Mutations of K-ras Oncogene and Absence of H-ras Mutations in Squamous Cell Carcinomas of the Lung

Jiří Vachtemich,2 Irena Horáková,
Hana Novotná, Petr Opálka,
and Helena Roubková
Laboratory of Molecular Biology [J. V., I. H., H. N.] and Department of Clinical Oncology [P. O., H. R.], Institute of Chest Diseases, 18071 Prague 8, Czech Republic

ABSTRACT

Mutations of the K-ras gene have been implicated in the pathogenesis of human lung adenocarcinomas. In most studies published so far, squamous cell lung cancers harbored ras mutations only exceptionally or no mutations were detected at all. We have examined 141 lung tumor DNA samples for mutations in codons 12, 13, and 61 of K-ras and H-ras oncogenes. A large panel of 118 squamous cell carcinomas was included in the study. For K-ras codon 12, we used a sensitive two-step PCR-restriction fragment length polymorphism method which detects <1% of mutated DNA in the sample. K-ras mutations were found in 17 tumors (12%; 14 in codon 12 and 3 in codon 13). Among 19 adenocarcinomas, mutation was revealed in 7 samples (37%). Of these, one sample harbored two point mutations in codon 12. Nine mutational events were found in squamous cell carcinomas (8%, one adenosquamous carcinoma included, all in codon 12). Of four large cell carcinomas, one contained a mutation. Mutant-enriched PCR products harboring mutations were directly sequenced. Fifteen mutational events were G→T transversions or G→A transitions, one was a G→C transition, and one sample revealed a frameshift deletion of one G from codon 12. Similar mutational spectrum was found in both squamous cell carcinomas and adenocarcinomas, suggesting similar carcinogenic pathways in both histological types of the tumor. The presence of mutations did not correlate with the stage of the disease. Moreover, we analyzed all samples for mutations in codons 12, 13, and 61 of the H-ras gene. We found only one mutation in codon 12. Thus, H-ras mutations apparently play an inferior role in lung carcinogenesis. We conclude that mutations of the K-ras oncogene can play a role in the development of not only lung adenocarcinomas but also of a subset (about 8%) of squamous cell carcinomas.

INTRODUCTION

The ras genes code for small membrane-bound proteins (p21 oncoprotein) that bind and hydrolyze GTP and participate, through a cascade of protein kinases, in transmitting signals into the nucleus. Activating point mutations in genes of the ras gene family that convert these proto-oncogenes into their transforming forms are well documented to occur in many human tumors. Most frequently, mutations of the c-Ki-ras-2 (K-ras) oncogene were detected in 70–95% of pancreatic cancers (1–4), in 30–60% of colon and rectal tumors (5–8), and in about 45% of ovarian tumors (9). K-ras was also reported to be activated in the neoplasms of the lung and other organs. Most of these mutations occur in codon 12 and only a minority of mutational events is located in codons 13 and 61. In many tumor cell types, K-ras mutations have been suggested to play a role in the multistep molecular pathogenesis of neoplastic transformation. For example, in carcinomas of the colon, K-ras appears to become mutated during the adenoma growth. Recently, although at lower frequencies, the mutations were detected even in normal colonic mucosa from patients suffering from the tumor (10, 11).

Both borderline and invasive ovarian carcinomas were also shown mutated in K-ras (9), implying that borderline tumors may represent a continuum between benign and invasive ovarian tumors. Also, K-ras mutations were found in 2 of 16 atypical endometrial hyperplasias, which are precursors of endometrial carcinomas (12). Pancreatic preneoplastic lesions (mucoepidermoid hyperplasias), the presumed precursors of the pancreatic carcinoma, have been also shown to contain K-ras mutations at high frequencies similar to those documented for ductal type of pancreatic carcinomas (13). All of these findings indicate that K-ras mutations may be relatively early genetic events in the formation of many human neoplasms.

About 80% of lung cancers are histologically classified as NSCLC,3 comprising the two most frequent types, adenocarcinoma and squamous cell carcinoma. It has been well documented that K-ras is mutated in a subset of lung adenocarcinomas. The frequency of mutations varied from 15 to 60% (14–25). Squamous cell carcinomas of the lung were reported to contain mutated K-ras only exceptionally. Of the 21 squamous cell lung tumors, 2 showed activated K-ras (24) and 2 of 6 cell lines established from squamous cell lung carcinomas contained K-ras mutations in codon 12 (21). In earlier studies, no mutations of K-ras in this type of tumor were detected (14, 17). Also, two mutations of H-ras, both in codon 61, were detected among 36 lung squamous cell carcinomas (19). In a recent study, however, the authors described an unusual distribution of K-ras activating mutations in lung neoplasms, the squamous cell tumors being mutated more frequently (8/38) than adenocarcinomas (322; Ref. 26). ras mutations are absent from small cell lung carcinomas (21) and neuroendocrine neoplasms of the lung (27). The clinical significance of K-ras mutations in lung car-

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: NSCLC, non-small cell lung carcinomas; RFLP, restriction fragment length polymorphism.
cinomas has also been investigated. Tumors harboring these mutations were found to have a generally worse prognosis and were associated with shortened survival (18, 26, 28).

In this work, we have undertaken the study on a large panel of squamous cell lung carcinomas to analyze the mutations in K-ras and H-ras oncogenes. In most studies carried out to date, activating mutations were detected by methods that do not enable revelation of the mutation if present in less than about 5–10% of cells (oligonucleotide hybridization, single-strand conformation polymorphism, and direct sequencing). Here we used a modified two-step mismatch, PCR method (29, 30) in which the detection of mutation in <1% of the tumor cell population is reliably accomplishable. In this method, the artificial restriction site is introduced by a mismatched primer. The normal allele is destroyed after the first PCR step by restriction enzyme digestion, enabling only the mutant allele to be amplified in the second PCR step (29, 30). We used this approach to screen changes in codon 12 of the K-ras gene, which is the most frequent mutational hot spot of ras genes in lung carcinomas and in most of other human solid tumors. For codons 13 and 61 of K-ras and all three codons (codons 12, 13, and 61) of H-ras, a single-step mismatch PCR assay was used. To determine the type of mutation, the mutant-enriched PCR products were directly sequenced.

MATERIALS AND METHODS

Tissue Samples, Preparation of DNA, and Control Cell Lines. Fresh tumor samples were obtained from surgically removed lungs immediately after resection. The patients were treated at the Institute of Chest Diseases (Prague, Czech Republic) and did not receive chemotherapy or radiotherapy before surgical resection. A fragment of tumor tissue adjacent to that used for histopathological diagnosis was frozen in liquid nitrogen and stored at -75°C. High molecular weight DNA was prepared by standard techniques using proteinase K digestion and phenol/chloroform extraction.

DNA extracted from cell lines (American Type Culture Collection) with known mutations served as controls. SW 480 cell line contains a homozygous mutation in codon 12 of K-ras (GGT→GTT). In lines Calu-1 and A-427, one allele is mutated at the same codon (GGT→GTG and GTG→GAT, respectively). LoVo cell line harbors GGC→GAC mutation in codon 13 of K-ras and NCI-H640 has a CAA→CAT mutation in codon 61 of K-ras. pT24C3 plasmid was a control for the screening of the H-ras (codon 12) mutations.

PCR. Genomic DNA (0.2 or 0.4 μg) was amplified in 20- or 40-μl volumes in a reaction containing primers (Genset), dNTPs (see below), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.01% (w/v) gelatin, and 1.5 mM MgCl₂. For codons 12 and 13 of the K-ras, the concentration of MgCl₂ was 2 mM. Table 1 gives the primers used in amplification reactions. Final concentration of primers in the reaction was 0.5 μM except for codon 13 H-ras where the concentration 1 μM was used. dNTPs were at the following final concentrations: 50 μM (K-ras, codons 12 and 13), 100 μM (K-ras, codon 61; H-ras, codon 61), or 200 μM (H-ras, codons 12 and 13). One unit of Taq polymerase (Perkin Elmer/Cetus) was added to the 40-μl reaction in hot start settings. Amplification mixtures were cycled 30 or 35 cycles in a thermal cycler (Perkin Elmer/Cetus), each cycle at 94°C for 1 min followed by 1 or 2 min at the annealing temperature and 1 or 2 min at 72°C. For codon 12 of K-ras, samples were amplified 16 cycles in the first step and then diluted 400 times in the second-step amplification. For single-step PCR screening of K-ras codon 12 mutations, primers for the second step were used (Table 1). The annealing temperatures were as follows: 55°C for K-ras, codon 12 (both steps); 52°C for K-ras, codons 13 and 61; 60°C for H-ras, codons 12 and 13; and 40°C for H-ras, codon 61.

The template for codon 13 of K-ras amplification was a 176-base pair PCR product amplified from genomic DNA by use of non-mismatched primers (K12S and K12A; Ref. 21) and diluted 10,000 times in the second amplification. For screening of codon 61 of H-ras, an approach described by the same authors was used: PCR product obtained by amplification of genomic DNA with primers 5'-ATGAGAGGTACCCAGGAGAG-3' and 5'-TCACGCGCGTTACCTGTTACT served as a template for amplification.

Restriction Enzyme Cleavage and Gel Electrophoresis. After amplification, reaction products were incubated with the appropriate restriction enzyme (Table 1) for a minimum of 4 h (3 units enzyme/sample) and electrophoresed through native 8% polyacrylamide gel or 3% NuSieve 3:1 agarose gel (FMC), stained in ethidium bromide, and photographed. Restriction enzymes were purchased from Fermentas (MvaI, BsuRI, BclI, HphII, and MspI), Sigma (HphI and AlwNI), and New England Biolabs (Earl). All samples showing mutant-specific band were processed twice to exclude a possible error introduced by Taq polymerase.

DNA Sequencing. For sequencing, the mutant allele-enriched PCR amplification products with mutations in codon 12 of the K-ras gene were purified by Magic PCR Preps kit (Promega) without restriction enzyme cleavage. The samples with mutations in codon 13 of K-ras were prepared for sequencing as follows: 176-base pair template (above) was first amplified with the antisense primer as in Table 1 and a sense primer (5'-TATTATAAGGGCTGCTGAAAATTG) having one mismatch in order to destroy the native BsuRI site, cleaved with BsuRI, diluted and amplified again with primers shown in Table 1 for K-ras, codon 13. The PCR product was then purified on a Minicon 30 (Amicon) microconcentrator.

Double-stranded PCR products were sequenced by Cir- cumpVent (New England Biolabs) or fmol (Promega) sequencing kits according to the conditions specified by the suppliers. 32P-labeled antisense primer (second step, Table 1) and the sense primer (Table 1) were used for sequencing PCR products to detect mutations in codon 12 and codon 13 of the K-ras gene, respectively.

Statistical Methods. Two-tailed Fisher’s exact test was used to examine the correlation of data (stage of the disease, histology, and presence or absence of mutations).

RESULTS

Mismatch PCR-RFLP Method for the Detection of Mutations in K-ras and H-ras Genes. A RFLP created through mismatched primers is a rapid and efficient method to detect point mutations and has been applied to the study of activation
of the ras gene group (21, 31). For codon 12 of K-ras, the primers used here in the two-step screening were the same as described (11). Sequences of all primers are summarized in Table 1. In most assays, a further mismatch is introduced into the primers to create a control restriction site. Because of this the sizes of PCR products differ from those obtained after restriction enzyme.

When the positive control DNA (SW 480, homozygously mutated K-ras codon 12) was diluted with normal DNA and processed by a two-step assay, the mutant allele-specific band was still discernible in the 1:333 dilution (not shown).

Frequency of Mutations in NSCLC and Single-Step versus Double-Step Assay for K-ras Codon 12. The non-small cell lung tumor samples analyzed in our study comprised 117 cases of squamous cell carcinomas, 1 adenosquamous cell carcinoma, 19 adenocarcinomas, and 4 large cell carcinomas. All tumors have been subjected to the two-step PCR-RFLP analysis to detect mutations in codon 12 of K-ras. Of 141 tumors 14 (10%) were found mutated in this codon (Fig. 1a). Four of these mutations were detected among 19 adenocarcinomas. Nine of 118 squamous cell carcinomas (1 adenocarcinoma included) and 1 of 4 large cell tumors harbored the mutation. The 14 samples were also screened by a less sensitive one-step procedure. As shown in Fig. 1b, the band intensity of mutated allele varied. In several samples, the mutated allele-specific band was very weak and in two samples (Fig. 1a, Lanes 4 and 5), the mutation was at the limit of detection. It means that, in mutation-positive tumors, at least 5% of the cells were mutated. This detection limit for the single-step method was estimated by a titration experiment with mutated (SW480) and control DNA (not shown). Of the 14 samples with K-ras codon 12 mutation, 1 mutation was found to be homozygous (Fig. 1, Lane 2). In this sample, either the normal allele was lost or both alleles had the same type of mutation. Normal allele-specific band was detectable in all other samples, along with the mutated one (Fig. 1b).

We screened codons 13 and 61 of the K-ras gene further for point mutations by a one-step PCR-RFLP procedure and found three mutations in codon 13 (Fig. 2). All of them occurred in adenocarcinomas. In the titration experiment for codon 13,
Mutations of very weak, indicating that only a minor portion of tumor cell was found in this codon. In the control DNA (NCI-H460), a finding of a mutation, no statistically significant correlation exists between the stage of the disease (ThM classification I-IV) and the presence or absence of K-ras mutations in all three codon 13 mutations were in stages III or IV. Although mutant-enriched PCR products were sequenced, only the mutated nucleotide bands or more prominent mutated bands besides residual normal bases are seen on sequence autoradiograms (Fig. 3). All of the mutational events, except for one, are G→T transversions (10 events) or G→A transitions (6 cases). One G→C transversion in the second position of codon 12 has been observed in a squamous cell carcinoma. In codon 12, the point mutations resulted in the following amino acid changes: from glycine (wild type) to cysteine (three samples), aspartic acid (four samples), valine (two samples), serine (two samples), alanine (one sample), and phenylalanine (two nucleotide changes, one sample). In addition, a single nucleotide deletion of one G in codon 12 of K-ras was detected in another squamous cell carcinoma (Fig. 3a, Lane 14). This mutation causes a frameshift resulting in a stop codon several bases downstream. The translation product of the transcribed RNA should therefore result in a short truncated peptide having apparently no transforming activity because most of the carboxy part of the protein should not be synthesized. The frameshift mutation in this sample was further confirmed by several independent amplifications of genomic DNA and sequencing both the antisense and sense strands (not shown). In codon 13, all three cases revealed a substitution from glycine to cysteine. Concerning both codons 12 and 13, nine- and eight-point mutations were in the first and the second position, respectively. Control cell lines were also processed by the same procedures as tumor DNAs and corresponding mutations were detected (not shown).

The overall mutational frequency was 17 (12%) of 141 in all NSCLC samples, 7 (37%) of 19 in adenocarcinomas, and 9 (8%) of 118 in squamous cell carcinomas (including 1 adenosquamous carcinoma), and 1 (25%) of 4 in large cell carcinomas. Therefore, it can be concluded that K-ras mutations occur not only in lung adenocarcinomas but also, at lower frequencies, in squamous cell carcinomas of the lung.

**DISCUSSION**

The two-step PCR-RFLP method was shown to be a reliable procedure to detect K-ras codon 12 point mutations present in only a small percentage of the tumor cells (4, 10, 11, 30). Here we screened 141 NSCLC DNA samples using this assay. All mutations were clearly recognized as prominent bands on the gel (Fig. 1). This method could be used in the screening of samples such as sputum, bronchoalveolar lavage, pancreatic juice, or feces, into which tumor cells are often shed from the tumor. Mutations of K-ras have been detected in sputum from patients with lung cancer and in pancreatic juice from patients.
Table 2  Types of K-ras mutations in NSCLC

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex, age</th>
<th>Histological diagnosis</th>
<th>Codon</th>
<th>Mutation</th>
<th>TNM stage</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>M, 64</td>
<td>Squamous cell carcinoma</td>
<td>12</td>
<td>GGT→TGT (Cys)</td>
<td>II</td>
</tr>
<tr>
<td>2</td>
<td>M, 46</td>
<td>Squamous cell carcinoma</td>
<td>12</td>
<td>GGT→GAT (Asp)*</td>
<td>III</td>
</tr>
<tr>
<td>3</td>
<td>M, 65</td>
<td>Squamous cell carcinoma</td>
<td>12</td>
<td>GGT→AGT (Ser)</td>
<td>II</td>
</tr>
<tr>
<td>4</td>
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<td>Adenocarcinoma</td>
<td>12</td>
<td>GGT→GAT (Asp)</td>
<td>I</td>
</tr>
<tr>
<td>5</td>
<td>M, 51</td>
<td>Squamous cell carcinoma</td>
<td>12</td>
<td>GGT→GT (Val)</td>
<td>I</td>
</tr>
<tr>
<td>6</td>
<td>M, 56</td>
<td>Adenosquamous carcinoma</td>
<td>12</td>
<td>GGT→TGT (Cys)</td>
<td>I</td>
</tr>
<tr>
<td>7</td>
<td>F, 65</td>
<td>Adenocarcinoma</td>
<td>12</td>
<td>GGT→GAT (Asp)</td>
<td>II</td>
</tr>
<tr>
<td>8</td>
<td>M, 52</td>
<td>Squamous cell carcinoma</td>
<td>12</td>
<td>GGT→GCT (Ala)</td>
<td>III</td>
</tr>
<tr>
<td>9</td>
<td>M, 68</td>
<td>Large cell carcinoma</td>
<td>12</td>
<td>GGT→GT (Val)</td>
<td>III</td>
</tr>
<tr>
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<td>F, 62</td>
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<td>12</td>
<td>GGT→AGT (Ser)</td>
<td>I</td>
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<tr>
<td>11</td>
<td>F, 54</td>
<td>Adenocarcinoma</td>
<td>12</td>
<td>GGT→TGT (Cys)</td>
<td>III</td>
</tr>
<tr>
<td>12</td>
<td>M, 64</td>
<td>Adenocarcinoma</td>
<td>12</td>
<td>GGT→TTT (Phe)*</td>
<td>I</td>
</tr>
<tr>
<td>13</td>
<td>M, 46</td>
<td>Squamous cell carcinoma</td>
<td>12</td>
<td>GGT→GAT (Asp)</td>
<td>II</td>
</tr>
<tr>
<td>14</td>
<td>M, 39</td>
<td>Squamous cell carcinoma</td>
<td>12</td>
<td>GGT→deletion of one G</td>
<td>III</td>
</tr>
<tr>
<td>15</td>
<td>F, 48</td>
<td>Adenocarcinoma</td>
<td>13</td>
<td>GCC→TGC (Cys)</td>
<td>III</td>
</tr>
<tr>
<td>16</td>
<td>M, 68</td>
<td>Adenocarcinoma</td>
<td>13</td>
<td>GCC→TGC (Cys)</td>
<td>III</td>
</tr>
<tr>
<td>17</td>
<td>F, 47</td>
<td>Adenocarcinoma</td>
<td>13</td>
<td>GCC→TGC (Cys)</td>
<td>IV</td>
</tr>
</tbody>
</table>

* Wild-type allele has not been present.

Fig. 3  Direct sequencing of mutant-enriched PCR products resulting from two-step amplification procedures. Examples of all types of mutations found in codon 12 (a) and codon 13 (b) of the K-ras gene are presented. For codon 12, antisense strand is shown (normal codon 12 sequence is ACC). The sense strand was synthesized in codon 13 sequencing reactions (normal codon 13 sequence is GGC). The numbers of tumor samples correspond to the numbers in Figs. 1 and 2 and in Table 2. Arrowheads, mutated bases. Normal codon triplet (sense strand) is also displayed under each sequence. Mismatched nucleotide is underlined in the sequence displayed on the left for each codon. Each sample was sequenced twice to verify the mutation.

with pancreatic cancer by another sensitive assay, namely, mutant-allele-specific amplification (33, 34). Very recently, mutated p53 and ras genes have been detected in archival sputum samples obtained before the clinical diagnosis of lung cancer (35). Also, K-ras mutations were found in the DNA isolated from the stool of patients with mutation-positive colorectal tumors (36) and suggested to be useful for early detection of colorectal tumors. In the case of bronchogenic carcinoma, preinvasive stages persist for years and classical screening methods are inefficient to detect the tumor at this stage (reviewed in Ref. 37). It remains to be elucidated the timing of appearance of K-ras mutations during the carcinogenesis of NSCLC and to test the diagnostic value of possible mutations of K-ras and other genes detected in sputum or bronchoscopic material.

Although all of the mutations of K-ras were well above the detection limit of the sensitive two-step PCR-RFLP method,
Mutations of K-ras in Lung Cancer

several samples were near or at the detection limit of the one-step assay. In other samples, most tumor cells were mutated. It can be inferred that K-ras mutations appear early during the progression of the tumor. However, if K-ras mutation occurs as an early event during the development of the squamous cell lung carcinoma, it probably does not confer a growth advantage to cells at least in a subset of mutated tumors. In a recent report, K-ras mutations were suggested to be acquired early in lung adenocarcinomas (38).

In this work, the pattern of K-ras mutations in NSCLC was similar to that reported previously. Among the samples analyzed, one revealed G→C transition, G→A transitions and G→T transversions were detected in all other mutated DNAs. These two types of mutations arise as a result of treatment by specific carcinogens in animal model systems. G→A transitions are detected at high frequency on application of methylating N-nitroso compounds such as methylnitrosourea, presumably specific carcinogens in animal model systems. G→T transversions were similar to that reported previously. Among the samples analyzed early in lung adenocarcinomas (38).

(a)pyrene, forming hydrophobic DNA adducts, produces mostly squamous cell lung carcinoma, it probably does not confer a mutation occurs as an early event during the development of the K-ras early during the progression of the tumor. However, if one-step assay. In other samples, most tumor cells were mutated in codon 13 were absent in squamous cell histological types of non-small cell lung cancer. On the other hand, mutations in codon 13 were absent in squamous cell carcinomas. In articles reporting the mutations of K-ras in squamous cell lung cancers (fresh tumors or cell lines, 12 samples total), no codon 13 mutations were described (19, 21, 24, 26). Either codon 12 or codon 61 mutations were present. Thus, our results, based on a large group of squamous cell lung tumors, further suggest that there is an absence of the mutations in codon 13 of K-ras in this type of tumor. Besides the K-ras codon 12 mutations, Rosell et al. (26) observed mutations in codon 61. Here, on the contrary, we were unable to detect any mutation in this codon among 141 NSCLC samples. K-ras mutations were reported to occur also in large cell lung carcinomas (21, 26). We found one mutation (codon 12) out of four of these tumors examined.

In more than a one-half of the NSCLC samples analyzed in this work, we have previously studied the methylation status of the specific MspI/HpaII sites at the 3' end of H-ras gene and loss of heterozygosity at the H-ras locus (39). We hypothesized that hypomethylation may contribute to allelic loss at the H-ras locus (39). Neither H-ras allelic loss nor H-ras hypomethylation (the latter change found in about one-third of tumor samples) correlated with K-ras mutations. An interesting fact, however, has been noted because the only sample with the loss of the shorter H-ras allele having no methylation change (five other DNAs with shorter allele loss were hypomethylated) displayed the homozygous mutation of K-ras described here (Fig. 1 and Table 2, patient 2). We have shown that the same types of mutations appear in both adenocarcinomas and squamous cell carcinomas of the lung. We have demonstrated on an extended number of squamous cell tumors that this histological type also harbors K-ras mutations, although at a frequency four times lower than that in adenocarcinomas. All mutations found in squamous cell tumors clustered in codon 12 of K-ras. One mutation was also noted in codon 12 of H-ras (squamous cell carcinoma) in a minority of tumor cells. H-ras mutations, therefore, do not have any importance in the formation of NSCLC. It remains to be investigated if screening of mutations in codon 12 of K-ras by a sensitive mutant allele-enriched method might become a contribution to an early diagnosis of NSCLC.

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