Nras and Kras mutation in Japanese lung cancer patients: Genotyping analysis using LightCycler

HIDEFUMI SASAKI, KATSUHIRO OKUDA, OSAMU KAWANO, KATSUHIKO ENDO, HARUHIRO YUKIUE, TOMOKI YOKOYAMA, MOTOKI YANO and YOSHITAKA FUJII

Department of Surgery II, Nagoya City University Medical School, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan

Received March 2, 2007; Accepted May 21, 2007

Abstract. Activating mutations of Ras gene families have been found in a variety of human malignancies, including lung cancer, suggesting their dominant role in tumorigenesis. Many studies have showed that the Kras gene is activated by point mutations in ~15-20% of non-small cell lung cancers (NSCLCs), however, there are only a few reports on Nras mutations in NSCLC. We have genotyped Nras mutation status (n=195) and Kras mutation status (n=190) in surgically treated lung adenocarcinoma cases. The presence or absence of Nras and Kras mutations was analyzed by real-time quantitative polymerase chain reaction (PCR) with mutation-specific sensor and anchor probes. EGFR mutation status at kinase domain has already been reported. Nras mutation was found in 1 of 195 patients. This mutation was a G-to-T transversion, involving the substitution of the normal glycine (GGT) with cystein (TGT) and thought to be a somatic mutation. The patient was male and a smoker. Kras mutant patients (11.1%; 21/190) had a significantly worse prognosis than wild-type patients (p=0.0013). Eighty-two Nras or Kras mutations at kinase domain had exclusively Nras or Kras mutations. Although Nras gene mutation might be one of the mechanisms of oncogenesis of lung adenocarcinoma, this was a very rare event. Further studies are needed to confirm the mechanisms of Nras mutations for the sensitivity of molecular target therapy for lung cancer.

Introduction

Lung cancer is the leading cause of cancer-related death in the world (1). Lung cancer was the leading indication for respiratory surgery (42.2%) in 1998 in Japan (2). More than 15000 patients underwent surgical operation at Japanese institutions in 1998 (2). Despite the advances made in surgery, chemotherapy, and radiotherapy, the overall 5-year survival rate still remains at only 15%. Other therapeutic targets and agents are needed for the management of non-small cell lung cancer (NSCLC).

Ras oncogenes encode a family of membrane-associated proteins that regulate signal transduction on binding to a variety of membrane receptors. They play important roles in the regulatory processes of proliferation, differentiation, and apoptosis (3,4). The ras inhibitor S-trans, trans-farnesylthiosalicylic acid chemosensitized human tumor cells (5). There are three functional Ras genes, Nras, Kras, and Hras, and all homologs carry constitutive activation of the Ras protein, which subsequently is held in GTP-bound status. These mutations have been described in various solid tumors. Kras mutation seems to be the most prominent ras mutations in patients with lung adenocarcinomas. Shigematsu et al reported that Kras gene mutations were present in 50 (8%) of the 617 non-small cell lung cancers and were more frequently found in adenocarcinomas than in other histologic types (12% versus 2%; p<0.001), in ever smokers than in never smokers (10% versus 4%; p=0.01), and in patients from the USA and Australia than in patients from Japan and Taiwan (12% versus 5%; p=0.0001) (6). A previous report from USA showed that two of 14 lung tumors from smokers had Nras mutation (7), and a Japanese study that 7% of NSCLC had Kras mutation, but there was no Nras mutation (8). However, these results were from a relatively small sample analysis.

The standard for experimental detection of mutations is direct sequencing of DNA samples from the tissues. However, for known mutations, real-time polymerase chain detection followed by melting curve analysis, using hybridization probes, is highly sensitive, rapid and an efficient alternative approach to mutation detection (9-11). To determine the Nras and Kras mutation status in Japanese lung carcinoma for screening and diagnostic purposes, we wanted to develop a faster and easy method to detect ras mutations. In this study, we investigated Nras mutation status at codon 12/13 and codon 61 by the real-time PCR assay using LightCycler (10), mutation-specific sensor and anchor probes. These probes were used in a previous study (12). We also investigated Kras mutation status at codon 12/13 by the real-time RT-PCR assay using LightCycler. The findings were compared to the clinicopathologic features of lung cancer.
Patients and methods

Patients. The study group included 195 lung adenocarcinoma patients who had undergone surgery at the Department of Surgery II, Nagoya City University Medical School Hospital between 1997 and 2006. All tumor samples were immediately frozen and stored at -80˚C until assayed. Genomic DNA was extracted from lung adenocarcinoma and matched with normal lung tissues using Wizard SV Genomic DNA purification kit according to the manufacturer's instructions. These samples were sequenced for EGFR kinase domain by ABI Prism 3100 analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan) (13-15) and analyzed by ABI Prism Seq Scape version 2.1.1.

The clinical and pathological characteristics of the 195 lung adenocarcinoma patients were: 116 cases at stage I, 22 at stage II, and 47 at stage III-IV (10 unknown). The mean age was 64.8 years (range, 38-88). Among the 195 lung cancer patients, 101 (51.8%) were male, 94 (48.2%) were female, 102 (52.3%) were non-smokers, and 93 (47.7%) were smokers. Written informed consent was obtained from the patients, and the study was approved by the institutional ethics committee of the Nagoya City University Medical School.

PCR assays for Nras. The genotyping PCR reactions were performed using LightCycler DNA Master hybridization probe kit (Roche Molecular Biochemicals, Mannheim, Germany) in a 20-μl reaction volume. The primer sequences for the Nras gene (codon 12/13) were: forward, 5-AG TACT GTAGTGTTGCTGCC-3 and reverse, 5-CCTCACCTCTATGGTGGAATC-3. For the genotyping, sensor (TTGGCACAGACCAATACATGCAGGA-phosphate) and anchor (LC Red 640 AAGGCACTGGCAATCCAAGCTAGCTAAATCCAGAGG phophate) probes were used. The primer sequences for the Nras gene (codon 61) were: forward, 5-CACGCCAGGAGGAGATACACATCCAGAAC-3 and reverse, 5-GATGGCGGAAATACACAGAAGG-3. For the genotyping, sensor (ATACAG CTGGCGGAGAGGAGGG-fluorescein) and anchor (LC Red 640 AGTGCGCGAGGAGGAGGG-fluorescein) probes were used.

AGTGGCGAGAAGAGAATACATGCAGGA-phosphate) probes were used. Conditions for real-time PCR reaction were previously reported (12).

PCR assay for Kras codon 12/13. Total RNA was extracted from lung cancer tissues and adjacent non-malignant lung tissues using Isogen kit (Nippon gene, Tokyo, Japan) according to the manufacturer's instructions. RNA concentration was determined by spectrophotometer and adjusted to a concentration of 200 ng/ml. RNA (1 μg) was reverse transcribed by Superscript II enzyme (Gibco BRL, Gaithersburg, MD) with 0.5 μg oligo (dT)12,18 (Amersham Pharmacia Biotech Inc. Piscataway, NJ). Initially, several DNA samples were also sequenced and Kras mutation was found (16). These sets of cDNA were used as a positive and negative control for genotyping. DNA concentration was determined by spectrophotometer and adjusted to a concentration of 50 µg/ml.

Figure 1. Detection of Kras mutation in cDNA extracted from lung cancer tissues. The negative derivative of the fluorescence (-dF/dT) versus temperature (Tm) graph shows peaks with different Tm. The wild-type product showed a single peak at 69˚C, whereas the mutant type showed an additional peak at 60˚C.

Figure 2. Kras mutant patients (11.1%; 21/190) had a significantly worse prognosis than wild-type patients (log-rank test, p=0.0013).
ng/ml. We used 1 μl of each DNA for LightCycler analyses. To ensure the fidelity of cDNA transcription, all samples were subjected to PCR amplification with oligonucleotide primers specific for the constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase. Five samples were excluded because of the quality of RNA. The Kras codon 12/13 mutation was analyzed by quantitative real-time RT-PCR, performed on LightCycler. The primer sequences for Kras gene (codon 12/13) were: forward, 5-AGA GAGGCCTGCTGAAAAT-3 and reverse, 5-AATTTGTTC TCTATAATGGTGAATATC-3. For the genotyping, sensor (LC Red 640-CTACGGCACCAGCTCAAAC) and anchor (TCCACAAAATGATTTCTGAATTAGCTGTATGCCTCA GGCACCTTTG-fluorescein) probes were used. Conditions for real-time PCR reaction were: one cycle of 95˚C for 2 min, 40 cycles of 95˚C for 1 sec, 55˚C for 10 sec, and 72˚C for 7 sec. At the end of the PCR reaction, samples were subjected to a melting analysis to confirm specificity of the amplicon. All positive samples from melting analysis were directly sequenced.

Statistical analyses. Statistical analyses were done using the Mann-Whitney U test for unpaired samples and the Wilcoxon signed rank-test for paired samples. Linear relationships between variables were determined by means of simple linear regression. Correlation coefficients were determined by rank correlation using Spearman’s test and χ² test. All analyses were done using the Stat-View software package (Abacus Concepts Inc., Berkeley, CA), and were considered significant when the p-value was <0.05.

Results

Sequence results for EGFR gene at kinase domain in lung cancer tissues. We analyzed the EGFR mutation status in kinase domain as previously reported (13-15). From the 195 lung cancer patients, 82 patients had the activating mutation. Twenty-eight were male (n=101) and 54 were female (n=94). Sixty-two were non-smokers (n=98) and 20 were smokers (n=97). Fifty-six patients were stage I (n=116) and 24 were stage II-IV (n=69). Thus, EGFR mutation status at kinase

<table>
<thead>
<tr>
<th>Factors</th>
<th>Kras codon 12/13 mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mutation patients</td>
</tr>
<tr>
<td>Mean age (yrs.) 65.5±9.3</td>
<td>21</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>≤60</td>
<td>5 (8.6 %)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>16 (12.1 %)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>15 (14.4 %)</td>
</tr>
<tr>
<td>Female</td>
<td>6 (7.0 %)</td>
</tr>
<tr>
<td>Pathological subtypes</td>
<td></td>
</tr>
<tr>
<td>Adeno</td>
<td>19 (10.2 %)</td>
</tr>
<tr>
<td>BAC</td>
<td>2 (50.0 %)</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>6 (6.5 %)</td>
</tr>
<tr>
<td>Moderately or Poorly</td>
<td>10 (15.6 %)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>14 (10.2 %)</td>
</tr>
<tr>
<td>N+</td>
<td>7 (13.2 %)</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>14 (14.7 %)</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>7 (7.4 %)</td>
</tr>
<tr>
<td>Pathological stages</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>8 (6.8 %)</td>
</tr>
<tr>
<td>II-IV</td>
<td>13 (17.8 %)</td>
</tr>
</tbody>
</table>

N+, lymph node metastasis positive; adeno, adenocarcinoma.

Table I. Clinicopathological data of 190 lung cancer patients.
domain was significantly correlated with gender (p<0.0001) and smoking status (p<0.0001). There was a tendency towards better prognosis in EGFR mutant patients than in EGFR wild-type patients (log-rank test, p=0.0747).

Genotyping of Kras codon 12/13 in lung cancer tissues. For genotyping, the anchor probe was matched for Kras wild-type. As shown in Fig. 1, the Kras wild-type PCR products showed a single peak at 68˚C, whereas the mutant-type showed an additional peak at 60˚C. From the 190 lung cancer patients, 21 patients had a Kras mutation (11.1%) (Table I). Fifteen were male and 14 were smokers. Kras mutation was significantly higher in advanced stage patients (stage II-IV, 13/73) than in stage I patients (8/117) (p=0.019). There was a tendency towards a higher Kras mutation ratio in male (p=0.1032). Kras mutant patients did not have Braf (17), erbB2 (15) (data not shown), and EGFR mutations at kinase domain. Thus, Kras mutation and EGFR mutation at kinase domain were exclusive. Of 21 samples, 19 were confirmed by direct sequencing. Of 19 Kras mutations, 11 (57.9%) were G-to-T transversions.

Kras mutant patients (11.1%; 21/190) had a significantly worse prognosis than wild-type patients (log-rank test, p=0.0013) (Fig. 2). Using the cox hazard regression model, pathological stage (p<0.0001) and Kras mutation (p=0.021) were independent prognostic factors.

Analysis of Nras mutations. For codon 12/13 genotyping, the anchor probe was matched for Nras wild-type. As shown in Fig. 3, the Nras wild-type products showed a single peak at 68˚C, whereas the codon 12 mutant type showed an additional peak at 63˚C. From the 195 lung cancer patients, only one patient had a Nras mutation. This was also confirmed by direct sequencing (Fig. 4). The adjacent matched-lung tissue was available and showed no additional peak from PCR assays, suggesting that these Nras mutations were somatic. This patient was a male and smoker. In total, 22 patients had ras mutations in adenocarcinomas. These ras and EGFR mutations at kinase domain were exclusive. There was a tendency towards a higher ras mutation ratio in smokers (p=0.0632) and in males (p=0.0714).

Discussion

We found only one Nras mutation in patients with adenocarcinoma. Somatic Nras or Kras mutations exclusively existed with EGFR kinase domain mutations. Recent studies have shown that EGFR gene mutations at kinase domain are common in lung cancers from never smokers and females with adenocarcinoma (13-15,18,19), and correlated with sensitivity for EGFR inhibitors (20,21). On the other hand, in this study, there was a tendency towards a higher ras mutation ratio in smokers (p=0.0632) and males (p=0.0714). Our analysis suggested that the population of somatic ras mutations and the EGFR mutations at kinase domain in lung cancers were different.
The mutational changes at critical codons of the ras genes result in loss of GTPase activity and gain of transforming ability in vitro and in vivo. Nras mutations are frequently observed in hematological malignancies. The incidence of Nras mutations is ~20-30% in acute myeloid leukemia (AML) as well as myelodysplastic syndromes and 10% in acute lymphoblastic leukemia (ALL) (22-25), predominantly at codon 61. The prognostic relevance of the Nras alteration is more obvious in AML than in ALL (25). Mutations in members of the ras gene family, especially Nras, have been identified in cutaneous melanomas, although their reported frequency varies over a wide range (26-29), and especially seen at codon 61. The ras gene family members are activated by point mutations at codon 12, 13 or 61 in ~20-30% of lung adenocarcinomas and 15-20% of all non-small cell lung cancers, but rarely in small cell lung cancers (30). Mutations in Kras account for >90% of ras mutations of lung adenocarcinomas with 85% of Kras mutations affecting codon 12 (8). Characteristically, ~70% of Kras mutations are G-to-T transversions, involving the substitution of the normal glycine (G) with either cysteine (T) or valine (GTT) (31). Uchiyama et al has shown that 7% of NSCLC had Kras mutation, but there was no Nras mutation (8).

Most ras and TP53 gene mutations in lung cancers are G-to-T transversions, molecular events that are linked to exposure to tobacco smoke carcinogens (32-34). Lung cancers that arise in never smokers rarely have Kras (G-to-T) gene mutations, and their TP53 gene mutations are seldom G-to-T transversions (32,35), suggesting that these cancers arise in response to exposure to carcinogens other than those present in tobacco smoke. G-to-T transversions are also the most frequently detected mutations in activated ras genes in benzo[a]pyrene-induced mouse lung tumors (36).

The findings presented support the hypothesis that there are distinct molecular pathways that are involved in the pathogenesis of lung adenocarcinomas, one involving EGFR tyrosine kinase domain mutations (non-smoker, female) and the other involving the ras gene mutations (smoker, male). K-ras mutant lung cancer has been reported to show poorer clinical outcome when treated with EGFr inhibitor (37).

A wide range of mutation detection methodologies exist, of which sequencing has been considered the gold standard because of its ability to identify the specific DNA sequence change that has occurred. However, dideoxy-sequencing is rarely sensitive below a 10% mutant allele frequency, which corresponds to a threshold of 20% tumor cells heterozygous for a mutation (38). Melting curve analysis represents a significant advantage for mutation detection in tumor samples. The developments in dye technology have played a major role in the emergence of this methodology.

Different base substitutions produce differences in melting behavior. An advantage of performing melting curve analysis on a real-time PCR machine is that the PCR amplification and analysis are performed in one run within an hour and the results are available for analysis at the end of the run.

The frequency of Kras mutations in our sample set was higher than previous Japanese reports (5,7,35). In our analysis, two melting curve positive patients were sequence wild-types. A previous report indicated that melting curve analysis could detect as little as 5-6% of mutant cell lines DNA diluted in normal DNA (39). Thus, melting curve analysis is a sensitive in-tube methodology to screen for mutations in clinical samples.

Acknowledgements

We would like to thank Mrs. Yuri Yamamoto for her excellent technicalassistances. This study was supported by AstraZeneca Research Grant 2004 and Grants-in-Aid for Science Research (Nos. 18659407, 18390381, 18790998) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

References


