LKB1 Loss Induces Characteristic Patterns of Gene Expression in Human Tumors Associated with NRF2 Activation and Attenuation of PI3K-AKT

Jacob M. Kaufman, PhD,* Joseph M. Amann, PhD,† Kyungho Park, BS,* Rajeswara Rao Arasada, PhD,† Haotian Li, BS,‡ Yu Shyr, PhD,§ and David P. Carbone, MD, PhD†

Introduction: Inactivation of serine/threonine kinase 11 (STK11 or LKB1) is common in lung cancer, and understanding the pathways and phenotypes altered as a consequence will aid the development of targeted therapeutic strategies. Gene and protein expressions in a murine model of v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (Kras)-mutant lung cancer have been studied to gain insight into the biology of these tumors. However, the molecular consequences of LKB1 loss in human lung cancer have not been fully characterized.

Methods: We studied gene expression profiles associated with LKB1 loss in resected lung adenocarcinomas, non–small-cell lung cancer cell lines, and murine tumors. The biological significance of dysregulated genes was interpreted using gene set enrichment and transcription factor analyses and also by integration with somatic mutations and proteomic data.

Results: Loss of LKB1 is associated with consistent gene expression changes in resected human lung cancers and cell lines that differ substantially from the mouse model. Our analysis implicates novel biological features associated with LKB1 loss, including altered mitochondrial metabolism, activation of the nuclear respiratory factor 2 (NRF2) transcription factor by kelch-like ECH-associated protein 1 (KEAP1) mutations, and attenuation of the phosphatidylinositol 3-kinase and v-akt murine thymoma viral oncogene homolog (PI3K/AKT) pathway. Furthermore, we derived a 16-gene classifier that accurately predicts LKB1 loss and attenuation of this signature.

Conclusion: Loss of LKB1 defines a subset of lung adenocarcinomas associated with characteristic molecular phenotypes and distinctive gene expression features. Studying these effects may improve our understanding of the biology of these tumors and lead to the identification of targeted treatment strategies.

Key Words: Genomics, LKB1, NRF2, PI3K/AKT.

(J Thorac Oncol. 2014;9: 794–804)
complete or nearly complete loss of LKB1 function, regardless of the underlying mechanism. Thus, we will refer to these processes collectively as instances of “LKB1 loss,” or as an “LKB1-deficient” tumor, with the presumption—that supported by our later findings—that these alterations yield similar tumor phenotypes.

Our analysis demonstrates that LKB1 loss is associated with a consistent pattern of gene expression across resected human NSCLC tumors and cell lines. A predictive signature derived from this pattern accurately classifies mutational and nonmutational loss of LKB1 in multiple validation sets. However, this pattern is not recapitulated in the murine model. Gene expression patterns and associations with other molecular features implicate activation of the forkhead box A2 (FOXA2), forkhead box O3 (FOXO3), cyclic-AMP responsive element binding protein (CREB), and NRF2 transcription factors and decreased PI3K/AKT signaling in tumors with LKB1 loss. By defining these dysregulated processes, this work will guide future research efforts aimed at understanding the complex effects of these pathways in determining phenotypic and clinical consequences in tumors that have lost LKB1.

**MATERIALS AND METHODS**

**Clinical and Molecular Data sets**

Preprocessed and normalized data for RNAseq gene expression, microRNA expression, copy number alterations, protein expression, somatic mutations, and clinical data for lung adenocarcinomas characterized by The Cancer Genome Atlas (TCGA) were obtained from the TCGA Web site. Processed gene expression data, somatic mutations, and clinical information for other publicly available data sets were downloaded from Gene Expression Ominibus (GEO) and ArrayExpress or from individual Web sites, as listed in Supplementary Table S1 (Supplementary Digital Content 1, http://links.lww.com/JTO/A585). In cases where data were presented as linear expression values, log2 transformed values were used. The status of LKB1 loss in NSCLC cell lines was taken from various previous studies given in Supplementary Data File 1 (Supplementary Digital Content 2, http://links.lww.com/JTO/A586).

Gene expression analysis of A549 and H2122 cell lines transduced with pBABE, LKB1, or LKB1-K78I was performed using HT Human Gene 1.1 ST PM16 array plate...
using a GeneTitan instrument (Affymetrix; Santa Clara, CA). They were then scanned on the Affymetrix GeneTitan AGCC v. 3.2.3 and then analyzed on Affymetrix Expression Console v. 1.1 using an robust multi-array average (RMA) normalization algorithm producing log2 results. These data are available from GEO data repository (GSE51266).

Statistical Analyses

Two-sided student’s T-tests were used to compare statistical differences in continuous variable distributions between groups of samples: for instance, gene expression differences between LKB1 wild-type and mutant tumors. For comparison of discrete variables such as the presence of somatic mutations, statistical significance was determined using Fisher’s exact test. Statistical associations between continuous variables such as LKB1 mRNA expression were determined using linear regression. Visualization of gene expression patterns by hierarchical clustering was performed with Cluster 3.0 and TreeView software packages. For the four expression clusters defined by hierarchical clustering, cluster “scores” were calculated for a given set of samples by averaging the standardized values of the respective genes. The score for the 16-gene LKB1-loss classifier, equivalent to the FOX/CREB cluster, was used for prediction of LKB1 status in clinical and cell line samples. LKB1-loss scores for NSCLC cell lines are given in Supplementary Data File 1 (Supplementary Digital Content 2, http://links.lww.com/JTO/A586).

Gene expression associations for the four transcriptional clusters were calculated using multivariable general linear regression models carried out with the ‘Limma’ package in R bioconductor software, (R foundation for statistical computing, Vienna, Austria). Transcription factor analysis was performed using the Broad Institute’s Molecular Signature Database,13,14 and initial perturbation analysis was performed using the Connectivity Map.15 Detailed information on data sources and statistical comparisons made for various analyses is given in Supplementary Table S1 (Supplementary Digital Content 3, http://links.lww.com/JTO/A587).

Cell Culture and Gene Transduction

A549 and H2122 cell lines were generously shared with us by John Minna and Luc Girard (University of Texas, Southwestern, Dallas, TX). Cell line identity was authenticated by DNA fingerprinting, and cell lines were tested to ensure that they were mycoplasma negative. Cells were cultured in Roswell Park Memorial Institute 1640 (RPMI1640) cell growth media, (GIBCO, Carlsbad, CA) containing 5% fetal bovine serum, without antibiotics. Empty pBABE viral plasmids, pBABE-LKB1 and pBABE-LKB1-K78I, were obtained from Addgene (Cambridge, MA). Phoenix cells were transfected with viral plasmids and retroviral particles were harvested from media supernatant 48 hours after transfection. Viruses were added to target cells with polybrene, and selection with 1 μg/ml puromycin was begun 48 to 72 hours after infection. Cells were selected under puromycin for 1 to 2 weeks before performing subsequent experiments, with experiments being completed within 2 months.

Immunoblots

Cell lysates were harvested while cells were in exponential growth phase in radioimmunoprecipitation assay (RIPA) lysis buffer containing phosphatase and protease inhibitors. Lysates were homogenized and run on precast sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels (BioRad, Hercules, CA). Phospho-ACC (s79), ACC, RS6, and LKB1 antibodies were obtained from Cell Signaling Technology, Danvers, MA.

CRE-Luciferase Reporter

We designed a dual-luciferase reporter driven by a ×3 cyclic-AMP responsive element (CRE) consensus-binding sequence in the promoter region in addition to a TATA box, which was inserted into a lentiviral construct upstream of luciferase. Luciferase activity from this reporter was compared with a control reporter that was identical but with mutated CRE sites. Cells were stably transduced to express CRE-wild-type or CRE-mutant reporters and ratios between the two were compared after subsequent perturbations.

More detailed descriptions of statistical and analytical procedures are available with Supplementary Methods online (Supplementary Digital Content 3, http://links.lww.com/JTO/A587).

RESULTS

LKB1 Loss Results in Consistent Gene Expression Changes in Human Tumors

The effects of signaling pathways are mediated in part by activation of transcription factors affecting the expression of downstream genes. Inferences drawn from the analysis of the dysregulated genes may disclose novel links between pathways and phenotypes that would otherwise be difficult to predict. We determined gene expression changes associated with LKB1 loss in lung adenocarcinomas characterized by the TCGA (Cancer Genome Atlas Research Network). Comprehensive molecular characterization of lung adenocarcinoma. Nature. 2014), as well as five additional studies of resected lung adenocarcinomas16–20 two large collections of NSCLC cell lines21,22 and data from two studies using the Lkb1/Kras murine model18 (Supplementary Table S1, Supplementary Digital Content 1, http://links.lww.com/JTO/A585). Genes with differential expression between LKB1-mutant and LKB1-wild-type samples were ranked by statistical significance for each data set (Supplementary Data File 2, Supplementary Digital Content 4, http://links.lww.com/JTO/A588). These LKB1-associated gene lists were then compared pairwise across all data sets, and statistical significance of overlapping genes was used to determine similarity (Fig. 2A; Supplementary Figure S1 [Supplementary Digital Content 5, http://links.lww.com/JTO/A589]; Supplementary Table S2 [Supplementary Digital Content 1, http://links.lww.com/JTO/A585]). A consistent pattern of gene expression is associated with LKB1 loss across human data sets (median p value = 3.8e-18 for 55 pairwise comparisons). Murine Lkb1 loss also resulted in a consistent gene expression signature across the two studies, but without significant overlap with the human studies, suggesting important differences in tumor
biology (Fig. 2A; Supplementary Figure S1 [Supplementary Digital Content 5, http://links.lww.com/JTO/A589]).

Having established that LKB1 loss results in consistent patterns of gene expression in human lung adenocarcinomas, we used two clinical data sets—one analysis of lung adenocarcinomas from University of Michigan (n = 178) and one characterized by Washington University (n = 41)—as training cohorts to identify 129 genes associated with LKB1 loss (Supplementary Data File 2, Supplementary Digital Content 4, http://links.lww.com/JTO/A588). Expression of these genes was visualized using hierarchical clustering, as shown for the Michigan training set and the TCGA validation set (Fig. 2B, C). Comparison of the correlation patterns in these two data sets allows identification of four consistently expressed transcriptional clusters (Fig. 2B, C; Supplementary Figure S2 [Supplementary Digital Content 5, http://links.lww.com/JTO/A589]). However, the correlation patterns of the down-regulated genes were not as reproducible across multiple data sets as the up-regulated genes, so we will focus our attention on the three clusters that show increased expression in LKB1-deficient tumors.

**Computational Approaches Identify Putative Drivers of Expression Clusters Associated with LKB1 Loss**

The pattern of gene expression associated with LKB1 loss may be reflective of biological processes altered in these tumors. We sought to identify transcription factors putatively associated with these genes using a bioinformatics approach. For each of the four clusters identified, ranked lists of gene expression association were determined using a multivariable linear model. The top 200 genes associated with each cluster were then used to generate hypotheses regarding the pathways or phenotypes that drive the expression of these clusters by mining public data sources, including predicted promoter transcription factor–binding sites, drug-induced perturbations characterized by the connectivity map project,15 and the diverse collections of predetermined genesets included in the molecular signature database.13,14

Two of the up-regulated clusters showed associations with tumor metabolic phenotypes. The “mTOR/mitochondria” cluster was associated with high expression of oxidative phosphorylation and mitochondria-associated genes and genes involved in protein translation (Supplementary Table S3, Supplementary Digital Content 1, http://links.lww.com/JTO/A585). The “NRF2” cluster contains oxidative stress response genes driven by the NRF2 transcription factor (Supplementary Table S4, Supplementary Digital Content 1, http://links.lww.com/JTO/A585). As LKB1 functions in conjunction with AMPK as a metabolic regulator,6,7,23 these phenotypes may either represent direct metabolic consequences of LKB1 loss or adaptive responses to compensate for loss of protective mechanisms.
Analysis of a third cluster of up-regulated genes implicated CREB, FOXO, and FOXA2 transcription factors (Supplementary Table S5, Supplementary Digital Content 1, http://links.lww.com/JTO/A585). Of the four gene clusters identified, the FOX/CREB cluster had the strongest association with LKB1 loss in the training cohort. Analysis of perturbed genes from the connectivity map also revealed induction of this cluster by colforsin, an adenylate cyclase stimulator that activates CREB, and by the typical antipsychotics thioridazine, prochlorperazine, and trifluoperazine, which have been identified as stimulators of FOXO transcription factors. We then searched the GEO and Array Express data repositories and found corroborating evidence for CREB and FOXO3 activation within this cluster. Furthermore, analysis of chromatin precipitation data showed significantly increased levels of FOXA2 promoter occupancy among these genes in A549 and HEPG2 cells. Thus, this cluster may represent the effects of a specific set of transcription factors that are dysregulated downstream of LKB1.

Wild-Type LKB1 Attenuates the LKB1-Associated Signature

To test whether LKB1 could exert direct effects on gene expression, we used an isogenic cell line model system in which LKB1 was stably expressed in LKB1-mutant NSCLC cell lines (A549 and H2122). Mutated LKB1-K78I and empty pBABE retrovirus were used as controls. LKB1 expression was confirmed by Western blot, and kinase activity was shown.

**FIGURE 3.** Restoring wild-type LKB1 attenuates the expression of the LKB1-deficient gene signature and the CREB transcription factor. A, Immunoblots of whole-cell lysates from A549 and H2122 stably expressing empty pBABE vector, LKB1 or K78I LKB1. Ribosomal protein S6 is used as a loading control. B and C, Changes in gene expression of A549 (B) or H2122 (C) cell lines after re-expressing wild-type or mutant LKB1 were compared with the gene lists for each of the four LKB1-associated clusters using a hypergeometric test. Log10 p values are indicated on the y axis, with positive values indicating induction of expression and negative values indicating repression. D, Activity of CRE-luciferase is shown for A549 and H2122 cell lines after stable expression of LKB1 or K78I LKB1. Reporter activations were determined relative to a control luciferase with mutated CRE sites and are shown relative to the pBABE control. p values show the significance of unpaired student’s t tests. LKB1, liver kinase B1; CREB, cyclic-AMP responsive element binding protein; pBABE, pBABE retrovirus; FOX, forkhead box protein; NRF2, nuclear respiratory factor 2.
by demonstrating that wild-type LKB1 resulted in phosphorylation of acetyl-CoA-carboxylase, a downstream target of AMPK (Fig. 3A) in A549 and H2122 cells.

Global gene expression analysis showed that genes directly altered by LKB1 expression in A549 and H2122 showed significant overlap with the differentially expressed genes from our analysis of clinical and cell line data sets (Fig. 2A; Supplementary Figure 1 [Supplementary Digital Content 5, http://links.lww.com/JTO/A589]). By comparing the overlap of LKB1-perturbed genes to each of the top 200 genes associated with the four gene clusters from our previous analysis, we show that genes affected directly by LKB1 expression showed the strongest association with the FOX/CREB gene cluster (hypergeometric test \( p \) values = 1.3e-30, 3.6e-45 for A549 and H2122; Fig. 3B, C), whereas mTOR/mitochondria and NRF2-associated clusters were relatively unaffected. Many of the 16 individual genes that originally identified the FOX/CREB cluster were down-regulated by twofold or more in response to LKB1 expression (Supplementary Figure S3, Supplementary Digital Content 5, http://links.lww.com/JTO/A589). Because this gene cluster was linked in part to CREB activity, we also tested CREB transcriptional activity in A549 and H2122 cell lines using a luciferase reporter driven by the CREB-consensus sequence, which showed a reduction in reporter activity of 30% to 40% (Fig. 3D; \( p < 0.05 \) for each cell line). This is consistent with previous studies showing CREB to be directly attenuated downstream of LKB1 due to effects on the CREB regulated transcription coactivator (CRTC) family of transcriptional coactivators.26–28

A 16-Gene Classifier Accurately Predicts LKB1 Mutations in Lung Adenocarcinoma

The FOX/CREB cluster showed a strong association with LKB1 mutations in the training cohort and its expression was also directly attenuated by the restoration of wild-type LKB1 in vitro. Thus, we tested the ability of a 16-gene classifier (AVPI1, BAG1, CPS1, DUSP4, FGA, GLCE, HAL, LKB1: Classifier: Mutant: 26 WT: 213 Sensitivity (%): 85 Specificity (%): 76 P-value: 2.8e-09 TCGA: Mut: 67 WT: 339 Sensitivity (%): 97 Specificity (%): 78 P-value: 2.5e-33 Cell lines: Mut: 39 WT: 46 Sensitivity (%): 93 Specificity (%): 91 P-value: 1.2e-16)

FIGURE 4. A 16-gene signature of LKB1 loss accurately predicts LKB1 mutations, loss of LKB1 expression, and loss of function. A, The population distribution is shown for numeric LKB1-loss scores calculated for 446 lung adenocarcinomas from the TCGA. Parameters are shown for calculations of best fit to a bimodal distribution; these parameters, and the two associated normal distributions, are shown. B, On the basis of the parameters for the two normal distributions determined for this population, the probability of a given score representing the high-expression LKB1 loss curve is shown. C, Sensitivity and specificity of the LKB1 classifier for prediction of LKB1 mutations across independent testing sets; \( p \) value represents the result of the Fisher’s exact test. D, Expression of LKB1 mRNA is shown for TCGA lung adenocarcinomas grouped by LKB1 mutation and LKB1-loss signature classification status. E, Expression of phospho-AMPK T172 is shown for the subset of TCGA lung adenocarcinomas with RPPA data, which were grouped by LKB1 mutation and LKB1-loss signature classification status. D and E, Each dot represents one tumor, with red bars indicating the median expression. \( p \) values represent results from the student’s \( t \) test comparing indicated groups. RPPA, reverse phase protein array; TCGA, The Cancer Genome Atlas; AMPK, adenosine monophosphate–activated protein kinase; LKB1, liver kinase B1; WT, wild-type; NS, not significant.

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Mutations in the pooled cohort, 65 of 67 in the TCGA cohort, and combined sensitivity of 93%; 22 of 26 somatic mutations were accurately predicted in each of the lung cancer validation cohorts with a mutation rate of 6.5% because of the region of overlap in the two underlying distributions (Fig. 4A, B). A cutoff value of 0.2 was selected from the training analysis and was used to classify tumors as LKB1 wild-type or LKB1 loss.

We then tested the ability of the signature to predict LKB1 mutations in independent validation sets: a pooled analysis of previously published resected lung adenocarcinomas, lung adenocarcinomas characterized by TCGA (Cancer Genome Atlas Research Network. Comprehensive molecular characterization of lung adenocarcinoma. Nature. 2014), and NSCLC cell lines. No samples from these validation sets were used at any point in the derivation of our signature from the stated training sets. LKB1 mutations were accurately predicted in each of the lung cancer validation cohorts with a combined sensitivity of 93%; 22 of 26 somatic LKB1 mutations in the pooled cohort, 65 of 67 in the TCGA cohort, and 36 of 39 in NSCLC cell lines (sensitivity 85%, 97%, and 92%; \(p = 2.8e^{-9}, 2.5e^{-33},\) and 1.2e-16; shown in Fig. 4C and Supplementary Figure S4, Supplementary Digital Content 5, http://links.lww.com/JTO/A589).

We further tested our LKB1-loss signature alongside previously reported studies by using gene set enrichment analysis to compare the various gene lists to the genes differentially expressed by LKB1 mutant tumors in the largest dataset - the TCGA cohort - and those directly perturbed by LKB1 in our in vitro study. The previous work available for comparison include three studies that examined the in vitro effects of LKB1 add-back in A549,1,29 H2126,1 and HeLa30 cells, a previous report of gene expression changes associated with LKB1 mutations,31 and expression profiles associated with the "magnoid" subtype of lung adenocarcinomas,18 which has been shown to have an increased prevalence of LKB1 mutations (Supplementary Table S6, Supplementary Digital Content 1, http://links.lww.com/JTO/A585). This analysis shows that LKB1 has consistent effects on gene expression in cell lines seen in our analysis and previous work, including similar changes in the nonlung HeLa cell line. The genes identified by Fernandez et al. do not seem to be reproducibly associated with LKB1 loss in our analysis. On the other hand, genes associated with the "magnoid" subtype show significant overlap with LKB1-associated genes, but to a much lesser extent than is observed for the LKB1-loss signature.

LKB1 Wild-Type Lung Adenocarcinomas That Express the LKB1-Loss Signature Show Evidence of LKB1 Inactivation

Twenty-three percent of tumors without known LKB1 mutations were classified as having LKB1 loss. However, some mutations may have been unrecognized, and there are multiple mechanisms by which tumor suppressors can be inactivated in addition to somatic mutation.10-12 Thus, we looked for additional evidence of LKB1 loss by examining LKB1 mRNA expression measured by RNAseq, and LKB1 protein expression and phosphorylation of AMPK-T172 in the subset of tumors that were also characterized by reverse phase protein array. Among the 67 tumors with identified mutations in LKB1, LKB1 mRNA expression and phospho-AMPK are strongly decreased (Fig. 4D, E; \(p = 4.7e^{-26}\) and 5.7e-11, respectively, by Student’s t test). Low pAMPK is consistent with the loss of LKB1 kinase activity, whereas decreased mRNA expression reflects the fact that of these 67 mutations, 49 (73%) are nonsense, splice site, or frameshift alterations that are expected to lead to reduced mRNA expression by nonsense-mediated decay.32 LKB1 protein is also decreased among these 67 mutant tumors (\(p = 0.030\)); the moderate statistical significance associated with this observation compared with that associated with phosphorylated AMPK may reflect differences in antibody specificity.

When the same analysis is applied to the 74 LKB1 wild-type tumors that express the LKB1-loss gene signature, these tumors exhibit the same characteristics of LKB1 loss: these tumors have low LKB1 mRNA expression (\(p > 7.3e^{-28}\); Fig. 4D), low LKB1 protein expression (\(p = 0.011\)), and show loss of LKB1 kinase activity as demonstrated by attenuated phosphorylation of AMPK-T172 (\(p = 1.7e^{-7}\); Fig. 4E). Moreover, when the 67 tumors with known LKB1 mutations are compared with the 74 LKB1-wild-type tumors that are predicted to have LKB1 loss, no significant differences are observed in LKB1 protein or mRNA expression or of AMPK phosphorylation (Fig. 4D, E). These tumors likely represent unrecognized cases of LKB1 loss that could occur by undetected mutation, intragenic deletion, chromosomal loss, or by an epigenetic mechanism. This finding suggests that the classifier may significantly surpass the observed specificity of 77% and shows that the LKB1-loss classifier is more sensitive than DNA sequencing for the detection of functional LKB1 loss.

Determination of Other Tumor Characteristics Associated with LKB1 Loss

Differentially expressed genes associated with LKB1 loss can be studied directly to infer patterns of transcription factor activation that may reflect underlying differences in pathway activation. To better understand other phenotypes of LKB1 loss, we performed statistical comparisons to determine differences in clinical characteristics, copy number alterations, microRNA expression, protein expression, and mutation prevalence between LKB1-wild-type lung adenocarcinomas and those with LKB1 loss (Fig. 5, Supplementary Tables S7–S10, Supplementary Digital Content 1, http://links.lww.com/JTO/A585). The 16-gene classifier was used to determine LKB1 status of TCGA-characterized lung adenocarcinomas for the purpose of these comparisons.

To uncover novel associations between LKB1 loss and alterations in other pathways, we studied the prevalence of somatic mutations in the TCGA cohort of lung adenocarcinomas using a defined set of cancer genes (Catalog of Somatic Mutations in Cancer [COSMIC] Cancer Gene Census). Of the 32 genes with at least a 5% mutation rate in this cohort, five
showed significant differences on the basis of LKB1 status. KRAS, KEAP1, and ATM were mutated more frequently in tumors that had lost LKB1, whereas EGFR and p53 mutations were less common (Fig. 5A). We were able to test associations with EGFR, KRAS, and p53 mutations independently using a pooled analysis of publicly available lung adenocarcinoma cohorts. Consistent with results from a prior study, this analysis confirmed fewer EGFR mutations (p = 1.9e-10) and increased prevalence of KRAS mutations (p = 0.00035) among tumors with LKB1 loss. No association with p53 loss was
observed in this pooled analysis, so this finding is of uncertain significance. No data set was available to directly confirm associations with ATM and KEAP1 mutations. However, tumors with KEAP1 loss exhibited high levels of the “NRF2 cluster” of gene expression, consistent with the direct role of KEAP1 in the degradation of the NRF2 transcription factor (Supplementary Figure S5, Supplementary Digital Content 5, http://links.lww.com/JTO/A589). Thus, expression of this cluster seems to be indirectly linked to LKB1 loss, reflecting instead the increased prevalence of KEAP1 loss in these tumors.

Associations between LKB1 loss and copy number alterations (Supplementary Table S7, Supplementary Digital Content 1, http://links.lww.com/JTO/A585), microRNA expression (Supplementary Table S8, Supplementary Digital Content 1, http://links.lww.com/JTO/A585), and protein expression (Supplementary Table S9, Supplementary Digital Content 1, http://links.lww.com/JTO/A585) were also determined using TCGA data. Findings from analysis of reverse phase protein array proteomic data may be of particular interest, because these can potentially reflect states of pathway activation that may be important for developing targeted interventions for LKB1-deficient tumors. Expression levels for 186 proteins and phosphorylated protein sites were compared between these groups using the Student’s t test. Several components of the PI3-kinase pathway were down-regulated among tumors with LKB1 loss, suggesting that this pathway may be attenuated in these tumors. Differentially expressed proteins and phosphorylations are mapped onto a schematic drawing representing key features of this pathway34–36 (Fig. 5B). Both the p85 and p110 subunits of PI3K showed significant decrease in expression (p = 0.00061 and 0.00047, respectively), as well as decreased phosphorylation of PDK1 at serine 241 (p = 0.00012), and decreased total Akt (p = 0.02) and phospho-S473 Akt (p = 0.018). Surprisingly, proteomic evidence did not suggest significant mTOR activation, showing only modest increase in eIF4E expression and decrease in 4E-BP1 (p = 0.037 and 0.032, respectively), with no significant differences in other components of mTOR signaling. A complete list of the significant associations seen in this analysis is given in Supplementary Table S9 (Supplementary Digital Content 1, http://links.lww.com/JTO/A585).

Finally, we show that LKB1 loss has no prognostic significance in lung adenocarcinoma (Fig. SC). There is no association with tumor stage or survival in either the TCGA or Director’s Challenge cohorts (Supplementary Table S10, Supplementary Digital Content 1, http://links.lww.com/JTO/A585). Smoking status was the only clinical characteristic associated with LKB1 loss, with tumors arising from never-smokers exhibiting a significantly lower prevalence of LKB1 loss. These findings are consistent with prior analysis of the clinical significance of LKB1 loss.11

DISCUSSION

Our work integrates detailed molecular characterizations from a number of sources, combining knowledge of mutations and other genetic alterations with analysis of gene and protein expression in lung adenocarcinomas. This gives the most comprehensive analysis to date of the consequences of LKB1 loss in lung adenocarcinomas, and the novel biological insights that we have uncovered will guide future experimentation and may suggest therapeutic strategies to target these tumors. In particular, we show that LKB1 loss in lung adenocarcinomas is associated with a characteristic pattern of gene expression changes, which can be used to accurately predict LKB1 loss and is also directly responsive to LKB1 activation in vitro. We integrate analysis of these gene expression patterns with data on somatic mutations and protein expression to describe novel associations between LKB1 and other oncogenic pathways, especially activation of the NRF2 pathway for reactive oxygen detoxification and dysregulation of the PI3K/AKT/FOXO3 pathway, both of which have important effects on multiple cellular phenotypes and can affect response to a variety of anticancer treatments. The identification of previously unknown phenotypes associated with LKB1 loss has also been a major rationale for the development and study of the murine model of Lkb1/Kras-mutant lung cancer. However, our meta-analysis of LKB1-associated gene expression shows that LKB1 loss produces distinctly different effects in murine tumors than in human lung adenocarcinomas and cell lines. It is unclear whether the dissimilarity in gene expression reflects differences in clinically relevant phenotypes. Further experimentation is warranted to explore these differences.

Our characterization of gene expression patterns allowed us to develop and validate a 16-gene LKB1-loss signature that sensitively detects 93% of LKB1 mutations in resected lung adenocarcinoma while also identifying tumors that have lost LKB1 by other mechanisms. Previous analysis of cell lines has shown that homozygous deletion and intragenic deletion are common mechanisms of LKB1 loss in addition to somatic mutation.10–12 Our work suggests that sequencing efforts to detect LKB1 mutations may fail to detect about half the instances of LKB1 loss. Thus, combination of direct sequencing with an expression-based classifier could enhance detection of LKB1 loss, for instance to assess the effect of LKB1 loss on response to mTOR inhibitors or other novel targeted agents in clinical trials.

In addition to the potential use of our signature as a clinical classifier, our study of the dysregulated genes associated with LKB1 loss has given insight into the biology of LKB1-deficient tumors. Other integrated molecular analyses have been applied to lung cancer (Cancer Genome Atlas Research Network. Comprehensive molecular characterization of lung adenocarcinoma. Nature. 2014)37,38 and have disclosed novel associations that would be difficult to appreciate with more targeted approaches, including association of LKB1 mutations with gene expression–defined subtypes of lung adenocarcinoma. Rather than taking a global approach used in many of these studies, our work focused specifically on LKB1 loss as a single phenotype of interest. Our subsequent analysis confirmed a role of CREB activation that has previously been shown to have important oncogenic effects in LKB1-deficient tumors and results from a well-understood mechanism involving LKB1-mediated regulation of CRTC transcriptional coactivators.26–28 Moreover, we identify additional transcription factors FOXO3, FOXA2, and NRF2 that
are active in LKB1-deficient tumors and may influence various phenotypes within these tumors. Our in vitro experiments also showed that restoring LKB1 expression led to down-regulation of significant subsets of these genes, demonstrating that LKB1 affects the activity of the corresponding transcription factors through direct downstream mechanisms.

Each of the factors we identify is known to influence key phenotypes in cancer. Further studies examining their effects and interactions in the context of LKB1 loss may be particularly informative. For instance, NRF2 is a key activator of the oxidative stress response and also plays a role in metabolic reprogramming of cancer cells.\(^3\)\(^,\)\(^4\)\(^,\)\(^6\) LKB1-deficient tumors have been shown to be susceptible to oxidative stress, because they are unable to make the appropriate adaptive responses in metabolism and biosynthesis.\(^3\)\(^,\)\(^4\)\(^,\)\(^1\) NRF2 is frequently activated by somatic mutations in KEAP1 in NSCLC,\(^2\)\(^,\)\(^3\)\(^,\)\(^4\)\(^,\)\(^2\)\(^,\)\(^4\)^ and our analysis of the TCGA lung adenocarcinomas shows that the odds of a tumor having a KEAP1 mutation are increased more than sixfold among tumors with LKB1 loss. This high level of overlap may suggest that selective pressure exists for the activation of NRF2 as a secondary protective mechanism to compensate for LKB1 loss.

Down-regulation of the PI3K/AKT pathway is also evident among resected lung adenocarcinomas with LKB1 loss. The mechanism by which LKB1 loss decreases PI3K/AKT signaling is unclear, but several possibilities are worth mentioning. One possibility is that mTOR activation resulting from LKB1 loss could result in feedback inhibition of PI3K/AKT signaling,\(^2\)\(^,\)\(^4\) analogous to the effects seen in tumors exhibiting TSC2 loss.\(^4\)\(^,\)\(^5\) Direct interaction of LKB1 and AKT has also been demonstrated,\(^4\) and LKB1 has been shown to facilitate AKT activation and exert antiapoptotic effects, including inhibitory phosphorylation of the proapoptotic FOXO3 transcription factor.\(^2\)\(^,\)\(^4\) Of note, activation of FOXO3 was also suggested by analysis of gene expression, showing that down-regulation of the PI3K/AKT pathway may result in alterations in downstream pathway activation. This work guides future functional experiments to explore the interplay of LKB1 with these various pathways and the resulting effects on clinically relevant phenotypes such as response to targeted inhibitors.

ACKNOWLEDGMENTS

The authors thank W. Pao, D. Kaufman, A. Russo, for their critical review of the article, and E. Kaufman, for her support. The authors thank the efforts of The Cancer Genome Atlas for their extensive molecular characterization of lung and other cancers and thank the many donors of tumor material who have made such resources possible.

Supported by the Strategic Partnering to Evaluate Cancer Signatures (SPECS) grant: NCI U01CA114771.

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