**LKB1 Mutations Frequently Detected in Mucinous Bronchioloalveolar Carcinoma**

Atsushi Osoegawa, Takuro Kometani, Kaname Nosaki, Kaoru Ondo, Motoharu Hamatake, Fumihiko Hirai, Takashi Seto, Kenji Sugio* and Yukito Ichinose

Department of Thoracic Oncology, National Kyushu Cancer Center, Fukuoka, Japan

*For reprints and all correspondence: Kenji Sugio, Department of Thoracic Oncology, National Kyushu Cancer Center, Notame 3-1-1, Minami-ku, Fukuoka 811-1395, Japan. E-mail: sugio.k@nk-cc.go.jp

Received April 6, 2011; accepted June 18, 2011

**Objective:** LKB1 mutations are common in patients with Peutz–Jeghers syndrome, which is characterized by mucocutaneous pigmentation, intestinal polyps and a high incidence of cancers at variable sites. This study investigated the status of the LKB1 gene in mucinous bronchioloalveolar carcinoma with or without Peutz–Jeghers syndrome.

**Methods:** Three mucinous bronchioloalveolar carcinoma tumors from two Peutz–Jeghers syndrome patients and seven tumors from sporadic mucinous bronchioloalveolar carcinoma patients were collected by surgery between 2002 and 2008, and high molecular weight genomic DNA was extracted from them. The nucleotide sequences in exons 1–9 of LKB1 were determined by genomic polymerase chain reaction-direct sequencing. The loss of heterozygosity was analyzed by high-resolution fluorescent microsatellite analysis using two microsatellite markers that encompass the LKB1 locus, D19S886 and D19S565. The mutations of KRAS, EGFR and p53 were also evaluated.

**Results:** The germline mutation of LKB1 in the Peutz–Jeghers syndrome patients was identified as G215D by analyzing genomic DNA from normal lung tissue specimens. Furthermore, two of the three mucinous bronchioloalveolar carcinomas from these Peutz–Jeghers syndrome patients exhibited additional somatic mutations. On the other hand, four of seven sporadic ‘non-Peutz–Jeghers syndrome’ mucinous bronchioloalveolar carcinomas had LKB1 mutations. Loss of heterozygosity analyses revealed allelic loss in two tumors with LKB1 mutations. As a result, 70% of the mucinous bronchioloalveolar carcinomas exhibited LKB1 mutations. KRAS, EGFR and p53 mutations were mutually exclusive and observed in four, two and one tumors, respectively. Among them, five mutations occurred concomitantly with LKB1 mutations.

**Conclusions:** The relatively high frequency of LKB1 mutations in mucinous bronchioloalveolar carcinoma patients may therefore suggest its involvement in lung carcinogenesis, at least in mucinous bronchioloalveolar carcinoma.

**Key words:** tumor suppressor gene – hereditary disease – bronchioloalveolar carcinoma – loss of heterozygosity

---

**INTRODUCTION**

Liver kinase B1 (LKB1) is a gene encoding a serine–threonine kinase, which was initially deposited in the database by Nezu et al. in 1996, without writing a published report, in a screen aimed at identifying new kinases. In 1997, Hemminki et al. (1) revealed that the locus responsible for Peutz–Jeghers syndrome (PJS) was located on chromosome 19p13.3, where they then found the locus encoding LKB1 with diverse mutations in PJS families (2). Another group also reported mutations in the LKB1 gene...
and called this enzyme serine–threonine kinase 11 (STK11) (3).

PJS is a rare autosomal-dominant disease characterized by mucocutaneous pigmentation and gastrointestinal hamartomatous polyposis (4,5). Furthermore, patients with PJS have an increased cancer risk, especially for cancers of gastrointestinal origin (6). Tumors arising from PJS are characterized by mucinous phenotypes, as are often observed in gastrointestinal tumors [i.e. adenoma malignum in the cervix (7), pancreatic adenocarcinoma (8) and intraductal pancreatic mucinous neoplasms (9)]. LKB1 mutations are not as common in sporadic cancers except for non-small cell lung cancers (NSCLC) (10), about a third of which exhibit LKB1 mutations (11–13). The representative mucinous NSCLC is mucinous bronchioloalveolar carcinoma (mBAC). mBAC is relatively rare among NSCLC, and there is only one case report of a PJS patient with mBAC (14).

The LKB1 mutations in two PJS patients with mBAC were herein evaluated, along with sporadic cases of mBAC.

PATIENTS AND METHODS

PATIENTS

Seventeen patients with mBAC were identified from 512 consecutive Japanese lung adenocarcinomas that underwent surgery at the Department of Thoracic Oncology, National Kyushu Cancer Center, from 2002 to 2008. Frozen tissue specimens were collected from 10 mBACs out of nine patients (four male and five female; age range: 43–79 years; median: 68 years; Table 1): 3 mBACs were derived from two PJS patients, who belonged to the same family. Tumor 1 was from a woman and Tumors 2 and 3 were from her daughter. Tumors 4–10 were from sporadic mBAC patients. Written informed consent was obtained from all patients, and ethical approval was obtained from the institutional review board of the National Kyushu Cancer Center. The histology of mBAC was determined based on hematoxylin and eosin staining according to the criteria of the World Health Organization (15). High-molecular-weight genomic DNA was extracted from the surgically resected specimens by standard phenol–chloroform methods and then was stocked in the bio-bank at our institute.

MUTATION AND LOSS OF HETEROZYGOSITY ANALYSIS OF THE LKB1 GENE

PCR primers were designed to cover all of the exons in the LKB1 gene (Table 2). Twenty-five nanograms of genomic DNA from the bio-bank were used for each PCR amplification. The sequences of the primers are listed in Table 2. Direct sequencing of the PCR products was performed using the ABI Prism 310 Genetic Analyzer (Perkin-Elmer). All sequencing reactions were performed in both forward and reverse directions. Mutations were confirmed by an analysis of at least two independent PCR amplifications.

Loss of heterozygosity (LOH) was analyzed by a high-resolution fluorescent microsatellite analysis (HRFMA)
using two microsatellite markers (D19S886 and D19S565) that encompass the LKB1 locus. Specific primers were designed for these microsatellite markers (Table 2). Separation was done with a four-color laser-induced fluorescence capillary electrophoresis system (ABI Prism 310 Genetic Analyzer, Perkin-Elmer). The collected data were evaluated using the Genescan analysis software package 310 Genescan v. 3.1.2 (16).

** Mutation Analysis of the EGFR, KRAS and p53 Genes **

Genomic PCR-direct sequencing was performed for exons 18–21 of the epidermal growth factor receptor (EGFR) gene, for codons 12–13 of the KRAS gene and for exons 5–9 of the p53 gene (17,18). The detailed sequences of the primers are available on request.

** Results **

**LKB1 Mutations in PJS Patients**

The LKB1 mutation in PJS was confirmed by analyzing the genomic DNA from normal lung tissue specimens. Both patients, the mother and her daughter, were proven to possess the G251D mutation. The same mutation was observed in the three tumors derived from those patients. In addition, the mother’s tumor had E223L, and one of the daughter’s tumors had Y60X. Both of these two tumors are thought to have compound heterozygotes. LOH were not informative in these three tumors (Table 1).

**LKB1 Mutations in Sporadic mBACs**

Four of the seven sporadic mBACs had LKB1 mutations: F354L in two and D194H and 63-stop in one each. LOH analyses revealed definite allelic loss in Tumor 4 (LOH was positive for both D19S886 and D19S565) and possible allelic loss in Tumor 5 (LOH was positive for D19S565 but not informative for D19S886). These results therefore explain one distinct (Tumor 4) and another possible (Tumor 5) biallelic inactivation of LKB1 (Fig. 1). The other two sporadic cases exhibited neither LKB1 mutations nor LOHs. Finally, 70% of mBACs exhibited LKB1 mutations. An analysis of the sporadic cases, excluding the PJS cases, revealed that the percentage was still as high as 57%.

**Mutations in KRAS, EGFR and p53**

A KRAS mutation was observed in four tumors: G12D in two and G12C and G12V in one each. The deletion in exon 19 (del E746-A750) of EGFR was observed in two tumors. The p53 mutation (Y220C) was observed in one tumor. The mutations in KRAS, EGFR and p53 were mutually exclusive (Table 1). Commonly, LKB1 mutations co-existed with

![Figure 1](https://academic.oup.com/jjco/article-abstract/41/9/1132/839529/figure1)
The mutation spectra of from PJS patients, although the frequency of dic lung tumors; it was found in all of the tumors derived mutation in mBAC to be relatively high (4/7; 57%) in spora-

The current study demonstrated the frequency of MUTATION SPECTRA OF p53 mutation in Tumor 5.
familiar PJS cases; EGFR mutations in Tumors 4 and 6; and KRAS mutations in Tumors 1 and 2, which are derived from single-base insertion and D53T-63X was a single-base deletion.

KRAS mutations in Tumors 1 and 2, which are derived from familar PJS cases; EGFR mutations in Tumors 4 and 6; and p53 mutation in Tumor 5.

MUTATION SPECTRA OF LKB1 IN mBAC

The somatic mutations identified were one G:C to A:T transition, three G:C to C:G transversions, one single-nucleotide insertion and one single-nucleotide deletion (Fig. 2). None of the other mutations were observed.

The mutation spectra of KRAS and p53 are G:C to T:A in two tumors, G:C to A:T in two tumors and T:A to C:G in one tumor. Both EGFR mutations were deletions.

DISCUSSION

The current study demonstrated the frequency of LKB1 mutation in mBAC to be relatively high (4/7; 57%) in sporadic lung tumors; it was found in all of the tumors derived from PJS patients, although the frequency of LKB1 mutation is reported to be 4–5% among lung cancers in Japan (19,20). LKB1 mutations in lung cancer were related with male sex, smoking history and KRAS mutations (10,13,19,20). No significance of these parameters was observed in the current series, other than the relationship between LKB1 mutation and mBAC. The mutations observed here have been reported in PJS patients, which inactivate kinase activity or impair farnesylation at the C-terminus (21,22).

Biallelic inactivation resulted from LKB1 mutations with LOHs or from compound heterozygotes recognized in PJS cases account for the importance of LKB1 mutation in mBAC tumorigenesis. LKB1 is a tumor suppressor gene that causes G1 arrest when overexpressed (23). Further studies have shown that LKB1 acts as a master kinase controlling cellular polarity via MAP/microtubule affinity-regulating kinase, energy metabolism and the mammary target of the rapamycin (mTOR) pathway via AMP-activated protein kinase (AMPK) (24). Enterocytes with wild-type LKB1 differentiate and gain polarity once attached to the basal layer or become confluent; nevertheless, mutant LKB1 cannot gain polarity (25).

LKB1’s role in polarity control was also confirmed from in vivo experiments by Shorning et al. (26). They also suggested the existence of possible relationships between LKB1 inactivation, polarity deregulation and excessive mucin production. They constructed a conditional knockout mouse model of LKB1. The epithelial cells of the mouse small intestine demonstrated an increased size and number of mucin-containing, goblet-cell-like cells when LKB1 was inactivated in the small intestines of mice by intraperitoneal injection of β-naphthoflavone. These undifferentiated cells showed features that were somewhere intermediate between Paneth and goblet cells (26). They concluded that these phenomena could explain the pathological aspect of polyp development in PJS patients, because alterations in goblet cells and elevated mucin production are commonly observed in hamartomas developing in PJS patients (27). Pathologically, similar features are observed in mBACs. mBAC cells are characterized by goblet cell dysplasia of the lining surface cells in the terminal respiratory unit, with excessive mucin production in the bronchioli and alveoli.

‘Pure mucinous’ colloid carcinomas, in which the immunohistochemical stains for luminal surface glycoproteins have shown inverted polarity, allow the colloid carcinoma cells to secrete mucin towards the stroma (28). It is therefore possible that such an LKB1 alteration would result in the deregulated polarity of mucin-producing cells, thereby leading to an overproduction of mucin, and an impaired differentiation of goblet cells, thus leading to uncontrolled proliferation. Further investigations are warranted to elucidate these hypotheses.

Homozygous Lkb1-deficient mice are lethal at midgestation by defects in the neural tube, mesenchymal cell death and vascular abnormalities (29). Heterozygous mice die of gastric polyps before forming carcinomas (30–32). However, heterozygous mice with other conditional mutants, such as Kras (12), p53 (33) or Pten (34), show a malignant potential once one of those switches has either been turned on or off. Several oncogene mutations were identified among the mBACs with LKB1 mutations. LKB1 loss could therefore be oncogenic even under heterozygous inactivation with other oncogenic mutations.

Several drugs have been tested against LKB1-causative tumors. Rapamycin, a macrolide antibiotic that inhibits the mTOR pathway, has been shown to reduce the gastric tumor burden in Lkb1(+/−) mice by oral administration (35). Metformin and phenformin, biguanides commonly used to treat diabetes, are some other candidates for treating those tumors. Biguanides inhibit ATP synthesis and thereby cause
a rise in the cellular AMP:ATP ratio, which in turn activates AMPK (36). Several models have also indicated the possible therapeutic use of biguanides for tumors caused by LKB1-AMPK insufficiency (34,37).

In conclusion, frequent LKB1 mutations were found in mBAC in both PJS cases and sporadic cases. LKB1 inactivation is therefore a possible cause of mBAC tumorigenesis. Furthermore, LKB1 may be a possible target of therapy for mBAC, using LKB1-targeted drugs, such as rapamycin and biguanides delivered either systemically or by airway inhalation.

Acknowledgements

We thank Yoko Takeda for her valuable technical assistance. We also thank Shinya Oda and Kenichi Taguchi, Department of Clinical Research, National Kyushu Cancer Center, for their critical comments and valuable technical advice.

Funding

This work was supported in part by a Grant-in-Aid for Cancer Research (16-1) from the Ministry of Health, Labour and Welfare of Japan and by a Grant-in-Aid for Young Scientists (B) No. 19790973 from the Japan Society for the Promotion of Science.

Conflict of interest statement

None declared.

References


22. Yoo LI, Chung DC, Yuan J. LKB1—a master tumour suppressor of the Colorectal Cancer (CRC) subfamily, including MARK/PAR-1. EMBO J 2004;23:833–43.


32. Yoo LI, Chung DC, Yuan J. LKB1—a master tumour suppressor of the Colorectal Cancer (CRC) subfamily, including MARK/PAR-1. EMBO J 2004;23:833–43.


