Detection of K-ras Mutations in Lung Carcinomas: Relationship to Prognosis

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ABSTRACT

The K-ras mutation is one of the most common genetic alterations found in human lung cancer. To evaluate the prognostic value of ras gene alterations in lung cancer in a U.S. population, we have screened 173 human lung tumors, which included 127 adenocarcinomas, 37 squamous carcinomas, and 9 adenosquamous carcinomas, for mutations in the K-ras gene using the combination of the PCR and denaturing gradient gel electrophoresis. Forty-three tumors contained K-ras mutations. Of these, 41 were identified among the adenocarcinomas (32%), 1 among the squamous carcinomas (2.7%), and 1 among the adenosquamous carcinomas (11%). Forty of these mutations were found in codon 12 and consisted of 24 G to T transversions, 12 G to A transitions, 2 G to C transversions, and 1 double GG to TT mutation. Two other G to T transversions were found in codon 13, and 1 A to C transversion was found in codon 61. The data showed that gender did not seem to affect the incidence and the types of the K-ras mutations or amino acid changes. Examination of the mutations in adenocarcinomas in relation to overall survival showed no difference in adenocarcinomas with K-ras mutations compared with K-ras-negative adenocarcinomas. However, the substitution of the wild-type GGT (glycine) at codon 12 with a GTT (valine) or a CGT (arginine) showed a strong trend (P = 0.07) toward a poorer prognosis compared with wild-type or other amino acid substitutions. Substitution of the wild-type glycine for aspartate (GAT) showed a strong trend (P = 0.06) for a better outcome than the valine or arginine substitution. Although these trends will require larger patient populations for verification, these data suggest that the prognostic significance of K-ras mutations may depend on the amino acid substitution in the p21ras protein.

INTRODUCTION

Mutations in the ras genes (K-, H-, and N-ras) genes are very common alterations identified in many types of human tumors and are implicated in the development of human cancer (1). These genes encode for similar Mr, 21,000 membrane-bound proteins (p21ras) which possess intrinsic GTPase activity and are involved in the cellular signal transduction pathways (2–6). Point mutations occurring at specific codons of the ras genes can give rise to proteins with reduced intrinsic GTPase activity and can seem to be associated with oncogenic activity of the proteins (1, 7, 8).

Mutations that activate the ras genes have been identified in several types of human tumors but are clustered in a small number of hot spots, usually in codons 12, 13, and 61. There is, however, a wide variation in the activation of the ras genes with certain types of tumors (1, 7, 8). For instance, K-ras gene mutations have been identified predominantly in lung, colon, and pancreas tumors, whereas the H-ras and N-ras genes are mutated commonly in bladder and breast tumors (9–15). In lung tumors, the most frequently mutated ras gene is K-ras, whereas N-ras and H-ras were also mutated but to a much lesser frequency (9, 16). There are four major histological types of lung cancer, including squamous cell carcinoma, adenocarcinoma, large cell carcinoma, and SCLC. The first three types are termed NSCLCs collectively and have different clinical features from SCLC. The mutated forms of the K-ras gene have been found frequently in NSCLC but not SCLC (9, 16–19).

In an attempt to associate the ras gene mutation with clinical and prognostic parameters, we screened 173 primary human lung tumors for mutations in the K-ras gene in NSCLC. We used the PCR (20, 21) in combination with DGGE (22) to screen for mutations in the K-ras gene. The mutational data are discussed in relation to clinical parameters, possible lung pathogenesis, and the results reported by other investigators.

MATERIALS AND METHODS

Tissue Samples. The tissues were fresh-frozen lung tumors obtained following lung resection at the University of Pittsburgh Medical Center and the Fox Chase Cancer Center (Philadelphia, PA). Tissues were collected and stored sequentially between 1989 and 1993. The diagnosis of carcinoma primary to the lung was confirmed by consulting surgical pathology reports for each patient. The cases examined for K-ras mutation included 127 adenocarcinomas (113 pure adenocarcinomas, 6 adenocarcinomas and squamous cell carcinoma, and 8 squamous cell carcinomas).

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3 The abbreviations used are: SCLC, small cell lung carcinoma; NSCLC, non-small cell lung carcinoma; DGGE, denaturing gradient gel electrophoresis.
nomas and 14 bronchioloalveolar carcinomas), 9 adenosquamous carcinomas, and 37 squamous carcinomas. Clinical records were consulted to determine the history and outcome for each patient with an adenoscarcinoma or adenosquamous carcinoma. This information is summarized in Table 2. Follow-up was not done on patients with squamous cell tumors because of the low incidence of K-ras mutations. Diagnosis and TNM staging were determined at the time of biopsy, and the follow-up period was determined from the date of diagnosis. The mean follow-up period for all patients was 20.0 (median, 20.5; range, 3–51) months. For the 75 patients who survived the follow-up period (censored patients), the mean follow-up time was 24.8 (median, 24.0) months. For the 51 patients who died during the follow-up period, the mean follow-up time was 12.9 (median, 11.0) months.

DNA Isolation and Amplification in Vitro. For DNA isolation, lung tissues were first cut into small slices and subjected to sonication. The minced tissue was then digested with RNase A1 and proteinase K followed by phenol-chloroform extraction using a standard published method (23). This method yields high purity DNA suitable for PCR consistently.

DNA amplification was performed in a 100-μl reaction mixture containing 1–2 μg genomic DNA in 10 mM Tris-HCl (pH 8.5), 3.0 mM MgCl₂, 50 mM KCl, 200 μM each deoxynucleotide triphosphate, 0.5 μM each primer, and 2.5 units Taq DNA polymerase (Perkin-Elmer, Norwalk, CT). About 30 cycles of PCR (94°C/1 min, 53°C/2 min, and 72°C/2 min) were carried out using a DNA thermal cycler (Perkin-Elmer Cetus). The primers used are: for exon 1, PKI1–1 (sense), 5′-TATTATAAGGCTCTGCTGAAA-3′; PKI2–1 (antisense), 5′-ATCAAA-GAAATGTTCTGAC-3′; for exon 2, PKI2–2 (sense), 5′-AAGTCTTCTGGCCATTTTTTA-3′; PKI3–2 (antisense), 5′-ATATTCAATTTAAAAACCACC-3′. The PCR products were purified from each reaction mixture and used for subsequent DGGE analysis.

DGGE Analysis. DGGE separates mutant alleles based on the melting difference of the duplex form compared with wild type as a consequence of mutation occurrence. To be suitable for analysis by DGGE, a duplex DNA fragment must contain two contiguous regions, high and low temperature melting domains (22). Such a structure can occur naturally. Otherwise, such as with exons 1 and 2 of the K-ras gene, an artificially high temperature melting domain or “clamp” must be added using PCR (23–25). Mutant alleles differing by only a fraction of the PCR-amplified fragments was found usually as a mutant/wild-type heteroduplex, which occurred during the last denaturation and reannealing step of PCR when DNA amplification was no longer at an exponential increase (26). For these reasons, when genomic DNA contained a mixture of wild-type and a sufficiently high fraction of mutant alleles, four major bands representing a mutant and a wild type homoduplex and the two respective mutant/wild-type heteroduplexes were usually detected by direct DGGE analysis.

For DGGE analysis, a 20-ng aliquot from each of the first round-amplified DNA was used as a template for a second round of PCR using the following primers: for exon 1, PKI1–1 and PKI1–1 clamp, 5′-GCCCGCTGCAAGCCGCCGCCGTGCCGCCGCCGCCGCCCGCGCGCCGTCTATTTCCCATATTA-3′; for exon 2, PKI2–2 (antisense), 5′-AAGAAAGCCTCCCAGTCCCTC-3′, and PKI2–2 clamp (sense), 5′-GCCGCTGCAAGCCGCCGCCCGCCCGCCGTCTATTTCCCATATTA-3′. PCR was performed in 50 μl containing 10 mM Tris-HCl (pH 8.5), 2.5 mM MgCl₂, 50 μM each deoxynucleotide triphosphate, 1 μM each primer, 0.25 μM [α-32P]dATP (3000 Ci/mmol; DuPont New England Nuclear, Boston, MA) and 1 unit Taq DNA polymerase. After 12 cycles, a 10-μl aliquot of each mixture was ethanol precipitated to eliminate most of the unincorporated radioisotope. The DNA was then migrated through a 12.5% polyacrylamide gel containing a 20–40% gradient of denaturants (urea plus formamide) for 12 h under the conditions described (23, 25). The gel was dried and subjected to autoradiography.

The mutant alleles that separated from the wild-type allele by DGGE were isolated from the gel and amplified further to a sufficient amount for sequencing analysis (23).

RESULTS

We examined the types and positions of mutations in the K-ras gene in 173 human lung tumors using PCR and DGGE (22). First, we carried out reconstruction experiments to test the level of sensitivity of this approach to detect K-ras mutants. Wild-type genomic DNA (10⁵ molecules) was mixed with different amounts of a known mutant genomic DNA containing a G to T transversion at the first G of codon 12. The results of DGGE analysis are shown in Fig. 1. Each lane shows the position of a major band corresponding to the homoduplex wild-type allele of exon 1 (wt). Furthermore, three additional bands representing the mutant homoduplex (hom.) and two mutant/wild-type heteroduplexes (het. 1 and het. 2) all appeared clearly in samples containing a mutant fraction of 10 and 5%. The mutant homoduplex band also can be detected in a sample containing 5% or more easily and may be able to detect mutant fractions as low as 2.5%.

Fig. 2 illustrates a DGGE screening of 15 of the 173 lung tumors for mutations in exon 1 of the K-ras gene. All of them,
except for samples 4, 5, 10–12, and 14, revealed a mutant homoduplex band (indicated by arrows) and two mutant/wild-type heteroduplex bands in addition to the wild-type band (wt). By comparing the patterns of the mutant homoduplex and the mutant/wild-type heteroduplexes between DNA samples analyzed in the same gel, one can identify those that contain the same mutations easily by the presence of the same patterns of mutant bands. This avoids the need for further analysis of all mutant samples by sequencing. Comparison of the patterns of the mutant bands showed that they contained four different mutations. Samples 1, 7, and 9 contained the same mutation, whereas samples 2, 3, 8, and 15 shared a second mutation, and samples 6 and 13 contained a third and fourth mutation. Sequencing analysis of the homoduplex mutants (indicated by arrows) showed that all four mutations identified involved codon 12. The wild-type codon (GGT) was converted to GAT for sample 6, and TGT for sample 13. The remaining three mutations consisted of two G to C transversions leading to two CTA (arginine) in codon 12 and one CAC (histidine) in codon 61.

Clinical histories, including follow-up information, were available for 126 patients with a diagnosis of adenocarcinoma or adenosquamous carcinoma. We did not seek clinical histories of patients with a diagnosis of squamous carcinoma because of the low incidence of K-ras mutations in these patients. Table 2 summarizes the clinical data for patients whose tumors contained a wild-type K-ras gene compared with those whose tumors contained a mutated K-ras allele. Multivariate analysis showed there were no significant differences between the two groups in distribution of age, gender, or stage, or in degree of differentiation. There also was no significant difference in the recurrence rates between the two groups. The overall survival for each type of K-ras mutation was compared with the others and with the overall survival curve for those with no mutations. A pronounced trend was observed toward shorter survival times of patients who displayed either a valine or arginine (GTT) or an arginine (CGT) mutation in codon 12 (P = 0.09 by the log-rank test; P = 0.15 by the Wilcoxon test). The overall survival for each type of K-ras mutation was compared with the others and with the overall survival curve for those with no mutations. A pronounced trend was observed toward shorter survival times of patients who displayed either a valine (GTT) or an arginine (CGT) mutation in codon 12 (P = 0.09 by the log-rank test; P = 0.07 by the Wilcoxon test; Fig. 6). Multivariate analysis was also performed by comparing these two mutation types with those of all other patients, and the same trend (P = 0.09) was found. These mutations were analyzed together, because a previous publication showed that these two amino acid substitutions had highly potent transforming activity compared with other substitutions (27; see “Discussion”). The shorter survival times of patients with the valine or arginine
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23456789 10 11 12 13 14 15

Fig. 2 Example of DGGE screening for mutations in exon 1 of the K-ras gene. The preparation of K-ras gene exon 1 from genomic DNA and its analysis by DGGE were described in "Materials and Methods." The figure shows the DGGE patterns of K-ras exon 1 amplified from 15 lung tumor samples (samples 1–15). Each sample shows a major band corresponding to the wild-type homoduplex (wt; except for samples 4, 5, 10, 11, 12, and 14), a mutant homoduplex (arrows), and two mutant/wild-type heteroduplexes. The mutant homoduplexes were subsequently isolated from the gel and sequenced to determine the types and positions of the mutations.

Fig. 3 Patterns in DGGE of different mutations identified in exon 1 of the K-ras gene. Eight different mutations were identified among the 43 mutations. Six mutations occurred in codon 12 (GGT), which resulted in AGT, CGT, GAT, GTT, TGT, and TTT, and two other mutations occurred in codon 13 (GGC) and resulted in GAC and TGC, as indicated. Another mutation occurring in codon 61 (CAA to CAG) is not shown.

of the 136 adenocarcinomas of the lung analyzed in this study, 77 were from males (56%), and 59 were from females (44%). Of these patients, 117 (73 males and 44 females) were smokers, 16 (13 females and 3 males) were nonsmokers, and 3 had unknown smoking histories. Among the smokers, K-ras

substitution were not accounted for by a difference in stage distribution between these patients and the patients with wild type K-ras. This effect on survival was not seen with any of the other mutations when analyzed separately (data not shown), and in fact, a pronounced trend toward longer survival times was observed with patients exhibiting an aspartate (GAT) substitution (Fig. 6; P = 0.006 by both log-rank and Wilcoxon tests compared with GTT and CGT mutations). Because of the small sample size, survival of patients with GAT mutations was not significantly different from that of those with the wild type. These trends are only suggestive and at this time do not reach significance at the 95% confidence level.

DISCUSSION

The combination of PCR and DGGE allowed us to screen for K-ras mutations from 173 primary human lung tumors. This approach was sufficiently sensitive to detect mutations present at a mutant frequency of 5% or higher clearly. This approach is also relatively simple, because DGGE allows detection of each base pair substitution in codon 12 or 13 of the K-ras gene as a unique and recognizable pattern of bands. Therefore, once a distinguishable pattern is identified by DGGE, there is no need for further characterization analysis of each individual mutant.

Our results showed a 31% frequency of K-ras mutations in adenocarcinomas, although only about 3% in squamous lung tumors. These frequencies coincide with several previous studies, which demonstrated that K-ras mutations were identified more frequently in lung adenocarcinomas (9, 16, 18, 28) and less in other histological phenotypes, including squamous carcinomas (9, 16, 18, 19). However, the frequencies of K-ras mutations in adenocarcinomas varied from 15–20% (16, 18, 29) to about 30% (9, 19) and even up to 57% (28). The reasons for these varied K-ras mutation frequencies in lung adenocarcinomas are unclear. The highest frequency (mutations in 12 of 21 lung adenocarcinomas) observed in the last above-mentioned study might be due to the small number of samples analyzed. The causal differences in the K-ras mutation frequencies in lung adenocarcinomas in the three Japanese studies and those in the European studies are not known, although geographical or genetic differences may play a role.

Of the 136 adenocarcinomas of the lung analyzed in this study, 77 were from males (56%), and 59 were from females (44%). Of these patients, 117 (73 males and 44 females) were smokers, 16 (13 females and 3 males) were nonsmokers, and 3 had unknown smoking histories. Among the smokers, K-ras
Table 1  Spectrum of K-ras mutations found in lung tumors

<table>
<thead>
<tr>
<th>Histology</th>
<th>Codon 12&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Codon 13&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Codon 61&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Total mutations</th>
<th>No. of cases analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TGT Cys</td>
<td>AGT Ser</td>
<td>CGT Arg</td>
<td>GGT Val</td>
<td>GAT Asp</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>11</td>
<td>2</td>
<td>2</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Adenosquamous</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Squamous</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> The normal sequence for codon 12 is GGT (Gly); for codon 13, it is GGC (Gly); and for codon 61, it is CAA (Gln).

Table 2  Clinical information on 126 lung adenocarcinoma<sup>b</sup> patients<sup>b</sup> used for K-ras mutational analysis for whom follow-up was available

<table>
<thead>
<tr>
<th></th>
<th>Wild-type K-ras</th>
<th>Mutated K-ras</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age, yr</td>
<td></td>
</tr>
<tr>
<td></td>
<td>65.5 ± 9.0</td>
<td>63.1 ± 11.8</td>
</tr>
<tr>
<td></td>
<td>(range 41–83)</td>
<td>(range 38–82)</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>(53%)</td>
<td>(59%)</td>
</tr>
<tr>
<td>Stage&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>(47%)</td>
<td>(41%)</td>
</tr>
<tr>
<td>Poor</td>
<td>35</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>(40.2%)</td>
<td>(28.2%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>29</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>(33.3%)</td>
<td>(41.0%)</td>
</tr>
<tr>
<td>Well</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>(23.0%)</td>
<td>(28.2%)</td>
</tr>
<tr>
<td>Unspecified</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(3.5%)</td>
<td>(2.6%)</td>
</tr>
<tr>
<td>No of recurrences</td>
<td>45 of 87 (51.7%)</td>
<td>21 of 39 (53.8%)</td>
</tr>
<tr>
<td>No of deaths during follow-up&lt;sup&gt;d&lt;/sup&gt;</td>
<td>37 (42.5%)</td>
<td>14 (35.9%)</td>
</tr>
<tr>
<td>No of survivors during follow-up</td>
<td>50 (57.5%)</td>
<td>25 (64.1%)</td>
</tr>
<tr>
<td>Mean follow-up time for deceased patients</td>
<td>13.8 ± 10.0</td>
<td>8.7 ± 6.0</td>
</tr>
<tr>
<td>Mean follow-up time for survivors</td>
<td>25.0 ± 11.1</td>
<td>23.0 ± 9.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Adenocarcinoma included the following confirmed diagnoses: adenocarcinoma, adenosquamous carcinoma, and bronchioloalevolar carcinoma. Sequential cases were collected between 1989 and 1993.

<sup>b</sup> Excluding three adenocarcinoma patients with a mutated K-ras gene and seven adenocarcinoma patients with a wild-type gene who were lost to follow-up.

<sup>c</sup> TNM pathological staging determined at time of biopsy.

<sup>d</sup> Follow-up is from time of biopsy.
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Fig. 4 Kaplan-Meier survival curves of adenocarcinoma and adenosquamous carcinoma patients with and without K-ras mutations. Survival curves were not significantly different ($P = 0.96$).

Fig. 5 Kaplan-Meier survival curves for stage I (node-negative) patients only, with and without K-ras mutations. The trend for shorter survival in patients with mutations was not significantly different from those with the wild type ($P = 0.14$).

Fig. 6 Kaplan-Meier survival curves of patients with a GAT mutation (aspartate) compared with patients with a GTT (valine) or CGT (arginine) mutation and with all other patients. There was a near-significant trend for shorter survival in the valine and arginine group ($P = 0.07$) compared with the wild-type group. There was also a near-significant difference ($P = 0.06$) in survival between this group and patients with a GAT (aspartate) substitution.

Transversions in several in vitro systems (23, 31, 32). The varying incidence of G to A transitions found in different studies may reflect differing carcinogenic exposure, such as to radon or nitrosamines, that can cause this type of mutation. Also, nicotine can be activated to the nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, which could contribute to G to A transversions in some smokers. In A/J mice, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone caused mutations in the K-ras gene of lung tumors that were almost exclusively G to A transitions in codon 12 (33).

Previously, a K-ras mutation at codon 18 was identified in lung adenocarcinomas and assumed to result in activation of the K-ras proto-oncogene (16). Among the 173 tumors analyzed, we did not find any mutation at that particular codon, although our approach would have allowed us to do so. An interesting finding was a double mutation, GGT to TTT, in codon 12, which transforms the wild-type glycine to a phenylalanine. Although this type of mutation had been found previously in a human mammary tumor cell line and had been thought to activate the K-ras gene specifically in mammary cells (34), it had never been identified before in lung tumors. Our present data indicate that the substitution of the glycine at codon 12 by a phenylalanine can occur in the lung also.

Our survival data do not replicate results from the European studies that showed a negative effect of K-ras mutations on survival in lung cancer (19), nor do they replicate results from the U.S. study that examined mutations in lung cancer cell lines (35). Two factors may influence our findings on survival: distribution of mutation types and stage of the patients displaying mutations. We found a relatively large number of aspartate mutations (26%), which are caused by the G to A transition. Furthermore, we found that survival of these patients as a group was better than that in patients with the wild type, although statistical significance was not reached because of the small number of individuals in the entire population displaying this.
mutation. In contrast, the survival of patients with the valine or arginine substitution was not as good as that of those with the wild type, although only marginal significance was achieved. The survival of patients with these two substitutions was also marginally worse than that of those with aspartate mutations. Again, marginal significance may be due to the small population size (14 individuals). These differing effects on survival could be due to the fact that substitution of the wild type glycine by different amino acids confers different properties on the resulting p21ras. This has been reported for the H-ras gene (27).

Substitution of different amino acids for glycine at codon 12 in the H-ras gene displayed a wide range of transforming activity based on the degree of cellular morphological transformation. Mutations that gave rise to gene products with the most potent transforming ability were those encoding valine, arginine, leucine, isoleucine, and threonine at position 12. Valine and arginine are the only mutations likely to be found commonly in tumors, because they require only one nucleotide substitution; the others (leucine, isoleucine, and threonine) require two or even three nucleotide substitutions. Compared with arginine and valine, cysteine, serine, and aspartate were considered moderately transforming (27). In a similar study, the transforming ability of several activating amino acid substitutions at codon 61 of the H-ras gene was examined based on the focus-forming activity of different mutants (36). H-ras mutants that encoded specific amino acids including valine and arginine at position 61 represented strong transforming mutants, which induced foci at high efficiencies. It will require an expanded study with a larger group of adenocarcinomas to determine whether these two specific mutations are also negative prognostic indicators for lung cancer. Regarding stage, an earlier Japanese study (18) suggested that survival differences could be found only in node-negative patients, whereas the survival rate for all patients was identical in mutated and wild type populations. We also found a trend toward shorter survival in stage I patients, all of whom were node negative. This may indicate that the mutated ras gene plays a role in progression.

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