic examination, with a tumor biopsy examination or a cytological approach. When a lesion is inaccessible to bronchoscopic biopsy or when the biopsy specimen is nondiagnostic, a diagnosis of cancer may be possible by cytological examination of the BALF, but this method is much less sensitive than the examination of a biopsy specimen (1). Cytological or pathological confirmation for small nodular lesions less than 2 cm in diameter is difficult; aggressive CT-guided aspiration cytology through the chest wall is often performed. However, many such lesions are resected without a diagnosis being made before the surgery.

Body fluids sometimes contain cells or cell debris bearing the oncogene mutations that characterize the related tumor, as has been shown for *ras* mutations in stool specimens from patients with colorectal tumors (2) and for *p53* mutations in urine from patients with bladder cancer (3). Similarly, mutations of K-*ras* that are associated with lung cancer have been detected in BALF cells (4). The clinical use 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 (5). In the largest study of *ras* mutations in human lung cancer, K-*ras* mutations in codon 12 predominated; they were found in 17% of 258 non-small cell lung cancer samples obtained by surgical resection, primarily (24%) in adenocarcinoma (6).

Cancer cells in BALF are always mixed with large numbers of genetically normal cells; therefore, the detection of *ras* mutations in BALF requires a sensitive assay such as PCR-PIREMA, which was developed to detect *ras* mutations (7, 8). It has been reported that the sensitivity and specificity of K-*ras* mutation detection in BALF samples for the diagnosis K-*ras* mutation-positive lung cancer were both 100% using PCR-PIREMA, and that this method detected K-*ras* mutations in BALF cells in 46% of adenocarcinomas of the lung (4). Therefore, we expected that we would frequently be able to detect the K-*ras* mutation in BALF cells from small lung lesions using the

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[3] The abbreviations used are: CT, computed tomography; BALF, bronchoalveolar lavage fluid; PCR-PIREMA, PCR-primer-introduced restriction with enrichment for mutation alleles.

Table 1 Patient characteristics

	No.
Total cases	33
Sex	
Male	18
Female	15
Age	
Mean	63
Range	45–79
Size of lesion[a] (cm)	
1.5–1.9	8
1.0–1.4	16
<1.0	9
Cytology[b]	
Negative	33

[a] The size of lesion was determined by chest CT.
[b] Cytological examination was performed by bronchoscopy.

Table 2 Final diagnosis of small pulmonary lesions

Diagnosis	No. of patients
Adenocarcinoma	20
Focal fibrosis	5
Pneumonia	5
Tuberculosis	1
Hamartoma	1
Swelling of lymph node	1

PCR-PIREMA method. We conducted a prospective study to determine whether the detection of K-*ras* mutation in BALF can aid the diagnosis of lung cancer in cases of a small pulmonary lesion that is cytologically negative on bronchoscopic examination.

MATERIALS AND METHODS

BALF Cell Collection. Between October 1995 and February 1998, patients with a nodular lesion of less than 2 cm in diameter in the peripheral lung that was subsequently diagnosed by biopsy specimen examination were enrolled in the present study. After chest roentgenography and CT, each patient underwent bronchoscopic examination to diagnose the cause of the lesion. Saline (50–100 ml) was injected into the target bronchus after transbronchial biopsy or brushing and lavage fluid specimens were obtained. One-half of the lavage fluid was used to make a cytological diagnosis, and the other half was used to test for K-*ras* mutation.

PCR-PIREMA Protocol. A modified PCR-PIREMA method was used to detect K-*ras* mutations in BALF cells (7). BALF cells (5×10^4) were washed and resuspended in 500 μl K-buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5% Tween 20, and 100 μg/ml proteinase K]. The cell suspension was incubated at 55°C for 1 h and then heated at 94°C for 10 min to inactivate proteinase K. These cell lysates were stored at −20°C until used for PCR.

Briefly, PCR around K-*ras* codon 12 was performed by using a mismatched primer (F primer, 5′-ACTGAATATAAACTTGTGGTAGTTGGACCT-3′; R primer, 5′-ACTCATGAAAATGGTCAGAGAAACCTTTAT-3′) that introduced a *Bst*NI restriction site into the PCR products derived from normal alleles. *Bst*NI digestion of the PCR products left only the PCR products derived from mutant alleles intact, after which further PCR selectively amplified the mutant PCR products. The first PCR reaction mixtures contained 10 μl of cell lysate, 8 μM concentrations of each nucleotide, 0.8 mM $MgCl_2$, and 5′-mismatched primer to introduce a *Bst*NI restriction site flanking the K-*ras* exon 1. The first PCR products were digested with *Bst*NI. When mutated K-*ras* was not detected after digestion of the first PCR products, which had been amplified by 30 cycles of PCR, a fresh aliquot of the samples was amplified by 10 cycles of PCR, and, after *Bst*NI digestion, the samples was amplified twice more. The second PCR reaction mixture contained 10 μl of the digest of the first PCR products (diluted 1:100), 4 μM concentrations of each nucleotide, and 0.6 mM $MgCl_2$. The second PCR products also were digested with *Bst*NI. The first and second PCR reactions were performed for 10 and 20 cycles, respectively, 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. The digest of the second PCR products (diluted 1:100) was then amplified under standard PCR conditions (each nucleotide at 200 μM, 1.5 mM $MgCl_2$, 55°C annealing, 34 cycles) using the same primers followed by repeat *Bst*NI digestion; these products were then electrophoresed on 2.5% agarose gels and stained with ethidium bromide. A digestion-resistant 192-bp band indicated the presence of a K-*ras* codon 12 mutation. Each sample was subjected to the entire PCR-PIREMA process at least twice. Extensive measures were taken to prevent cross-contamination of samples. A normal control sample and a known mutation sample were included in all of the experiments.

Statistical Analysis. The χ^2 test was used to analyze the differences in the frequency of K-*ras* mutation between lung cancers and nonmalignant lesions.

RESULTS

Between October 1995 and February 1998, 56 patients with small nodular lesions in the peripheral lung that were less than 2 cm in diameter visited the Kanagawa Cancer Center. Fifteen of the patients were diagnosed with lung cancer cytologically by bronchoscopic examination, and eight of them did not receive a definite diagnosis and did not undergo surgical resection. The other 33 patients, for whom transbronchial examination did not reveal lung cancer cytologically although definite diagnosis was made later by surgical resection, entered the present study. The diameter of the lesion was less than 2 cm, and there was no lymph node swelling on chest CT for all of the 33 patients. The patient characteristics are shown in Table 1. The diameter of the lesion was between 1.5 cm and 1.9 cm for 8 patients, between 1.0 cm and 1.4 cm for 16 patients, and less than 1.0 cm for 9 patients. In all of the 33 cases, lung cancer was suspected based on the radiological findings, and the lesion was resected. The diagnoses were 20 cases of adenocarcinoma, 5 of focal fibrosis, 5 of pneumonia, 1 of tuberculosis, 1 of hamartoma, and 1 of lymph node swelling (Table 2). Data for K-*ras* mutation in BALF cells in four representative patients are shown in Fig. 1. Only normal K-*ras* was observed after the first amplification and digestion with the restriction enzyme for all of the four patients. We detected mutation in K-*ras* in the BALF cells from two patients after the third amplification and digestion with the

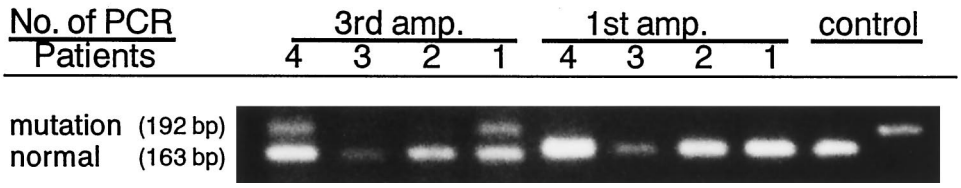

Fig. 1 PCR-PIREMA analysis of BALF cell lysates. *1st amp.*, PCR products of the first amplification (30 cycles) after digestion with *Bst*NI. *3rd amp.*, PCR products of the enriched screening step (three PCR amplifications followed by digestion with *Bst*NI: there were 10, 20, and 34 cycles in the first, second, and third amplifications, respectively.) The results for BALF cells from four representative patients are shown. Only the normal 163-bp band was observed after the first amplification in all of the cases, but a mutated K-*ras* 192-bp band was detected after the third amplification in cases 1 and 4. The diagnoses were adenocarcinoma (cases *1* and *4*) and pneumonia (cases *2* and *3*).

Table 3 Prevalence of K-*ras* codon 12 mutations in small pulmonary lesions by diagnosis

Detection of k-*ras* mutation in lung cancer was significantly higher than that in nonmalignancy ($P = 0.012$).

	No. of cases	
Diagnosis	Mutation (%)	Normal (%)
Lung cancer[a]	15 (75)	5 (25)
Nonmalignancy	4[b] (31)	9 (69)

[a] Adenocarcinoma (20).
[b] Focal fibrosis (3), pneumonia (1).

restriction enzyme. The lesions of the two patients with mutated K-*ras* were adenocarcinoma, and those of the other two patients, with normal K-*ras*, were pneumonia. The K-*ras* mutation results for all of the 33 patients are shown in Table 3. Only normal K-*ras* was observed after the first amplification and digestion for all of the 33 samples, and three sequential amplifications and digestions were performed for all of the samples. We detected mutation of K-*ras* in the BALF cells from 15 of the 20 lung cancer patients (75%) and in cells from only 4 of the 13 patients without a malignant lesion (31%). The nonmalignant lesions with a K-*ras* mutation were three cases of focal fibrosis and one of pneumonia. The detection rate of K-*ras* mutation in lung cancer was significantly higher than that in nonmalignant lesions ($P = 0.012$).

DISCUSSION

PCR-PIREMA is easily applied to BALF cells from patients undergoing diagnostic bronchoscopy as reported by Mills *et al.* (4, 7), and this method was reported to detect ras mutations at a higher rate than other methods. PCR-PIREMA has detected *ras* mutations in 46% of adenocarcinomas of the lung and detected one mutated allele in K-*ras* per 10^6 normal alleles. Most small lung cancers treated at our hospital are adenocarcinomas, and all of the lung cancer patients included in the present study had adenocarcinoma. Therefore, we examined whether detection of K-*ras* mutation using PCR-PIREMA aids the diagnosis of small pulmonary lesions.

Many genetic changes have been identified in lung cancer, but little is known about the chronology of their development. Some genetic changes may represent early activation events, whereas others are more likely to accompany late events related to invasion and metastasis. On the basis of the limited evidence, some investigators have suggested that in some human tumor types, including lung cancer, *ras* mutations may fall into the former category. The present study demonstrated that a codon 12 K-*ras* mutation was present in 15 (75%) of 20 lung cancer cases, which is higher than the previously reported frequencies. We analyzed very small lung cancer lesions, which in all 20 of the cases were adenocarcinoma. Our results may indicate that the K-*ras* mutation occurs more frequently in the early stage of adenocarcinoma compared with advanced adenocarcinoma. Analysis of the K-*ras* mutation in small resected lung cancers of less than 2 cm in diameter will verify this hypothesis.

Making cell lysates from BALF cells is easy and the PCR-PIREMA assay is rapid, nonradioactive, and readily adaptable to processing large numbers of clinical samples. The assay can also be used to detect all of the activating mutations of K-*ras.* However, three PCR amplifications are required to detect one mutant allele in 10^6 normal alleles, and there is a high misincorporation rate of Taq polymarase. One error occurs per 10^4 bases under standard PCR conditions. To minimize the PCR error, we used the protocol reported by Mills *et al.* (4), but we decreased the cycle number for the first and second amplifications.

Fifteen of 20 adenocarcinomas had a mutated K-*ras* in the BALF cells, which is significantly higher than the mutated K-*ras* frequency that we saw in patients with nonmalignant lesions (4 of 13). Therefore, the detection of mutated K-*ras* in BALF cells may indicate lung cancer of the target lesion in spite of a pathological or cytological negative finding. K-*ras* mutation was detected in BALF cells from four patients with nonmalignant lesions: three had focal fibrosis, and one had pneumonia. Atypia of cells was observed in a lesion removed from one the focal fibrosis patients, and active inflammation was observed in the lesion from the patient with pneumonia. Therefore, a K-*ras* mutation may present in nonmalignant lesions such as atypia and active inflammation, and the detection of a K-*ras* mutation does not always correlate with cytological detection of cancer cells. Nevertheless, the frequency of a K-*ras* mutation in BALF cells is about 2.5 times greater in cases of lung cancer, and, therefore, the K-*ras* mutation in BALF cells is a clinically useful biomarker for lung cancer. In conclusion, detection of a K-*ras* mutation in BALF cells using PCR-PIREMA aids the diagnosis of lung cancer in patients with small pulmonary lesions. Our results suggest that surgical resection should be considered for patients with a K-*ras* mutation in their BALF cells even if the cytological finding is negative.

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