Clinical Cancer Research

Immunohistochemical Loss of LKB1 Is a Biomarker for More Aggressive Biology in *KRAS*-Mutant Lung Adenocarcinoma

Antonio Calles[1], Lynette M. Sholl[2], Scott J. Rodig[2], Ashley K. Pelton[2], Jason L. Hornick[2], Mohit Butaney[1], Christine Lydon[1], Suzanne E. Dahlberg[3], Geoffrey R. Oxnard[1,4], David M. Jackman[1,4], and Pasi A. Jänne[1,4,5]

Abstract

Purpose: *LKB1* loss is common in lung cancer, but no assay exists to efficiently evaluate the presence or absence of LKB1. We validated an IHC assay for LKB1 loss and determined the impact of LKB1 loss in *KRAS*-mutant non–small cell lung cancer (NSCLC).

Experimental Design: We optimized and validated an IHC assay for LKB1 (clone Ley37D/G6) using a panel of lung cancer cell lines and tumors with known *LKB1* mutations, including 2 patients with Peutz–Jeghers syndrome (PJS) who developed lung adenocarcinoma. We retrospectively analyzed tumors for LKB1 using IHC from 154 *KRAS*-mutant NSCLC patients, including 123 smokers and 31 never-smokers, and correlated the findings with patient and tumor characteristics and clinical outcome.

Results: LKB1 expression was lost by IHC in 30% of *KRAS*-mutant NSCLC (smokers 35% vs. never-smokers 13%, $P = 0.017$). LKB1 loss did not correlate with a specific *KRAS* mutation but was more frequent in tumors with *KRAS* transversion mutations ($P = 0.029$). *KRAS*-mutant NSCLC patients with concurrent LKB1 loss had a higher number of metastatic sites at the time of diagnosis (median 2.5 vs. 2, $P = 0.01$), higher incidence of extrathoracic metastases ($P = 0.01$), and developed brain metastasis more frequently (48% vs. 25%, $P = 0.02$). There was a nonsignificant trend to worse survival in stage IV *KRAS*-mutant NSCLC patients with LKB1 loss.

Conclusions: LKB1 IHC is a reliable and efficient assay to evaluate for loss of *LKB1* in clinical samples of NSCLC. LKB1 loss is more common in smokers, and is associated with a more aggressive clinical phenotype in *KRAS*-mutant NSCLC patients, accordingly to preclinical models. *Clin Cancer Res; 21(12); 2851–60.* ©*2015 AACR.*

Introduction

The methodology for classifying non–small cell lung cancer (NSCLC) is evolving from use of histology alone to incorporating genomics. This change is based on the discovery of genomic alterations that define molecular subtypes, which can be targeted therapeutically (1). Lung cancer patients with identified driver genomic alterations treated with a targeted therapy survive longer than those who did not receive a targeted therapy (2). *KRAS* mutations are the most prevalent oncogenic driver mutation, detected in 30% of smokers and 10% of never-smokers with lung adenocarcinoma in Western populations (3–5). In contrast, for patients with *KRAS* mutations, targeted therapies have been largely unsuccessful and chemotherapy remains the current standard of care.

Therapeutic efforts at targeting *KRAS* have focused on inhibition of its intrinsically activated GTPase activity (6, 7), the use of farnesyl transferase inhibitors to impair the association of KRAS with the cell membrane where KRAS mediates its action (8–10), or synthetic lethality by inhibiting targets that impair the function of dependent genes (11–13). More recently, inhibition of MEK, the immediate downstream effectors of KRAS-mediated signaling, either alone or in combination with chemotherapy, has been evaluated both in preclinical models and in clinical trials (14, 15). In a randomized phase II clinical trial, previously treated patients with *KRAS* mutations who were treated with the MEK inhibitor selumetinib in combination with docetaxel had clinically meaningful improvement in progression-free survival, tumor responses, and patient-reported outcomes, compared with patients who were treated with docetaxel alone (16). Currently, this combination is being evaluated in a phase III clinical trial compared with docetaxel alone (ClinicalTrials.gov identifier: NCT01933932). Another MEK inhibitor, trametinib, has also been compared with docetaxel in previously treated advanced *KRAS*-mutant NSCLC patients (17). In this study, both trametinib and docetaxel had a similar response rate (12%) and progression-free survival (17).

The clinical efficacy of single-agent trametinib or the selumetinib/docetaxel combination is only observed in a subset of

[1]Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts. [2]Department of Pathology, Brigham and Women's Hospital, Boston, Massachusetts. [3]Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Boston, Massachusetts. [4]Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts. [5]Belfer Institute for Applied Cancer Science, Dana-Farber Cancer Institute, Boston, Massachusetts.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Prior presentation: Presented in part at the Annual Meeting of the American Society of Clinical Oncology, Chicago, IL; May 30–June 3, 2014.

Corresponding Author: Pasi A. Jänne, Lowe Center for Thoracic Oncology Dana-Farber Cancer Institute, HIM223, 450 Brookline Avenue, Boston, MA 02115. Phone: 617-632-6076; Fax: 617-582-7683; E-mail: pjanne@partners.org

doi: 10.1158/1078-0432.CCR-14-3112

Translational Relevance

KRAS is the most commonly mutated oncogene in non–small cell lung cancer (NSCLC). *KRAS*-mutant NSCLC is heterogeneous, detected in both smokers and never-smokers, and *LKB1* loss co-occurs in approximately 30% of these tumors. The presence of *KRAS* mutations with *LKB1* loss confers worse outcomes in preclinical murine models and predicts for resistance to selumetinib and docetaxel. *LKB1* is inactivated through a broad variety of genomic mechanisms, limiting the ability to efficiently evaluate it using routine clinical specimens. In the current study, we optimized an LKB1 IHC assay using cell lines and tumors with known *LKB1* genomic inactivation. This assay can be used to evaluate the clinical and therapeutic impact of LKB1 loss in tumors from *KRAS*-mutant NSCLC patients, especially those undergoing treatment with a MEK inhibitor. Prospective studies incorporating LKB1 IHC can now be conducted to evaluate whether LKB1 loss confers a similar impact in *KRAS*-mutant NSCLC patients.

KRAS-mutant patients and suggests that there may be heterogeneity among *KRAS*-mutant lung cancers. Genetic mutations that coexist with the *KRAS* mutations, especially inactivation of tumor suppressors, may contribute to both tumor aggressiveness and response to treatment. In a genetically engineered mouse model (GEMM) of *Kras* G12D NSCLC, concurrent inactivation of the tumor suppressor liver kinase B1 (*Lkb1*$^{-/-}$) confers a more aggressive phenotype, and leads to more tumor metastases compared with cancers in *Kras* G12D GEMMs (18). Consistent with the clinical findings, the selumetinib/docetaxel combination was more effective than docetaxel alone in *Kras* G12D GEMMs of lung cancer (19). However, in *Kras* G12D/Lkb1$^{-/-}$ GEMMS, selumetinib/docetaxel was significantly less effective (19). Collectively these findings highlight *LKB1* status as a relevant marker in the biologic heterogeneity of *KRAS*-mutant NSCLC.

LKB1, also known as the serine/threonine protein kinase 11 (*STK11*), regulates cell energy homeostasis, cell polarization, and apoptosis (20, 21). *LKB1* comprises 9 exons located on chromosome 19p13.3. Germline mutations in *LKB1* cause the autosomal dominant Peutz–Jeghers syndrome (PJS; OMIM 175200; refs. 22, 23). PJS is characterized by the development of hamartomatous polyps in the gastrointestinal (GI) tract, mucocutaneous pigmentation and an increased risk of developing cancer. *LKB1* functions as a tumor suppressor gene, and has been associated with several different types of sporadic cancer. Within lung cancer, *LKB1* ranks as the third most common mutation found in lung adenocarcinoma (24–27). Both *LKB1* alleles are somatically inactivated in 30% of lung adenocarcinomas and more commonly within *KRAS*-mutant NSCLC compared with *EGFR*-mutant NSCLC (24–27). Similarly to *KRAS*-mutant NSCLC, *LKB1* mutations are linked to smoking history (24).

The role of *LKB1* loss-of-function mutations as either a prognostic or predictive marker within human *KRAS*-mutant NSCLC is currently unknown. One potential reason for this observation is the wide range of *LKB1* genomic alterations observed in sporadic cancers, making it a challenge to develop one single assay capable of detecting all such alterations. Several types of somatic mutations in *LKB1* in sporadic cancers have been identified, including insertions, deletions (including large intragenic deletions), nonsense, frameshift, missense, and splice site alterations, and more infrequently hypermethylation of the promoter (28). Moreover, homozygous deletions of *LKB1* have been frequently reported in NSCLC (29, 30). Detection of genomic loss by DNA sequencing of clinical specimens is challenging because of the admixture with DNA from nontumor cells. However, most of the genomic alterations in *LKB1* result in either a truncated protein (and therefore inactive) or the absence of the protein (28). Thus, an IHC assay represents a potentially reliable, simple, and cost-effective method for evaluating for *LKB1* loss by querying the expression status of LKB1.

We report on the optimization and validation of an IHC assay for detection of LKB1 expression that can be applied to clinical NSCLC tumor specimens. We used this assay to determine the frequency of *LKB1* loss in *KRAS*-mutant NSCLC, and to understand the clinical characteristics and the prognostic implications of *LKB1* in advanced *KRAS*-mutant NSCLC.

Materials and Methods

Study population

We identified patients with *KRAS*-mutant NSCLC through an institutional database of patients that had undergone genotyping as previously described (31, 32). Systematic genotyping for *KRAS* mutations for all advanced nonsquamous NSCLC was initiated at DFCI in 2008 and is performed in a CLIA-certified laboratory (32). We manually reviewed the list of the selected cases and the staging workup at the time the patient was identified to have systemic disease. Brain metastasis either pathologically confirmed or detected by imaging (CT scan or brain MRI) at any point of the evolution of the disease was considered for the analysis. All patients were consented to an Institutional Review Board (IRB)-approved protocol allowing collection of specimens and clinical data. The tumor content of each of the specimens was confirmed to be sufficient for IHC analyses. Pack-years of smoking was defined as (average number of cigarettes per day/20) $\times$ years smoking. Former smokers were defined as those who had quit smoking $\geq$1 year from the time of diagnosis of lung cancer. Never-smokers were defined as patients who had smoked $\leq$ 100 cigarettes in their lifetime.

Cell lines and cell culture

Lung cancer cell lines A549, H460, DMS-53, UMC-11, H2122, H23, 11-18, H322, HCC827 were obtained from ATCC and grown in RPMI-1640 supplemented with 10% FBS and 1% 1$\times$ antibiotic-antimycotic (Life Technologies, cat# 15240-062). DMS-53 cells were grown in Waymouth Medium (Life Technologies, cat# 31220-023) and supplemented as above. Ectopic LKB1 expression in the *LKB1*-mutant A549 human NSCLC was performed by lentiviral transfection as previously described (33). All cells were cultured at 37°C in a humidified incubator with 5% CO2 and tested negative for mycoplasma infection. All cell lines were authenticated in September 2014 using the Promega Geneprint 16 cell ID system and were performed at the Research Technology Support Facility at Michigan State University (East Lansing, MI).

IHC

Four-micron thick formalin-fixed, paraffin-embedded (FFPE) tissue sections on charged glass slides were prepared per standard

protocols for IHC. Epitope retrieval was performed on the Leica Bond III autostainer at pH 9 for 20 minutes. LKB1 antibody (Abcam, cat# ab15095, clone Ley 37D/G6) was added at 1:15,000 for 30 minutes at room temperature and antibody detected using the Bond Polymer Refine Detection kit, cat# DS9800. Slides were counterstained with hematoxylin. LKB1 antibody clone Ley37D/G6 is a murine monoclonal IgG2b antibody anti-human LKB1, raised against recombinant LKB1 of human origin targeting an epitope located at the C-terminal region of the protein (34). For antibody optimization studies, A549 and A549 LKB1 cells were FFPE into a block. Sections from the FFPE block were subject to IHC analogous to tumor sections.

For clinical specimens, LKB1 expression was evaluated in the background nonneoplastic tissue, for example, alveolar macrophages or respiratory epithelium in lung specimens, providing an internal negative staining control. LKB1 staining was scored as intact or lost, with any degree of expression qualifying as intact. In some cases, the staining was heterogeneous, which was also noted. All the cases tested in the optimization and validation set showed cytoplasmic staining, and the cellular localization of protein expression in the clinical set was hence not further classified.

High-throughput genotyping

Mass-spectrometric–based cancer gene mutation profiling was performed in 440 lung adenocarcinoma specimens as part of an institutional genotyping platform, termed OncoMap, as previously described (35). *LKB1* mutations were found in six cases and we used these specimens for IHC optimization purposes. For IHC validation, clinical next-generation sequencing (NGS) was performed in FFPE clinical tumor samples from newly diagnosed NSCLC patients in our CLIA-certified laboratory as part of an IRB-approved research protocol. We utilized high-throughput exon capture and massively parallel sequencing, termed OncoPanel, to detect single nucleotide variants, insertion-deletion events, and copy number alterations, as previously described (36).

Statistical analysis

Associations between categorical data were tested using the Fisher exact test; Wilcoxon rank-sum test was used to test for association with continuous measures. Using the Fisher exact test, this study had 80% power for comparing a rate of LKB1 loss of 35% among smokers with a rate of 10% among never smokers while testing at the one-sided 0.025 level. Overall survival is defined as the time in months from date of diagnosis of metastatic disease to death from any cause, and patients not experiencing an event are censored at their last date of follow-up. Event time distributions were compared using the log-rank test, and Cox proportional hazards models were fitted to estimate HRs. All tests were conducted at the two-sided 0.05 level, and no adjustments have been made for multiple comparisons.

Results

KRAS-mutant patients

Between August 2008 and January 2014, we identified 514 *KRAS*-mutant patients among 1818 patients who had undergone genotyping (28%). Forty-two (8.2%) were never-smokers. We retrieved archival FFPE tumor specimens from never-smokers and smokers in a 1:3 ratio. We prioritized tumors from smokers with *KRAS*-mutant NSCLC with surgical specimens amenable to IHC. From the original 42 never-smokers, tissue could not be collected in 11 patients (tumor block exhausted/not located = 7 patients, lack of consent = 2 patients, confirmed remote story of light-smoking = 2 patients). The clinical and molecular characteristics of the patients and tumors selected for further analyses did not differ significantly from the remainder of the *KRAS*-mutant patients as two of the categories (sex and histology) approached *P* value of 0.05 (Supplementary Table S1).

LKB1 IHC assay optimization and validation in FFPE tissue using genetically annotated cell lines and human tumor specimens

We first set to optimize and validate the LKB1 IHC assay by using both lung cancer clinical samples and cell lines with known *LKB1* mutations. *LKB1*-mutant cell lines included DMS-53 (harboring the most common missense mutation, located in the kinase domain), and the H23 cells (nonsense mutation at exon 8; predicted to encode for a shorter LKB1 protein that lacks the C-terminal region; Supplementary Table S2; ref. 37). A549 cells (*KRAS* G12S, *LKB1* p.Q37*) and its variant transfected with active *LKB1* were used as positive and negative controls, respectively (Supplementary Table S2). *EGFR*-mutant cell line HCC827 was also used as negative control because it contains an intact *LKB1* (27). We detected a high degree of correlation with *LKB1* loss and lack of LKB1 protein expression by IHC (Fig. 1A and 1B and Supplementary Table S2). The one exception was the H322 cells in which LKB1 was detected by IHC despite an *LKB1* mutation. These cells are known to harbor an interstitial in-frame deletion of exons 2 and 3, which is expected to encode for a smaller protein (38), preserving the C-terminal portion of the protein, making it possible to be detected by IHC. A549/LKB1 cells demonstrated positive staining by IHC compared with A549 cells and staining was both nuclear and cytoplasmic (Fig. 1A and 1B). This dual cellular localization of LKB1 has been previously described for ectopic expression of LKB1 (39) and has been postulated being related to the lack of STRADα required for cytosolic localization of LKB1 complex (34). Some cell lines only demonstrated staining in the Golgi, characterized by a paranuclear dot-like staining, and were categorized as LKB1 negative (Supplementary Table S2).

Next, we tested the LKB1 IHC assay in a panel of FFPE specimens from patients with NSCLC. We first used tumor and nontumor tissue sections from two PJS patients who developed lung adenocarcinoma (Fig. 1D–I). Both patients had previous resections of hamartomatous polyps from the GI tract. LKB1 expression was detected in the cytoplasm of the epithelium but not in the stroma of the GI polyps (Fig. 1D and 1G). Similarly, positive staining was observed in the normal respiratory epithelium and the alveolar macrophages in their lung cancer surgical specimens (Fig. 1E, F, H, and I, respectively). In contrast, LKB1 expression was lost in the lung adenocarcinomas of both patients (Fig. 1F and 1I). The presence of LKB1 expression in nontumor tissue is likely due to the presence of one remaining intact *LKB1* germline allele and is restricted to previously known patterns of expression while in the tumor cells both alleles were lost (40). We next tested for LKB1 expression in 6 *LKB1*-mutant tumors from patients with NSCLC where the mutation was detected by allelotyping (OncoMap; ref. 35; Supplementary Table S3). Two specimens with p.P281L and p.E57fsX7 mutations, respectively, revealed weak staining and were considered as positive. The lack of information on other *LKB1* allele did not allow further interpretation of these results. In

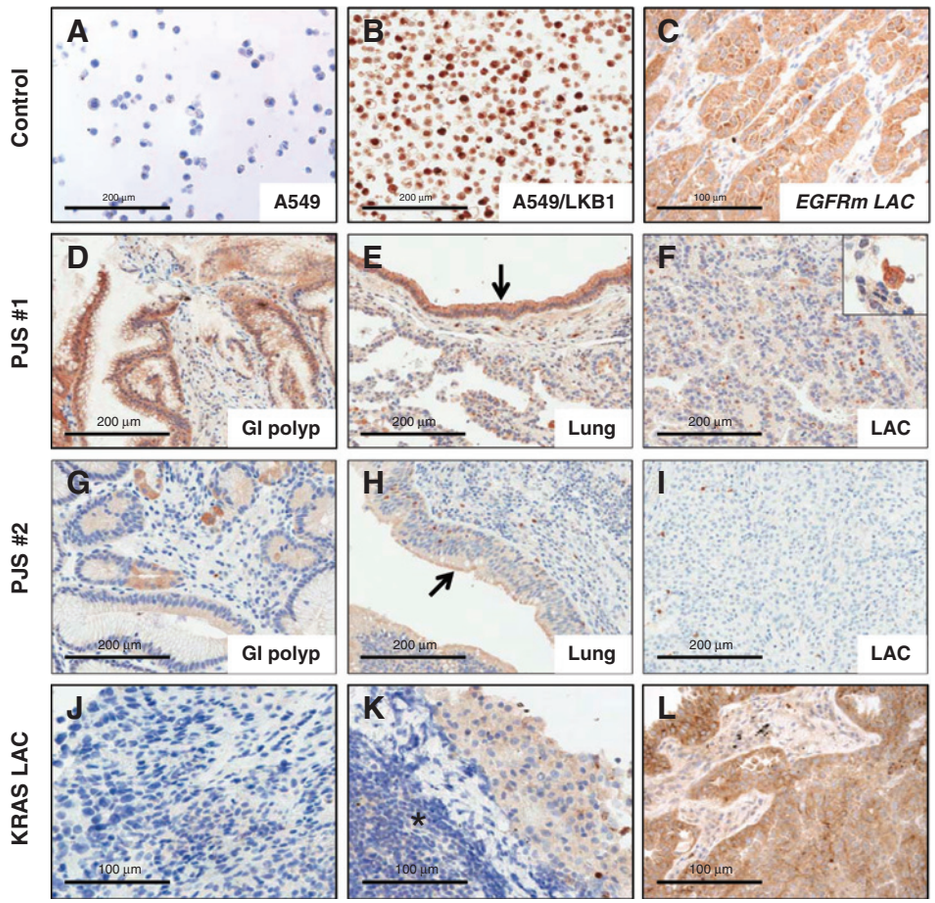


Figure 1.
Expression of LKB1 in FFPE cell lines and clinical specimens with known genetic alterations in *LKB1*. A, A549 cell line (*LKB1* p.Q37* mutant) showed no immunostaining for LKB1. B, A549 cell line was transfected with *LKB1* (A549/LKB1) and showed positive immunostaining (nuclear and cytoplasmic). C, *EGFR* L858R-mutant lung adenocarcinoma with intact expression of LKB1. D–I, two patients with PJS showing LKB1 expression in normal epithelia in previously resected hamartomatous GI polyps (D and G), and in the respiratory tract (E and H, arrows) from their surgically resected lung adenocarcinoma (F and I). In both patients, lung adenocarcinoma showed loss of LKB1 expression (F, with higher background staining compared with I, but similar to their correlative samples; detail of LKB1 intact staining in an alveolar macrophage—F, upper right corner). J–L, three *KRAS*-mutant lung adenocarcinoma specimens showing different LKB1 expression: J, loss (biallelic inactivation of *LKB1* by NGS); K, weak staining (lymph node metastasis, normal lymph node tissue in the left lower corner marked with an asterisk); L, strong staining (weak background in the stroma). All the IHC carried out with anti-LKB1 clone Ley37D/G6 (Abcam) 1:15,000. LAC, lung adenocarcinoma.

addition, two cases had equivocal staining, although one of them did not contain any cells that could be used as an internal negative control. The positive control—a tumor specimen with a biallelic deletion of *LKB1* by NGS—had loss of LKB1 expression, whereas the negative control—an *EGFR* mutant lung adenocarcinoma—showed strong LKB1 staining (Fig. 1J and 1C, respectively).

To further study our LKB1 IHC assay using clinical samples, we tested a clinical set of *KRAS*-mutant NSCLC specimens that had undergone targeted NGS (36). Between June and December 2013, we identified 27 cases harboring *KRAS* mutations and correlated their *LKB1* mutation status with the LKB1 IHC staining results (Table 1). No *LKB1* mutation was detected by NGS in 16 of 27 (59%) samples, whereas *LKB1* genetic alterations were identified in 11 of 27 (41%) of the samples. LKB1 expression was detected in 14 of 16 *LKB1* wild-type samples. In 2 of 16 specimens, no LKB1 expression was detected despite an intact *LKB1* locus. Both samples lacked internal negative controls (e.g., macrophages or respiratory epithelium) and it is possible that the assay failed in these two specimens. Alternatively, these cancers may contain other genomic methods of *LKB1* inactivation, such as methylation, not captured by NGS. In cases with intact LKB1 staining, there were different degrees of protein expression, varying from weak to strong cytoplasmic staining (data not shown). Among the 11 *LKB1*-mutant specimens, no LKB1 expression was detected in eight cases (73%). In two cases, LKB1 loss was considered heterogeneous, consisting of some weak staining with areas of multifocal loss of LKB1 expression. In a third case, which harbored a single copy deletion of the *LKB1* gene, LKB1 expression was intact by IHC (Table 1). As complete loss of LKB1 expression by IHC requires biallelic inactivation, these mismatches in the LKB1 staining with the *LKB1* genotype may be explained by the presence of a wild type *LKB1* allele responsible for some degree of protein expression, similar to the PJS patients showed previously. Of note, in three cases, we detected genomic evidence of biallelic inactivation of *LKB1*, consisting of deletion of one allele and a mutation in the other allele (Table 1). In all of these 3 patients, LKB1 expression was lost by IHC.

Evaluation of LKB1 loss using LKB1 IHC in smokers and never-smokers with *KRAS*-mutant NSCLC

We used the LKB1 IHC assay to evaluate the clinical and molecular features of smokers and never-smokers with *KRAS*-mutant NSCLC. We obtained FFPE tumor specimens on 154 patients (31 from never-smokers, 123 smokers), including the 27 patients previously sequenced used for validation. The demographic features of the patients are summarized in Table 2. Smokers and never-smokers patients were similar in terms of age, gender, race, histology, and stage. A 2:1 predominant female patient distribution is consistent with previous studies in *KRAS*-mutant population (3, 41). The G12C (40%) and G12V (18%) mutations were the most common *KRAS* mutation

Table 1. Summary of the NGS results (OncoPanel) on 27 *KRAS*-mutant NSCLC patients comparing *LBK1* genotype with LKB1 IHC staining

Tumor specimen			*KRAS* status			*LKB1* status					
Biopsy type	Tissue of origin	Tumor content (%)	Type of mutation	Allele fraction (%)	Coverage	*LKB1* DNA change	Exon	Allele fraction (%)	Coverage	AA change	LKB1 IHC
Surgical	Lung	70	G12V	24	138	wt					Intact
Surgical	Lung	40	G12C	9	174	wt					Intact
Surgical	Lung	70	G12C	26	140	wt					Intact
Surgical	Lung	30	G12C	12	277	wt					Lost[a]
Surgical	Lymph node	60	G12C	43	223	wt					Lost[a]
Core bx	Lung	80	G12V	9	135	wt					Intact
Surgical	Adrenal	80	Q61H	23	323	wt					Intact
Surgical	Lung	60	G12V	32	81	wt					Intact
Surgical	Lung	35	G12F	40	123	wt					Intact
Surgical	Pleura	60	G12D	29	99	wt					Intact
Surgical	Lung	70	G12S	28	260	wt					Intact
Cytology	Lung	30	G12C	15	123	wt					Intact
Surgical	Lung	70	G12C	65	294	wt					Intact
Surgical	Lung	60	G12C	25	212	wt					Intact
Surgical	Lung	30	G12C	31	177	wt					Intact
Surgical	Brain	50	G12C	42	171	wt					Intact
Surgical	Pleura	50	G12V	13	109	c.465_splice + Single copy deletion (19p13.3)		19	31	p.G155_splice	Lost
Surgical	Lung	90	G12C	45	177	c.291_splice + Single copy deletion (19p13.3)		26	87	p.K97_splice	Lost
Surgical	Lymph node	80	G13C	17	252	c.164_165TG>T + Single copy deletion (19p13.3)	1	33	148	p.L55fs	Lost
Cytology	Pleural fluid	60	G12C	21	197	c.157_158GA>G	1	21	165	p.D53fs	Lost
Surgical	Lung	50	G12D	26	210	c.129_130CA>C	1	46	396	p.A43fs	Focal loss
Surgical	Lymph node	60	G12A	19	163	c.580G>T	4	9	65	p.D194Y	Lost
Surgical	Lymph node	70	G12C	39	133	c.836_836G>GC	6	43	95	p.G279fs	Lost
Surgical	Lung	90	G12V	4	188	c.464_splice		50	18	p.G155_splice	Lost
Cytology	Lung	30	G12C	23	296	c.598_splice		17	135	p.A200_splice	Lost
Surgical	Lung	70	G12V	25	176	Single copy deletion (19p13.3)					Focal loss
Surgical	Lymph node	80	G12D	9	131	Single copy deletion (19p13.3)					Intact

[a]No internal control staining.

Table 2. Clinical and molecular characteristics by smoking status in *KRAS*-mutant NSCLC patients tested for LKB1 IHC ($n = 154$)

Characteristic	*KRAS*-mutant NSCLC		
	Smokers *n* (%)	Never-smokers *n* (%)	*P*
Total (*n*)	123 (80)	31 (20)	
Median age, y (range)	64 (41–84)	65 (20–84)	0.60
Sex			
Female	86 (70)	23 (74)	0.83
Male	37 (30)	8 (26)	
Smoking history			
Former	91 (74)	—	
Current	32 (26)	—	
Pack-years, median (range)	30 (2–120)	—	
Race			
Caucasian	114 (93)	27 (88)	0.08
Asian	0 (0)	2 (6)	
Afro-American	5 (4)	2 (6)	
Hispanic	1 (1)	0 (0)	
Declined/unknown	3 (2)	0 (0)	
Histology			
Adenocarcinoma	109 (89)	30 (97)	0.92
Squamous	0 (0)	0 (0)	
Adenosquamous	3 (2)	0 (0)	
Large cell	3 (2)	0 (0)	
NOS	8 (7)	1 (3)	
Stage (AJCC 7th edition)			
I	33 (27)	8 (26)	0.24
II	14 (11)	6 (19)	
III	10 (8)	5 (16)	
IV	66 (54)	12 (39)	
Type of *KRAS* mutation			
Transversion	101 (82)	11 (35)	<0.001
Transition	22 (18)	20 (65)	
KRAS mutation			
G12C	49 (40)	2 (6)	<0.001
G12V	22 (18)	4 (13)	
G12D	19 (15)	18 (58)	
G13X	5 (4)	1 (3)	
Q61X	7 (6)	0 (0)	
Multiple	5 (4)	1 (3)	
Others	16 (13)	5 (16)	

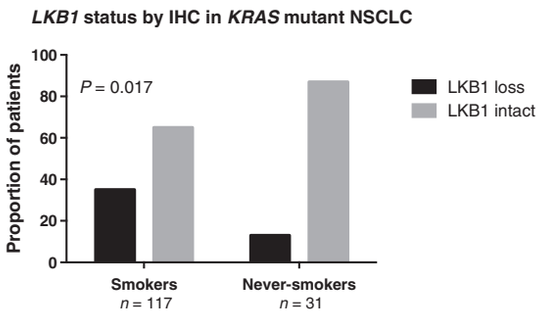


Figure 2.
LKB1 expression by smoking status in *KRAS*-mutant NSCLC patients (n = 154). The overall incidence of LKB1 loss assessed by IHC was 30% (95% CI, 24%–38%) and was higher in smokers than in never-smokers (35% vs. 13%, $P = 0.017$) since genetic alterations in *LKB1* are known to be smoking related. IHC failed in six cases (4%).

subtype in smokers, whereas G12D (58%) was most common in never-smokers ($P < 0.001$). Never-smokers were more likely to harbor *KRAS* transition mutations rather than transversion mutations, while the converse was true for smokers (82% transversion mutations vs. 18% transition mutations, $P < 0.001$; Supplementary Fig. S1). The most common nucleotide change was G>T in smokers and G>A in never-smokers ($P < 0.001$). This pattern of *KRAS* mutations is in accordance with previously reported studies on specific subtypes of *KRAS* mutations in smokers and never-smokers (4, 42).

Of the 154 samples for LKB1 IHC assay, six cases failed IHC (4%); in three, the tumor tissue quality was not sufficient for staining and in the remaining three cases, we could not interpret the staining due to a lack of internal control. Using our IHC assay, the overall incidence of LKB1 loss was 30% (95% confidence intervals; CI, 24%-38%) and was higher in smokers than in never-smokers (35% vs. 13%, $P = 0.017$; Fig. 2). Among the cases in which we observed LKB1 staining, the intensity of staining varied from weak to strong (data not shown), and the staining was universally cytoplasmic (Fig. 1). Any degree of LKB1 staining by IHC was considered positive. Heterogeneous staining, composed of areas of intact and lost staining, was observed in eight cases, all of which were classified as LKB1 intact. Overall, we obtained successful staining in a wide variety of clinical biopsies (surgical specimens 73%, core biopsies 16%, and cytology specimens 11%).

There were no differences in LKB1 loss by age, sex, race, histology, or pack-years of smoking (Supplementary Table S4). There was a trend toward LKB1 loss in stage IV patients. Although LKB1 was not associated with the type of *KRAS* mutations, LKB1 loss was preferentially found in tumors with *KRAS* transversion mutations as opposed to transition mutations (ratio 5:1, $P = 0.029$). This is in contrast with the never-smokers where the absence of LKB1 expression was almost mutually exclusive with the presence of a *KRAS* transition mutation. Only one case of 20 with *KRAS* transition mutations showed loss of LKB1, whereas 5 out of 13 cases with transversion mutations showed loss of LKB1 expression ($P = 0.025$).

Loss of expression of LKB1 confers worse prognosis features in advanced *KRAS*-mutant NSCLC

Preclinical mouse models demonstrate that *Kras* G12D/*Lkb1*$^{-/-}$ tumors have a more aggressive phenotype, characterized by increased metastases, compared with *Kras* G12D–only mice (18). To evaluate whether this same observation could also occur in humans, we evaluated the clinical features of stage IV *KRAS*-mutant patients based on their LKB1 status. We included patients with both initially diagnosed stage IV disease and those who developed recurrent disease during the follow-up after being diagnosed for early-stage NSCLC. In total, 126 patients were evaluable (74 initially diagnosed with stage IV disease, 52 with recurrent disease). Patients with both a *KRAS* mutation and LKB1 loss by IHC had higher number of metastatic sites at the time of diagnosis of stage IV disease (median 2.5 vs. 2.0, $P = 0.01$) and developed brain metastasis more frequently (48% vs. 25%, $P = 0.02$; Table 3). Patients with intact LKB1 tumors were more likely to have intrathoracic metastases ($P = 0.02$), whereas those with LKB1 loss tumors had significantly higher incidence of extrathoracic metastases ($P = 0.01$, Table 3). The higher number of metastatic sites together with the higher frequency of extrathoracic metastasis suggests that LKB1 loss may promote metastasis beyond the lung. Altogether, our data support the preclinical evidence that LKB1 loss confers features of worse prognosis in patients with stage IV *KRAS*-mutant lung adenocarcinoma.

Table 3. Clinical characteristics and metastatic profile in stage IV *KRAS*-mutant NSCLC patients by LKB1 IHC status ($n = 126$)

Characteristic	LKB1 intact *n* (%)	LKB1 loss *n* (%)	*P*
Total (*n*)	84 (67)	42 (33)	
Median age, y (range)	64 (30–84)	63 (37–80)	0.15
Sex			
Female	59 (70)	28 (67)	0.69
Male	25 (30)	14 (33)	
Smoking history			
Never-smoker	17 (20)	4 (10)	0.29
Smoker			
Former	50 (60)	27 (64)	
Current	17 (20)	11 (26)	
Pack-year, median (range)	30 (0–120)	28 (0–80)	0.94
Race			
Caucasian	75 (89)	40 (95)	0.84
Asian	1 (1)	0 (0)	
Afro-American	4 (5)	2 (5)	
Hispanic	1 (1)	0 (0)	
Declined/unknown	3 (4)	0 (0)	
Histology			
Adenocarcinoma	76 (90)	37 (88)	0.31
Squamous	0 (0)	0 (0)	
Adenosquamous	3 (4)	0 (0)	
Large cell	1 (1)	0 (0)	
NOS	4 (5)	5 (12)	
Type of *KRAS* mutation			
Transversion	58 (69)	35 (83)	0.13
Transition	26 (31)	7 (17)	
KRAS mutation			
G12C	26 (31)	17 (40)	0.99
G12V	11 (13)	9 (21)	
G12D	21 (25)	7 (17)	
G13X	3 (4)	2 (5)	
Q61X	6 (7)	0 (0)	
Multiple	4 (5)	1 (2)	
Others	13 (15)	6 (14)	
Median of sites of metastasis (range)	2 (1–6)	2.5 (1–6)	0.01
Visceral metastasis	64 (76)	34 (81)	0.65
Lung	54 (64)	22 (52)	0.25
Brain	21 (25)	20 (48)	0.02
Liver	6 (7)	5 (12)	0.50
Adrenal	3 (4)	6 (14)	0.06
Nonvisceral metastasis	56 (67)	34 (81)	0.14
Lymph node	42 (50)	26 (62)	0.26
Bone	27 (32)	19 (45)	0.17
Pleura	24 (29)	7 (17)	0.19
Soft tissue	4 (5)	5[a] (12)	0.16
Intrathoracic metastasis	66 (79)	24 (57)	0.02
Extrathoracic metastasis	39 (46)	30 (71)	0.01

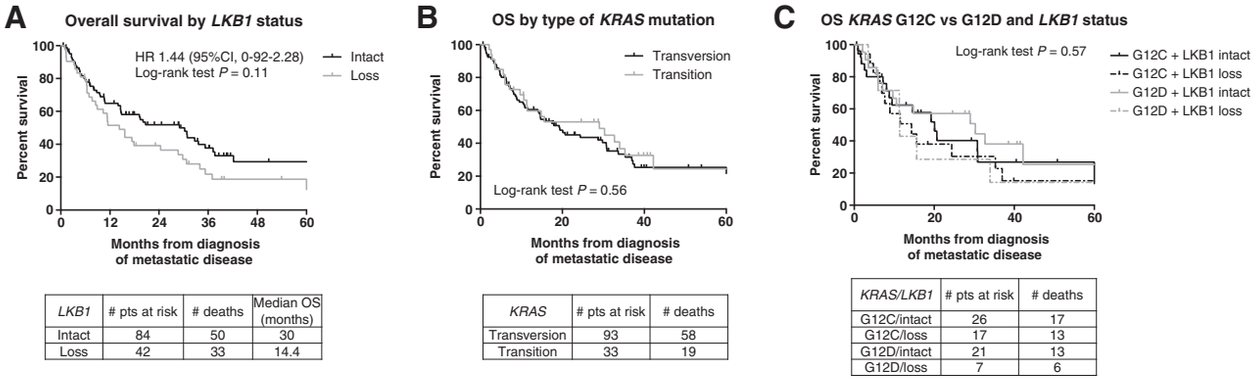
[a]Three cases with subcutaneous metastasis.

To confirm whether LKB1 loss has an impact on survival, we analyzed the survival outcomes of the 126 stage IV *KRAS*-mutant NSCLC patients in which we evaluated LKB1 status by IHC (LKB1 intact 84 patients, LKB1 loss 42 patients; Table 3). The groups were similar in age, gender, race, smoking history, histology, and *KRAS* genotype (Table 3). The Kaplan–Meier survival curves showed a tendency toward a shorter overall survival in patients with LKB1 loss compared with those with intact LKB1, although this difference was not statistically significant (median OS in LKB1 loss 14.4 vs. LKB1 intact 30.0 months, log-rank $P = 0.11$) with a HR = 1.44 (95% CI, 0.92–2.28; Fig. 3A). There was no significant difference in outcomes based on the class of KRAS mutation (transversion vs. transition) or type of *KRAS* genotype (Fig. 3B and Supplementary Fig. S2). The survival effect of LKB1 loss seems to be of the same magnitude regardless of the class (transversion vs. transition) or type of KRAS mutation (G12C/G12V; Fig. 3C and Supplementary Fig. S2, respectively). However, these data did not reach statistical significance in our cohort of patients.

Discussion

LKB1 is a tumor suppressor gene that is commonly lost in cancers from patients with NSCLC. Previous studies have demonstrated that *LKB1* loss is more common in *KRAS*-mutant compared with in *EGFR*-mutant lung cancers (24–27). Preclinical murine studies demonstrate that *Kras*-mutant tumors with *Lkb1* loss are more aggressive and less likely to respond to docetaxel/selumetinib treatment (18, 19). The impact of *LKB1* loss in human lung cancer is currently not known. Given the increased number of clinical studies focusing on *KRAS*-mutant NSCLC, including a phase III clinical trial of docetaxel/selumetinib versus docetaxel (ClinicalTrials.gov identifier: NCT01933932), it is critical to be able to evaluate the presence of LKB1 loss and to correlate the findings with outcome. However, no systematic tools exist to evaluate *LKB1* loss. In part, this is due to the complexity of *LKB1* loss, which can occur through multiple different genomic and non-genomic mechanisms. To overcome this limitation, we evaluated an LKB1 IHC assay as a simple and cost-effective method that can be applied to clinical NSCLC specimens.

We optimized our assay using a variety of complementary methods, including evaluation of cell lines and patient specimens that were known to be intact for *LKB1* or contain *LKB1* mutations. Despite the accuracy of the IHC assay shown in our genotyped samples, this method has also limitations. Although we used NGS of clinical specimens for comparative purposes, genotyping including NGS cannot be considered as gold standard to address for *LKB1* loss given the multiple mechanisms leading to its loss. Hence, this limits the ability to determine the sensitivity and specificity of our assay. Second, we cannot extrapolate the accuracy of this assay for all of the different *LKB1* mutations beyond the ones included in our panel. For instance, some *LKB1* missense mutations that result in functional inactivation of LKB1 could still result on LKB1 protein expression, and hence be misclassified as LKB1 intact using IHC. Third, testing for loss of expression of a protein by IHC may be more challenging than confirming its presence. The interpretation on LKB1 staining depends on the tissue background staining and the presence of internal negative controls: an excessive background or the lack of internal control may lead to either equivocal results or not valid results. In our studies, we observed this in 5% of the cases but we could not assume the same incidence in other conditions and requires further confirmation. In addition, we observed the heterogeneous staining in some cases, likely reflecting the tumor heterogeneity, which cannot be effectively captured with genotyping only. The biologic significance of this heterogeneity is currently unknown and may represent the evolutionary pattern of LKB1 loss as a tumor suppressor gene in the progression of the tumor. Overall, we were able to apply a simple dichotomous and easily reproducible scoring system with cytoplasmic LKB1 either present, or absent, that is preferable other scoring methods, including H-scores, for evaluation of tumor suppressor gene loss (43).



LKB1	# pts at risk	# deaths	Median OS (months)
Intact	84	50	30
Loss	42	33	14.4

KRAS	# pts at risk	# deaths
Transversion	93	58
Transition	33	19

KRAS/LKB1	# pts at risk	# deaths
G12C/intact	26	17
G12C/loss	17	13
G12D/intact	21	13
G12D/loss	7	6

Figure 3.
Kaplan–Meier curves for comparison of overall survival (OS) in stage IV *KRAS*-mutant NSCLC. A, OS in stage IV *KRAS*-mutant NSCLC according to LKB1 status. B, OS of *KRAS* tranversion vs. transition mutations. C, OS by different *KRAS* mutations (G12C vs. G12D) and LKB1 status.

We observed a lower incidence of LKB1 loss in never-smokers with *KRAS*-mutant NSCLC, consistent with studies suggesting LKB1 is a smoking-induced alteration (28). Moreover, the almost mutually exclusive presence of transition mutations with LKB1 loss could have therapeutic implications. *KRAS* G12D, the most common mutation in never-smokers, is reported to have better outcomes and responses to selumetinib/docetaxel when LKB1 is intact in murine preclinical models, while the reverse is true when LKB1 is inactivated (19). Thus, it is possible that *KRAS*-mutant never-smokers may have a differential clinical response to MEK inhibitors or MEK inhibitor combinations (14, 19, 44). The incorporation of tumor LKB1 testing into current and future clinical trials is necessary to help confirm the preclinical observations. Never-smokers with *KRAS* mutations are usually an underestimated subgroup because of the general belief that *KRAS* mutations are only found in smokers. Although less common than *EGFR* mutations, *KRAS* mutations are detected in 10% of Caucasian never-smokers and are similar in incidence to anaplastic lymphoma kinase rearrangements (3, 4, 45, 46).

Our results using LKB1 IHC are consistent with the murine preclinical observations, which demonstrate an increase in metastases and a worse outcome in the *Kras* G12D/*Lkb1*$^{-/-}$ model (15, 19, 47). The clinical and molecular profiles of the *KRAS*-mutant patients and their relationship with smoking status are similar to those previously reported (3, 4, 25, 48). We observed a 30% incidence of LKB1 loss in our study, more commonly in smokers, expected for this population suggesting that our large subset of *KRAS*-mutant patients is representative and consistent with prior studies (18, 26, 29, 30, 47). In patients with LKB1 loss, there was an increase in both the frequency and types of metastases, which translated into a trend toward a worse survival. These observations suggest that LKB1 loss may have a prognostic role, a finding that will require additional prospective validation. Our findings suggest that a simple dichotomous assay for IHC LKB1 loss is both simple and fast and is appropriate for routine use in clinical pathology specimens and/or as part of future prospective trials. If validated, LKB1 status may need to be incorporated into clinical trials of *KRAS*-mutant patients both for its prognostic and predictive roles.

Disclosure of Potential Conflicts of Interest

G.R. Oxnard is a consultant/advisory board member for AstraZeneca, Genentech, and Novartis. P.A. Jänne is a consultant/advisory board member for Abbot, AstraZeneca, Boehringer Ingelheim, Chugai, Clovis, Genentech, Pfizer, and Sanofi. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: A. Calles, S.J. Rodig, P.A. Jänne
Development of methodology: A. Calles, S.J. Rodig, J.L. Hornick
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Calles, S.J. Rodig, A.K. Pelton, J.L. Hornick, M. Butaney, G.R. Oxnard, D.M. Jackman, P.A. Jänne
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Calles, L.M. Sholl, S.J. Rodig, M. Butaney, S.E. Dahlberg, P.A. Jänne
Writing, review, and/or revision of the manuscript: A. Calles, L.M. Sholl, J.L. Hornick, S.E. Dahlberg, G.R. Oxnard, D.M. Jackman, P.A. Jänne
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Calles, M. Butaney, C. Lydon, G.R. Oxnard, D.M. Jackman
Study supervision: P.A. Jänne

Grant Support

A. Calles is a grant recipient from the Spanish Medical Oncology Society. S.E. Dahlberg received funding from the Gallup funds to support her collaboration in this work.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 2, 2014; revised February 1, 2015; accepted February 23, 2015; published OnlineFirst March 3, 2015.

References

1. Meador CB, Micheel CM, Levy MA, Lovly CM, Horn L, Warner JL, et al. Beyond histology: translating tumor genotypes into clinically effective targeted therapies. Clin Cancer Res 2014;20:2264–75.
2. Kris MG, Johnson BE, Berry LD, et al. Using multiplexed assays of oncogenic drivers in lung cancers to select targeted drugs. JAMA 2014;311:1998–2006.

3. Dogan S, Shen R, Ang DC, Johnson ML, D'Angelo SP, Paik PK, et al. Molecular epidemiology of EGFR and KRAS mutations in 3,026 lung adenocarcinomas: higher susceptibility of women to smoking-related KRAS-mutant cancers. Clin Cancer Res 2012;18:6169–77.
4. Riely GJ, Kris MG, Rosenbaum D, Marks J, Li A, Chitale DA, et al. Frequency and distinctive spectrum of KRAS mutations in never smokers with lung adenocarcinoma. Clin Cancer Res 2008;14:5731–4.
5. Yamaguchi N, Vanderlaan PA, Folch E, Boucher DH, Canepa HM, Kent MS, et al. Smoking status and self-reported race affect the frequency of clinically relevant oncogenic alterations in non–small-cell lung cancers at a United States-based academic medical practice. Lung Cancer 2013; 82:31–7.
6. Lim SM, Westover KD, Ficarro SB, Harrison RA, Choi HG, Pacold ME, et al. Therapeutic targeting of oncogenic K-Ras by a covalent catalytic site inhibitor. Angew Chem Int Ed Engl 2014;53:199–204.
7. Ahmadian MR, Zor T, Vogt D, Kabsch W, Selinger Z, Wittinghofer A, et al. Guanosine triphosphatase stimulation of oncogenic Ras mutants. Proc Natl Acad Sci U S A 1999;96:7065–70.
8. Adjei AA, Cohen RB, Franklin W, Morris C, Wilson D, Molina JR, et al. Phase I pharmacokinetic and pharmacodynamic study of the oral, small-molecule mitogen-activated protein kinase kinase 1/2 inhibitor AZD6244 (ARRY-142886) in patients with advanced cancers. J Clin Oncol 2008; 26:2139–46.
9. Adjei AA, Mauer A, Bruzek L, Marks RS, Hillman S, Geyer S, et al. Phase II study of the farnesyl transferase inhibitor R115777 in patients with advanced non–small-cell lung cancer. J Clin Oncol 2003;21: 1760–6.
10. Riely GJ, Johnson ML, Medina C, Rizvi NA, Miller VA, Kris MG, et al. A Phase II trial of salirasib in patients with lung adenocarcinomas with KRAS mutations. J Thorac Oncol 2011;6:1435–37.
11. Puyol M, Martín A, Dubus P, Mulero F, Pizcueta P, Khan G, et al. A synthetic lethal interaction between K-ras oncogenes and Cdk4 unveils a therapeutic strategy for non–small cell lung carcinoma. Cancer Cell 2010;18:63–73.
12. Steckel M, Molina-Arcas M, Weigelt B, Marani M, Warne PH, Kuznetsov H, et al. Determination of synthetic lethal interactions in KRAS oncogene-dependent cancer cells reveals novel therapeutic targeting strategies. Cell Res 2012;22:1227–45.
13. Stephen Andrew G, Esposito D, Bagni Rachel K, McCormick F. Dragging ras back in the ring. Cancer Cell 2014;25:272–81.
14. Engelman JA, Chen L, Tan X, Crosby K, Guimaraes AR, Upadhyay R, et al. Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. Nat Med 2008; 14:1351–6.
15. Ji H, Wang Z, Perera SA, Li D, Liang MC, Zaghlul S, et al. Mutations in BRAF and KRAS converge on activation of the mitogen-activated protein kinase pathway in lung cancer mouse models. Cancer Res 2007;67: 4933–9.
16. Janne PA, Shaw AT, Pereira JR, Jeannin G, Vansteenkiste J, Barrios C, et al. Selumetinib plus docetaxel for KRAS-mutant advanced non–small-cell lung cancer: a randomised, multicentre, placebo-controlled, phase 2 study. Lancet Oncol 2013;14:38–47.
17. Gandara DR, Hiret S, Blumenschein GR, Barlesi F, Delord JP, Madelaine J, et al. Oral MEK1/MEK2 inhibitor trametinib (GSK1120212) in combination with docetaxel in KRAS-mutant and wild-type (WT) advanced non–small cell lung cancer (NSCLC): A phase I/Ib trial. J Clin Oncol 31, 2013 (suppl; abstr 8028).
18. Ji H, Ramsey MR, Hayes DN, Fan C, McNamara K, Kozlowski P, et al. LKB1 modulates lung cancer differentiation and metastasis. Nature 2007;448: 807–10.
19. Chen Z, Cheng K, Walton Z, Wang Y, Ebi H, Shimamura T, et al. A murine lung cancer co-clinical trial identifies genetic modifiers of therapeutic response. Nature 2012;483:613–17.
20. Oakhill JS, Steel R, Chen ZP, Scott JW, Ling N, Tam S, et al. AMPK is a direct adenylate charge-regulated protein kinase. Science 2011;332:1433–5.
21. Lizcano JM, Goransson O, Toth R, Deak M, Morrice NA, Boudeau J, et al. LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. EMBO J 2004;23:833–43.
22. Hemminki A, Markie D, Tomlinson I, Avizienyte E, Roth S, Loukola A, et al. A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. Nature 1998;391:184–7.
23. Jenne DE, Reimann H, Nezu J, Friedel W, Loff S, Jeschke R, et al. Peutz-Jeghers syndrome is caused by mutations in a novel serine threonine kinase. Nat Genet 1998;18:38–43.
24. Sanchez-Cespedes M. The role of LKB1 in lung cancer. Fam Cancer 2011; 10:447–53.
25. Imielinski M, Berger Alice H, Hammerman Peter S, Hernandez B, Pugh Trevor J, Hodis E, et al. Mapping the Hallmarks of Lung Adenocarcinoma with Massively Parallel Sequencing. Cell 2012;150:1107–20.
26. Ding L, Getz G, Wheeler DA, Mardis ER, McLellan MD, Cibulskis K, et al. Somatic mutations affect key pathways in lung adenocarcinoma. Nature 2008;455:1069–75.
27. Koivunen JP, Kim J, Lee J, Rogers AM, Park JO, Zhao X, et al. Mutations in the LKB1 tumour suppressor are frequently detected in tumours from Caucasian but not Asian lung cancer patients. Br J Cancer 2008;99: 245–52.
28. Sanchez-Cespedes M. A role for LKB1 gene in human cancer beyond the Peutz-Jeghers syndrome. Oncogene 2007;26:7825–32.
29. Gill RK, Yang SH, Meerzaman D, Mechanic LE, Bowman ED, Jeon HS, et al. Frequent homozygous deletion of the LKB1/STK11 gene in non–small cell lung cancer. Oncogene 2011;30:3784–91.
30. Fang R, Zheng C, Sun Y, Han X, Gao B, Li C, et al. Integrative genomic analysis reveals a high frequency of LKB1 genetic alteration in chinese lung adenocarcinomas. J Thorac Oncol 2014;9:254–58.
31. Cardarella S, Ogino A, Nishino M, Butaney M, Shen J, Lydon C, et al. Clinical, pathologic, and biologic features associated with BRAF mutations in non–small cell lung cancer. Clin Cancer Res 2013;19:4532–40.
32. Cardarella S, Ortiz TM, Joshi VA, Butaney M, Jackman DM, Kwiatkowski DJ, et al. The introduction of systematic genomic testing for patients with non–small-cell lung cancer. J Thorac Oncol 2012;7:1767–74.
33. Gao Y, Xiao Q, Ma H, Li L, Liu J, Feng Y, et al. LKB1 inhibits lung cancer progression through lysyl oxidase and extracellular matrix remodeling. Proc Natl Acad Sci 2010;107:18892–7.
34. Carretero J, Medina PP, Blanco R, Smit L, Tang M, Roncador G, et al. Dysfunctional AMPK activity, signalling through mTOR and survival in response to energetic stress in LKB1-deficient lung cancer. Oncogene 2007;26:1616–25.
35. MacConaill LE, Campbell CD, Kehoe SM, Bass AJ, Hatton C, Niu L, et al. Profiling critical cancer gene mutations in clinical tumor samples. PLoS ONE 2009;4:e7887.
36. Wagle N, Berger MF, Davis MJ, Blumenstiel B, Defelice M, Pochanard P, et al. High-throughput detection of actionable genomic alterations in clinical tumor samples by targeted, massively parallel sequencing. Cancer Discov 2012;2:82–93.
37. Carretero J, Medina PP, Pio R, Montuenga LM, Sanchez-Cespedes M. Novel and natural knockout lung cancer cell lines for the LKB1/STK11 tumor suppressor gene. Oncogene 2004;23:4037–40.
38. Matsumoto S, Iwakawa R, Takahashi K, Kohno T, Nakanishi Y, Matsuno Y, et al. Prevalence and specificity of LKB1 genetic alterations in lung cancers. Oncogene 2007;26:5911–8.
39. Tiainen M, Vaahtomeri K, Ylikorkala A, Makela TP. Growth arrest by the LKB1 tumor suppressor: induction of p21(WAF1/CIP1). Hum Mol Genet 2002;11:1497–504.
40. Conde E, Suarez-Gauthier A, García-García E, Lopez-Rios F, Lopez-Encuentra A, García-Lujan R, et al. Specific pattern of LKB1 and phospho-acetyl-CoA carboxylase protein immunostaining in human normal tissues and lung carcinomas. Hum Pathol 2007;38:1351–60.
41. Yu HA, Sima CS, Shen R, Kass S, Gainor J, Shaw A, et al. Prognostic impact of KRAS mutation subtypes in 677 patients with metastatic lung adenocarcinomas. J Thorac Oncol 2014;10:431–7.
42. Slebos RJ, Hruban RH, Dalesio O, Mooi WJ, Offerhaus GJ, Rodenhuis S. Relationship between K-ras oncogene activation and smoking in adenocarcinoma of the human lung. J Natl Cancer Inst 1991;83: 1024–7.
43. Chaux A, Peskoe SB, Gonzalez-Roibon N, Schultz L, Albadine R, Hicks J, et al. Loss of PTEN expression is associated with increased risk of recurrence after prostatectomy for clinically localized prostate cancer. Mod Pathol 2012;25:1543–9.
44. Mahoney CL, Choudhury B, Davies H, Edkins S, Greenman C, Haaften G, et al. LKB1/KRAS mutant lung cancers constitute a genetic subset of NSCLC with increased sensitivity to MAPK and mTOR signalling inhibition. Br J Cancer 2009;100:370–5.

45. Kim HR, Shim HS, Chung JH, Lee YJ, Hong YK, Rha SY, et al. Distinct clinical features and outcomes in never-smokers with nonsmall cell lung cancer who harbor EGFR or KRAS mutations or ALK rearrangement. Cancer 2012;118:729–39.
46. Takamochi K, Oh S, Suzuki K. Differences in and mutation spectra in lung adenocarcinoma of never and heavy smokers. Oncol Lett 2013;6:1207–12.
47. Marcus AI, Zhou W. LKB1 regulated pathways in lung cancer invasion and metastasis. J Thorac Oncol 2010;5:1883–6.
48. Varghese AM, Sima CS, Chaft JE, Johnson ML, Riely GJ, Ladanyi M, et al. Lungs don't forget: comparison of the KRAS and EGFR mutation profile and survival of collegiate smokers and never smokers with advanced lung cancers. J Thorac Oncol 2013;8:123–5.