LKB1 gene mutations in Japanese lung cancer patients

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Mutation of the *LKB1* gene (also known as *STK11*) is regarded as a cause of Peutz–Jeghers syndrome. In Caucasian patients, *LKB1* somatic mutations occur in approximately one-third of lung adenocarcinomas. The aim of the present study was to examine the *LKB1* gene in Japanese patients with lung cancer and to evaluate its clinical and pathological implications. We sequenced the *LKB1* gene in 22 lung cancer cell lines and 100 Japanese patients with lung cancer (including 81 adenocarcinomas, 14 squamous cell carcinomas and five other histological types) who had undergone curative pulmonary resection. We also determined expression levels of the *LKB1* gene by quantitative real-time reverse transcription–polymerase chain reaction and correlated these results with the clinical and pathological features of patients. Among the 22 cell lines, four had mutations and three of these were in adenocarcinoma cells. Of 100 primary lung cancers, only three had *LKB1* gene mutations (3%). All of them were male smokers with adenocarcinomas. Hence, when confined to this subset of patients, the mutation frequency was 9% (3/33). No significant correlation was observed between the expression level of *LKB1* and patient clinicopathological features. In conclusion, *LKB1* gene mutations were relatively rare in Japanese patients with lung cancer compared with Caucasian patients. *LKB1* gene mutations appear to be frequent in male, smoking patients of Caucasian origin, in contrast to *EGFR* or *HER2* mutations that are frequent in non-smoking, female patients of Asian origin. (*Cancer Sci* 2007; 98: 1747–1751)

Mutations of the *LKB1* gene (also known as *STK11*; locus 19p13.3) are regarded as a cause of Peutz–Jeghers syndrome (PJS),[1,2] because germline mutations of the *LKB1* gene are present in 50–70% of PJS families.[3–5] PJS is an autosomal dominantly inherited disorder, characterized by mucocutaneous hyperpigmentation and multiple benign gastrointestinal hamartomatous polyps. Patients with PJS have a 10–18-fold increased risk of cancer, most commonly those affecting the gastrointestinal tract as well as those of the pancreas, lung, breast, uterus, cervix, testis and ovary. The relative risk for all cancers in PJS patients aged 15–64 years is reported to be 15.2 and that for lung cancer is as high as 17.0.[6]

LKB1 is a tumor suppressor gene, encoding a protein of 436 amino acids with a serine–threonine kinase domain (residues 50–319),[2] similar to those of the sucrose non-fermenting (SNF)/adenosine monophosphate (AMP)-activated protein kinase family.[7] Overexpression of LKB1 leads to cell growth inhibition by G_1 cell-cycle arrest,[8] and is associated with Brahma protein homolog 1 and cellular tumor antigen p53.[9,10] Its involvement with the vascular endothelial growth factor signaling pathway and the phosphatase and tensin homolog (PTEN) pathway has also been demonstrated.(11,12) Somatic mutations of the LKB1 gene are relatively rare in sporadic cancers. For example, frequencies of somatic mutation were as follows: pancreatic cancer 4% (4/100); billary cancer 6% (1/16); hepatocellular carcinoma 1% (1/80); colon cancer 0% (0/20); and testicular tumor 4% (1/28).[13–15] The only exception seems to be lung adenocarcinomas. Sanchez-Cespedes *et al*. first reported that mutational inactivation of the *LKB1* gene is found in one-third of pulmonary adenocarcinomas in Caucasian patients.[16] However, the biological and clinical implications of *LKB1* gene alterations in Japanese patients with lung cancer have not been well established. Therefore, we decided to examine the *LKB1* gene in Japanese patients with lung cancer, although very recently Matsumoto *et al*. reported the prevalence and specificity of *LKB1* genetic alterations in Japanese lung cancer patients,[17] while we were preparing this manuscript.

Materials and Methods

Lung cancer cell lines. Twenty-two lung cancer cell lines (eight adenocarcinoma, six squamous cell carcinoma, three large-cell carcinoma and five small-cell carcinoma) were available for this study. Twelve lung cancer cell lines were of Japanese origin, and the others were of Caucasian origin (Table 1).

Tumor specimens. Primary lung tumors were collected from 100 unselected patients diagnosed with lung cancer. They had undergone potentially curative pulmonary resection at the Department of Thoracic Surgery, Aichi Cancer Center Hospital, between January 2001 and February 2002, after obtaining the appropriate approval from the institutional review board and the patients' written informed consent. None of the patients showed any clinical manifestations of PJS. All patients were Japanese; 59 were men and 41 were women, with an age at diagnosis ranging from 36 to 89 years (median 66 years). Fifty-seven patients had stage I disease, 13 had stage II, 27 had stage III, and three had stage IV. There were 81 adenocarcinomas, 14 squamous cell carcinomas, one adenosquamous carcinoma, two large-cell carcinomas, one small-cell carcinoma and one carcinoid. Thirty-nine patients had never smoked and 61 were current or former smokers. We had previously determined the epidermal growth factor receptor (*EGFR*), v-ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) and tumor protein p53 (*TP53*) mutations in this cohort.[18–20]

RNA and DNA extraction. Tumor samples were obtained at the time of surgery, frozen rapidly in liquid nitrogen and stored at –80°C. Frozen tumor tissue specimens were grossly dissected by a surgical pathologist (Y. Y.) to enrich the tumor cells as much as possible. Total RNA was isolated using the RNeasy Kit (Qiagen, Valencia, CA, USA) in 97 cases; extraction was

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Table 1. Characteristics of lung cancer cell lines and results of sequencing analysis

Histology	Cell line	Race	Mutation	Amino acid change
Adenocarcinoma	A549	Caucasian	109C→T	Q37Ter[†]
	H23	Caucasian	996G→A	W332Ter[†]
	SK-LU-1	Caucasian	Wild type	
	SK-LC-3	Caucasian	Wild type	
	VMRC-LCD	Japanese	–1G→T(IVS3)	Del 466–598 (133 bp) Stop codon at 286
	ACC-LC-94	Japanese	Wild type	
	ACC-LC-319	Japanese	Wild type	
	RERF-LC-MT	Japanese	Wild type	
Squamous cell carcinoma	Calu1	Caucasian	Wild type	
	SK-MES-1	Caucasian	Wild type	
	ACC-LC-176	Japanese	1062C→G	F354L[§]
	PC-1	Japanese	Wild type	
	PC-10	Japanese	Wild type	
	RERF-LC-AI	Japanese	Wild type	
Large-cell carcinoma	Calu6	Caucasian	Wild type	
	NCI-H460	Caucasian	109C→T	Q37Ter[‡]
	SK-LC-6	Caucasian	Wild type	
Small-cell carcinoma	SK-LC-2	Caucasian	Wild type	
	ACC-LC-48	Japanese	Wild type	
	ACC-LC-49	Japanese	Wild type	
	ACC-LC-80	Japanese	Wild type	
	ACC-LC-172	Japanese	Wild type	

Reported by [†]Sanchez *et al.*[16], [‡]Carretero *et al.*[21] and [§]Launonen *et al.*[22]

impossible in three cases (all three cases were males with adenocarcinomas). Genomic DNA was extracted using proteinase K.

Analysis of *LKB1* gene mutations. Genomic DNA (100 ng) was used for exon amplification. The genomic DNA sequence of the *LKB1* gene was obtained from GenBank (accession number NC000019). Polymerase chain reaction (PCR) of genomic DNA was carried out using AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) for exons 1, 2, 6, 7 and 8, and Platinum *Taq* Polymerase (Invitrogen, Carlsbad, CA, USA) and the PCR × Enhancer System (Invitrogen) for exons 3, 4–5 and 9. PCR primers were as follows: exon 1, 5′-AAATTTTGGAGA-AGGGAAGTCG-3′ (forward) and 5′-GGAGGAGAGAAGGA-AGGAAGAC-3′ (reverse); exon 2, 5′-TTCTCTCTAGGGAAGG-GAGGAG-3′ (forward) and 5′-ATTGCCACAATGGCTGACTT-3′ (reverse); exon 3, 5′-CTCCAGAGCCCCTTTTCTG-3′ (forward) and 5′-CAGTGTGGCCTCACGGAAAGGA-3′ (reverse); exons 4–5, 5′-CCTGGACTTCTGTGACTTCC-3′ (forward) and 5′-GAGTGTGCGTGTGGTGAGTG-3′ (reverse); exon 6, 5′-CTC-CTAGGGCGTCAACCAC-3′ (forward) and 5′-ACACCCCCA-ACCCTACATTT-3′ (reverse); exon 7, 5′-CTTAGGAGCGTCC-AGGTATCAC-3′ (forward) and 5′-CTCAACCAGCTGCCC-ACAT-3′ (reverse); exon 8, 5′-GAGCTGGGTCGGAAAACTG-3′ (forward) and 5′-AGAAGCTGTCCTTGTTGCAGA-3′ (reverse); and exon 9, 5′-CTGGGCAGCAGCTGTAAGT-3′ (forward) and 5′-TGACGGTCACCATGACTGACTA-3′ (reverse).

The PCR conditions were: exons 1, 2, 6, 7 and 8, one cycle of 95°C for 10 min, 35 cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 40 s, and one cycle of 72°C for 10 min; exons 3 and 9, one cycle of 95°C for 2 min, 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 40 s, and one cycle of 72°C for 2 min; and exons 4–5, one cycle of 95°C for 2 min, 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 50 s, and one cycle of 72°C for 2 min. PCR products were diluted and cycle sequenced using the BigDye Terminator Cycle Sequencing Kit v. 3.1/1.1 (Applied Biosystems). Sequencing reaction products were separated electrophoretically on an ABI PRISM 3100 apparatus (Applied Biosystems). Both the forward and reverse sequences obtained were analyzed with BLAST and by manual review. Mutations were confirmed with a second independent analysis.

For cell lines, we also analyzed total RNA. Total RNA (2 ng) was used for amplification. The cDNA sequence of the *LKB1* gene was obtained from GenBank (accession number NM000455). Exons 1–9 were divided into three sections and each section was amplified with the Qiagen OneStep RT-PCR Kit (Qiagen) and primers as follows: exons 1–3, 5′-AGTCGGAACACAAGGA-AGGAC-3′ (forward) and 5′-CTGGCTATGCAGGTACTCCAG-3′ (reverse); exons 4–7, 5′-GAGAAGCGTTTCCCAGTGTG-3′ (forward) and 5′-CTTCAGCCGGAGGATGTTT-3′ (reverse); and exons 8–9, 5′-GAAAGGGATGCTTGAGTACGAA-3′ (forward) and 5′-AACCGGCAGGAAGACTGAG-3′ (reverse). The reverse transcription (RT) conditions were one cycle of 50°C for 30 min, 95°C for 15 min, 40 cycles of 94°C for 40 s, 62°C for 40 s, 72°C for 1 min, and one cycle of 72°C for 10 min. Sequence analysis was carried out as for the DNA analysis.

Relative quantitation by real-time RT-PCR analysis. Expression levels of the *LKB1* gene were determined by quantitative real-time RT-PCR using the SYBR Green method (QuantiTect SYBR Green RT–PCR Kit; Qiagen) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Primer sequences were 5′-TCCATGCACTTTATGTGGAGAC-3′ (forward) and 5′-AAAAGAAAATGGAACCAACCAA-3′ (reverse). Quantitation was carried out in duplicate, and the expression levels of 18S ribosomal RNA were used as the internal control. The expression value for each sample is given as a relative value compared with that of the control sample (Calu6, large-cell carcinoma cell line).

Statistical analysis. The χ^2-test was used to compare proportions. The two-sided significance level was set at $P < 0.05$. Multivariate analysis for the association between *LKB1* expression levels and clinical and pathological factors were done using a logistic regression model. The Kaplan–Meier method was used to estimate the probability of survival, and survival differences were analyzed with the log-rank test. All analyses were carried out using StatView software (version 5; SAS Institute, Cary, NC, USA).

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Table 2. *LKB1* gene changes in lung cancers of Japanese patients

Sample number	Mutation	Amino acid change	Sex	Histology (differentiation)	pStage	Smoking status	*EGFR*	*TP53*	*KRAS*
1221†	125G→T	R42L	Male	AD (Mod)	IIB	Smoker	G719S	Wild type	Wild type
1097	842C→T	P281L	Male	AD (Mod)	IIIA	Smoker	Exon19 deletion	Wild type	Wild type
1188	842delC	Stop codon 286	Male	AD (Por)	IB	Smoker	Wild type	R248L	Wild type
1136	923G→T	W308L	Male	AD (Por)	IIIA	Smoker	Wild type	Wild type	G12S
1185†	1190C→T	A397V	Male	AD (Mod)	IIIB	Smoker	Wild type	Wild type	G12R
1129†	1276C→T	R426W	Male	AD (Mod)	IIB	Smoker	L858R	Wild type	Wild type

†Polymorphism. AD, adenocarcinoma; EGFR, epidermal growth factor receptor; Mod, moderately differentiated; Por, poorly differentiated.

Results

***LKB1* gene mutations in lung cancer cell lines.** We detected five *LKB1* gene alterations in 22 lung cancer cell lines (Table 1). Of them, mutations of three cell lines (A549, H460 and H23), have been reported previously, and we were able to confirm them.[16,21] We detected base substitution at the 3′ splice site of intron 3 (–1G→T) in VMRC-LCD, disrupting the splice acceptor consensus sequence. As a result, abnormal mRNA splicing was expected to occur and indeed, we confirmed by sequencing of cDNA that exon 3 was spliced directly to exon 5, skipping exon 4. Recently, this mutation was reported independently by Matsumoto *et al*.[17] Another alteration, F354L (Phe to Leu at codon 354), was reported to be a rare polymorphism (0.3% in Finnish colorectal cancer patients, and 6.3% in Korean colorectal cancer patients).[22] In summary, *LKB1* mutations were found in 18% (4/22) of lung cancer cell lines, and in 38% (3/8) of adenocarcinoma cell lines. All cell lines harboring mutations but one (VMRC-LCD) were of Caucasian origin. Additionally, we also sequenced cDNA confirming all of the mutations detected by the genomic DNA sequencing.

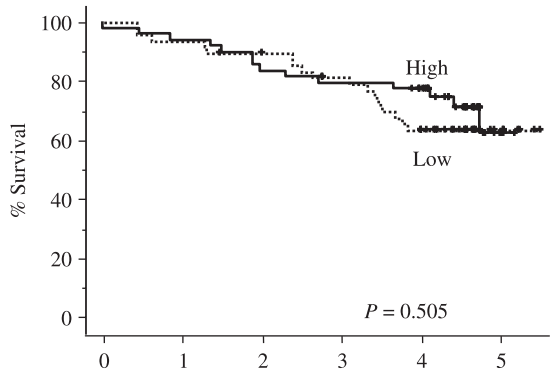
***LKB1* gene mutations in resected lung cancer specimens.** We detected six alterations in 100 lung cancer specimens (Table 2). All six alterations were confirmed by the second independent PCR. Among them, one was a deletion mutation (842delC) and five were base substitutions resultiong in amino acid changes (Arg to Leu at codon 42 [R42L], Pro to Leu at codon 281 [P281L], Trp to Leu at codon 308 [W308L], Ala to Val at codon 397 [A397V], and Arg to Trp at codon 426 [R426W]). DNA from matched normal lung tissues of these six patients was investigated to verify whether the detected alteration was a somatic or germline change. In four patients, the same alterations (R42L, P281L, A397V and R426W) that were found in tumor DNA were present in the corresponding normal tissues. All of these alterations except P281L lay outside the kinase domain and were regarded as polymorphisms. However, because P281L is located within the kinase domain and has been reported as a somatic mutation in several cancer types,[14,23,24] it was not possible to simply conclude that P281L was also a polymorphism. Rather, the presence of P281L in adjacent lung tissue raises the possibility of a somatic mutation occurring in normal-appearing lung tissue as a result of field cancerization. However, it was not possible to test this hypothesis because this patient had died. At this time, we considered P281L as a somatic mutation. In summary, we identified three mutations (P281L, 842delC and W308L) (3%), one of which, W308L, was novel. All patients with mutated *LKB1* genes were male smokers with adenocarcinomas. The incidence of the *LKB1* gene mutations in male smokers with adenocarcinoma was 9% (3/33). Of the three mutated cases, one had stage IB disease and two had stage IIIA. One was moderately differentiated and the other two were poorly differentiated adenocarcinomas. Each of the three patients had either mutation of the *EGFR*, *TP53* or *KRAS* gene (Table 2).

Table 3. Characteristic clinicopathological factors according to level of *LKB1* gene expression

Characteristic	Low		High		*P* (univariate)	*P* (multivariate)
	n	%	*n*	%		
No. patients	49		48			
Sex						
Male	32	56	25	44	0.186	0.827
Female	17	43	23	57		
Age						
≤66 years	24	49	25	51	0.760	0.945
>66 years	25	52	23	48		
Smoking status						
Non-smoker	16	42	22	58	0.184	0.947
Smoker	33	56	26	44		
pStage						
I	26	46	30	54	0.347	0.566
II–IV	23	56	18	44		
Histology						
AD	36	46	42	54	0.082	0.476
Non-AD	13	68	6	32		
LKB1						
Mutated	1	33	2	67	0.617	0.573
Wild type	48	51	46	49		
EGFR						
Mutated	14	37	24	63	0.044	0.201
Wild type	33	58	24	42		
TP53						
Mutated	22	58	16	42	0.243	0.607
Wild type	27	46	32	54		
KRAS						
Mutated	4	50	4	50	0.976	0.959
Wild type	45	51	44	49		

The patients were divided into two groups (low or high) by median value for *LKB1* gene expression. AD, adenocarcinoma; EGFR, epidermal growth factor receptor.

Expression analysis of the *LKB1* gene. Quantitative real-time RT-PCR analysis was carried out with 97 primary lung cancer specimens, because total RNA was not available in three cases. The three patients were male with adenocarcinomas, and wild-type *LKB1*. *LKB1* mutations were not associated with the expression levels of the *LKB1* gene. When the patients were divided into two groups according to the median level of gene expression, *LKB1* expression levels were not associated with any clinicopathological factors. This was also the case when we divided patients by tertile, quartile or quintile level. *LKB1* expression was higher in tumors harboring *EGFR* mutations with borderline significance ($P = 0.044$) in univariate analysis but not in multivariate analysis (Table 3). Furthermore, expression of the *LKB1* gene did not affect the patient's survival after surgery (Fig. 1).



Number of patients at risk						
High expression	48	45	40	38	37	34
Low expression	49	46	44	40	32	32

Fig. 1. Effect of *LKB1* gene expression on the survival of patients with lung cancer, calculated from the day of surgery.

Discussion

We identified four *LKB1* gene mutations in 22 lung cancer cell lines. When confined to adenocarcinoma, the incidence of *LKB1* mutations was 38%. This incidence was comparable to those reported by Carretero *et al*.[21] and Matsumoto *et al*.[17] They detected *LKB1* mutations in 54% (6/11) and 42% (13/31) of lung adenocarcinoma cell lines, respectively. However, *LKB1* mutations do occur in histological types of lung cancer other than adenocarcinomas, albeit to a lower incidence. Indeed, one of four cell lines with *LKB1* mutation was a large-cell carcinoma. This is consistent with a previous report showing that *LKB1* mutations are present in all histological types of lung cancer, including small-cell lung cancer.[17]

We found only three *LKB1* mutations in 100 lung cancer specimens. All of them were adenocarcinomas of male smokers. Hence, the incidence of *LKB1* mutation was 4% (3/81) in adenocarcinomas and 9% (3/33) in male smokers with adenocarcinoma. This is in very good agreement with the recent report by Matsumoto *et al*. They reported that *LKB1* mutations were found in seven of 91 (8%) male smokers but in none of 64 females and non-smokers with adenocarcinomas.[17]

Matsumoto *et al*. showed that large deletions in the *LKB1* gene were detected in 11 of 70 (16%) lung cancer cell lines, but not in resected lung cancer specimens. They concluded that the mutational frequency might be underestimated in resected specimens because of contaminating normal cells.[17] Although we could not detect large deletions even in our cell lines in the present study, underestimation of the incidence of *LKB1* mutations is possible.

It was originally reported that one-third of primary lung adenocarcinomas harbor *LKB1* mutations.[16,25] Compared to the present report, the incidence of *LKB1* mutations in Japanese patients appears lower. This contrast is analogous to a difference in the incidence of *KRAS* mutations between Japanese patients and Caucasian patients. *KRAS* mutations are also known to be more frequent in smokers.[26] In our previous work, *KRAS* mutations were found in 13% of Japanese adenocarcinomas,[18] whereas they were present in 33% of Dutch cases.[27] In contrast, it is now well established that the incidence of *EGFR* or *HER*2 mutations is much higher in East-Asian patients with adenocarcinoma than in those of Caucasian origin.[28] These differences were at least partially due to differences in smoking status between Japanese lung cancer patients and Caucasian patients. In our cohort, 83% of female patients and 10% of male patients were never-smokers,[18] whereas only 15% of US female and 6% male patients with lung cancer were never-smokers.[29]

There is a lot of evidence to show that the *LKB1*, *TP53* and *EGFR–KRAS* pathways interact with each other. For example, PTEN is required for a response to EGFR kinase inhibitors,[30–32] and germline mutation of the *PTEN* gene is a cause of Cowden's disease, another hamartomatous polyposis syndrome like PJS. It is also known that mutations of the *TP53* gene are rare in PJS carcinoma, indicating that loss of *LKB1* bypasses the need to mutate *TP53* in PJS tumor development.[33] However, in lung cancer, there appeared to be no interrelationship between *LKB1* mutations and other gene mutations. Although we only found three mutations in our resected lung cancer specimens, each of them had either *KRAS*, *EGFR* or *TP53* mutation. In agreement with this observation, it is reported that there was no significant relationship between *LKB1* mutations and *KRAS* or *TP53*,[16] or between *LKB1* mutations and *KRAS*, *EGFR* or *TP53*.[17] This is in contrast with the fact that *KRAS*, *EGFR* and *HER2* mutations have a mutually exclusive relationship.[28] Tumors with *LKB1* mutation tended to be poorly differentiated adenocarcinoma. Two of the three tumors in our study, and six of the seven tumors in Matsumoto's study with *LKB1* mutation were poorly differentiated adenocarcinomas.[17] Shen *et al*. have reported that low expression of LKB1 protein is associated with worse overall survival in patients with breast cancer.[34] However, low-level expression of *LKB1* did not appear to affect patient survival in our study.

In conclusion, *LKB1* gene mutations were relatively rare in Japanese patients with lung cancer compared with Caucasian patients. *LKB1* mutations appear to be frequent in male, smoking patients of Caucasian origin, in contrast to *EGFR* or *HER2* mutations that are frequent in non-smoking, female patients of Asian origin.

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