K-ras Mutations Are a Relatively Late Event in the Pathogenesis of Lung Carcinomas

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Abstract

We investigated preneoplastic lesions associated with lung cancer to determine at what stage in lung carcinogenesis K-ras mutations appear. We selected sixty archival lung cancer resection cases that had K-ras mutations. We precisely microdissected 74 relevant areas from paraffin-embedded sections. K-ras mutations at codons 12, 13, and 61 were determined by the designed restriction fragment length polymorphism method using mismatched nested primers and confirmed by direct sequencing. All samples of invasive and metastatic cancers had K-ras mutations, as did four of five lesions of noninvasive cancer. Mutations were detected in only 1 of 12 dysplastic lesions and were absent from hyperplastic and normal-appearing cells. In all cases, the specific point mutation and the mutational pattern in the tumors, metastases, and the corresponding noninvasive lesions were identical. These results indicate that K-ras mutations arise relatively late in the pathogenesis of lung cancer and may be associated with the appearance of the malignant phenotype.

Introduction

As with other epithelial tumors, squamous cell lung carcinomas develop after a series of sequential preneoplastic changes which include hyperplasia, metaplasia, atypical hyperplasia (or dysplasia), and carcinoma in situ (1). While the sequence for peripherally arising cancers such as adenocarcinoma or large cell carcinoma has not been established, they may be accompanied by hyperplastic and dysplastic changes in peripheral airway cells (2, 3). Presumably, adenocarcinomas must go through a noninvasive (CIS) stage prior to becoming invasive. However, because of the tendency of peripheral adenocarcinomas to grow along preexisting alveolar and bronchiolar walls (lepidic growth; Ref. 4), it is difficult to be certain whether the noninvasive components represent CIS or lepidic extensions of the invasive component. In this sequence of the development of lung cancers, multiple genetic alterations are involved in carcinogenesis, including activation of dominantly acting cellular oncogenes and inactivation of tumor suppressor genes. Some of these changes have been documented in preneoplastic lesions (5). Ras mutations, one of the common genetic alterations in various types of human cancers, occur frequently in adenocarcinomas primary to the pancreas, colorectum, and lung (6–9). A point mutation is found in approximately 20% of NSCLC, mainly in adenocarcinomas, while ras mutations have not been detected in any small cell lung cancer tumor or cell line (6, 7, 9). In adenocarcinoma of the lung, more than 90% of ras mutations occur in the K-ras gene, and 80% of these are in codon 12. Ras gene mutations have been implicated as an early event in neo-

Materials and Methods

Samples and Cell Lines. Twenty-four NSCLC tumors were screened for the presence of ras mutations. Six cases positive for mutations and having extensive areas of preneoplastic changes were selected. Serial 5-μm-thick sections were cut, and all slides in the series were stained with hematoxylin and eosin. Every first slide was covered with a coverslip to confirm the presence of the preneoplastic lesions and the tumors, and other slides lacking coverslips were used for microdissection. Cell lines identified previously as having ras mutations (Ref. 7; Table 2) were used as positive controls.

Microdissection of Materials from Stained Slides and DNA Extraction. We used our modification of a previously described precise microdissection technique (12) to collect cells under direct microscopic observation from hematoxylin and eosin-stained slides of paraffin-embedded materials. Microdissection was performed with an inverted microscope using a micropipette with a fine tip that was pulled to a fine capillary tube with a micropipette puller (Narishige; PB-7) and with a joystick-operated hydraulic micromanipulator (Nikon-Narishige; SO-188). The dissected cells were allowed to adhere to the microcapillary tip and collected in 0.5-ml siliconized microcentrifuge tubes. The materials were digested in 10–50 μl of buffer consisting of 20 mM Tris (pH 8.0), 1 mM EDTA, 0.5% Tween 20, and 200 μg/ml proteinase K for 24–48 h at 37°C, and then incubated for 15 min at 95°C to inactivate the proteinase K. Preliminary examination using polymorphic markers demonstrated that at least 40 cells were necessary for consistent detection of both alleles for each PCR reaction (data not shown). Therefore, we used 5 μl of the digested sample extract for each PCR reaction, which included DNA from at least 50 cells.

Detection of K-ras Mutations Using PCR-based Designed RFLP. We optimized our previously described designed RFLP method (7) for analysis of formalin-fixed, paraffin-embedded archival materials. Exons 1 and 2 of the K-ras gene were amplified by nested PCR methods as described previously (7). Details of methodology and the sequences of all the primer pairs used in the screening and identification steps for detecting mutations at codons 12, 13, and 61 have been deposited with the Editorial Office of the Journal. They may be obtained from the Office or from the authors. Mutations detected by the designed RFLP method were confirmed by direct sequencing. For direct sequencing, we used a biotinylated primer for the PCR reaction to isolate single-stranded DNA using streptavidin-coated magnetic beads (Dynabeads, Dynal, Norway).
Table 1  Incidence of the K-ras mutations in various stages of lung carcinogenesis

<table>
<thead>
<tr>
<th>Pathological stage</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
<th>Case 4</th>
<th>Case 5</th>
<th>Case 6</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>0/2</td>
<td>0/4</td>
<td>0/2</td>
<td>0/1</td>
<td>0/2</td>
<td>0/1</td>
<td>0/12</td>
</tr>
<tr>
<td>Normal bronchiole</td>
<td>0/1</td>
<td>0/2</td>
<td>0/2</td>
<td>0/1</td>
<td>0/2</td>
<td>0/1</td>
<td>0/4</td>
</tr>
<tr>
<td>Hyperplasia Bronchiole</td>
<td>0/7</td>
<td>0/4</td>
<td>0/1</td>
<td>0/2</td>
<td>0/1</td>
<td>0/16</td>
<td>0/2</td>
</tr>
<tr>
<td>Bronchiole Alveolar type II</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>Squamous metaplasia</td>
<td>0/2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dysplasia Bronchiole</td>
<td>0/4</td>
<td>0/1</td>
<td>0/1</td>
<td>0/2</td>
<td>1/4</td>
<td>1/2</td>
<td>1/12 (8)</td>
</tr>
<tr>
<td>Bronchiole Alveolar type II</td>
<td>0/4</td>
<td>0/2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noninvasive cancer</td>
<td>2/2</td>
<td>0/1</td>
<td>0/1</td>
<td>1/1</td>
<td>1/1</td>
<td>4/5</td>
<td>1/12 (8)</td>
</tr>
<tr>
<td>Invasive cancer</td>
<td>8/8</td>
<td>6/6</td>
<td>2/2</td>
<td>1/1</td>
<td>2/2</td>
<td>22/21</td>
<td>21/21 (100)</td>
</tr>
<tr>
<td>Metastatic cancer</td>
<td>1/1</td>
<td>1/1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2/2 (100)</td>
</tr>
</tbody>
</table>

- The incidence shown is number of lesions positive/number of lesions examined.
- Adeno, adenocarcinoma; Sq, squamous cell carcinoma.
- Wt sequence of codons 12 and 13 are GGT (Gly) and GGC (Gly), respectively.

Results

Screening of Tumors for K-ras Mutations. Our initial screening revealed 9 cases of K-ras mutations in DNA from 23 frozen NSCLC specimens (37%). Seven cases had codon 12 mutations, of which three cases were TGT (Cys), two were GCT (Ala), one was GTT (Val), and one was GAT (Asp). In two cases with codon 13 mutations, one was TGC (Cys) and one was GAC (Asp) substitution. We selected five mutation-positive cases which had extensive preneoplastic lesions. An additional case was selected because it had a previously identified mutation. Five of the six selected cases were peripheral adenocarcinomas, while one case was a centrally arising squamous cell carcinoma.

PCR-based Designed RFLP Analysis Using Mismatched Primers in Microdissected Materials. We precisely identified areas of normal bronchiolar epithelium, preneoplastic lesions (in bronchi, bronchioles, and alveoli), noninvasive, invasive, and metastatic cancer by microscopic observation. We microdissected 50–500 cells in each area, and at least 50 cells were used for each PCR reaction. In total, 74 lesions were microdissected from 6 cases (Table 1).

We screened for the presence of mutations in codons 12, 13, and 61 in microdissected materials. All microdissected materials provided suitable template DNA for use in PCR reactions, resulting in the amplification of a 97-base pair product specific for codon 12 of the K-ras gene. Mutations in codon 12 abolish the BstNI site, leaving an intact PCR product of 97-base pair size after enzyme digestion (Fig. 1A). In cases 1, 2, 3, 5, and 6 which had codon 12 mutations in DNA derived from fresh tumor tissues, identical mutations were found in all microdissected foci of invasive and metastatic tumor. Codon 12 mutations were also identified in foci of noninvasive carcinoma of cases 1, 5, and 6 (Fig. 2). However, the single focus of noninvasive carcinoma identified in case 2 had a wt gene. By contrast, no mutation was found in 11 dysplastic lesions from cases 1, 2, 3, and 5, except for...
K-RA S MUTATIONS IN PATHOGENESIS OF LUNG CARCINOMAS

Fig. 2. a, photomicrograph of normal bronchiolar epithelium (N), noninvasive (P), and invasive (IN) carcinoma from Case 1 (hematoxylin and eosin, X 300). These areas were individually microdissected. The areas of noninvasive and invasive carcinoma contained both mutant and wt alleles, while the adjacent nonmalignant epithelium contained only wt allele. b, dysplastic lesion of type II alveolar pneumocytes from Case 5 that had a codon 12 mutation. No tumor cells were identified in this or in adjacent fields (hematoxylin and eosin, X 300).

one lesion from case 5 (dysplastic type II alveolar pneumocytes). Other preneoplastic lesions, including 16 hyperplastic and 2 squamous metaplastic lesions and 4 foci of normal bronchiolar epithelium, had the wt ras gene. All foci from cases 1, 2, 3, 5, and 6 had wt alleles at codons 13 and 61. In case 4, microdissected samples from adenocarcinoma had a codon 13 mutation, but one hyperplastic and two dysplastic lesions had wt (Table 1). In summary, only one of six dysplastic lesions (17%), which originated from type II alveolar pneumocytes, was positive for a K-ras mutation, but four of five foci of noninvasive carcinoma (80%) were positive. In all cases, the specific mutation in the invasive cancer, metastases, and the corresponding preneoplastic lesions were identical. These mutations were confirmed by direct sequencing.

Pattern of ras Mutations. The designed RFLP method using mismatched primers enables simultaneous visualization of both mutant and normal alleles in heterozygous cell populations. Analysis of tumors and cell lines indicates that in about 80% of the cases with ras mutations, both mutant and wt bands are present at about equal intensities (Fig. 3, Pattern I). However, in the remaining cases, there is a gross imbalance in the ratios of intensities of the mutant and wt bands. The mutant band may be in great excess (Fig. 3, Pattern Ia), or the wt band may be completely absent (Fig. 3, Pattern Ib). These patterns are not artifacts of the PCR reaction, as they are completely reproducible. In five cases, Pattern I was present in the tumors and preinvasive lesions. However, in case 3, Pattern Ib was present in the invasive and metastatic tumor (lepidic growth of the carcinoma was not identified in this case; Fig. 1).

Discussion

Most adenocarcinomas arise from the precursor cells of the bronchioles and alveoli (Clara cells and type II alveolar pneumocytes) or
K-RAS MUTATIONS IN PATHOGENESIS OF LUNG CARCINOMAS

from metaplastic mucous cells (4). Molecular analyses of peneplastic lesions found in bronchioles or alveoli provide us with an understanding about the pathogenesis of these peripherally arising tumors. We have developed methodologies for the analysis of molecular lesions in small numbers of cells (about 50) accurately microdissected from formalin-fixed, archival paraffin-embedded tissues. K-ras mutations were not found in foci of hyperplasia, metaplasia, or dysplasia (except for a single focus of dysplastic type II alveolar cells). However, ras mutations were present in three of four cases in which a noninvasive tumor component (CIS) was identified. In all six cases, the precise base substitution and the mutational pattern were identical in samples of preinvasive, invasive, and metastatic carcinoma. By contrast, K-ras mutations are an early event in the pathogenesis of some malignant tumors, such as colorectal, pancreatic, or endometrial cancer (11, 13).

Our results suggest that K-ras mutations occur mainly at the noninvasive carcinoma stage and that they may be involved in the conversion of dysplastic cells to preinvasive cancer cells. The growth pattern of peripheral lung adenocarcinomas along preexisting structures (alveoli and bronchioles) prevents us from determining whether these lesions are true in situ carcinomas or whether they simply represent a growth pattern of the invasive component. In either case, K-ras mutations are relatively late events in the pathogenesis of lung adenocarcinomas. The findings in the solitary squamous cell carcinoma examined were identical to those in the adenocarcinomas. Finding identical point mutations and mutational patterns in all samples of the tumor cells (preinvasive, invasive, and metastatic) from individual cases indicates that the mutations occurred prior to clonal expansion. Li et al. (14) found that ras mutations had a homogeneous topographical distribution throughout eight lung adenocarcinomas, suggesting that ras mutations arose prior to clonal expansion of the tumor cells. Their findings are consistent with ours. In addition, findings similar to ours have been reported recently by Oshima et al. (15). As most adenocarcinomas are believed to arise from the precursors of the peripheral airways, we examined all regions of the respiratory epithelium including bronchi, bronchioles, and alveoli. Of interest, the only nonmalignant peneplastic lesion in which we found a ras mutation was in a focus of atypical adenomatous hyperplasia of the lung (4). Molecular analyses of preneoplastic foci (5). In summary, we have identified that K-ras mutations are associated with the appearance of the malignant phenotype, indicating that the mutations are relatively late event in lung cancer pathogenesis and that they may be associated with the transformation of dysplastic cells into neoplastic cells.

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References


Table 2 K-ras mutation patterns in NSCLC cell lines as determined by the designed RFLP analysis

<table>
<thead>
<tr>
<th>Mutation pattern</th>
<th>Cell line</th>
<th>Codon</th>
<th>Base substitution&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pattern I</td>
<td>NCI-H157</td>
<td>12</td>
<td>CGT</td>
</tr>
<tr>
<td></td>
<td>NCI-H358</td>
<td>12</td>
<td>TGT</td>
</tr>
<tr>
<td></td>
<td>NCI-H2009</td>
<td>12</td>
<td>GCT</td>
</tr>
<tr>
<td>Pattern IIa</td>
<td>NCI-H727</td>
<td>12</td>
<td>GTT</td>
</tr>
<tr>
<td>Pattern IIb</td>
<td>AS49</td>
<td>12</td>
<td>AGT</td>
</tr>
<tr>
<td></td>
<td>NCI-H647</td>
<td>13</td>
<td>GAC</td>
</tr>
<tr>
<td></td>
<td>NCI-H650</td>
<td>61</td>
<td>CTA</td>
</tr>
<tr>
<td></td>
<td>NCI-H460</td>
<td>61</td>
<td>CAT</td>
</tr>
</tbody>
</table>

<sup>a</sup> Base substitution is shown in boldface.

Fig. 3. Patterns of the K-ras codon 12 mutation as identified by the designed RFLP method (BstNl digestion). Pattern I, both mutant (upper band, 97 bp) and wt allele (lower band, 77 bp) bands are present at about equal intensities (H157). Pattern IIa, the mutant band is in great excess (H727). Pattern IIb, the wt band is absent (AS49). WT, wild-type pattern.

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