

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

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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 *EGFR* 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.

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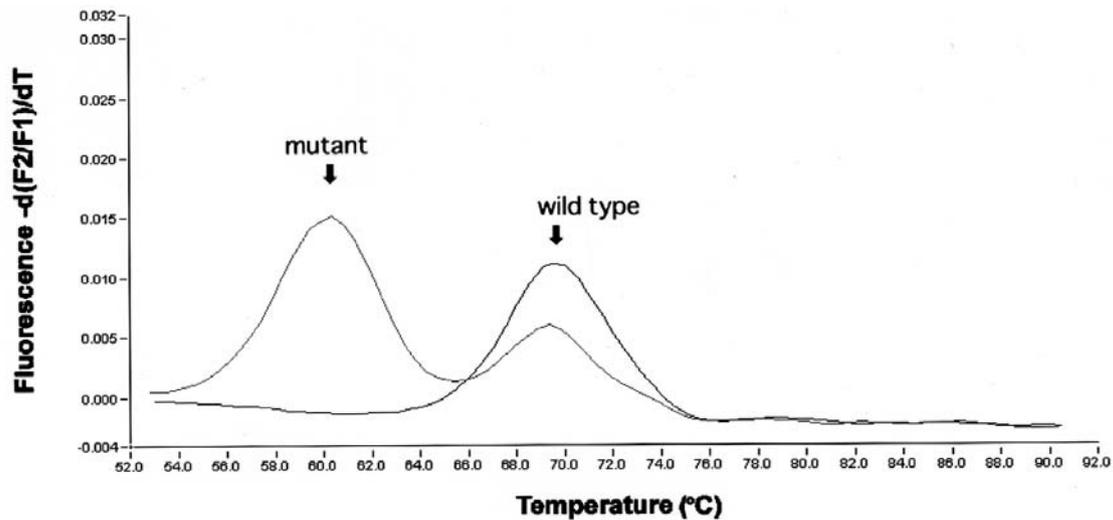


Figure 1. Detection of *Kras* mutation in cDNA extracted from lung cancer tissues. The negative derivative of the fluorescence ($-dF/dT$) versus temperature (T_m) graph shows peaks with different T_m . The wild-type product showed a single peak at 69°C, whereas the mutant type showed an additional peak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

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-AGTACTGTAGATGTGGCTCGCC-3 and reverse, 5-CCTCACCTCTATGGTGGGATC-3. For the genotyping, sensor (TTG GAGCAGGTGGTGTGGG-fluorescein) and anchor (LC Red 640 AAGCGCACTGACAATCCAGCTAATCCAGAA CC-phosphate) probes were used. The primer sequences for the *Nras* gene (codon 61) were: forward, 5-CACCCCCAG GATTCTTACAGA-3 and reverse, 5-GATGGCAAATAC ACAGAGGAAGC-3. For the genotyping, sensor (ATACAG CTGGACAAGAAGAG-fluorescein) and anchor (LC Red 640

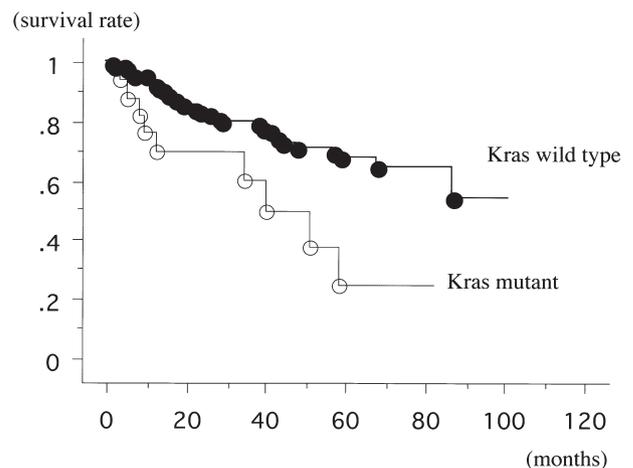


Figure 2. *Kras* mutant patients (11.1%; 21/190) had a significantly worse prognosis than wild-type patients (log-rank test, $p=0.0013$).

AGTGCCATGAGAGACCAATACATGAGGA-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)₁₂₋₁₆ (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

Factors	Kras codon 12/13 mutations		p-value
	Mutation patients	Wild-type patients	
Mean age (yrs.) 65.5±9.3	21	169	
Age			
≤60	5 (8.6 %)	53 (91.4%)	0.4785
>60	16 (12.1%)	116 (87.9%)	
Gender			
Male	15 (14.4%)	89 (85.6%)	0.1032
Female	6 (7.0%)	80 (93.0%)	
Pathological subtypes			
Adeno	19 (10.2%)	167 (89.8%)	0.0611
BAC	2 (50.0%)	2 (50.0%)	
Differentiation			
Well	6 (6.5%)	87 (93.5%)	0.0619
Moderately or Poorly	10 (15.6%)	54 (84.4%)	
Lymph node metastasis			
N0	14 (10.2%)	124 (89.8%)	0.5557
N+	7 (13.2%)	46 (86.8%)	
Smoking status			
Smoker	14 (14.7%)	81 (85.3%)	0.1053
Non-smoker	7 (7.4%)	88 (92.6%)	
Pathological stages			
I	8 (6.8%)	109 (69.7%)	0.019
II-IV	13 (17.8%)	60 (82.2%)	

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

ng/ml. We used 1 μ l 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 GAGGCTGCTGAAAAT-3 and reverse, 5-AATTTGTTC TCTATAATGGTGAATATC-3. For the genotyping, sensor (LC Red 640-CTACGCCACCAGCTCCAAC) and anchor TCCACAAAATGATTCTGAATTAGCTGTATCGTCAA GGCCTCTTG-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 χ^2 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

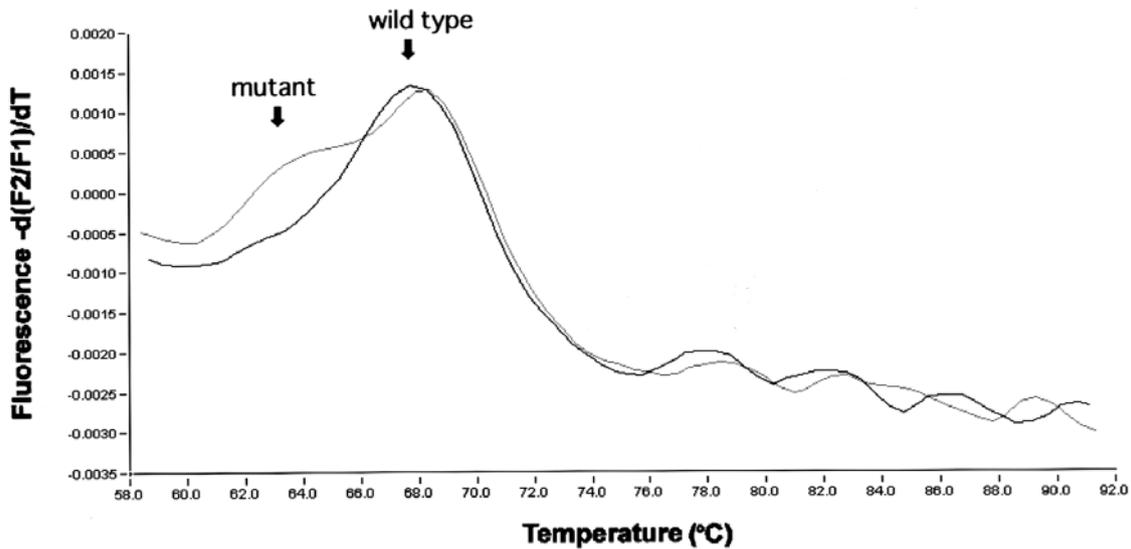


Figure 3. Detection of *Nras* mutation in DNA extracted from lung cancer tissues. The negative derivative of the fluorescence ($-dF/dT$) versus temperature (T_m) graph shows peaks with different T_m . The wild-type product showed a single peak at 68°C, whereas the mutant type showed an additional peak at 63°C.

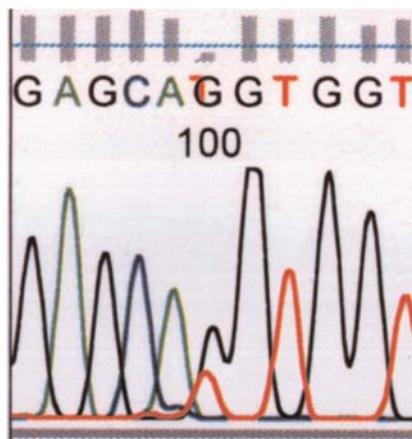


Figure 4. The sequencing data for *Nras* gene using RT-PCR (direct sequencing) method. The PCR products showed glycine (GGT) to cysteine (TGT) transition at codon 12.

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 69°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.



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mutational changes at critical codons of the ras genes 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 (GGT) with either cysteine (TGT) 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.

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