Prevalence and specificity of *LKB1* genetic alterations in lung cancers

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Abstract

Germline *LKB1* mutations cause Peutz–Jeghers syndrome, a hereditary disorder that predisposes to gastrointestinal hamartomatous polyposis and several types of malignant tumors. Somatic *LKB1* alterations are rare in sporadic cancers, however, a few reports showed the presence of somatic alterations in a considerable fraction of lung cancers. To determine the prevalence and the specificity of *LKB1* alterations in lung cancers, we examined a large number of lung cancer cell lines and lung adenocarcinoma (AdC) specimens for the alterations. *LKB1* genetic alterations were frequently detected in the cell lines (21/70, 30%), especially in non-small cell lung cancers (NSCLCs) (20/51, 39%), and were significantly more frequent in cell lines with *KRAS* mutations. Point mutations were detected only in AdCs and large cell carcinomas, whereas homozygous deletions were detected in all histological types of lung cancer. Among lung AdC specimens, *LKB1* mutations were found in seven (8%) of 91 male smokers but in none of 64 females and/or nonsmokers, and were significantly more frequent in poorly differentiated tumors. The difference in the frequency of *LKB1* alterations between cell lines and tumor specimens was likely to be owing to masking of deletions by the contamination of noncancerous cells in the tumor specimens. These results indicate that somatic *LKB1* genetic alterations preferentially occur in a subset of poorly differentiated lung AdCs that appear to correlate with smoking males.

Keywords

LKB1; lung cancer mutation; deletion; smoking; poor differentiation

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Introduction

Germline mutations of the *LKB1* gene (also known as *STK11*) cause Peutz–Jeghers syndrome (PJS), an autosomal dominant disorder characterized by gastrointestinal hamartomatous polyposis and mucocutaneous melanin pigmentation (Hemminki *et al.*, 1998; Jenne *et al.*, 1998). PJS patients also have an increased risk for intestinal and extraintestinal malignancies, including breast, ovarian, pancreatic and lung cancers (Hemminki, 1999; Westerman *et al.*, 1999). The majority of germline *LKB1* mutations result in truncation of the protein, leading to its dysfunction, thus, the *LKB1* gene has been considered to act as a tumor suppressor in PJS. The *LKB1* gene is implicated in the regulation of multiple biological processes, including cell-cycle arrest, p53-mediated apoptosis and induction of cell polarity (Baas *et al.*, 2004; Alessi *et al.*, 2006). Therefore, LKB1 has been also predicted to have a potential of a tumor suppressor in sporadic tumors. However, somatic *LKB1* gene alterations has been identified in a small fraction of those tumors, such as malignant melanomas, pancreatic and breast cancers (Bignell *et al.*, 1998; Avizienyte *et al.*, 1999; Guldberg *et al.*, 1999; Su *et al.*, 1999). Recently, it was reported that half of lung adenocarcinoma (AdC) cell lines and one-third of primary lung AdCs harbor somatic *LKB1* gene alterations, whereas other histological types of lung cancer, including squamous cell carcinoma (SqC) and small cell carcinoma (SCLC) do not (Sanchez-Cespedes *et al.*, 2002; Carretero *et al.*, 2004). A more recent study showed that large cell carcinoma (LCC) cell lines also had *LKB1* genetic alterations (Zhong *et al.*, 2006). Given the results from these studies, somatic *LKB1* gene alterations might occur preferentially in lung AdCs and LCCs. However, the pathogenic significance of *LKB1* genetic alterations in lung cancers is not well understood. Thus, we investigated a larger number of lung cancer cell lines as well as primary and metastatic lung AdCs for the genetic alterations. We further analysed the correlation of the genetic alterations with clinicopathological characteristics of patients with lung AdC.

Results

Genetic alterations of the *LKB1* gene in lung cancer cell lines

We first examined 70 lung cancer cell lines for *LKB1* genetic alterations. The cell lines consisted of 31 AdCs, 2 adenosquamous carcinomas (AdSqCs), 11 SqCs, 7 LCCs and 19 SCLCs. Exons 1–9 covering the entire coding region and exon–intron boundaries of the *LKB1* gene were analysed for mutations and deletions/insertions by genomic polymerase chain reaction (PCR) and direct sequencing. Homozygous deletions of regions upstream of the *LKB1* gene were also examined. Point mutations and homozygous deletions were detected in nine and 13 cell lines, respectively. One cell line, Ma29, had both a point mutation and a homozygous deletion. Thus, in total, 21 of the 70 cell lines (30%) showed genetic alterations of the *LKB1* gene (Table 1). Point mutations were detected only in AdCs and LCCs, whereas homozygous deletions were detected in all histological types of lung cancer.

The types of genetic alterations are summarized in Table 2. Point mutations detected in PC7, Ma29 and PC13 were missense mutations, and those in A549, H23 and Ma25 were nonsense mutations. HCC44 had a point mutation in combination with a 1-bp insertion, resulting in a missense mutation coupled with a frame-shift mutation. The remaining two-point mutations, detected in VMRC-LCD and HCC515, respectively, were splice site mutations. In all the cell lines with point mutations, only the sequences for mutant allele were detected and those for the wild-type allele were not detected. The sequence analysis also revealed 1-bp homozygous deletions leading frameshift mutations in two other AdC cell lines, H1395 and H2122. Large homozygous deletions, excluding the 1-bp deletions, were detected in 11 cell lines (Figure 1). In seven (Ma29, A427, 11–18, HCC366, SQ5, HCC15 and H1184) of the

11 cell lines, the deleted regions commonly included exon 1 and/or a 0.5 kb upstream region, which was considered as a promoter region of the *LKB1* gene (Hearle *et al.*, 2005). The remaining four cell lines, Ma24, H2126, Lu65 and H157, had intragenic deletions. In Lu65, exon 5 was not amplified and exon 4 was amplified with a shorter size. Sequencing of the shorter-sized product revealed that Lu65 had a 93-bp deletion from exon 4 to exon 5.

Altered *LKB1* expression in lung cancer cell lines

To evaluate the expression status of the *LKB1* gene in lung cancer cell lines, reverse transcription–PCR (RT–PCR) analysis was performed in all 70-lung cancer cell lines. Normal-sized and shorter-sized RT–PCR products were found in 53 (76%) and eight cell lines (11%), respectively. The remaining nine cell lines (13%) showed the absence of the products, implying no or decreased expression of LKB1 mRNA (Figure 2). The nine cell lines without RT–PCR products included the seven cell lines, in which exon 1 and/or a 0.5 kb upstream region of the *LKB1* gene were homozygously deleted, suggesting that the L*KB1* gene is not expressed owing to the lack of promoter sequence in these cell lines (Table1 and Figure 1). Ma17 and HCC78 also showed the absence of RT–PCR products, although their genetic alterations were unknown.

To confirm genetic alterations and to investigate causative genetic changes for the expression of mutated and truncated transcripts, the normal-sized and shorter-sized RT–PCR products were sequenced. The intraexonal point mutations in seven cell lines and the 1-bp deletions in two cell lines were all confirmed in this analysis. In two cell lines with splice site mutations, abnormal mRNA splicing was observed. In VMRC-LCD carrying a point mutation at the 3′ splice site of intron 3, exon 3 was spliced directly to exon 5 skipping exon 4. In HCC515 carrying a point mutation at the 5′ splice site of intron 5, exon 5 was spliced to exon 6 skipping the terminal 29 bp in exon 5. Both the aberrantly spliced transcripts led to frameshifts, thus, were expected to synthesize truncated proteins. In four cell lines with intragenic homozygous deletions, interstitially deleted sequences were also shown in cDNA. In Ma24, in agreement with a deletion of exons 2–7 in genomic DNA, cDNA also lacked the sequence for these exons. In H2126, RT–PCR products were also shorter and lacked the sequence for exons 4–6. The abnormal cDNA sequence in H2126 defined in this study was identical to that in a previous report (Carretero *et al.*, 2004). In Lu65, the breakpoint for deletion in genomic DNA was consistent with that in cDNA. In H157, cDNA lacked the sequence for exons 2 and 3, whereas only exon 2 was homozygously deleted in genomic DNA. The sequence for exons 2 and 3 was also deleted in H322 and H1437, although no genetic alteration was detected in these cell lines. This interstitial deletion was in-frame, thus, was expected to synthesis a small protein.

Deletion mapping of the *LKB1* gene in lung cancer cell lines

To more precisely determine deleted regions in the *LKB1* locus, large deletions detected in 11 cell lines were mapped at a 1 ~ 3-kb resolution by genomic PCR (Figure 3a). Most deletions, excluding a deletion in Ma29, were located in the 45-kb region between 29-kb upstream and intron 7 of the *LKB1* gene. The region 0.5–2.5-kb upstream of the *LKB1* gene was deleted in seven of the 11 cell lines, suggesting that this is a common region of deletions in lung cancer.

To elucidate the molecular process of interstitial deletions in the *LKB1* locus, DNA sequences of break-points for such deletions were determined. DNA fragments including breakpoint junctions were amplified by genomic PCR using primers recognizing regions flanking the breakpoints. DNA sequences of breakpoint junctions were able to be determined in A427, Ma24, H2126 and H157 in addition to Lu65 (Figure 3b). Of the five junctions, 2- or 3-bp nucleotides overlapped at three junctions. At two other junctions,

DNAs were directly joined or were joined with insertion of two nucleotides. There were no junctions joined by homologous recombination utilizing repetitive sequences.

Both a point mutation in exon 6 and a homozygous deletion of exon 1 and an upstream sequence were detected in the Ma29 cell line. No sequence for the wild-type allele was detected in this cell line. Thus, it was deduced that both a point mutation in exon 6 and an interstitial deletion including exon 1 had occurred in one allele, and the other allele was deleted by a larger deletion including exon 6 and this interstitial deletion. As no LKB1 mRNA was detected in Ma29, the biological significance of the mutation was unclear. In five other cell lines with homozygous deletions, breakpoint junctions were successfully determined. Thus, it was revealed that interstitial deletions had occurred in one allele and the other allele was lost by larger deletions including their respective interstitial deletions. In the remaining six cell lines with homozygous deletions, the nature of homozygous deletions was unclear.

Genetic alterations of the *LKB1* gene in primary and metastatic lung AdCs

We further examined primary and metastatic lung AdC cases for the genetic alterations (Table 3). First, 106 stage I primary lung AdCs and 25 brain metastases were examined. In stage I tumors, only one tumor (1%) had a mutation, a missense mutation coupled with a frameshift mutation owing to 1-bp insertion in exon 1. On the other hand, *LKB1* mutations were detected in three (12%) of the 25 metastases; two nonsense mutations in exons 4 and 6, respectively, and one frameshift mutation owing to a 1-bp deletion in exon 6. Next, an additional set of primary lung AdCs, for which both RNAs and genomic DNAs were available for analysis, was examined for interstitial deletions as well as mutations in the *LKB1* gene. In 24 cases, a cDNA fragment covering the entire coding region of LKB1 was amplified by nested RT–PCR and directly sequenced. If mutations were detected in RT–PCR products, the presence of identical mutations in genomic DNA was confirmed by genomic PCR and direct sequencing. The cases consisted of 13 cases pathologically diagnosed as stage I, 5 as stage II and 6 as stage III. LKB1 mutations were detected in three (13%) of the 24 cases. These three cases (100T, 170T and 171T) were pathologically diagnosed as stages IB, IA and IIB, respectively. In case 100T, a lack of the terminal 151-bp in exon 1 was shown in RT–PCR products, and the presence of a point mutation at the 5′ splice-donor site of intron 1 was revealed by genomic DNA sequencing. Therefore, the lack of the terminal 151-bp in exon 1 was concluded to be a consequence of abnormal mRNA splicing owing to a splice donor site mutation. Case 170T showed three base substitutions coupled with a 1-bp insertion leading a frameshift, and case 171T had a nonsense mutation identical to that detected in a lung cancer cell line, A549. As interstitial deletions in the *LKB1* gene were not identified by RT– PCR analysis in the tumor specimens, long-range PCR analysis was performed on genomic DNA to explore large deletions in the *LKB1* gene in these 24 AdCs. However, no deletions were identified in the tumors. All of the nucleotide changes detected in tumor specimens were confirmed to be somatic mutations by sequencing DNA from the corresponding noncancerous tissues.

Correlation of *LKB1* mutations with clinicopathological characteristics of patients with lung AdC

We further analysed the correlation of *LKB1* mutations with several clinicopathological characteristics of patients with lung AdC. In particular, information on gender, age, smoking history and differentiation of tumors was available in all cases subjected to *LKB1* mutation analysis. Thus, an association study of *LKB1* mutations with these variables was performed among 155 AdC cases (Table 4). *LKB1* mutations were present only in males and smokers, and not in females and nonsmokers. Moreover, *LKB1* mutations were significantly more frequent in poorly differentiated AdCs than in well or moderately differentiated ones.

Correlation of *LKB1* genetic alterations with *EGFR, KRAS* or *p53* mutations

We also analysed the correlation of *LKB1* genetic alterations with epidermal growth factor receptor (*EGFR*), *KRAS* and *p53* mutations in 70 lung cancer cell lines. Types of *EGFR, KRAS* and *p53* mutations as well as those of *LKB1* genetic alterations detected in these cell lines are summarized in Supplementary Information (Supplementary Table S1). *EGFR* mutations and *KRAS* mutations showed mutually exclusive correlations, as reported (Kosaka *et al.*, 2004). *LKB1* genetic alterations were significantly more frequent in cell lines with *KRAS* mutations (8/12, 67%) than those without (13/58, 22%), whereas there was no significant correlation or inverse correlation between *LKB1* genetic alterations and *EGFR* or *p53* mutations (Table 5). A correlation between *LKB1* and *KRAS* alterations was also significant, when the analysis was limited to the 31 AdC cell lines only. *LKB1* genetic alterations were detected in six of eight (75%) AdC cell lines with *KRAS* mutations but only in seven of 23 (30%) AdC cell lines without ($P = 0.04$). We also examined all 155 AdC cases for *EGFR* and *KRAS* mutations, and the 106 stage I AdCs and the 25 brain metastases for *p53* mutations. Among the seven cases with *LKB1* mutations, one case had both a *KRAS* mutation and a *p53* mutation, and two other cases had *p53* mutations. *EGFR* mutations were not detected in any of the seven cases. However, no significant correlation or inverse correlation between *LKB1* mutations and the other gene mutations was observed in these AdC cases (Table 4).

Discussion

In the present study, 70 lung cancer cell lines and 155 lung AdC cases were examined for *LKB1* genetic alterations. Of the lung cancer cell lines examined, *LKB1* genetic alterations were detected in 21 cell lines (30%). The alterations were more frequently detected in NSCLCs (20/51, 39%) than in SCLCs (1/19, 5%). The high incidence of *LKB1* genetic alterations in NSCLC cell lines was previously reported by Carretero *et al.* (2004). They reported the presence of *LKB1* alterations only in AdCs among various histological types of lung cancer cell lines. Another study showed that two LCC cell lines, H157 and H460, had *LKB1* genetic alterations (Zhong *et al.*, 2006), although H157 was histologically classified as a SqC in this study, and H460 as an AdC in Carretero *et al.* (2004). The present study revealed that point mutations commonly occur in AdC and LCC, whereas homozygous deletions occur not only in AdC and LCC but also in other histological types, such as SqC and SCLC. Thus, this is the first report, to our knowledge, demonstrating the presence of *LKB1* genetic alterations in SqC and SCLC. Among 21 lung cancer cell lines with the genetic alterations, missense mutations were detected only in three cell lines. As, in other sporadic cancers, missense mutations of the gene were reported to be relatively frequent compared with nonsense mutations or insertions/deletions (Launonen, 2005), the high frequencies of nonsense mutations and insertions/deletions are considered distinctive of lung cancer. The difference in the types of *LKB1* genetic alteration, in addition to that in the frequencies of the alterations, between lung cancer and other sporadic cancers raises a possibility that *LKB1* alterations might be induced by environmental factors, such as smoking. This is supported by a previous report showing that allelic losses or gains of chromosome 19p, on which the *LKB1* gene maps, were more frequent in lung AdCs from smokers than those from nonsmokers (Sanchez-Cespedes *et al.*, 2001).

Mutations of the *LKB1* gene were also detected in tumor specimens of lung AdCs. The frequency of the genetic alterations was 1% of stage I cases (1/106), 13% of another stage IIII cases (3/24) and 12% of brain metastases (3/25). All of the genetic alterations detected were point mutations or a 1-bp deletion, and large deletions were not detected. The difference in the frequency of large deletions between tumor specimens and cell lines is likely to be owing to masking of large deletions by the contamination of noncancerous cells in the tumor specimens. Recently, *LKB1* deletions, which cannot be detected by

conventional methods, were reported to be detected by a highly sensitive method, called multiplex ligation-dependent probe amplification, in 14–16% of PJS patients (Hearle *et al.*, 2006; Volikos *et al.*, 2006). Thus, it is possible that such deletions may occur in a considerable fraction of primary lung AdCs. The frequencies of point mutations and 1-bp deletions were also lower in tumor specimens than in cell lines. As LKB1 plays a role in the regulation of cell cycle and apoptosis, cell lines might be preferentially established from *LKB1*-mutated lung cancer cells, which are likely to acquire proliferative and survival advantages *in vitro* by LKB1 inactivation.

In the present study, *LKB1* genetic alterations were detected in pathological stage I or II cases. Thus, the genetic alterations are not necessarily a late event during lung AdC progression. This result was consistent with a previous immunohistochemical study showing marked reduction of LKB1 protein in 10% of AAH, a precursor lesion of lung AdCs (Ghaffar *et al.*, 2003). As all of the *LKB1* genetic alterations detected in our analysis of tumor specimens were nonsense mutations or frameshift mutations, the expression of truncated LKB1 proteins was also predicted to occur relatively early in the development of lung AdCs. Furthermore, *LKB1* genetic alterations were detected only in males and smokers, indicating a correlation between the occurrence of genetic alterations and tobacco exposure. Additionally, *LKB1* genetic alterations were more frequently detected in poorly differentiated AdCs. Therefore, defects in differentiation of AdC cells with *LKB1* genetic alterations might be associated with the functional loss of LKB1 proteins in the cells. Consistent with our result, a recent study revealed that a lung cancer cell line, A427, with a large deletion in the *LKB1* gene, possessed a greater invasive potential compared with a cell line expressing the wild-type *LKB1* gene, and forced expression of wild-type LKB1 in A427 led to reduction of this potential (Upadhyay *et al.*, 2006).

Frequent alterations of the *LKB1* gene in lung cancer have made it possible for us to analyse the correlation of the alterations with the status of *p53, EGFR* and *KRAS* mutations. *LKB1* genetic alterations were significantly frequent in lung cancer cell lines with *KRAS* mutations. This correlation was particularly observed in lung AdC cell lines. As *KRAS* mutations are considered to be relevant to smoking, the frequent coexistence of *LKB1* genetic alterations and *KRAS* mutations supports our result of frequent *LKB1* genetic alterations in lung AdCs from smokers. In tumor specimens, a case with a *KRAS* mutation also had a *LKB1* mutation. However, there was no tumor carrying both *LKB1* genetic alterations and *EGFR* mutations. Because *EGFR* mutations occur frequently in lung AdCs of females and nonsmokers (Kosaka *et al.*, 2004; Shigematsu and Gazdar, 2006), lung AdCs with *LKB1* genetic alterations, all of which were detected in males and smokers, are likely to be a different subset from those with *EGFR* mutations. On the other hand, several studies revealed that LKB1 leads to cell growth inhibition owing to G1 cell-cycle arrest through a p53-dependent mechanism (Tiainen *et al.*, 2002), and to apoptosis through its physical interaction with p53 (Karuman *et al.*, 2001). Among 21 lung cancer cell lines and four lung AdC specimens with *LKB1* genetic alterations, 17 (81%) and three (75%) had *p53* mutations, respectively. The frequent coexistence of *LKB1* genetic alterations and *p53* mutations, as well as that of *LKB1* genetic alterations and *KRAS* mutations, are suggested to be involved in aggressiveness of lung cancer. Further analyses are needed to elucidate the pathogenic significance of the interaction of these genes in lung carcinogenesis.

We demonstrated here that *LKB1* genetic alterations occur frequently in lung cancers, especially in NSCLCs with *KRAS* mutations and with smoking histories, and are significantly associated with poorly differentiated phenotypes of lung AdCs. Our results indicate the involvement of somatic *LKB1* genetic alterations in the development of a subset of poorly differentiated lung AdCs that appear to correlate with smoking males.

Materials and methods

Cell lines

Seventy lung cancer cell lines, consisting of 51 NSCLCs and 19 SCLCs, were used as listed in Supplementary Table S1. The 51 NSCLCs consisted of 31 AdCs, two AdSqCs, 11 SqCs and seven LCCs. Additionally, in six AdCs (H1395, H1437, H2009, H2087, H2122 and H2347) and four SCCs (H209, H2107, H2141, and H2171), corresponding lymphoblast cell lines were available for analysis. Detailed information about H- and HCC-series cell lines have been described elsewhere (Burbee *et al.*, 2001). PC-, Lu- and Ma-series cell lines were provided by Drs Y Hayata (Tokyo Medical University, Tokyo, Japan), T Terasaki (Kanagawa Institute of Technology, Kanagawa, Japan) and S Hirohashi (National Cancer Center Research Institute, Tokyo, Japan) and M Takada (National Hospital Organization Kinkichuo Chest Medical Center, Osaka, Japan), respectively. Cell lines were also obtained from the American Type Culture Collection (Manassas, VA, USA), the Japanese Collection of Research Bioresources (Tokyo, Japan), and the RIKEN BioResource Center (Tsukuba, Japan). Genomic DNA was isolated as described previously (Kishimoto *et al.*, 2005), and polyadenylated RNA was extracted with a Fast Track mRNA isolation kit (Invitrogen, San Diego, CA, USA).

Tumor specimens

One hundred thirty primary tumors, 25 brain metastases and corresponding noncancerous tissues were obtained at surgery or at autopsy from lung AdC patients treated at the National Cancer Center Hospital, Tokyo, Japan. Primary tumors consisted of a set of 106 stage I primary tumors, for which genomic DNAs were available for analysis, and another set of 24 stage I–III primary tumors, for which both genomic DNAs and RNAs were available for analysis. All of the 130 primary tumors, 16 of the 25 brain metastases and corresponding noncancerous tissues were macrodissected and stored at −80°C until DNA or RNA extraction. Genomic DNAs were prepared described previously (Kishimoto *et al.*, 2005). Total RNA was extracted with an RNeasy kit (Qiagen, Valencia, CA, USA). The remaining nine brain metastases and corresponding noncancerous tissues were fixed with methanol and embedded in paraffin. Cancerous and noncancerous cells of these nine cases were microdissected using the Pixcell Laser Capture Microdissection system (Arcturus Engineering, Mountain View, CA, USA), and their genomic DNAs were extracted as described previously (Matsumoto *et al.*, 2006a).

Mutation and deletion analysis of the *LKB1* gene

PCR primers were designed to amplify each exon in the *LKB1* gene. Ten nanograms of DNA from the cell lines and the macrodissected materials were used for PCR amplification. For the microdissected materials, nested PCR was carried out after initial PCR using 100 pg of DNA. PCR products were cycle-sequenced using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3700 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). PCR primers were also set to examine the cell lines for homozygous deletions of upstream regions of the *LKB1* gene. Homozygous deletions were confirmed by multiplex PCR using the *IRF1* locus on chromosome arm 5q as a reference (Kishimoto *et al.*, 2005). PCR primer sequences and PCR conditions are available on request.

Expression analysis of the *LKB1* gene

Polyadenylated RNA or total RNA was reverse-transcribed to cDNA by using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). cDNA conversion mixtures were subjected to PCR amplification of the entire coding region of

LKB1 for 35 cycles. Primers used for the amplification were 5′-GGACTGACGTGTAGAACAAT-3′ in the noncoding region of exon 1 and 5′-CGTGGTCGGCACAGAAG CA-3′ in exon 10. PCR products were directly sequenced in both directions. The gene encoding glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was amplified to estimate the efficiency of cDNA synthesis.

Mutation analysis of the *EGFR, KRAS* and *p53* genes

All of the 106 stage I primary lung AdCs and 19 of the 25 brain metastases of lung AdC were previously examined for mutations of exons 18–21 in the *EGFR* gene and of exons 1 and 2 in the *KRAS* gene (Matsumoto *et al.*, 2006a, b). The remaining tumors and all the 70 cell lines were also examined for these mutations by genomic PCR and direct sequencing as described previously (Matsumoto *et al.*, 2006a). Mutations of exons 2–11 in the *p53* gene were previously analysed using the 106 stage I cases and 38 of the 70 cell lines (Fujita *et al.*, 1999; Tomizawa *et al.*, 2002). All of the 25 brain metastases and the remaining cell lines were examined for mutations of exons 4–8 in the *p53* gene by genomic PCR and direct sequencing as described previously (Park *et al.*, 2003).

Statistical analysis

Fisher's exact test was used to assess the association of *LKB1* genetic alterations with clinicopathological factors or mutations of the *EGFR, KRAS* and *p53* genes. A *P*-value of <0.05 was considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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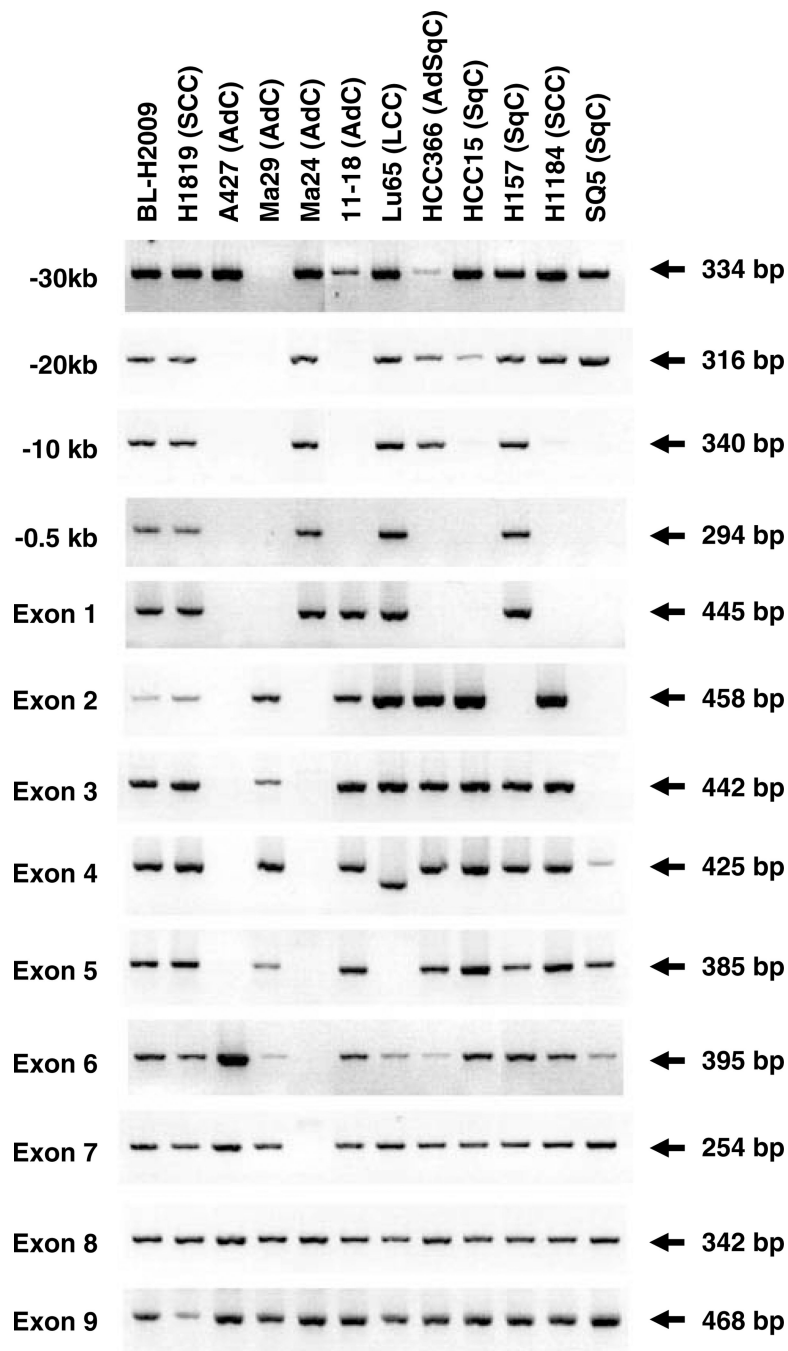
Figure 1.
Homozygous deletions of the *LKB1* gene in lung cancer cell lines. DNA fragments for exons and upstream regions of the *LKB1* gene amplified by genomic PCR were fractionated by a 3% agarose gel. Sizes of the fragments are shown on the right. A homozygous deletion in H2126, which was demonstrated in a previous report (Carretero *et al.*, 2004), is not shown.
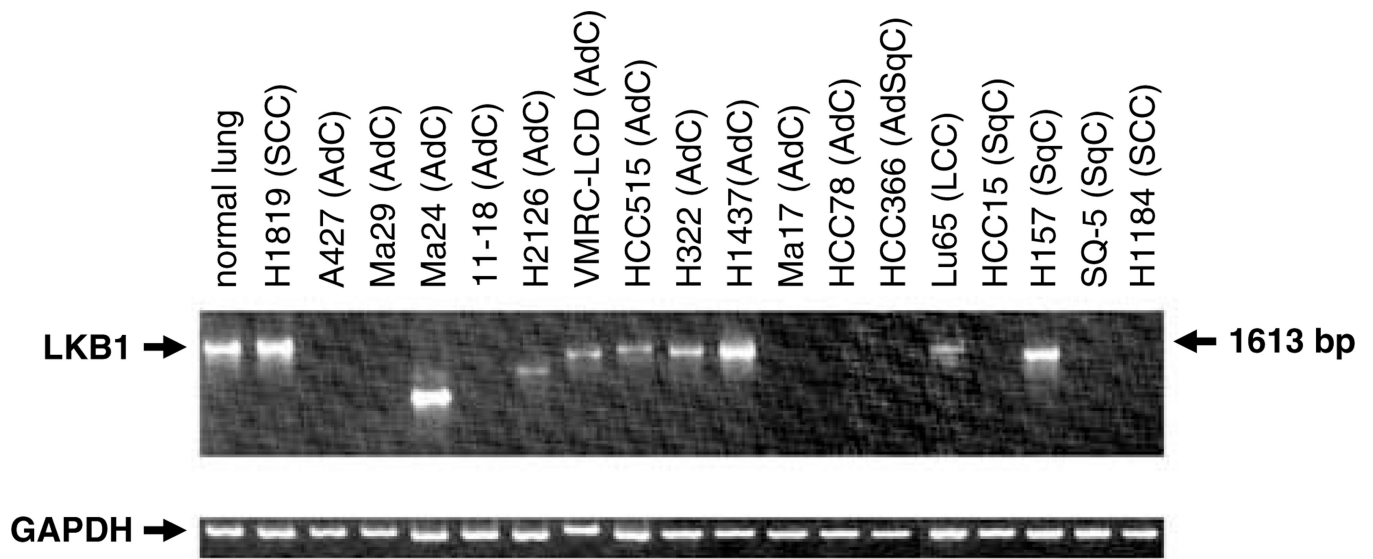
Figure 2.
Expression of the *LKB1* gene in lung cancer cell lines. RT–PCR was performed as described in Materials and methods. RT–PCR products were resolved on a 1% agarose gel. GAPDH amplification was used for a loading control.
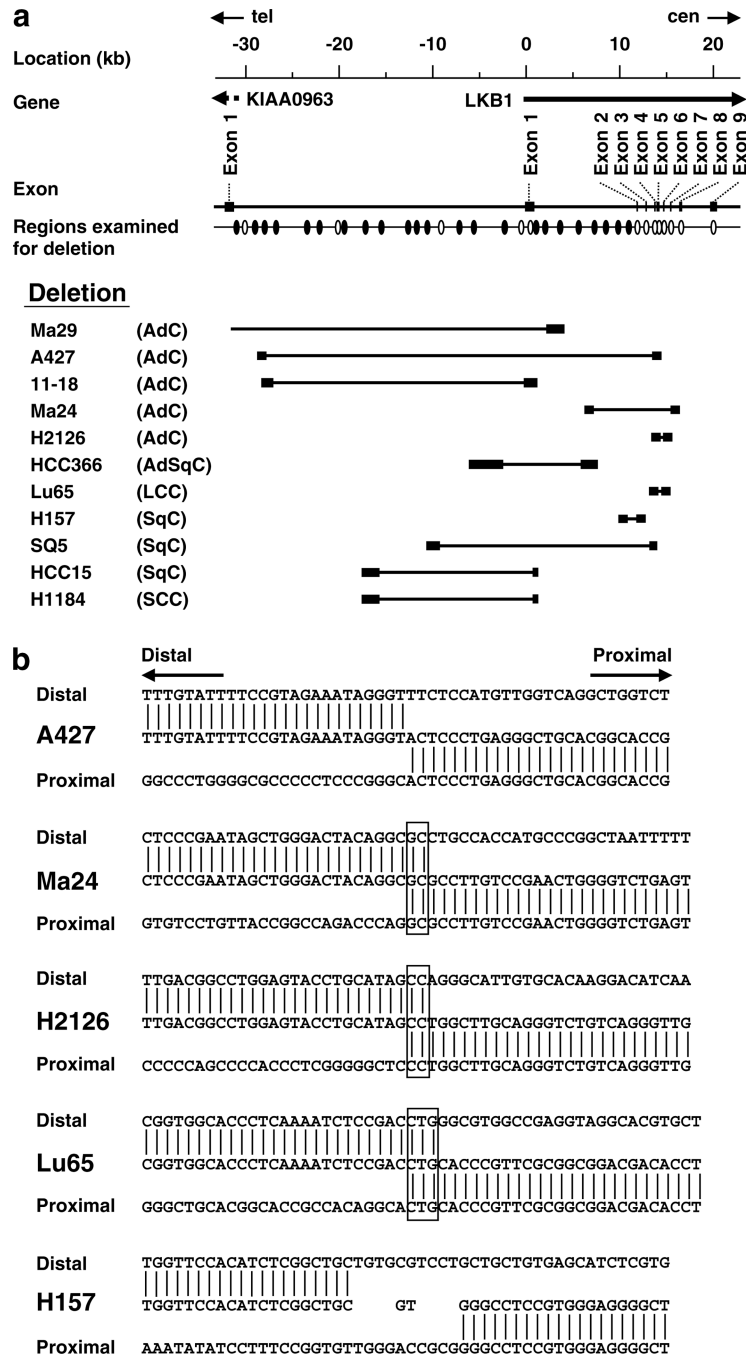
Figure 3.
(**a**) Deletion map of the *LKB1* locus. Regions analysed by genomic PCR are shown by ovals. Results of the PCR analysis for the regions indicated by open ovals are shown in Figure 1. Distal and proximal breakpoints for deletions are shown by closed boxes, respectively. The bar put between the closed boxes indicates the extent of deletion in each cell line. In Ma29, the distal breakpoint was undetermined. (**b**) DNA sequences of breakpoint junctions for deletions in or upstream of the *LKB1* gene. Junctions without insertion or overlap of nucleotides (A427), with overlaps of 2- or 3-bp nucleotides (Ma24,

H2126 and Lu65) and with insertion of 2-bp nucleotides (H157) are shown, respectively. Overlapped nucleotides are boxed.

Table 1

Frequencies of LKB1 genetic alterations in lung cancer cell lines

Histological type	Frequency (%)		
	Point mutation	Homozygous deletion	Total
AdC	7/31 (23)	7/31 (23)	13/31 (42)[a]
AdSqC	0/2 (0)	1/2 (50)	1/2 (50)
SqC	0/11 (0)	3/11 (27)	3/11 (27)
LCC	2/7 (29)	1/7 (14)	3/7 (43)
SCLC	0/19 (0)	1/19 (5)	1/19 (5)
Total	9/70 (13)	13/70 (19)	21/70 (30)

Abbreviations: AdC, adenocarcinoma; AdSqC, adenosquamous carcinomas; LCC, large cell carcinoma; SCLC, small cell carcinoma; SqC, squamous carcinoma.

[a]In cell line Ma29, both a point mutation and a homozygous deletion were detected.

Table 2

Table 2 of LKB1 alterations in lung cancer cell lines

Genetic alteration	Cell line	Histological type	Site/region	Nucleotide change	mRNA expression	Effect
Point mutation	A549	AdC	exon 1	109C > T	+	Q37 Ter
	HCC44	AdC	exon 1	153G > C + 1C ins.	+	M51I, 52 → 162 stop
	PC7	AdC	exon 6	747A > C + 749G > T	+	T250P
	Ma29[a]	AdC	exon 6	847C > G	−	L283V
	H23	AdC	exon 8	996G > A	+	W322 Ter
	VMRC-LCD	AdC	IVS 3	−1G > T	+	Y156-E199 del., 200 → 286 stop
	HCC515	AdC	IVS 5	+1G > T	+	V236-L245 del., 246 → 265 stop
	PC13	LCC	exon 5	668A > T	+	E223V
	Ma25	LCC	exon 6	782C > G	+	Y261 Ter
Deletion	H1395	AdC	exon 1	165–169 1G del.	+	56 → 63 stop
	H2122	AdC	exon 6	837–842 1C del.	+	279 → 286 stop
	Ma29[a]	AdC	~ exon 1		−	
	A427	AdC	−29 kb ~ exon 4	43 308 bp del.	−	
	11–18	AdC	−25 kb ~−0.5 kb		−	
	Ma24	AdC	exon 2 ~ exon 7	9058 bp del.	+	K97N, E98-S307 del.
	H2126	AdC	exon 4 ~ exon 6	1194 bp del.	+	Y156-G268 del., 269 → 271 stop
	HCC366	AdSqC	−2.5 kb ~ exon 1		−	
	Lu65	LCC	exon 4 ~ exon 5	93 bp del.	+	G196-L201 del.
	H157	SqC	exon 2	1498 bp del.	+	E98-G155 del.
	SQ5	SqC	−9kb ~ exon 3		−	
	HCC15	SqC	−15 kb ~ exon 1		−	
	H1184	SCLC	−15 kb ~ exon 1		−	
Unknown	H322	AdC			+	E98-G155 del.
	H1437	AdC			+	E98-G155 del.

[a]This cell line has both a point mutation and a large deletion.

Table 3

LKB1 gene alterations in primary and metastatic lung adCs

| Tumor | Material | Frequency (%) | | Sample (Stage) | Types of LKB1 gene alterations | | | |
		Total	Smokers		Exon or intron	Nucleotide change		Effect
Primary tumor								
Set 1 (Stage I)	DNA only	1/106 (1)	1/62 (2)	10–22 (IA)	exon 1	150G > T, 152T > A, 153–157 1Gins.		M51K, 53 → 162 stop
Set 2 (stage I–III)	DNA/RNA	3/24 (13)	3/10 (30)	100T (IB)	IVS 1	+1G > T		G47-K96 del., 97 → 102 stop
				170T (IA)	exon 1	150 1Tins., 152T > G, 156–157GG > TT		51 → 162 stop
				171T (IIB)	exon 1	109C > T		Q37Ter
Brain metastases	DNA only	3/25 (12)	3/19 (16)	N011T	exon 4	475C > T		Q159Ter
				N111T	exon 6	766G > T		E256Ter
				BM6	exon 6	837–842 1C del.		279 → 286 stop

Table 4

Correlations of *LKB1* genetic alterations with clinicopathological characteristics of patients with lung adC

Clinicopathological feature	Subset	Number	LKB1 mutation (%)		P-value[a]
			+	−	
Gender	Male	96	7 (7)	89 (93)	0.04
	Female	59	0 (0)	59 (100)	
Age	<62	73	3 (4)	70 (96)	>0.99
	62	82	4 (5)	78 (95)	
Smoking history	Smoker	91	7 (8)	84 (92)	0.04
	Nonsmoker	64	0 (0)	64 (100)	
Differentiation	Well	73	1 (1)	72 (99)	(Well *vs* mod.) > 0.99
	Moderately	48	0 (0)	48 (100)	(Well *vs* poorly) < 0.01
	Poorly	34	6 (18)	28 (82)	(Mod. *vs* poorly) < 0.01
EGFR mutation	+	54	0 (0)	54 (100)	0.10
	−	101	7 (7)	94 (93)	
KRAS mutation	+	15	1 (7)	14 (93)	0.52
	−	140	6 (4)	134 (96)	
p53 mutation	+	52	3 (6)	49 (94)	0.30
	−	79	1 (1)	78 (99)	

[a] Fisher's exact test.

Table 5

Correlations of *LKB1* genetic alterations with *EGFR*, *KRAS* or *p53* mutations in lung cancer cell lines

Gene	Mutation	Number	*LKB1* mutation/deletion (%)		P-value[a]
			+	−	
EGFR	+	5	2 (40)	3 (60)	0.63
	−	65	19 (29)	46 (71)	
KRAS	+	12	8 (67)	4 (33)	< 0.005
	−	58	13 (22)	45 (78)	
p53	+	56	17 (30)	39 (70)	> 0.99
	−	14	4 (29)	10 (71)	

Abbrevaition: EGFR, epidermal growth factor receptor

[a]Fisher's exact test.