Clinical Cancer Research

LKB1 Expression Correlates with Increased Survival in Patients with Advanced Non–Small Cell Lung Cancer Treated with Chemotherapy and Bevacizumab

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Abstract

Purpose: LKB1 is a key sensor of metabolic stress, including hypoxia and glucose deprivation, two features of the tumor microenvironment exacerbated by antiangiogenic therapy. We investigated the role of LKB1 as a potential predictive marker of sensitivity to bevacizumab in advanced non–small cell lung cancer (aNSCLC).

Experimental design: We retrospectively analyzed LKB1 expression by IHC in 98 samples from 125 patients with aNSCLC, including 59 patients treated with chemotherapy and 39 treated with chemotherapy plus bevacizumab. IHC intensity was recoded in two classes (negative/weak vs. moderate/intense) and correlated with outcome according to treatment arm. Patient-derived tumor xenografts (PDXs) were used to investigate mechanisms involved in preclinical models.

Results: In the whole study population (125), median OS and PFS were 11.7 [95% confidence interval (CI), 9.1–15.3] and 6.7 (95% CI, 5.7–7.2) months, respectively. Differential impact of the marker on outcome of the 98 patients was highlighted according to the treatment. Patients with negative/weak LKB1 status did not have a statistically significant benefit from bevacizumab added to chemotherapy (HR for patients treated with bevacizumab: 0.89; 95% CI, 0.51–1.56; $P = 0.6803$), whereas patients expressing moderate/intense LKB1 and receiving bevacizumab had significant lower risk of death compared with those not receiving bevacizumab (HR, 0.26; 95% CI, 0.10–0.64; $P = 0.0035$). Loss of LKB1 was associated with reduced AMPK activation in PDXs and increased tumor necrosis following bevacizumab administration, highlighting impaired control of the metabolic stress caused by this antiangiogenic drug.

Conclusions: Our data hint at a possible predictive impact of LKB1 expression in patients with aNSCLC treated with chemotherapy plus bevacizumab. *Clin Cancer Res; 23(13); 3316–24. ©2017 AACR.*

Introduction

Liver kinase B1 (LKB1, also called STK11), a serine-threonine protein kinase of the calcium calmodulin family, is ubiquitously expressed in several tissues. As a stress-responsive gene (1), *LKB1* displays varying effects under cell stress conditions, such as glucose deprivation or hypoxia. One of the best known functions of LKB1 is regulation of cell metabolism, accomplished in part by its effects on mTOR, a key regulator of protein synthesis and cell growth (2) and AMP-activated protein kinase (AMPK), a cellular sensor of ATP levels that supports cell viability in response to energy stress (3).

Our group previously observed that ovarian cancer xenografts bearing a defective *LKB1* gene fail to activate AMPK and become largely necrotic after administration of the anti-VEGF mAb A4.6.1, which caused a dramatic depletion of glucose and exhaustion of ATP levels in tumors (4). Moreover, in preclinical models, loss of LKB1/AMPK activation was associated with sustained cell proliferation and rapidly acquired resistance to antiangiogenic therapy, compared with tumors bearing a functional LKB1/AMPK pathway (5).

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

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Translational Relevance

Inhibition of angiogenesis is one of the strategies to improve the outcome of patients with advanced non–small cell lung cancer (aNSCLC), but no predictive markers are currently available to select patients who will benefit most from the treatment. LKB1 has a fundamental role in the cell response to stress conditions and may be involved in tumor cell adaptation to metabolic perturbations induced by angiogenesis inhibition. This retrospective analysis indicates LKB1 as a potential predictive marker of increased benefit from antiangiogenic treatment in aNSCLC. Differential impact on overall survival of the marker according to the administration of bevacizumab was highlighted.

On the basis of these findings, we speculated that LKB1 coordinates reduction of cell proliferation and suppression of anabolic metabolism in tumors treated with antiangiogenic drugs, putatively by acting in concert with additional regulators of cell-cycle progression, such as mTOR, AMPK, TP53, and p27Kip1 (6). Supporting this hypothesis, we recently reported that phosphorylated AMPK (p-AMPK) and ACC (p-ACC) levels are associated with favorable overall survival in patients with colorectal cancer treated with chemotherapy and bevacizumab (7). However, as in this retrospective study, all patients received bevacizumab, it was not possible to conclude about the predictive role of LKB1/AMPK in the response to bevacizumab.

These considerations motivated us to further investigate the role of LKB1 in the modulation of tumor response to anti-VEGF therapy. Non–small cell lung cancer (NSCLC) is a relevant malignancy for this study, as *LKB*1 genetic inactivation has been reported in approximately 30% of cases, especially lung adenocarcinoma (8), and LKB1 has a demonstrated role in lung tumorigenesis (9, 10). In previous studies, LKB1 expression was not prognostic in NSCLC (11), although some recent findings indicate that mutations in *LKB1* exons 1–2 could be associated with worse prognosis (12). In nonsquamous advanced NSCLC (aNSCLC), the association of platinum-based chemotherapy and bevacizumab is one of the options for first-line treatment in the absence of driver mutations, while more recently antiangiogenic drugs, including also ramucirumab and nintedanib, have been studied in combination with taxanes as second-line treatment (13–15). No molecular markers have been validated to individuate patients most likely to benefit from these antiangiogenic treatments.

Here, we present results of a retrospective clinical study aiming to assess the possible role of LKB1 as a biomarker of sensitivity to bevacizumab in aNSCLC.

Materials and Methods

Patients

We retrospectively selected 125 consecutive patients affected by aNSCLC and treated between 2011 and 2014 at four clinical centers in the North of Italy: Istituto Oncologico Veneto IOV-IRCCS (Padova, Italy), Azienda Ospedaliera Integrata (Verona, Italy), Azienda Ospedaliera Santa Maria degli Angeli (Pordenone, Italy), and Ospedale dell'Angelo (Mestre, Venezia, Italy). Clinical data and tissue samples were collected. The main inclusion criteria were histologically confirmed diagnosis of aNSCLC (stage IV or IIIB not subjected to radical-intent radiotherapy), first-line treatment with platinum-based chemotherapy or platinum-based chemotherapy plus bevacizumab followed by bevacizumab maintenance, available tumor tissue, and clinical follow-up. Tissue samples were therefore collected before chemotherapy or palliative radiotherapy. Study design and molecular analyses were presented and approved by the IOV Institutional Review Board. The informed consent form to be signed by patients for molecular analyses and data collection was approved by the Ethics Committee of each local center.

IHC

Five-micron-thick formalin-fixed, paraffin-embedded (FFPE) tumor samples were stained by IHC, using mouse anti-LKB1 polyclonal antibody (Ley37D/G6, Santa Cruz Biotechnology) and rabbit anti-pAMPK mAb (Thr172, Cell Signaling Technology), according to the manufacturer's instructions. Staining conditions for LKB1 and pAMPK were previously reported (4, 7). IHC was performed using a Leica Bond III Autostainer (Leica). IHC results were evaluated by one experienced pathologist with no prior knowledge of clinical data. Immunoreactivity was scored semiquantitatively considering the intensity for LKB1: intensity was scored as 0–3 (no staining 0, light staining 1, moderate staining 2, and strong staining 3). For statistical analysis, samples were recoded in two class: LKB1 negative/weak staining as negative and moderate/intense as positive.

Genetic analyses

When tissue was available, genomic DNA (gDNA) was extracted, and *LKB1* genotype was studied by Sanger sequencing. Tumor areas were identified by a pathologist, and the sample was subjected to macrodissection to obtain a tumor fraction >50% for subsequent molecular studies. gDNA was extracted by QIAamp DNA Micro Kit (Qiagen), according to the manufacturer's instructions. A total of 50 ng of gDNA was used for PCR to amplify all exons of *LKB1* gene, using primers listed in Supplementary Table S1. Exons were amplified through Taq enzyme activation for 10 minutes at 95°C and 45 cycles, composed of 1 minute at 95°C, 1.5 minutes at 60°C for exon 3 or at 62°C for exons 1, 2, 4–9, and 2 minutes at 72°C, followed by 10 minutes at 72°C.

EGFR and *KRAS* status were determined either by Sanger sequencing or by pyrosequencing. To this end, exon 2–3 of *KRAS* gene and exons 18–21 of *EGFR* gene were amplified through PCR using primers, sequences, and amplification conditions previously reported (16). Pyrosequencing analysis for *KRAS and EGFR* mutations was performed using the anti-EGFR mAb response (*KRAS* status) and EGFR sensitivity (*EGFR* status) kit (Diatech Pharmacogenetics, Iesi, Italy) and run on PyroMark Q96 ID. ALK status was assessed by FISH, according to routine procedures.

Patient-derived xenograft studies

Procedures involving animals and their care conformed to the institutional guidelines that comply with National and International laws and policies (EEC Council Directive 86/609, OJ L 358, 12 December, 1987). Patient-derived xenografts (PDXs) were generated at Istituto Nazionale Tumori - INT (Milan, Italy) by subcutaneous implantation in SCID mice of minced tumor samples obtained from patients with lung adenocarcinoma, as detailed elsewhere (17).

Anti-human VEGF mAb (bevacizumab) was administered intraperitoneally at 100 μg/dose biweekly, and mice were sacrificed 48 hours after the last dose. Control mice received intraperitoneal injections of PBS.

Quantification of necrosis in tumor sections was carried out by calculating the percentage of the necrotic area in the entire tumor section by using a light microscope equipped with digital camera and MODEL software (Leica Microsystems). Tumor vessels were labeled with rat anti-CD31 mAb (Becton Dickinson) followed by staining with a goat anti-rat 546 secondary antibody (Invitrogen) and quantification of microvessel density (MVD) as detailed elsewhere (5). For each sample, six to nine fields (×200) were scored using an integrated grid.

Statistical analysis

The Clinical Trials and Biostatistics Unit at IOV managed data collection and their analysis. Continuous and ordinal variables were summarized as median and interquartile range (IQ), categorical variables were reported as counts and percentages. The relationship between variables was assessed with χ^2 or Fisher exact test, as appropriate. Outcome was analyzed in terms of progression-free survival (PFS) and overall survival (OS). PFS was calculated from the date of diagnosis to relapse or death for any cause. OS was measured from the date of diagnosis to death for any reason. Patients who did not develop the event at the end of the study were censored at the date of last observation. The survival probability was computed using the Kaplan–Meier method and heterogeneity in survival rates among strata was assessed using the log-rank test. The 1-year PFS and OS were reported with their 95% confidence intervals (CIs). Multivariable analysis was conducted using the Cox proportional hazards regression method to identify factors present at diagnosis that were associated with the risk of death. A stepwise variable selection procedure was applied to identify a subset of covariates for the final model, considering in a first step all variables with a P value of at least 0.05 at univariate analysis and evaluating each of the nonsignificant variables in a second step, also testing the interaction terms. After checking the proportional hazards assumption, HRs with the 95% CIs, calculated according to the Wald method, were computed.

Results obtained from PDX analysis were expressed as mean ± SD. Statistical comparison between two sets of data was done by using either the unpaired Student t test (two-tailed) or the Mann–Whitney test (two-tailed), depending on the distribution of values. Differences were considered statistically significant at $P < 0.05$.

Statistical analyses were performed using the SAS statistical package (SAS, rel. 9.4; SAS Institute Inc.).

Results

Patient characteristics and outcome of the study population

Clinical data of the 125 patients included in the study are summarized in Table 1. Median age of the study population was 64 years, with a prevalence of male (68%) and current (43%) or former (41%) smokers. The majority of cases were diagnosed as lung adenocarcinoma (78%), and only six (5%) patients had Eastern Cooperative Oncology Group (ECOG) performance status (PS) superior to 1. Forty-two patients (34%) received first-line treatment for advanced disease with platinum-based chemotherapy for four cycles, followed by bevacizumab as maintenance treatment. No patient received any other antiangiogenic drug as a further line of systemic treatment. Details about chemotherapy treatment are depicted in Table 1. Clinical features of 98 patients with samples available for biomarker analysis were similar to the overall study population (data not shown).

Table 1. Clinical–pathologic characteristics of patients

	All patients ($n = 125$) (%)	Patients with LKB1 data ($n = 98$) (%)
Gender		
Female	40 (32.0)	30 (30.6)
Male	85 (68.0)	68 (69.4)
Age at diagnosis		
Median (IQ range)	64.0 (58.0–71.0)	64.0 (56.0–70.0)
≤70	90 (72.0)	74 (75.5)
>70	35 (28.0)	24 (24.5)
Histology		
Adenocarcinoma	98 (78.4)	76 (77.5)
Squamous cell carcinoma	15 (12.0)	10 (10.2)
Large cell carcinoma	4 (3.2)	4 (4.1)
Other/NOS	8 (6.4)	8 (8.2)
PS (ECOG)		
0	46 (36.8)	36 (36.7)
1	73 (58.4)	56 (57.1)
2	6 (4.8)	6 (6.1)
Smoking status		
Current	54 (43.2)	40 (40.8)
Former	51 (40.8)	42 (42.9)
Never	20 (16.0)	16 (16.3)
First-line treatment		
Platinum doublet plus bevacizumab	42 (33.6)	39 (39.8)
Cisplatin-gemcitabine	*21 (50.0)*	*19 (48.7)*
Carboplatin-paclitaxel	*15 (35.7)*	*14 (35.9)*
Cisplatin-pemetrexed	*5 (11.9)*	*5 (12.8)*
Carboplatin-gemcitabine	*1 (2.4)*	*1 (2.6)*
Platinum-doublet	83 (66.4)	59 (60.2)
Carboplatin/gemcitabine	*24 (28.9)*	*18 (30.5)*
Cisplatin-gemcitabine	*5 (6.0)*	*4 (6.8)*
Carboplatin/pemetrexed	*41 (49.4)*	*28 (47.4)*
Cisplatin/pemetrexed	*12 (14.4)*	*8 (13.5)*
Carboplatin/paclitaxel	*1 (1.2)*	*1 (1.7)*

After a median follow-up for living patients of 35.4 (96% CI, 6.0–64.9) months, the median OS of the study population was 11.7 (95% CI, 9.1–15.3) months, whereas median PFS was 6.7 (95% CI, 5.7–7.2) months (data not shown). Response rate (RR) according to the RECIST v1.1 criteria was 43%.

Multivariable analysis showed that ECOG PS <1 and nonsquamous histology appeared to be positive prognostic factors, affecting OS and PFS with statistical significance (Supplementary Tables S2A–S2D).

LKB1 expression and correlation with genetic markers

Because of inadequate representation of tumor or loss of tissue section during IHC analysis, the number of cases with evaluable LKB1 status was 98, including 59 samples from patients treated with chemotherapy and 39 from patients treated with chemotherapy plus bevacizumab.

We initially investigated the correlation between LKB1 protein expression and the mutational status of *LKB1* in a cohort of 18 samples for which both tissue slides and DNA samples were available. Statistical analysis revealed significant association between LKB1 status by IHC and results of genetic analysis (Fisher exact test, $P = 7.145E{-}05$): in fact, all samples mutated in *LKB1* (5, 27%) were negative by IHC staining. Moreover, all but two LKB1-positive samples by IHC had wild-type *LKB1* sequence

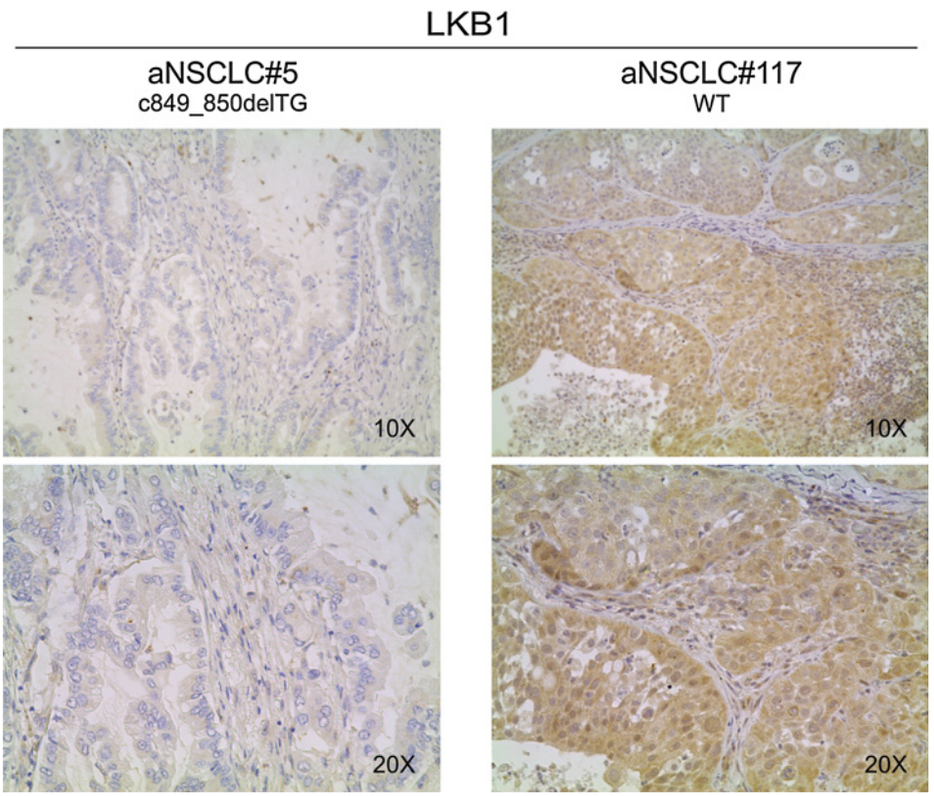

Figure 1.
Representative patterns of LKB1 expression in advanced non–small cell lung cancer (aNSCLC) samples. Microphotographs of one LKB1-negative (#5) and one LKB1-positive (#117) tumor sample by IHC analysis; ×100 and ×200 magnifications are shown. Genetic analysis disclosed LKB1-mutated and wild-type sequences in sample #5 and #117, respectively.

(Supplementary Table S3). In two samples, LKB1 IHC was negative despite these samples bearing a wild-type *LKB1* sequence. Epigenetic regulation of LKB1 or deletions of the *LKB1* gene without mutations could possibly explain this discrepancy. These results indicate that IHC will likely score negative in a larger proportion of tumor samples, compared with sequence analysis. Because of the limited availability of genomic DNA for sequencing, in most samples we could investigate LKB1 status only by IHC and tested its influence on outcome.

Sixty-five (66%) samples expressed negative/weak levels of LKB1 protein and were classified as LKB1 negative ($LKB1^-$), whereas 33 (34%) samples had moderate/intense staining and were classified as LKB1 positive ($LKB1^+$). As expected, LKB1 was mainly localized in the cytoplasm of tumor cells (Fig. 1). We found no association between protein expression and main clinicopathologic features including histology, PS, sex, and smoking status (data not shown).

The samples had been routinely evaluated for the presence of *EGFR*-sensitizing mutations, and five cases were positive for *EGFR* mutations: three patients carried exon 21 mutations, and in two cases, exon 19 deletions were detected. Two of five *EGFR*-mutated cases were evaluable for LKB1 expression; they were all categorized as positive and received bevacizumab.

ALK rearrangements were detected in six patients, five of them were evaluable for LKB1 IHC scoring assignment. At the time of data analysis, three of them had received treatment with crizotinib. No *ROS1* rearrangements were detected in the study population. All five *ALK*-positive cases expressed high/moderate levels of LKB1, and none of them received bevacizumab in a first-line setting.

KRAS status could be investigated in 62 samples. We detected *KRAS* mutations in 20 samples (30%). Details of the somatic mutations found are reported in Supplementary Table S4. Although in other studies, *LKB1* mutations were enriched in *KRAS*-mutated tumor samples (18, 19), in our cohort, no association between *KRAS* status and LKB1 expression was found (χ^2 test, $P = 0.3989$; data not shown).

LKB1 expression and outcome

In the study population with available IHC results (98 patients), LKB1 protein expression had no impact on outcome. Kaplan–Meier curves of OS and PFS are shown in Supplementary Fig. S1.

Gender, age category (≤70, >70), histology (squamous vs. nonsquamous cell carcinoma), PS (0 vs. 1/2/3), smoking status (current vs. never/former), treatment, and LKB1 expression were investigated in univariate and multivariable analysis for their impact on PFS and OS. Moreover, we also considered the interaction between the biomarker and antiangiogenic treatment in the Cox multivariable model. Table 2 shows the variables included in the final model: PS, LKB1 expression, treatment, and the interaction term. The LKB1 status is a significant modifier of the effect of treatment ($P = 0.0199$). The HR for patients with LKB1 moderate/intense who did not receive bevacizumab was 3.84 (95% CI, 1.56–9.48) compared with those that received chemotherapy plus bevacizumab ($P = 0.0035$). Differently, the HR for patients with LKB1-negative/weak treated with first-line platinum-based chemotherapy without bevacizumab was 1.12 (95% CI, 0.64–1.97) compared with those who received bevacizumab ($P = 0.6803$). The different impact of antiangiogenic treatment on the outcome for the two levels of expression of the

Table 2. Cox regression analysis for overall survival (multivariable analysis) in patients analyzed for LKB1 protein expression

	Patients ($N = 98$)	Events ($N = 81$)	HR (95% CI)	*P*
PS (ECOG)				0.0315
0	36	26	1[a]	
1, 2	62	55	1.76 (1.05–2.95)	
First-line treatment				0.6803
LKB1 status IHC				0.0297
LKB1 negative/weak				
No bevacizumab			1.12 (0.64–1.97)	0.6803
vs. bevacizumab				
LKB1 moderate/intense				
No bevacizumab			3.84 (1.56–9.48)	0.0035
vs. bevacizumab				

[a]Reference category.

biomarker was also highlighted in the Kaplan–Meier curves. We noticed that among patients expressing negative/weak levels of the marker, no benefit was observed following the administration of antiangiogenic treatment in addition to platinum-based chemotherapy (Fig. 2C). On the contrary, Fig. 2D shows statistically different outcome in terms of OS according to the administration of bevacizumab among patients expressing moderate/intense LKB1 ($P = 0.0004$).

In another way, when we considered patients with aNSCLC treated with first-line platinum-based chemotherapy without bevacizumab, LKB1 protein expression affected OS with statistical significance. In this treatment cohort ($n = 59$), median OS of LKB1-negative patients was 9.1 (95% CI, 6.4–15.2) months, whereas median OS was 4.8 (95% CI, 3.7–6.2) months in the presence of LKB1 ($P = 0.01$, Fig. 2A). One-year OS was 37.5 (95% CI, 22.9–52.1) for patients with negative/weak LKB1 and 15.8 (95% CI, 3.9–34.9) for patients with moderate/intense level of LKB1 staining ($P = 0.01$). On the contrary, among 39 patients treated with chemotherapy plus bevacizumab, median OS of patients expressing moderate/intense LKB1 was 30.5 (95% CI, 5.7–ND) months, whereas patients with negative/weak biomarker expression had a median OS of 12.6 (95 CI, 8.3–27.9) months (Fig. 2B). In this group, 1-year OS was 56.0 (95% CI, 34.8–72.7) for patients with negative/weak LKB1 and 78.6 (95% CI, 47.2–92.5) for patients with moderate/intense level of LKB1. The difference between the two groups of patients is not statistically significant ($P = 0.10$), but the comparison of Kaplan–Meier curves depicted in Fig. 2B shows the opposite trend with respect to the biomarker effect observed among patients treated without bevacizumab and the curves become distinctly different at 6 months. Finally, to rule out a potential confounding effect of histology, we performed analysis excluding patients with squamous histology, obtaining comparable results, as shown in Supplementary Fig. S2.

LKB1 modulates AMPK activation in aNSCLC

Speculatively, one possible mechanism downstream of LKB1 accounting for the positive outcome of patients with aNSCLC treated with bevacizumab could be the activation of AMPK, an established LKB1 target known to reduce metabolic demand and cell proliferation under stress conditions (20). Intriguingly, we recently reported a positive prognostic role of AMPK activation in patients with advanced stage colorectal cancer treated with chemotherapy plus bevacizumab (7). To test this hypothesis, we investigated expression of the phosphorylated form of AMPK (pAMPK, a marker of AMPK activation) in tumor sections from patients with aNSCLC. Because of the limited amount of tissue available, analysis was limited to 30 tumor samples, including 19 samples disclosing negative/weak levels of LKB1 and 11 samples with moderate/intense levels of LKB1 expression.

Statistical analysis revealed a significant association between LKB1 status and pAMPK expression (Fisher exact test, $P = 0.0465$). Two representative cases are depicted in Supplementary Fig. S3.

To further investigate the impact of LKB1 on the response to anti-VEGF therapy, we generated PDXs from patients with NSCLC with characterized *LKB1* genetic status. By comparing LKB1 and pAMPK expression in the PDX66 and PDX111 models, representative of *LKB1* mutated and wild-type tumors, respectively, we observed that *LKB1* mutation was associated with a lack of AMPK activation in tumors (Fig. 3A). These results were confirmed in four additional PDX models (not shown). Moreover, we analyzed the effects of bevacizumab in the PDX66 and PDX111 models. We found that MVD was significantly reduced in both models, in line with the antiangiogenic activity of this drug, but LKB1-deficient tumors developed significantly larger necrotic areas, compared to LKB1-proficient tumors (Fig. 3B). These results suggest an impaired capacity of LKB1-deficient tumors to adapt to the metabolic stress caused by the anti-VEGF drug, likely due to the lack of AMPK activation and consistent with our previous observations (4).

Discussion

Medical treatment of lung cancer is prevalently not guided by the molecular tumor characteristics, both when using chemotherapy and when using antiangiogenic drugs like bevacizumab (21–24).

Several efforts have been made to identify predictive biomarkers of sensitivity to bevacizumab, but none of them has been validated in clinical practice (25). This is likely related to the complexity of antiangiogenic effects both on tumor cells and on microenvironment (24). Remarkably, the possibility that a predictive biomarker of response to antiangiogenic therapy could be restricted to a specific tumor type has not been considered so far.

Our approach differs from previously published studies on biomarkers in patients treated with antiangiogenics as it does not analyze factors directly involved in angiogenesis. On the contrary, we focus on the capacity of tumor cells to sense and adapt to metabolic perturbations induced by antiangiogenic treatment. This process has been initially described and characterized in preclinical models, where it seems to be mediated by the LKB1/AMPK pathway (4, 5, 26). According to our preclinical findings (4, 5) and the known role of LKB1/AMPK in tumor cell response to stress conditions (27–29), we hypothesize that hypoxia and nutrition deprivation caused by angiogenesis inhibition activate LKB1/AMPK and induce metabolic rewiring leading to reduced cell growth. We speculate that impairment of this pathway, as would happen in NSCLC lacking LKB1 expression, will affect response to antiangiogenic drugs, possibly by favoring tumor necrosis, as we found in PDX models (Fig. 3). Although the association between tumor necrosis and outcome of antiangiogenic therapy is not firmly established in patients, in preclinical models, necrosis often leads to increased recruitment tumor of macrophages and other specialized subsets of myeloid cells, which can promote tumor angiogenesis and lead to escape from VEGF blockade and tumor progression (30).

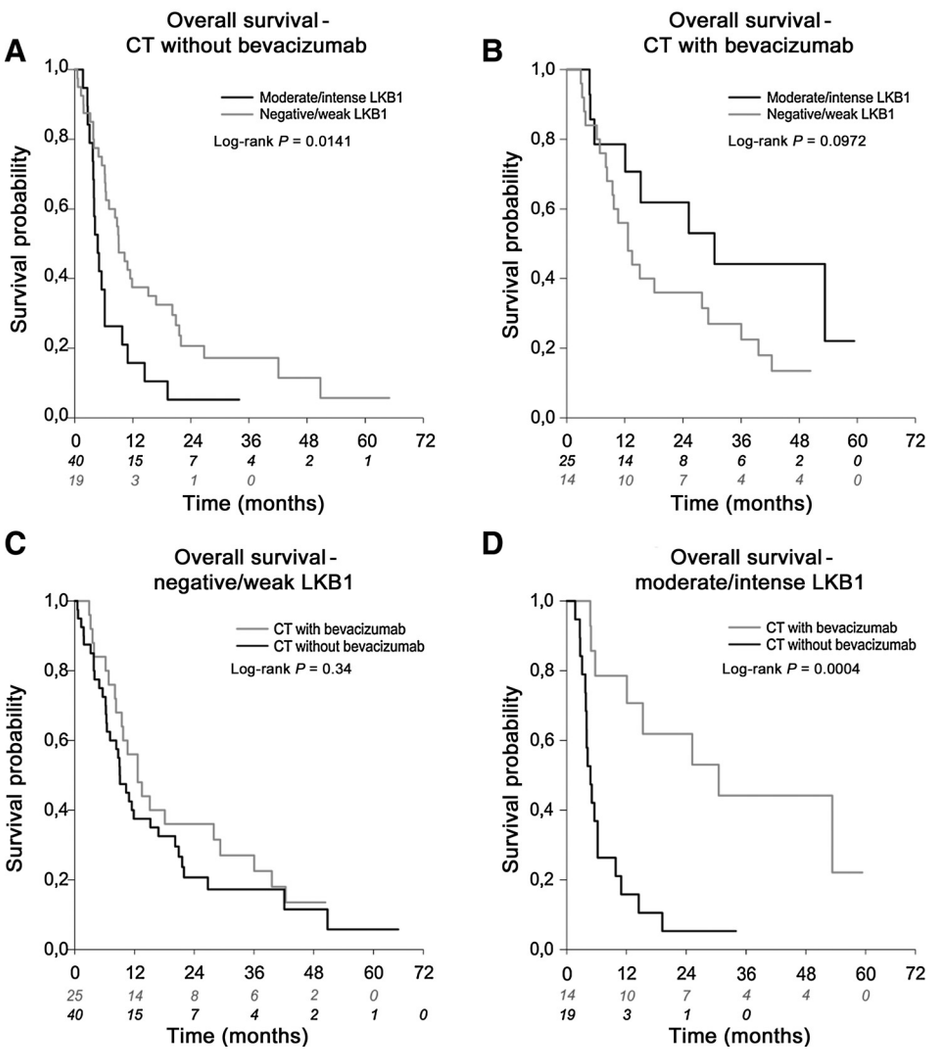

Figure 2.
Differential impact of bevacizumab on the outcome according to LKB1 expression. Log-rank test comparing overall survival of patients treated with first-line platinum-based chemotherapy (CT) with or without bevacizumab according to LKB1 protein expression levels (moderate-high vs. weak-null). Among patients treated only with platinum-based chemotherapy, median OS was 9.1 (95% CI, 6.4–15.2) months when LKB1 was negative, 4.8 (95% CI, 3.7–6.2) months when the marker was expressed, $P = 0.014$ (**A**). Among patients treated with platinum-based chemotherapy plus bevacizumab, median OS was 12.6 (95% CI, 8.3–27.9) months in the presence of negative LKB1 and 30.5 (95% CI, 5.7–ND) months when LKB1 was positive ($P =$ ns, **B**). **C** and **D,** Log-rank test comparing overall survival of patients treated with or without antiangiogenic drug in the presence of negative LKB1 or in the presence of positive LKB1 expression, respectively. Among patients expressing moderate/high levels of LKB1 protein, median OS was 30.5 (95% CI, 5.7–ND) when treated with chemotherapy plus bevacizumab, 4.8 (3.7–7.2) months for patients treated with chemotherapy only ($P = 0.0004$).

In this pilot study, we investigated the prognostic and predictive role of LKB1 in tumor samples from patients treated with first-line platinum-based chemotherapy with or without bevacizumab. While LKB1 had no prognostic value (Supplementary Fig. S1), expression of LKB1 had statistically significant interactions with treatment and identified patients who are more likely to benefit from the bevacizumab- chemotherapy combination, as compared to standard chemotherapy (Figure 2, Table 2).

Intriguingly, patients treated with first-line platinum-based chemotherapy without the antiangiogenic drug had improved outcome when LKB1 was not expressed. This finding, which is at odds with the observations in the patients who received bevacizumab, may be explained by considering the well-established role of LKB1 as a genomic sensor participating in the DNA damage response triggered by oxygen radicals (31–33). In our experience, the lack of functional LKB1 renders tumor cells more vulnerable to DNA-damaging agents capable of inducing oxidative stress *in vitro*, such as platinum (data not shown).

The main limit of the study is its retrospective nature. We collected clinical data and pathologic samples from patients treated with or without bevacizumab according to clinical practice. In the overall study population, the administration of bevacizumab seems to slightly affect the outcome of patients (Supplementary Fig. S4), but this is likely to be at least partially due to clinical selection. Together with the impact of the marker in the overall study population, the results obtained in the control group of patients treated without bevacizumab confirmed that the potential role of the marker is strictly related to the treatment administrated. The differential effect of the biomarker was highlighted when analyzing OS results. PFS analysis is not shown but the retrospective nature of the analysis, the lack of detailed standardization in radiologic evaluation timing, and the potential impact of antiangiogenic treatment on postprogression survival are elements that may explain the lack of predictive significant impact of the biomarker on PFS. On the other hand, the effect on OS is not likely to be affected by postprogression treatments that were homogeneous in patients treated with or without bevacizumab in first-line. The impact of second and third lines on OS is also limited by the rarity of targetable genetic alterations in the study population (detailed in the Results section).

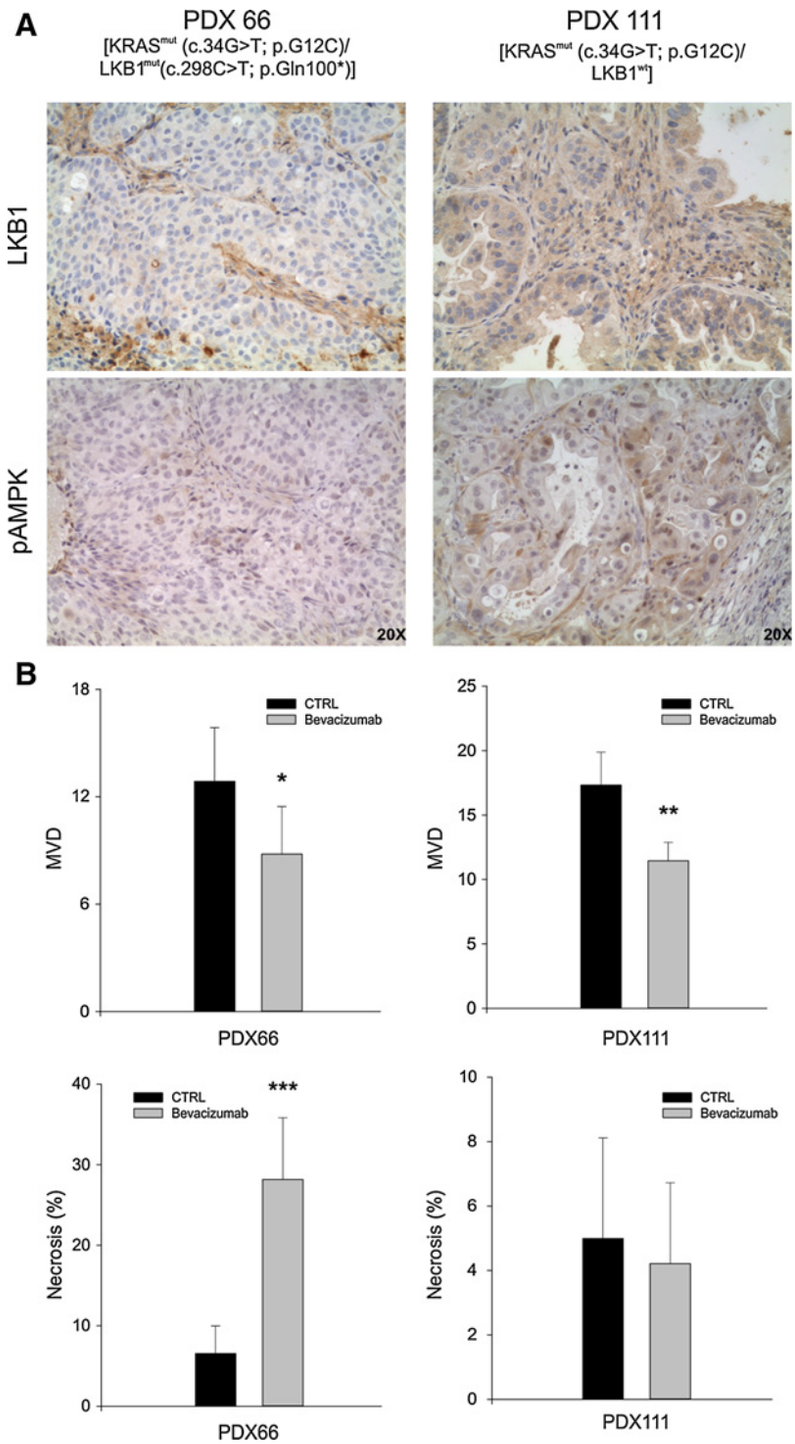

Figure 3.

Anti-VEGF therapy increases necrosis of LKB1^{-} PDXs. **A,** LKB1 and phosphorylated AMPK (pAMPK) expression in tumor samples from xenografts derived from patients with aNSCLC with known *LKB1* genetic status. **B,** Analysis of vascularization and necrosis in PDX66 and PDX111 tumors after anti-VEGF mAb (100 μg/dose, administered every 2 days, for 4 weeks). Top, microvessel density (MVD) values, calculated following staining of tumor sections with the endothelial cell marker anti-CD31. Bottom, analysis of necrosis in tumor samples. Columns indicate quantitative analysis in $n = 5$ different tumors per group; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

In our study population, more than one-half of tumor samples were classified as negative/weak by IHC, and this is substantially higher than the expected *LKB1* gene–mutated fraction, based on previous studies (8, 34). However, it should be considered that, given the intrinsic difficulties of genetic analyses of a gene whose mutations are variable and widespread (8, 10), the frequency of *LKB1* mutations may be underestimated. Moreover, loss of heterozygosity, homozygous deletion of the chromosome 19p at the *LKB1* locus, or *LKB1* promoter hypermethylation represent known alternative mechanisms of tumor suppressor gene inactivation (8, 35, 36), which may contribute to explain these discrepancies. In our analysis, we arbitrarily chose to aggregate LKB1-negative and weakly positive samples in one category, so to also take into account these additional mechanisms of LKB1 inactivation which could attenuate but likely not completely abrogate the expression of LKB1 protein. This decision was grounded on

the hypothesis that very low protein levels would impair LKB1 biological activity under metabolic stress conditions, similar to complete loss of LKB1. In future studies, it will certainly be important to determine whether assessment of LKB1 status by IHC or sequencing are equivalent in terms of predictive value for response to bevacizumab and to further investigate the most appropriate threshold for clinical interpretation of LKB1 IHC results. In addition, it will be interesting to correlate LKB1 status to mutations in other genes involved in the regulation of metabolism, including TP53 and KRAS, frequently mutated in NSCLC and potentially able to affect the outcome (37, 38).

Potential applications of the findings, specifically in the field of lung cancer treatment, include improved selection of patients for first-line treatment, but also optimization of patients for second-line treatment. As a matter of fact, recently, the therapeutic options for second-line treatment of nononcogene addicted aNSCLCs have greatly increased and the addition of antiangiogenic drugs including ramucirumab, nintedanib, and bevacizumab to taxanes has been demonstrated to have a role in this clinical setting (13–15, 39–42).

Finally, as LKB1 is commonly inactivated in lung and cervical cancer but not in other malignancies, our results underscore the possibility that so far vanishing biomarkers of response to antiangiogenic drugs might not be universal but rather restricted to specific tumor types.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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