Original Article

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LKB1, *TP16*, *EGFR*, and *KRAS* somatic mutations in lung adenocarcinomas from a Chiba Prefecture, Japan cohort

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ABSTRACT: The discovery of somatic mutations in cancer-related genes has been applied to understand the genetic basis of cancer. Here we report somatic mutations of two tumor suppressor genes: *LKB1* (exons 1, 4, and 8) and *TP16* (*CDKN2A*) (exons 1 and 2); and two oncogenes: epidermal growth factor receptor *EGFR* (exons 18-21) and *KRAS* (exon 2) in 97 lung adenocarcinoma tissues in a cohort from the Kujukuri coast area of Chiba, Japan. In the *LKB1* gene, only F354L substitutions were observed in 14 of the 97 tissue samples (14.4%). In the *TP16* gene, only two deletions were observed in contrast to previous reports. On the other hand, the *EGFR* gene was highly mutated (38.1%) and mainly L858R substitutions occurred (23.7%) as well as insertions and deletions. In the *KRAS* gene, 10 substitutions at codon 12 were observed (10.3%). Co-occurrence of *EGFR* and *KRAS* somatic mutations was identified in one patient, those of *EGFR* and *LKB1* were in three patients, and those of *KRAS* and *LKB1* were in four patients. The lower rates of *LKB1*, *TP16*, and *KRAS* somatic mutations in lung adenocarcinomas are characteristic of the Kujukuri cohort as compared to Caucasians.

Keywords: Chromatograms, tumor suppressor genes, oncogenes, mTOR pathway, RAS pathway, co-occurrence of mutations

1. Introduction

The *LKB1* tumor suppressor gene, which encodes serine-threonine kinase 11 (STK11), has been shown to regulate cell cycle progression, apoptosis, and cell polarity (*1*). Previous reports have suggested a *LKB1* somatic mutation rate as high as 30% in non-small cell lung carcinoma (NSCLC) tumors derived from Caucasian patients (*2,3*), while being infrequent in NSCLC Asian patients (3%) (*4*). Another review (*5*) reported that *LKB1* somatic mutations were observed in 20.18% of lung adenocarcinoma by analyzing the Catalog of Somatic Mutations in Cancer (COSMIC) (*6*), a large-scale database curated by the Wellcome Trust Sanger Institute. The *TP16* gene (*CDKN2A*, cyclin-dependent kinase inhibitor 2A) is also a tumor suppressor gene and has been reported to show a high rate of deletions as a major type of somatic mutation in cancer, consisting of 24.57% of lung adenocarcinomas in the COSMIC database (*5*). In contrast to these tumor suppressor genes, *EGFR* and *KRAS* oncogenes play a central role in tumorigenesis. The tyrosine kinase activity of EGFR phosphorylates tyrosine residues of target proteins, including KRAS, to initiate multiple signaling pathways resulting in cell proliferation, migration, metastasis, resistance to apoptosis, and angiogenesis (*7,8*). Small-molecule inhibitors of EGFR tyrosine kinase activity (TKIs) such as gefitinib and erlotinib provide a good concept for anticancer drugs. However, clinical trials have revealed significant variability in the TKI response depending on *EGFR* and *KRAS* gene mutations (*9*). Interestingly, somatic *EGFR* and *KRAS* gene mutations, which are upstream and downstream of the RAS pathway, respectively, have been reported to be mutually exclusive. Thus, concurrent *EGFR* and *KRAS* mutations have been very rare (*5*).

Lung adenocarcinoma is the most common form of lung cancer, which has an average 5-year survival rate of 15%, mainly because of late-stage detection and paucity of late-stage treatment options (*10,11*). To determine the genetic basis of lung adenocarcinoma in a cohort from the Kujukuri coast area in Chiba prefecture, Japan, we analyzed exons 1, 4, and 8 of *LKB1*, exons 1 and 2 of *TP16* which includes almost the entire sequence of the protein coding region, exons 18-21 of *EGFR*, and exon 1 of *KRAS*, according to the histogram of somatic mutations in *LKB1*, *TP16*, *EGFR* and *KRAS* from the COSMIC database (*6*).

2. Materials and Methods

2.1. *Patients*

Primary tumor samples from patients with lung adeno-

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carcinoma were obtained from 97 randomly selected patients (54 males and 43 females) with lung cancer who were diagnosed and had undergone surgical resection at Asahi General Hospital, Asahi-Shi, located in the Kujukuri coast area, Chiba prefecture, Japan from September 2004 to March 2009. This study was conducted according to the principles expressed in the Declaration of Helsinki. Asahi General Hospital obtained written informed consent from all subjects. The Asahi General Hospital Institutional Review Board approved use of human tissue in this study according to the Ethical Guidelines of the Ministry of Health, Labour, and Welfare of Japan. The data were analyzed anonymously. All patients were pathologically diagnosed as having primary lung adenocarcinoma and subclassified into histological subtypes by examining more than 10 histological slides. The age distribution was: 5 patients under 50-years old; 12 patients 50- to 59-years-old; 28 patients 60- to 69-years-old; 39 patients 70- to 79-years-old; and 13 patients 80 or over (average = 69.11, S.D. = 9.94).

2.2. *DNA extraction and sequencing analysis*

Genomic DNA extraction was performed using a simple 96-well plate based DNA extraction. A few milligrams of the frozen specimen was digested with Proteinase K at 55°C for 2 h in 100 μL of extraction buffer (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.5% Tween 20) followed by heat inactivation at 95°C for 10 min. The preparation was centrifuged at 3000 × g for 15 min, and 2-5 μL of the supernatant was used as a PCR template.

Mutations were detected using PCR-based direct sequencing of three *LKB1* exons (exons 1, 4, 8), two *TP16* exons (exons 1, 2), four *EGFR* exons (exons 18-21), and exon 1 of the *KRAS* gene. The primers for PCR amplification and sequencing analysis are shown in Table 1. PCR amplification was done in 50 μL reactions containing 0.2 μM of each primer pair and 0.25 units of Pfu DNA polymerase (Celestar-Lexico Sciences, Chiba, Japan).

LKB1 exons were amplified by initial denaturation at 94°C for 5 min followed by 40 cycles of 10 sec at 98°C, 30 sec at 68°C, and a 1 min final extension at 72°C. *TP16* exons were amplified by initial denaturation at 94°C for 5 min followed by 40 cycles of 10 sec at 98°C, 30 sec at 65°C, 30 sec at 72°C, and a 1 min final extension at 72°C. *TP16* exon 2 amplification was performed with 5% of DMSO. *EGFR* exons were amplified by initial denaturation at 94°C for 1 min followed by 35 cycles of 5 sec at 98°C, 30 sec at 65°C, 1 min at 72°C, and a final extension at 72°C for 1 min. The exon 1 region of *KRAS* was amplified by initial denaturation at 94°C for 1 min followed by 40 cycles at 98°C for 5 sec, 50°C for 30 sec, and 72°C for 30 sec, and a final extension at 72°C for 5 min.

The PCR products were purified using a GFX96 PCR purification kit (GE Healthcare Life Sciences, Little Chalfont, UK) and sequenced directly using a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) with 1 μM of each sequencing primer.

Several samples that were difficult to determine the mutation status by the direct sequencing method were cloned into pUC118 before sequencing.

3. Results

Somatic mutations in *LKB1*, *TP16*, *EGFR*, and *KRAS* genes observed in the cohort from the Kujukuri coast area of Chiba prefecture are summarized in Table 2. The sequence chromatograms are shown in Figures 1, 2, and 3 for single nucleotide substitutions in *LKB1*, *EGFR*, and *KRAS*; two *TP16* deletions; and *EGFR* deletions and insertions, respectively.

Table 1. PCR primer sequences and sequencing primer sequences for exons 1, 4 and 8 of *LKB1*, exons 1 and 2 of *TP16*, exons 18-21 of *EGFR*, and exon 1 of *KRAS*

Gene	Exon	PCR primer sequences	Sequencing primer sequences
LKB1	exon 1	F 5'-TGGAGAAGGGAAGTCGGAACACAAGG-3'	F 5'-GGGAAGTCGGAACACAAGGAAGGAC-3'
		R 5'-GCCAGACGGGTCCAGCTCAG-3'	R 5'-GAACCATCAGCACCGTGACTGGC-3'
	exon 4	F 5'-CCAGCTGGGCCTGTGGTGTTTG-3'	F 5'-CCCCTGTGAGGGGCAGGGAG-3'
		R 5'-CTGGTCCGGCAGGTGTCGTC-3'	R 5'-AACGGGTGCAGTGCCTGTGG-3'
	exon 8	F 5'-AGAGGACATGGCTGAGCTTCTGTGG-3'	F 5'-CAGAGGAGCTGGGTCGGAAAACTGG-3'
		R 5'-GGGACGTGGGATTGGCCACC-3'	R 5'-TGCAGACAGGCAGGCACCCTG-3'
TP16	exon 1	F 5'-AGGGTCGGAGGGGGCTCTTC-3'	F 5'-AAGAGGAGGGGCTGGCTGGTC-3'
		R 5'-CTGATTCCAATTCCCCTGCAAACTTCG-3'	R 5'-CTCCAGAGTCGCCCGCCATC-3'
	exon 2	F 5'-GGGCTTGTGTGGGGGTCTGC-3'	F 5'-TGGCGGTGAGGGGGCTCTAC-3'
		R 5'-GGCGCGTGAGCTGAGGCAAG-3'	R 5'-TCCCGGGCTGAACTTTCTGTGC-3'
EGFR	exon 18	F 5'-TGTCCTTCCAAATGAGCTGGCAAGTG-3'	F 5'-GTGTCCTGGCACCCAAGC-3'
		R 5'-GGAGTTTCCCAAACACTCAGTGAAACAAAG-3'	R 5'-CCCCACCAGACCATGAGAG-3'
	exon 19	F 5'-GGGTGCATCGCTGGTAACATCC-3'	F 5'-GCAGCATGTGGCACCATCTC-3'
		R 5'-GATGTGGAGATGAGCAGGGTCTAG-3'	R 5'-CAGAGCAGCTGCCAGACATG-3'
	exon 20	F 5'-CTCAAGATCGCATTCATGCGTCTTCAC-3'	F 5'-GCATTCATGCGTCTTCACCTG-3'
		R 5'-CACACACATATCCCCATGGCAAACTC-3'	R 5'-CATATCCCCATGGCAAACTCTTG-3'
	exon 21	F 5'-GTCAGCAGCGGGTTACATCTTCTTTC-3'	F 5'-CAGCCATAAGTCCTCGACGTG-3'
		R 5'-CTCACCCAGAATGTCTGGAGAGC-3'	R 5'-TCCTCCCCTGCATGTGTTAAAC-3'
KRAS	exon 1	F 5'-AGGTACTGGTGGAGTATTTGATAGTG-3'	F 5'-TTGATAGTGTATTAACCTTATG-3'
		R 5'-CATACTCCCAAGGAAAGTAAAGTTCC-3'	R 5'-TGGTCCTGCACCAGTAATATGC-3'

F, forward. R, reverse.

Table 2. Somatic mutations in exons 1, 4 and 8 of *LKB1*, exons 1 and 2 of *TP16*, exons 18-21 of *EGFR*, and exon 1 of *KRAS* observed in the Kujukuri cohort

Gene/exon	Somatic mutations	Number	Male	Female
LKB1 (exons 1, 4, 8)	ttc/ttg (F354L)	14	9	5
TP16 (exons 1, 2)	del549c	1	1	0
	del666-673	1	1	0
EGFR (exons 18-21)	ctg/cgg (L858R)	23	11	12
	D770-N771 ins SVD	1	0	1
	cgc/tgc (R776C), ctg/cgg (L858R)	1	0	1
	746ELREA deletion	7	6	1
	746ELREA deletion, ctg/cgg (L858R)	1	1	0
	ggc/gcc (G719A)	1	0	1
	747LRE deletion, A750P, ctg/cgg (L858R)	1	0	1
	P772-H773 ins YNP, H773Y	1	0	1
	gtg/ctg (V834L), ctg/cgg (L858R)	1	0	1
KRAS (codon 1)	ggt/gtt (G12V)	1	0	1
	ggt/tgt (G12C)	4	2	2
	ggt/gat (G12D)	3	2	1
	ggt/gat (G12A)	2	2	0

The two right columns show the numbers related to gender.

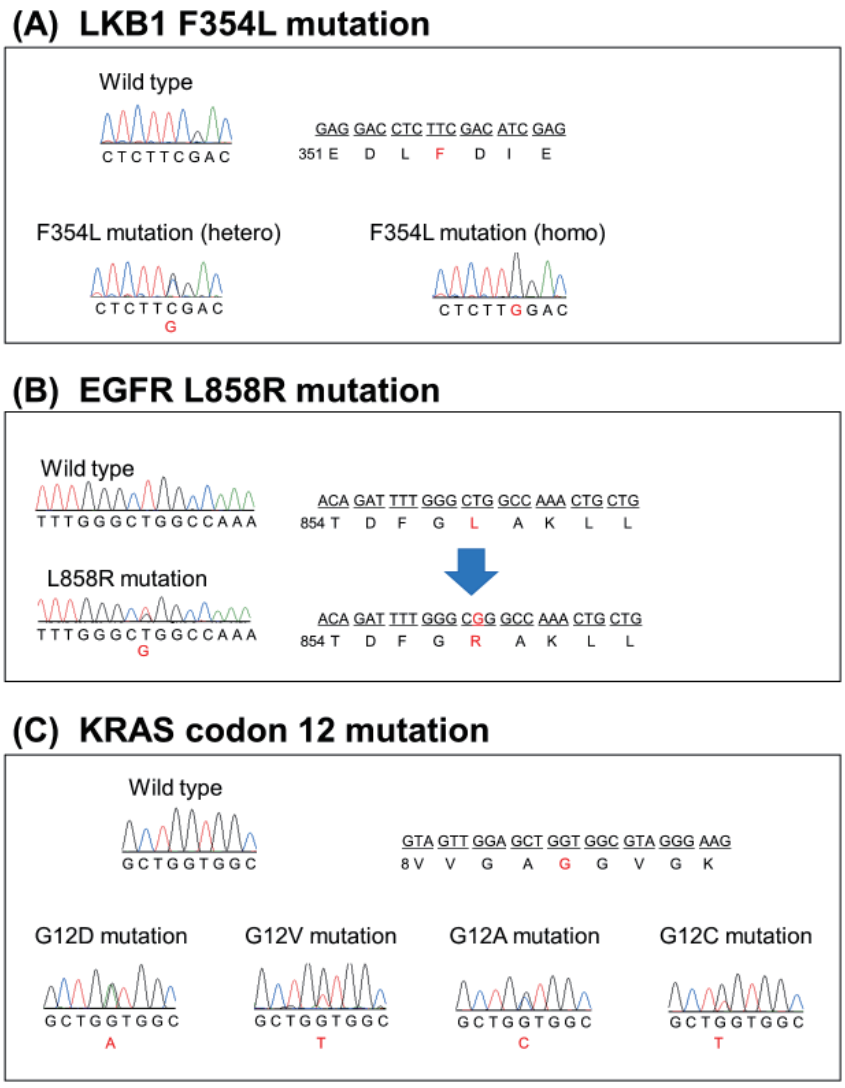

Figure 1. Wave charts of analyzed nucleotide sequences and corresponding amino acid sequences. (A) *LKB1* wild type nucleotide sequence and base substitutions (hetero and homo) at F354L. **(B)** *EGFR* wild type nucleotide sequence and base substitution at F858R. **(C)** *KRAS* wild type nucleotide sequence and base substitutions at codon 12.

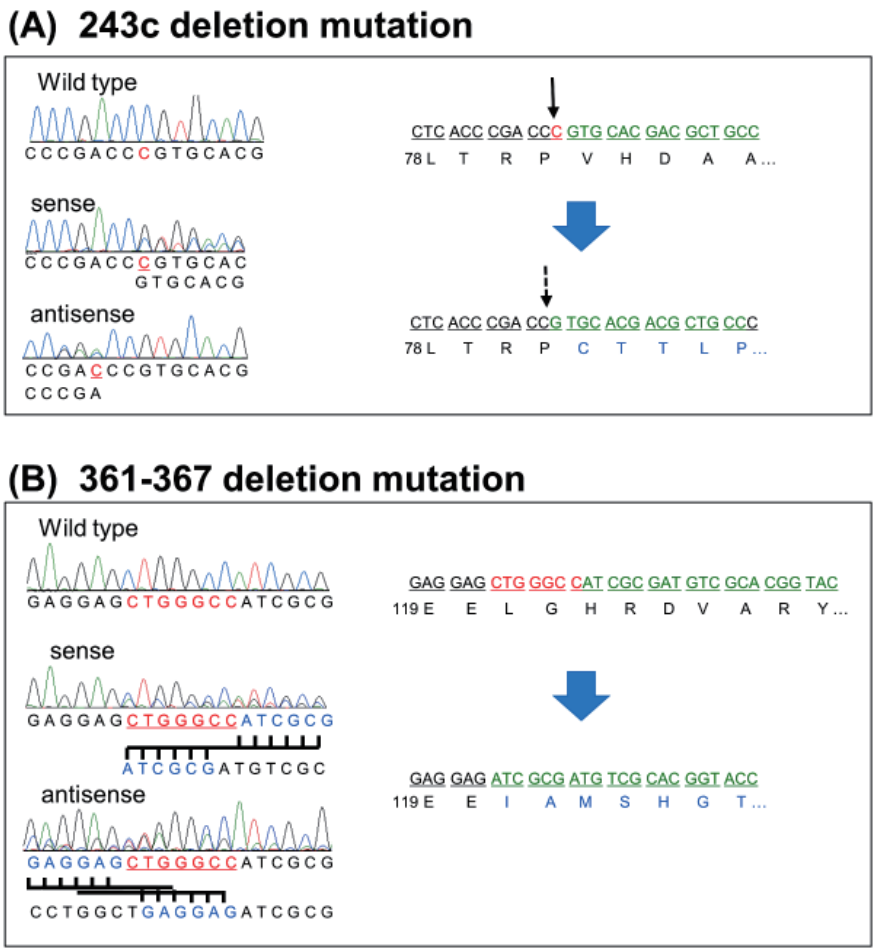

Figure 2. Wave charts of nucleotide sequences and corresponding amino acid sequences of wild type and two deletions observed in the *TP16* gene. (A) 549C deletion mutation. **(B)** 666-673 deletion mutation.

3.1. *LKB1 and TP16 mutations*

We analyzed the sequence of exons 1, 4, and 8 of the *LKB1* gene and the sequence of the *TP16* gene (exons 1 and 2). Of the 97 lung adenocarcinoma tissue samples, only 14 samples (14.4%) were observed to contain F354L substitutions in exon 8 of the *LKB1* gene, of which two samples showed biallelic mutations. Figure 1A shows the three types of *LKB1* sequences observed in chromatograms: wild type, hetero F354L mutation, and biallelic (homo) F354L mutation. On the other hand, only 2 of the 97 tissue samples (2.1%) showed deletions in exon 2 of the *TP16* gene (cytosine deletion at 549 changed the amino acid sequence after 82Val; and the deletion from 666 to 673 changed the amino acid sequence after 121Leu; the numbers of nucleotides and residues are from NM_000077.4 and NP_000068.1 in the NCBI database), as shown in Figures 2A and 2B.

3.2. *EGFR and KRAS mutations*

The major somatic mutations of the *EGFR* gene observed in the cohort were L858R substitutions at exon 21, as shown in the Figure 1B chromatogram. There were 23 samples of the 97 tissue samples (23.7%) that showed only this substitution and four samples that showed other types of mutations in addition to this substitution (Table 2). Another major mutation was the 746-ELREA-750 deletion at exon 19 that was observed in 7 samples (7.2%). The sequencing result chromatogram showed this deletion in Figure 3A. Figures 3B and 3C showed the sequencing results of insertion mutations. Figure 3B showed the insertion of three amino acids YNP between 772Pro and 773His in exon 20, and the histidine residue was substituted by Tyr. Figure 3C showed the insertion of three amino acids SVD between 770Asp and 771Asn in exon 20. The other mutations observed are summarized in Table 2. In total, 37 of 97 samples (38.1%) displayed somatic mutations in the *EGFR* gene.

The somatic mutations observed in the *KRAS* gene were only substitutions at codon 12 of amino acid residue Gly to Asp (3 samples), to Val (1 sample), to Ala (2 samples), and to Cys (4 samples). A total of 10 samples showed somatic mutations in *KRAS* (10.3%). Figure 1C shows the sequences of the somatic mutations at *KRAS* codon 12 on chromatograms.

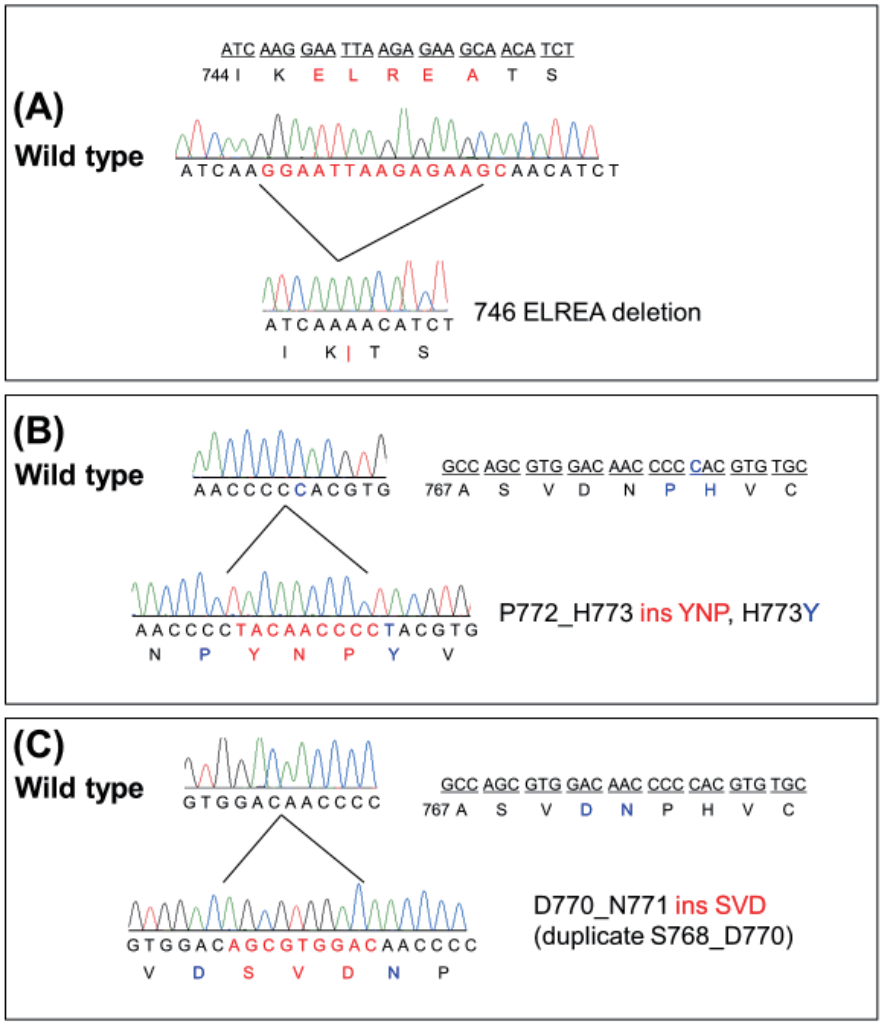

Figure 3. Wave charts of nucleotide sequences and corresponding amino acid sequences of wild type, deletions, and insertions observed in the *EGFR* gene. (A) 746ELREA deletion. **(B)** P772_H772 insertion YNP with H773Y. **(C)** D770_N771 insertion SVD (duplication S768_D770).

3.3. *Concurrency of mutations*

The concurrent *EGFR* and *KRAS* mutations have been reported to be very rare (*5*), meaning the occurrence of those mutations were mutually exclusive in lung adenocarcinoma. We observed that only one of the 97 tissue samples harbored the concurrent *EGFR* and *KRAS* mutations. In contrast, co-occurrence of *EGFR* and *LKB1* mutations were observed in three samples, while co-occurrence of *KRAS* and *LKB1* mutations were observed in four samples.

4. Discussion

We analyzed somatic mutations in tumor suppressor genes *LKB1* and *TP16*, and in oncogenes *EGFR* and *KRAS*, using 97 lung adenocarcinoma tissue samples in a cohort from the Kujukuri coast area of Chiba prefecture, Japan. Compared with the review paper that analyzed the COSMIC database (*5*), we showed the rate of *TP16* somatic mutations in the Kujukuri cohort to be very small (2.1%), while the COSMIC database showed a 24.57% mutation rate. The *LKB1* mutation rate was 14.4% from our observations, while that of the COSMIC database was 20.18%. In particular, only F354L substitutions in the *LKB1* gene were observed in the Kujukuri cohort.

As for *EGFR*, the somatic mutation rate was 38.1% from our observations, which is a little bit higher than the rate from the COSMIC database (35.85%). *KRAS* also showed a lower somatic mutation rate in the Kujukuri cohort (10.3%) than that from the COSMIC database (23.02%). The lower rates of somatic mutations for *LKB1*, *TP16*, and *KRAS* genes in lung adenocarcinomas are characteristic of the Kujukuri cohort.

EGFR mutations have been more commonly found in tumors from patients who never smoked cigarettes (*12*), while *KRAS* mutations have been present in those with significant tobacco exposure (*9,13*). Unfortunately, we could not follow the history of smoking or non-smoking patients from clinical records of the cohort,

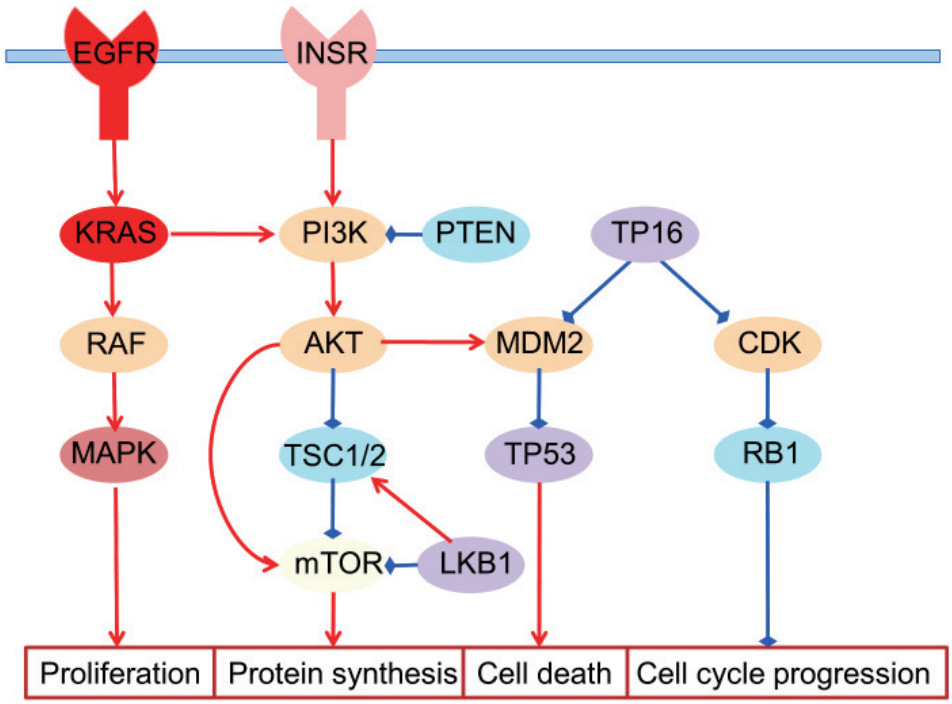

Figure 4. Pathways relating to *LKB1*, *TP16*, *EGFR*, and *KRAS*. The pathways were summarized from Kyoto Encyclopedia of Genes and Genomes (KEGG). Red lines represent activation and blue lines represent inhibition.

except for the 11 patients who were never-smokers. Among the 11 patients, we found four *EGFR* mutations (36.3% = 4/11) that included three L858R substitutions and one insertion, and a single *KRAS* mutation (9.1% = 1/11). The percentages of *EGFR* and *KRAS* mutations in the 11 never-smoking patients were not significantly different from those in the remaining 86 patients (*EGFR*: 38.3% = 33/86, and *KRAS*: 10.5% = 9/86), suggesting that *EGFR* and *KRAS* mutations in the cohort are not associated with tobacco use. Contrary to previous reports describing the association between *EGFR* mutations and non-smoking history (*12*), recent papers have reported no association with smoking or non-smoking history in Asian patients (*14-16*).

Gender also has been reported to be unconnected with *EGFR* mutations in Asian patients (*14,15*). We also found no association between gender and any type of *EGFR* mutations in the cohort. However, focusing on the 746ELREA deletions, 6 out of 7 patients with this type of deletion were male patients. Moreover, we investigated the distribution of mutations in the four genes across different clinicopathological subgroups based on tumor size, clinical stage, and histological subtypes including acinar, papillary, and bronchioloalveolar carcinoma patterns. However, no significant association with clinicopathological subgroups was found (data not shown), which is consistent with a previous report (*17*).

One interesting topic in analyzing genetic somatic mutations is the co-occurrence of mutations in two genes. Co-occurrence of *EGFR* and *KRAS* mutations, which lie upstream and downstream of the RAS pathway respectively, has been reported to be mutually exclusive (*5*). In the review paper that analyzed the COSMIC database (*5*), the co-occurrence of *TP16* and *KRAS* mutations was reported in pancreatic cancer, lung large cell cancer, colon cancer, kidney tumors, and lung adenocarcinoma. However, in the present study, co-occurrence of *TP16* and *KRAS* mutations was not observed because of the lower mutation rate of *TP16* in the Kujukuri cohort. On the other hand, as to *LKB1* we co-sequenced *LKB1* and *KRAS* in the 97 lung adenocarcinomas tissue samples, and found four co-occurring mutations in these genes. So, mutations in these genes may not be mutually exclusive and therefore these genes may not be located on the same pathway. Co-occurrence of *LKB1* and *EGFR* mutations was also observed in three samples. Taken together, *LKB1* might act separately from the RAS pathway to suppress tumorigenesis. According to the known pathways summarized from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Figure 4) related to the *LKB1*, *TP16*, *EFGR*, and *KRAS* genes pathways, *LKB1* attenuates protein synthesis by inhibiting the mTOR pathway (*18*), that is separated from the RAS pathway.

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