Low mutational rate of K-ras codon 12 in singular bronchoscopy specimens in suspected lung cancer*

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Mutations of the K-ras gene are found in a subset of non-small-cell lung carcinomas (NSCLC). The aim of our study was to determine the K-ras codon 12 mutation in the first, singular bronchoscopy specimen in parallel with the cytological examination for the diagnosis of lung cancer.

Samples were obtained by diagnostic bronchoscopy in 140 patients with suspected lung tumors. The analysis of K-ras mutations was carried out by a sensitive two-step mutation-enriched polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. This method has been confirmed earlier to be positive for mutated tumor cells and negative for normal lung parenchyma and bronchus.

Of the 140 patients with suspected cancer, 93 were diagnosed as NSCLC by cytology or histology in either the same specimen used for the detection of K-ras mutation or in later biopsies. However, only four K-ras codon 12 mutations were detected in the first bronchoscopic material: one in adenocarcinoma, two in squamous cell tumors, and one mutation was found in a patient with dysplasia which was diagnosed later as a squamous cell carcinoma.

Our findings indicate that although the K-ras (codon 12) mutation is a gene lesion infrequently detectable in a singular specimen taken at the first bronchoscopy examination in cases of clinically suspected lung cancer, the detection of this mutation can help to confirm the cytological diagnosis of NSCLC or may be even diagnostic in cytologically negative cases.

Key words: lung carcinoma, K-ras, codon 12, bronchoscopy

Lung cancer is one of the leading causes of cancer-related death in the industrialized world and a successful treatment depends on an early diagnosis followed by surgical resection. Little progress has been made in decreasing lung cancer mortality by using conventional methods of early diagnosis and screening. Large randomized controlled trials of radiological and cytological screening for lung cancer showed that periodic chest X-rays and/or sputum cytology have no impact on lung cancer mortality and such screening is generally not recommended [6, 7]. Similar randomized trial carried out in Czech male population revealed that results of lung cancer therapy were not improved by earlier tumor detection, and programs of screening by regular X-ray examination conferred no benefit [11].

Clinically useful tests for lung cancer include both screening tests, used in asymptomatic population, and diagnostic tests, used in patients clinically suspected of having lung cancer. Both screening and diagnostic tests must be able to identify patients at an early, surgically resectable stage of the disease. The presence of genetic changes in preneoplastic lesions of the airway epithelium from metaplasia and dysplasia to carcinoma *in situ*, and the clinical correlations of these changes are therefore important for the development of reliable molecular screening tests for lung cancer [18, 20, 26, 27, 36].

Enormous number of published data implicate point mutations of the K-ras gene as the most prevalent mutations found amongst ras oncogenes in lung cancer, developing early in a

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subset of NSCLC, and as such can be used as sensitive biomarkers for the diagnosis of NSCLC [17, 22, 27, 35]. Adenocarcinomas of the lung generally show higher mutational frequencies of K-ras than squamous cell carcinomas, ranging from 15 to 60 per cents [2, 16, 23, 28, 31, 33]. Almost 90% of all K-ras mutations are located in codon 12. K-ras point mutations were suggested to be early events acquired before clonal expansion of the tumor [10, 13, 14, 25]. However, along with the detection of mutated K-ras in NSCLC, positivity was also seen in a relatively large proportion of normal lung tissue or bronchial epithelium specimens from cancer patients [4, 9, 21, 34]. In contrast to these results, in some experimental settings, no K-ras mutations were found in normal bronchial and lung parenchymal samples from patients with NSCLC when mutations were present in the tumor [32, 33]. The presence or absence of mutations in normal lung apparently depends on the sensitivity of the assay used for the detection of mutations.

In this study, we detected K-ras codon 12 mutations in parallel with the cytological and/or histological examination of samples obtained by bronchoscopy in 140 patients being evaluated for suspected lung cancer. We used a mutation-enriched polymerase chain reaction-restriction fragment length polymorphism method for the detection of mutations. This method was designed to be specific for the tumor tissue; although it is sufficiently sensitive, no positive results were seen in the normal lung [33]. We employed this assay to elucidate whether the parallel processing of a specimen for both the cytology and the detection of K-ras codon 12 mutation could be an aid in the early diagnosis of NSCLC. We analyzed only a singular bronchoscopic sample taken at the first examination in suspected lung cancer patients to investigate a possible utility of the K-ras mutation as a simple, parallel routine test in the primary clinical material along with cytology/histology.

Patients and methods

Patient selection. The study included 140 patients (118 males and 22 females; age range, 42 to 86 years; average age 64.2 years) who underwent diagnostic bronchoscopic procedure for radiologically or clinically suspected lung cancer at the Clinic of Pneumology and Thoracic Surgery (University Hospital in Prague-Bulovka). The Institutional Ethical Committee approved the study. Analysis of point mutations at codon 12 of the K-ras was carried out independently of the results of the cytological and/or histological examination of bronchial tissue. None of the patients had undergone primary radiotherapy or chemotherapy. All except two patients were current or former smokers.

Tumor specimens. Patients were referred to bronchoscopy examination from their primary care physician because of clinical suspection of lung cancer. Using flexible fiberoptic bronchoscopy, three bronchial aspirates were taken from the tumor-suspected area in all patients regardless of the endoscopic finding and a portion of the last sample was used for K-ras mutation analysis. In addition, where the tumor-suspected area was visible in the endoscopic view (in 24 patients), a biopsy sample was excised. For K-ras analysis, samples were washed in PBS and stored at –75 °C until processed for DNA analysis. DNA was isolated by standard techniques (incubation with Proteinase K and phenol/chloroform extraction). Usually at least several hundred cells were available and DNA isolation always resulted in a sufficient quantity of undegraded DNA.

Analysis of point mutations in codon 12 of K-ras. Mutations at codon 12 of K-ras were examined in 140 bronchial aspirates and additional tumor biopsy specimens from 24 patients by a sensitive two-step mutation-enriched polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) as previously described [33], and the mutations were verified by direct sequencing of the PCR products. The PCR-RFLP method is sensitive and detects one mutated cell among at least 1000 normal cells. No mutation has been detected in any of the non-tumoral lung parenchyma samples [33] and thus this mutation assay is specific for the tumor tissue.

Results

Table 1 shows the final histological diagnosis and the number of patients in which the tumor was diagnosed from the first cytology/histology. Of the 140 patients undergoing evaluation, 85 patients did have a diagnosis of lung cancer (or other cancers) as revealed by cytology/histology from the material obtained from the first bronchoscopy. Fifty five samples were negative at the first cytological (or cytological + histological) examination and of these negative samples, normal cells were found in 28, metaplasias in 8, and dysplasias in 9 specimens. In 10 cases, no tumor was confirmed during the following 6 months. Thus, of the 55 patients in which tumor cells were not found in the first bronchoscopic sample, 45 cases were diagnosed as neoplasias later.

Detection of mutations by PCR-RFLP revealed only four mutations of the K-ras codon 12 (Tab. 1). One mutation was found in adenocarcinoma and two mutations in squamous cell carcinomas. The fourth mutation was found in the sample cytologically read as dysplasia in which squamous cell carcinoma was diagnosed later. The mutations were detected by the double-step PCR-RFLP assay (Fig. 1) and confirmed by sequencing of mutation-enriched PCR products as described [33] (not shown). The data of four patients with K-ras codon 12 mutations detected by sequencing are summarized in Table 2. The mutations changed glycine to cysteine, valine, aspartic acid and serine, respectively, and were detected in bronchial aspirates; no mutation was found in 24 patients who underwent parallel bronchoscopic biopsies. All mutations were identified in male smokers. No mutations were found in large cell carcinomas or small cell carcinomas, and

Table 1. K-ras codon 12 mutations in bronchoscopic samples

Final diagnosis		Tumor cells diagnosed in the examined sample*	K-ras codon 12 mutation
Squamous cell carcinoma	69	52	3
Adenocarcinoma	17	13	1
Small cell carcinoma	26	16	–
Large cell carcinoma	7	4	–
Other tumors**	11	–	–
No tumor	10	–	–
All	140	85	4

*bronchial aspirates or biopsy specimens taken at the first diagnostic bronchoscopy; **carcinoids, metastases from kidney and other organs, sarcomas.

Table 2. Clinical data of patients with K-ras mutation

Age/Sex	Material evaluated	Cytological diagnois	Bronchoscopy mutation	Final dg.	TNM/Stage	K-ras codon 12
55/M	b. wash.*	AD	mass	AD	pT2N0MO/I	GGT>TGT
71/M	b. wash.	dysplasia	infiltrate, hemo.	SQ	T3N2M0/IIIa	GGT>GTT
76/M	b. wash.	SQ	effusion	SQ	T4N0M1/IV	GGT>GAT
56/M	b. wash.	SQ	mass	SQ	pT2N1M0/II	GGT>AGT

*b. wash. – bronchial washing, AD – adenocarcinoma, SQ – squamous cell carcinoma, hemo. – hemoptysis.

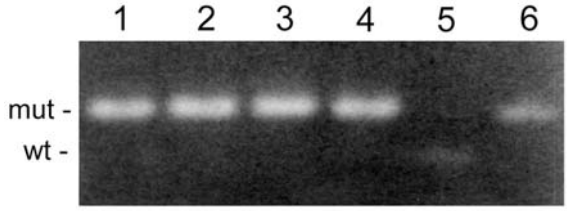

Figure 1. K-ras codon 12 mutations analyzed by double-step PCR-RFLP. Lanes: 1, positive sample (adenocarcinoma); 2, positive sa mple (dysplasia, diagnosed later as squamous cell carcinoma); 3, 4, two positive samples (squamous cell carcinomas); 5, one of the negative samples; 6, positive control (cell line SW480). Upper bands correspond to the mutated K-ras allele and lower band is the normal (wild type) allele (indicated on the left).

no mutations were found in patients not having a tumor, as expected.

Discussion

Numerous reports addressed the question as to whether mutations in the K-ras gene could be used as a complement to the examinations used in the early diagnosis of lung carcinomas [1, 4, 5, 10, 12–14, 21, 25, 29, 34]. Sensitive methods such as allele-specific amplification, two-step PCR-RFLP, ligase chain reaction (LCR), point-EXACCT technique, or cloning of PCR products are needed to detect the mutations in a minority of cells in specimens like sputum, bronchoalveolar lavage (BAL) fluid, or small biopsies [1, 3, 5, 8, 9, 14, 15, 22, 24, 29, 30]. We investigated here the utility of the K-ras codon 12 mutation detection in bronchoscopy specimens obtained at the first diagnostic examination in clinically suspected cases. Although the most sensitive tests can detect K-ras mutations in histologically normal bronchial epithelium or in malignant precursor tissues, and such a detection can justify subsequent clinical monitoring, the positivity in these assays lowers their expected diagnostic value. The aim of our study was to ascertain whether K-ras mutations could be used as a diagnostic test in a singular bronchoscopy specimen and to compare the results with routine cytology. We used a sensitive assay based on a mutation-enrichment PCR step which was demostrated to be negative in all nontumor specimens [33]. We detected total four samples harboring mutations, three of which were also tumor-positive in cytology and epithelial dysplasia was detected in the fourth (in which the squamous cell carcinoma was diagnosed from a later biopsy). The mutational rate found here is surprisingly low, especially in adenocarcinomas which are known to contain higher frequency of K-ras mutations. In most of cases, lung cancer diagnosis is based on the finding of tumor cells in the cytological smears. The number of cancer cells may vary greatly among samples, as has been also the case in samples described here (from a small cell group to more than a half of tumor cells in the sample). Therefore, we can not entirely exclude that some samples had a portion of mutated cells that was below the detection limit of the assay and might have escaped detection. Nevertheless we feel this is a less likely explanation of the low frequency of mutations, corroborated by the observation that no mutation was found in a tumor tissue available from several subsequent surgical resections in operable patients (5 adenocarcinomas and 4 squamous cell tumors, data not shown). Furthermore, all 24 bioptic samples which contained higher fraction of malignant cells revealed no mutation, confirming the negativity in the bronchoscopic aspirates (Tab. 1).

We have previously analyzed K-ras and H-ras mutations in resected NSCLC [33] and found the frequency 8% in squamous cell carcinomas. Most of the tumors analyzed in the present study were squamous cell types and the frequency of K-ras codon 12 mutations was 4.4% (3/69, Tab. 1). Only one mutation was detected among 17 adenocarcinomas (5.9%). Although the analyzed groups of patients are different, the frequencies of mutations detected in the brochoscopic material are about 2-times and 8-times lower than those seen in fresh resected squamous cell tumors and adenocarcinomas, respectively. However, due to a relatively low number of patients, the difference between these two in-

dependent groups of patients (tumor tissue taken by surgery versus bronchoscopic sample) is not statistically significant ($p<0.05$, Fisher's test). Moreover, codons 13 and 61 of K-ras might have also contained a mutation that was not analysed in the present study. It is known that SCLC cells never contain K-ras mutations [19]. Here, as expected, we likewise did not detect any mutation among 26 SCLC specimens diagnosed cytogically or histologically from the same or later bronchoscopic material (Tab. 1).

In conclusion, we have investigated the possibility of using the screening of K-ras codon 12 mutations during the first diagnostic steps by examining just the first bronchoscopic specimen. The results show that, despite the availability and applicability of a sensitive, specific and rapid molecular screening method, the analysis of K-ras codon 12 mutations as a widely used assay in the early diagnosis of lung cancer seems to be limited due to the relatively low frequency of K-ras mutation found in a single bronchoscopic sample of NSCLC. Therefore, it seems to be impractical to use the detection of K-ras mutations as a method applicable only to the first bronchoscopic sample in suspected lung cancer patients. This suggests that other approaches, such as parallel examination of bronchoalveolar lavage fluid and sputum may be needed to detect more diagnostic mutations.

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