Published OnlineFirst October 15, 2014; DOI: 10.1158/1078-0432.CCR-14-0506

Biology of Human Tumors

Clinical Cancer Research

RhoB Determines Tumor Aggressiveness in a Murine EGFR^{L858R}-Induced Adenocarcinoma Model and Is a Potential Prognostic Biomarker for Lepidic Lung Cancer

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Abstract

Purpose: A crucial event in lung adenocarcinoma progression is the switch from an aerogenous spread toward an infiltrating tumor. Loss of RhoB expression has been suggested to be critical for lung cancer invasion. Here, we tested RhoB expression as a prognostic biomarker in non–small cell lung cancer (NSCLC) with a special focus on lepidic pattern.

Experimental Design: We analyzed RhoB expression using both IHC and RT-qPCR in two series of operated patients ($n = 100$ and 48, respectively) and in a series of advanced lepidic adenocarcinoma ($n = 31$) from different hospitals. Next, we examined the role of RhoB in lung cancer progression in transgenic mice that express inducible *EGFR*^{L858R} crossed with *Rhob* null mice.

Results: We identified that loss of RhoB expression was strongly associated with worse survival ($P = 0.0001$) and progression-free survival ($P < 0.001$) in the first series. We then confirmed these results after multivariate analyses of the second series. In the series of adenocarcinoma with lepidic features issued from a clinical trial (IFCT-0401), we showed that loss of RhoB expression was associated with higher aggressiveness of stage IV. Finally, we showed that *EGFR*^{L858R}/*Rhob*$^{+/+}$ mice developed mainly diffuse lung tumors with a lepidic pattern, whereas *EGFR*^{L858R}/*Rhob*$^{+/-}$ and *EGFR*^{L858R}/*Rhob*$^{-/-}$ developed a greater number of tumors, and aggressive adenocarcinomas with invasive properties.

Conclusions: We showed that RhoB is not only a strong prognostic factor in NSCLC but it is also critical for the acquisition of an aggressive phenotype of adenocarcinoma. *Clin Cancer Res; 20(24); 6541–50. ©2014 AACR.*

Introduction

Lung cancer remains the leading cause of cancer-related death worldwide (1). Surgery is the primary treatment modality for patients with early-stage operable non–small cell lung cancer (NSCLC) associated with adjuvant chemotherapy for stages II and III patients (2). Nevertheless, approximately half of these patients eventually experience a relapse after resection. Currently, a patient's tumor–node–metastases (TNM) stage is the main clinical variable that provides prognostic information. However, the information on TNM (or the specific tumor histopathologic subtype) does not predict which patients within a TNM stage category will derive increased-survival benefit from surgery and adjuvant chemotherapy. There is a need for additional biomarkers to help clinicians better predict tumor prognostics and to optimize postoperative strategies.

Research into the molecular basis of lung cancer has revealed insights into various critical pathways that are deregulated in lung tumorigenesis and are associated with the clinical course of lung cancer (3) Among them, markers associated with DNA repair (ERCC1 and RRM1), cell cycle (CDK4, CDK6, and cyclin D1), apoptosis (caspase-3, Bcl-2, and p53), and major oncogenes (KRas and EGFR) have been reported. More recently, gene signatures, including a combination of mRNA (4) or of microRNA (5), have been shown to improve prediction of lung cancer prognostic but are difficult to perform routinely. We (6) and others (7) have

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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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doi: 10.1158/1078-0432.CCR-14-0506

Published OnlineFirst October 15, 2014; DOI: 10.1158/1078-0432.CCR-14-0506

Translational Relevance

We aimed to decipher the potential role of RhoB, a small Rho-GTPase and a tumor suppressor, as a prognostic marker in lung cancer, with a focus on adenocarcinoma with lepidic features. On the basis of three independent series of patients, we identified that loss of RhoB expression was strongly associated with a worse outcome and particularly with higher aggressiveness in adenocarcinoma with lepidic component. We confirmed our findings in a mouse model of *EGFR*-driven adenocarcinoma with a lepidic pattern crossed with RhoB knockout (KO) mice. We conclude that the RhoB status may help define the prognosis of adenocarcinoma with lepidic features.

reported that RhoB is involved in lung carcinogenesis and might be of interest as a prognostic marker for lung cancer.

RhoB belongs to the Rho-GTPase family involved in many cell functions, such as survival, migration, and angiogenesis and is implicated in tumorigenesis and metastasis (8–10) RhoB is likely acting as a tumor suppressor (8) demonstrated by four lines of evidence: ectopic expression of RhoB in cancer cell lines suppresses tumorigenesis (6, 11–13), *Rhob* null mice show higher frequency of tumor formation (14), RhoB expression is frequently down regulated in cancer tumors (6, 7, 15, 16), and *in vivo* restoration of RhoB expression leads to tumor regression (17). Interestingly, we reported that RhoB protein expression decreases as lung cancer progress (6) and that RhoB loss relates to the acquisition of invasiveness mediated by the PI3K/AKT pathway (18), positioning RhoB as a candidate gene for lung tumor progression. This is particularly obvious for adenocarcinoma that has lepidic features (formerly "bronchiolo-alveolar carcinoma") for which the key step of progression is migration and invasion through the basal membrane (19). These lepidic adenocarcinomas generally express RhoB whereas invasive carcinomas do not (6).

We tested the hypothesis that loss of RhoB expression was associated with a worse prognosis in lung cancer with a particular focus on adenocarcinoma with lepidic features. We analyzed RhoB expression in three independent series of patients issued from two different hospitals and a clinical trial (IFCT-0401), of which one issued from a clinical trial dedicated to lepidic adenocarcinoma. We then used a transgenic mouse model of an activated mutant of EGFR-dependent lung cancer (20), to demonstrate the critical role of RhoB in lung cancer progression reinforcing its relevance as lung cancer prognostic biomarker.

Materials and Methods

Patients and ethical considerations

Patients were issued from three independent series. The first one included patients ($n = 100$) who had undergone surgery in the Thoracic Oncology Department (Toulouse University Hospital, France), and whose biopsy samples were analyzed in the Pathology Department. The details of these patients have been previously described (21) and are also provided in Supplementary Table S1. Immediately after surgery, the lung tumors were frozen and stored at −80°C. Patients who had received any radiotherapy or chemotherapy treatment before surgery were excluded from the analyses. The second series included patients ($n = 48$) who had undergone surgery in the Thoracic Surgery Department (Strasbourg University Hospital, Strasbourg, France) for stages I to III lung cancer. Details of this series are shown in Supplementary Table S1. Immediately after surgery, biopsies from lung tumors were frozen and stored at −80°C for further analyses in the Biological Resource Centre. The third series included samples from 31 patients out of the IFCT-0401 study (NCT00198380; ref. 22). This phase II trial was evaluating the efficacy and safety of gefitinib as a first-line treatment for patients with advanced bronchiolo-alveolar carcinoma (now called adenocarcinoma with lepidic component). All paraffin-embedded samples were collected and centralized in Tenon Hospital (Paris, France) for subsequent IHC analysis using the same antibody and procedures as in series 1. Details of this series are shown in Supplementary Table S1.

For all patients, diagnoses were assessed by a lung cancer pathologist by applying the latest WHO classification (23); the stage was assigned according to the TNM classification (24, 25). Patients were treated and followed-up in each respective institution.

All patients signed an informed consent permitting analyses of tissues. All informed consents were collected and stored in the respective pathology departments. This study was approved by the Ethics of Human Research Committee at the Pathology Department, Toulouse Hospital (Toulouse, France). For the series 3, which is ancillary of the IFCT04-01 trial, all patients signed an informed consent allowing analysis on their tumor.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were used for the IHC procedures for patients from series 1 to 3. After rehydratation, deparaffinized sections were pretreated by microwave epitope retrieval. Endogenous peroxidase activity was quenched before a blocking step of nonspecific binding sites performed in 5% goat normal serum in TBS-Tween (Wash buffer; Dako). Sections were incubated with a RhoB monoclonal antibody (C-5; Santa Cruz Biotechnology Inc.; 1:75) overnight at 4°C. After washes, sections were then incubated with the polyclonal Envision complex (DAKO) and the peroxidase activity was demonstrated by diaminobenzidine. Sections were eventually washed in water, lightly counterstained with hematoxylin, dehydrated and mounted in DPX. Tissues expressing different levels of RhoB were included in each immunohistochemical run to unify any possible discordance in intensity. Two observers (I. Rouquette and I. Raymond-Letron), blinded to the patients' status, independently evaluated the extension and intensity of the staining. For RhoB, the extension was scored

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as the percentage of positive cells (0%–100%), and the intensity of staining was compared with a known external positive control (0, none; 1+, mild; 2+, moderate; 3+, intense). Any discordant independent readings were resolved by simultaneous reviews by both observers.

RT-qPCR

Gene-expression profiling was performed on RNA isolated from the human tumor tissue specimens in series 2, then reverse transcripted using the iScript cDNA Synthesis Kit, following the manufacturer's instructions (Bio-Rad Laboratories). Quantitative real-time PCR of RhoB and PPIA mRNA was performed with a CFX96 detection system (Bio-Rad), using iQ SYBR Green Supermix (Bio-Rad) and the sequence-specific primers for RhoB (forward, 5-TTGTGCCTGTCCTAGAAGTG-3′; reverse, 5-CAAGTGTGGTCAGAATGCTAC-3′) and PPIA (Hs_PPIA_3_SG QuantiTect primer assay; Qiagen). The relative RhoB mRNA expression was calculated according to the $2^{-\Delta C_q}$ method, normalized to the PPIA mRNA levels.

Molecular biology

KRas and EGFR mutations were analyzed using HRM (high-resolution melting) analysis followed by direct sequencing after microdissection of tumor-rich areas and genomic DNA extraction. All samples were sequenced to detect *EGFR* mutations in exons 18, 19, 20, and 21, and KRas mutation in exons 1 and 2. The sequenced data were visualized using Seqscape v5.2 (Applied Biosystems) and were independently analyzed by two scientists. HRMs results were compared with sequencing results to validate the HRM analysis.

Mouse experiments

Lung-specific tetracycline-inducible human $EGFR^{L858R}$ bi-transgenic mice (CCSP-rtTA; tetO-$EGFR^{L858R}$) were crossed into *Rhob* null ($Rhob^{-/-}$), heterozygous ($Rhob^{+/-}$), and wild-type (WT; $Rhob^{+/+}$) strains. Both mouse strains have been described previously (14, 20). Approval from the Claudius Regaud Institute Animal Ethics Committee (# ICR-2009-021) was obtained for the use of mice in this animal model and for the study protocols. All procedures involving animals and their care conformed to institutional guidelines for the use of animals in biomedical research.

Five- to 6-week-old mice were fed *ad libitum* with food pellets that contained doxycycline (1 g/kg) for 8 weeks, and were then sacrificed by cervical dislocation. The lungs were excised and inflated via intratracheal infusion with 4% buffered formaldehyde, and immersion fixed for 24 hours at room temperature before dehydration and paraffin embedding. Paraffin sections (4 μm) were used for hematoxylin and eosin (H&E) staining and IHC using standard procedures. The proliferating index was determined by Ki67 staining (SP6; Thermo Fisher Scientific), and the lung vessels were stained with CD34 antibody (MEC 14.7 AbD; Serotec). Transgene expression was evaluated with an anti-$EGFR^{L858R}$ antibody (Cell Signaling Technology). Digital slides were blind evaluated by two operators of whom a veterinary pathologist, according to reference articles (20, 26). Detailed methods for tumor grading and quantification of the tumor/lung-area ratio are described in Supplementary Materials.

Statistical analysis

Continuous variables are presented as their means (SDs) or their medians (interquartile range) according to their distributions. The χ^2 or Fisher exact test was used to compare categorical variables, and the Student *t* test, variance analysis or a nonparametric test was used for continuous variables. Paired measurements of continuous data were compared using the Wilcoxon matched-pairs signed-rank test.

Overall survival (OS) was defined as the time between diagnosis and death. Patients alive were censored at the last available time the subject was known to be alive. For series 2, progression-free survival (PFS) was calculated from the date of diagnosis to the date of progression or to the date of last follow-up. These endpoints were analyzed using the Kaplan–Meier method, the log-rank test, and Cox models. To address confounding, a multivariate Cox regression model was performed. The initial model included all variables that were associated to progression or death in a bivariate analysis with a conservative *P* value of 0.2. We used a backwards method, controlling for confounders at each step to obtain a reduced model. Variables found to be statistically associated with a *P* value lower than 5% remained in the final model. First-order interactions were tested and the proportionality assumption was verified by evaluating Cox-Snell residuals and log–log plot.

For series 2, a minimum *P* value approach, referring to OS, was used to determine the cutoff value of RhoB in RT-qPCR.

Tests were two-sided and *P* values <0.05 were considered significant. All analyses were conducted using Stata version 9.0.

Results

RhoB expression in lung cancer

Our primary aim was to analyze RhoB expression by IHC in a series of 100 surgical patients with NSCLC (series 1). We first tested RhoB antibody specificity on formalin-fixed paraffin-embedded specimens. RhoB staining was tested on normal human bronchial epithelial BEAS-2B cells (ATCC-CRL-9609) transduced either by the RhoB adenovirus or shRNA against RhoB as previously described (18). The staining of RhoB was strongly increased in cells overexpressing RhoB whereas it was reduced when RhoB expression was inhibited by shRNA-mediated silencing, thus demonstrating RhoB antibody specificity (Supplementary Fig. S1A and S1B).

Immunohistochemical analysis of series 1 showed that 20% of the tumors were negative for RhoB, 48% had mild staining (+), 25% had moderate staining (++), and 7% had intense staining (+++; as described in the Materials and Methods section; Supplementary Fig. S1C).

Published OnlineFirst October 15, 2014; DOI: 10.1158/1078-0432.CCR-14-0506

RhoB was equally expressed regarding gender, age, and tobacco use (Table 1). RhoB appeared to be more expressed in early-stage tumors (stage I compared with more advanced stages, stages II and III), but without reaching significance ($P = 0.12$). Loss of RhoB seemed to be also associated with larger tumors ($P = 0.09$; Table 1).

We next analyzed the histologic features. We did not find any difference in RhoB expression between the various histologic subtypes or according to the presence of tumor emboli. In contrast, we found that RhoB expression was significantly associated with the absence of necrosis ($P = 0.04$) and the presence of a lepidic component ($P = 0.0003$; Table 1).

Correlation between RhoB and oncogenic drivers in lung cancer

We and others showed that RhoB is able to regulate of many growth factor receptors, among which EGFR (27) and to interact with the KRas signaling pathway (12, 18). EGFR and KRas are crucial markers in bronchioloalveolar carcinoma development (28). We thus analyzed these biomarkers along with RhoB. We observed that RhoB expression is independent from TTF1 ($P = 0.85$), KRas mutation ($P = 0.59$), and EGFR expression ($P = 0.76$). Interestingly, RhoB expression is associated with EGFR mutation as all mutated patients displayed an overexpression of RhoB ($P = 0.0005$; Supplementary Table S2).

Predictive and prognostic values of RhoB in lung cancer

Next, we attempted to identify the prognostic value of numerous clinical and biologic parameters in the first series of surgical patients who did not receive any preoperative treatment. We identified, in univariate analysis, that the number of tumors and lymphatic emboli were associated with worse OS and PFS whereas tumor stage was only associated with the PFS (Table 2). Neither tobacco use, age at the time of diagnosis, necrosis, presence of lepidic components, nor postoperative chemotherapy were prognostic factors. In contrast, a none-to-mild RhoB expression was strongly associated with shorter survival ($P = 0.0008$; Table 2). Five-year survival was 91% for patients with moderate-to-strong RhoB expression versus 53% for patients with negative-to-mild RhoB expression (Fig. 1A). After multivariate analyses, low RhoB expression remained associated with shorter survival times [adjusted HR, 8.13; 95% confidence interval (CI), 1.91–34.55; $P = 0.005$). PFS was also strongly influenced by RhoB expression. In univariate and multivariate analyses (HR, 5.24; 95% CI, 2.23–12.28; $P < 0.0001$ and adjusted HR, 5.58; 95% CI, 2.36–13.19; $P < 0.001$, respectively), none-to-mild RhoB expression was associated with shorter PFS, 22 months in this group of patients, whereas the median PFS was not reached in patients with moderate-to-intense RhoB expression (Fig. 1B).

We further analyzed a second series of 48 patients with NSCLC from another hospital (series 2) by performing quantitative RT-qPCR. We observed differential RhoB expression between control tissues and tumor tissues among the 48 patients: from 0.021 (range, 0.002–1.203) to 0.015 (range, 0.00–0.268; $P = 0.0001$). We also observed that loss of RhoB expression was associated with a shorter PFS ($P = 0.05$) but this did not reach significance for OS ($P = 0.12$; Fig. 1C and D).

Table 1. RhoB expression according to clinical and pathologic characteristics

Item	$RhoB^{-}$	$RhoB^{+}$	$RhoB^{++}$	$RhoB^{+++}$	*P*
Gender: M/F (%)	55/45	50/50	52/48	29/71	0.716
Age (median, y)	58.1	59.5	61.8	62.1	0.565
Smokers (%)	90	88	80	71	0.476
Tobacco (median packs/y)	37.5	40	40	40	0.813
Stage (%)					
I	30	48	68	71	
II	55	37	20	14	0.124
III	15	15	12	14	
Tumor size (median, mm)	4	3.5	2.7	2.5	0.09
ADC/SqCC (%)	80/20	90/10	92/8	100/0	0.556
Emboli (%)	65	50	44	29	0.343
Necrosis (%)	50	69	40	29	0.04
Lepidic component					
0%	70	75	36	42	
0<%<10	15	10	40	0	0.0003
10<%<70	15	13	20	29	
>70%	0	2	4	29	

Abbreviations: ADC/SqCC, adenocarcinoma/squamous-cell carcinoma; M/F, male/female; packs/y, packs year.

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Table 2. Prognostic value of clinical and biologic characteristics

	OS		PFS	
Item	***P***	**HR (95% CI)**	***P***	**HR (95% CI)**
Gender (M/F)	0.486	0.771 (0.371–1.605)	0.822	0.934 (0.544–1.621)
Age	0.922	1.037 (0.500–2.151)	0.898	0.965 (0.560–1.664)
Tobacco use	0.604	1.322 (0.459–3.814)	0.5811	1.289 (0.603–2.754)
Adjuvant CT	0.869	0.938 (0.439–2.006)	0.402	1.268 (0.726–2.215)
Number of tumors	0.009	2.823 (1.246–6.395)	0.016	2.232 (1.139–4.371)
Necrosis	0.49	1.297 (0.619–2.718)	0.249	1.384 (0.794–2.413)
Emboli	0.0021	2.318 (1.113–4.824)	0.005	2.179 (1.253–3.787)
TNM				
Stage 0	0.512	1.0	0.011	1.0
Stage IA–B		1.039 (0.330–3.277)		2.463 (0.881–6.886)
Stage IIA–B		1.546 (0.536–4.456)		4.265 (1.620–11.226)
Stage IIIA–B		2.088 (0.636–6.853)		3.793 (1.268–11.342)
At least 10% of lepidic component	0.503	0.770 (0.358–1.657)	0.0156	0.659 (0.369–1.177)
None to mild RhoB expression	0.0008	7.923 (1.882–33.349)	<0.0001	5.236 (2.233–12.281)

Abbreviation: CT, chemotherapy.

RhoB in lepidic carcinoma

As mentioned above, we observed a striking difference in the expression of RhoB according to the presence of lepidic components in the first series (Table 1). More precisely, RhoB expression was directly correlated with the proportion of lepidic features in all operated adenocarcinomas (Fig. 2). Twenty-nine percent of tumors that presented high levels of RhoB (+++) had at least 70% lepidic component, whereas proportion of lepidic component was significantly lower in tumors that showed moderate (++) and low (+) levels of RhoB and nearly absent in RhoB negative tumors (Fig. 2A).

We then analyzed a third independent series of patients harboring advanced adenocarcinoma with lepidic features enrolled in a clinical trial (IFCT-0401; ref. 22). Interestingly, loss of RhoB expression was associated with shorter survival time ($P = 0.009$), as also observed in the two other series (Fig. 2B).

Loss of RhoB expression in a mouse model of bronchiolo-alveolar carcinoma

The correlation between RhoB expression and the prognosis of adenocarcinoma with lepidic features prompted us to assess the impact of RhoB loss *in vivo* by crossing *Rhob* WT, heterozygous and null mice with a mouse model known to develop this subtype of cancer through an inducible lung-specific $EGFR^{L858R}$ mutation (20). RhoB expression level in healthy and tumor tissues was assessed by Western blot analysis in $EGFR^{L858R}/Rhob^{+/+}$, $EGFR^{L858R}/Rhob^{+/-}$ (half-level), and $EGFR^{L858R}/Rhob^{-/-}$ (no expression) mice, with a slight but not significant decrease of RhoB in lung tumors of RhoB WT and heterozygous mice (Supplementary Fig. S2A and S2B). Consistent with observations previously reported for the model of inducible lung-specific $EGFR^{L858R}$ mutation, the mice developed focal or diffuse epithelial hyperplasia, atypical adenomatous hyperplasia with lepidic pattern (Supplementary Fig. S2Ca), multifocal lung adenomas (Supplementary Fig. S2Cb) and, in some cases, adenocarcinomas (Supplementary Fig. S2Cc–d). Tumors were $EGFR^{L858R}$-positive (Supplementary Fig. S2A and S2Dg–h), mucin-negative as determined by PAS-diastase staining (Supplementary Fig. S3), and the presence of intraalveolar macrophages was commonly observed in lesions.

Degree of cellular and nuclear atypia in atypical adenomatous hyperplasia and overall lung macrophage content were not influenced by the *Rhob* status.

In contrast, quantification of the tumor/total lung ratio revealed a significant extension of tumor lesions in $EGFR^{L858R}/Rhob^{-/-}$ and $EGFR^{L858R}/Rhob^{+/-}$ mice compared with $EGFR^{L858R}/Rhob^{+/+}$ mice (Fig. 3A). This was correlated with a significant increase in both number and size of tumor nodules (Fig. 3B and C) and with a greater proliferating index, as determined by Ki67 staining (Fig. 3D and Supplementary Fig. S2Dk–l).

We further evaluated the aggressiveness of these tumors by establishing a 4-grade classification modified from reference histopathologic criteria (26). We observed according to this classification a slight but not significant increase in the number of grade 1 (Fig. 4Aa–b) and 2 (Fig. 4Ac–d) tumors in $EGFR^{L858R}/Rhob^{-/-}$ and $EGFR^{L858R}/Rhob^{+/-}$ mice compared with $EGFR^{L858R}/Rhob^{+/+}$ mice (Fig. 4B). In contrast, the number of lesions with grade 3 (Fig. 4Ae–f) increased dramatically in $EGFR^{L858R}/Rhob^{-/-}$ and $EGFR^{L858R}/Rhob^{+/-}$ mice, whereas tumors with the highest grade 4 (Fig. 4Ag–h), corresponding to adenocarcinomas in our classification, were exclusively detected in $EGFR^{L858R}/Rhob^{-/-}$ and $EGFR^{L858R}/Rhob^{+/-}$ mice (Fig. 4B).

Because recent findings related RhoB to angiogenesis (29), we investigated whether RhoB could influence neo-

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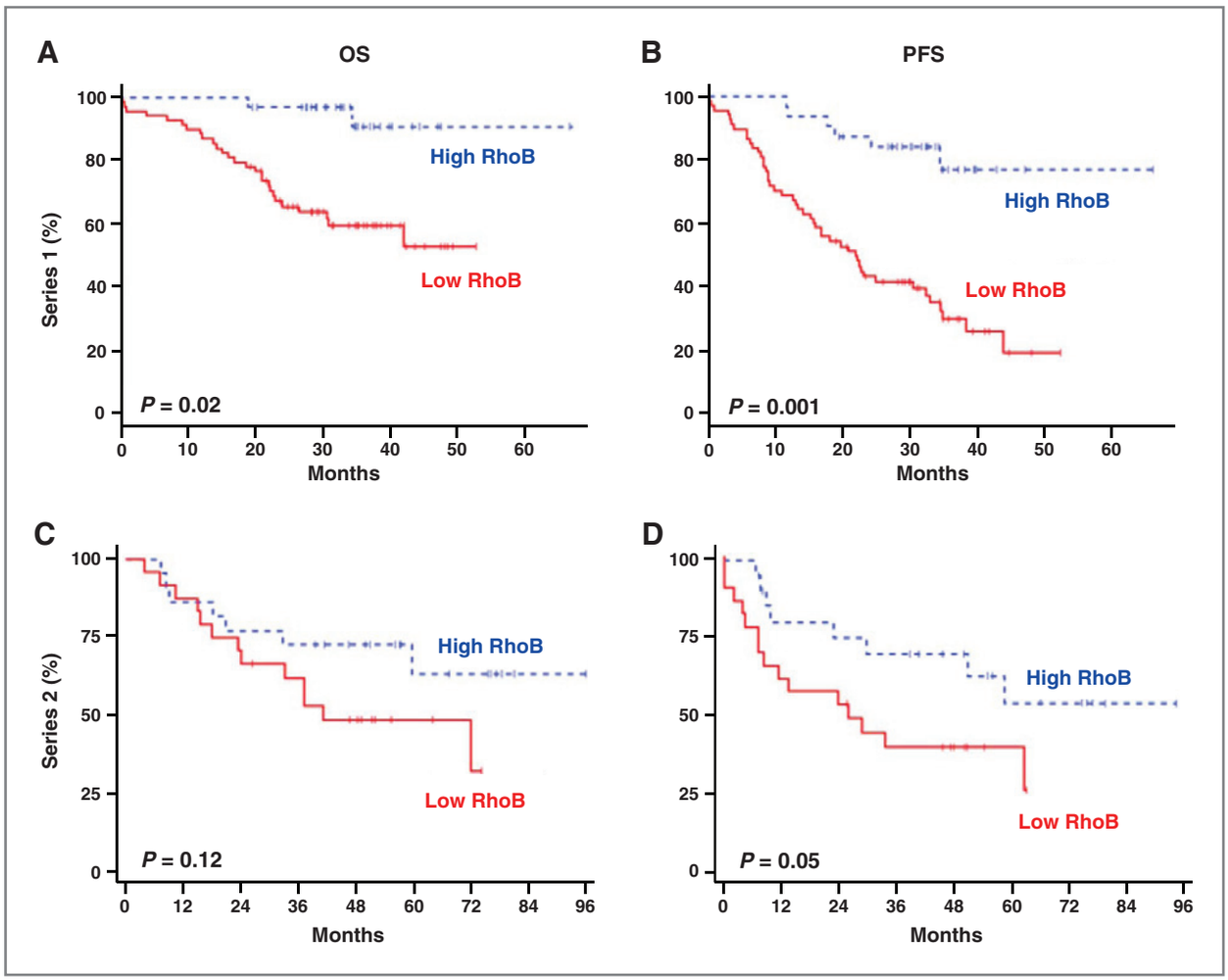

Figure 1. Prognostic value of RhoB in NSCLC. OS (A and C) and PFS (B and D) of operated patients according to RhoB expression assessed by IHC from series 1 (0 and + vs. ++ and +++; A and B), or by RhoB mRNA expression (RT-qPCR) from series 2 (C and D).

vessel formation in this model. CD34 immunostaining highlighted the tumor vascular supply but was consistent with the preexisting alveolar vascular network and did not suggest a significant neoangiogenesis (Supplementary Fig. S4). Moreover, no difference in vessel content was found between the three *Rhob* genotypes (Supplementary Fig. S4).

We also analyzed downstream signaling pathways of EGFR by Western blot analysis with antibodies directed against phosphorylated Akt (ser473) and phosphorylated ERK1/2, in all our mice models. We first confirmed that both kinases were over activated in $EGFR^{L858R}$-driven tumors, although increase of phospho-Erk was almost but not quite statistically significant in $EGFR^{L858R}/Rhob^{+/+}$ mice. Activation of Erk between healthy and tumor tissues was more important in RhoB heterozygous and KO mice compared with *RhoB* WT mice, and no differences on Akt activation were observed between the three RhoB genotypes (Supplementary Fig. S5).

Discussion

Herein, we have identified RhoB as a potential prognostic factor in patients operated from lung cancer. In the first series of stages I to III NSCLC, we observed by IHC that RhoB loss of expression was associated with a shorter PFS and OS after multivariate analysis. We next wanted to confirm our findings obtained in this training set. We analyzed a second independent series of operated patients by RT-qPCR excluding that the results might have been affected by the immunohistochemical process or may have been biased because of the monocentric design of the study. The use of RT-qPCR appears relevant as a confirmatory procedure as we already found a correlation between RhoB mRNA and protein expression in several lung tumor cell lines. Moreover, RhoB is an immediate early gene whose expression is often first regulated at transcriptional levels (30, 31). All these analyses were performed on chemonaïve patients. Because it is known that RhoB expression can be influenced by chemotherapy (32), which may explain the discrepancies with another study in which RhoB was rather

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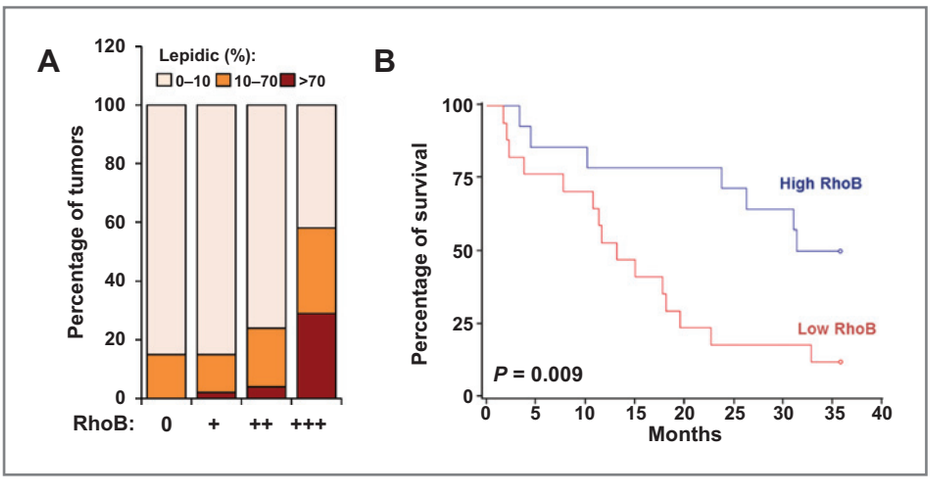

Figure 2. RhoB expression and prognostic value in adenocarcinoma with lepidic component. A, the percentage of lepidic component in adenocarcinomas according to RhoB expression. B, OS according to RhoB expression in series 3: that is, stage IV adenocarcinoma with lepidic features.

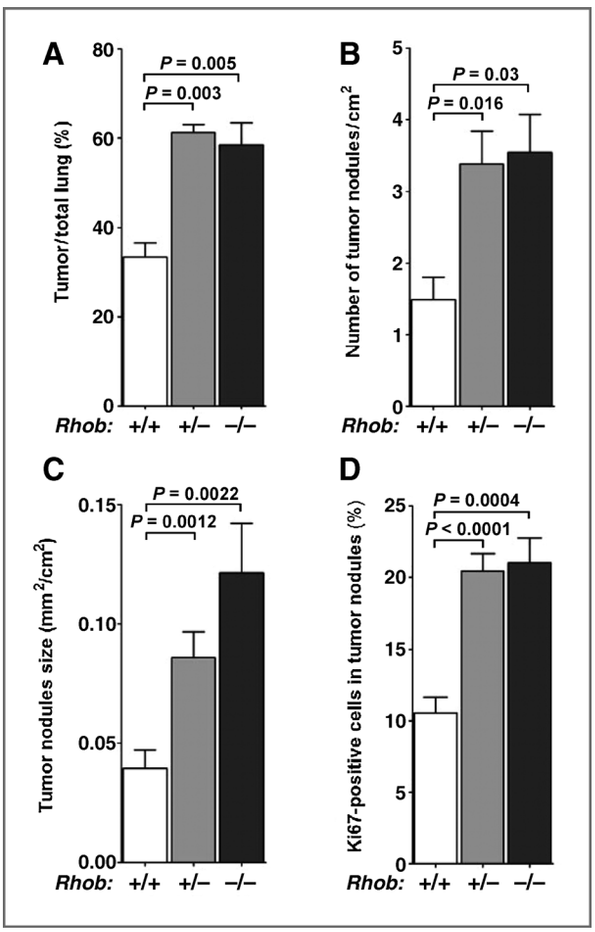

Figure 3. Impact of RhoB expression on development of lung tumors in a murine model of adenocarcinoma with lepidic features. A, quantification of the tumor/lung ratio. B, quantification of the number of tumor nodules per cm^2 of total lung. C, quantification of the tumor nodules size, expressed in mm^2 per cm^2 of total lung. D, the percentage of Ki67-positive cells in tumor nodules. Histograms, means ± SEMs; ($EGFR^{L858R}$/$Rhob^{+/+}$, white bar, $n = 6$; $EGFR^{L858R}$/$Rhob^{+/-}$, light gray bar, $n = 7$; $EGFR^{L858R}$/$Rhob^{-/-}$, dark gray bar, $n = 6$).

associated with a worse outcome, but the survival data were reported in a small subgroup of patients (treated adenocarcinoma, $n = 38$; ref. 33). It is noteworthy that we confirmed our results in a third series of 31 advanced adenocarcinoma, suggesting that the prognostic value of RhoB is not limited to early stages. Despite being conducted in three independent cohorts, the variations in the procedures and the small number of patients prevent us from drawing definitive conclusion about the prognostic role of RhoB in lung cancer. Most of the patients of our study had adenocarcinoma (11% and 34% of the patients presented with a squamous cell carcinoma in series 1 and 2, respectively). Subgroup analysis performed in adenocarcinoma subgroups showed identical results.

We hypothesized that the loss of RhoB gave the tumor a more aggressive phenotype, as already shown in preclinical studies (18) and as suggested by the loss of RhoB expression between preinvasive and invasive stages in the lung biopsies (6).

We confirmed our hypothesis in a transgenic murine model of lung cancer induced by an activating EGFR mutation, which is known to lead to adenomas and adenocarcinomas (20) crossed with *Rhob* WT, heterozygous, or null mice (14). Of interest, we report that loss of RhoB, even partially (as no significant differences were observed between heterozygous and KO mice), was associated with a greater extension of tumor tissue consistent with an increased number of lung tumor nodules. Erk activation was more pronounced in the lung tumors from *Rhob* heterozygous and null mice compared with WT mice, suggesting that this pathway could play a role in the development of tumor aggressiveness caused by RhoB loss. We also observed that loss of RhoB was correlated with a more aggressive pattern of these tumors with a greater Ki67 index, which is fully consistent with our previous observations in human invasive carcinomas (6), and with susceptibility to carcinogens in KO mice (14). RhoB loss has already been shown to be associated with the development of lung tumors in nude mice (18) or intraperitoneal tumors in syngeneic mice (14), whereas the *in vivo* restoration of RhoB

Published OnlineFirst October 15, 2014; DOI: 10.1158/1078-0432.CCR-14-0506

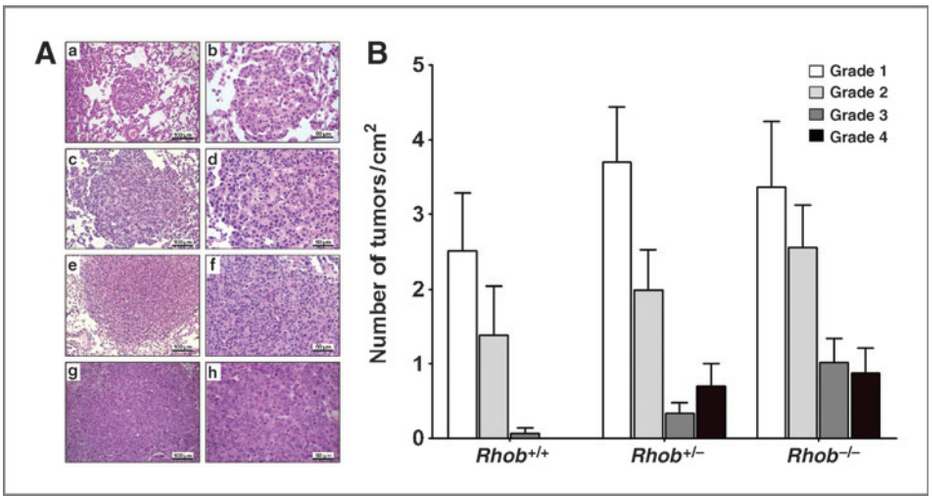

Figure 4. Impact of RhoB expression on lung tumor grade in a murine model of adenocarcinoma with lepidic features. A, histologic grades of lung tumors, highlighting the different stages of tumor progression; a and b, grade 1 lesion, consisting of small-size focal-condensed nodules of atypical adenomatous hyperplasia; c and d, grade 2 tumor (low-grade adenoma) composed of well-differentiated, more "plump" cuboidal cells; e and f, grade 3 tumor (high-grade adenoma) characterized by an increase in cell density, cell size, and nuclear to cytoplasmic ratio; g and h, grade 4 tumor (adenocarcinoma) of larger size, showing greater cytologic atypia, increased frequency of mitoses and invasive features on surrounding lung parenchyma. Nodular tumors were mostly developed continuous to diffuse epithelial hyperplasia changes. H&E, original ×200 and ×400 magnification for all 4 grades, with scale bar. B, quantification of the number of tumors per cm^2 of lung, according to the tumor grade and the *Rhob* status. Histograms, means ± SEMs; ($EGFR^{L858R}/Rhob^{+/+}$, $n = 6$; $EGFR^{L858R}/Rhob^{+/-}$, $n = 7$; $EGFR^{L858R}/Rhob^{-/-}$, $n = 6$).

expression leads to ovarian tumor regression (17). Although classical tumor-suppressor genes generally require mutation or loss of both alleles to facilitate tumor progression, we showed here that *rhob* heterozygosity is sufficient to increase lung tumorigenicity in an EGFR-mutated context. This original result could, therefore, propose the murine gene *rhob* as a new potential haplo-insufficient tumor-suppressor gene, such as $p27^{Kip}$ (34) or PTEN (35), among others. In this particular model, RhoB haplo insufficiency reflects that the partial diminution of RhoB protein levels, observed during lung tumor progression (6), is sufficient to promote tumor invasiveness. However, this apparent haplo insufficiency must not be generalized as other studies using *Rhob* KO and heterozygous mice did not report this effect (36) that may be restricted to some types of cancer and/or oncogenic stimuli. Interestingly, a recent study using a similar mouse model showed that the *Nkx2-1* murine gene haplo insufficiency induced pulmonary tumors in an oncogenic $Kras^{G12D}$ context, but not in combination with oncogenic $EGFR^{L858R}$ (37). This observation confirms that partial loss of a specific gene does not have the same effect according to the oncogenic background, and then strengthens the close relationship between RhoB and EGFR.

An important challenge in the field of thoracic oncology is the management of adenocarcinoma with lepidic features. This subtype of carcinoma is associated with good survival when it is diagnosed at an early stage, but it can rapidly become invasive, spreading along the bronchial tree, invading through the basal membrane, and eventually leading to a very aggressive phenotype with a poor outcome (38–41). The crucial event in the progression of this type of cancer is the switch from a "horizontal" spread toward "vertical" invasion of the basal membrane. Acquisition of an epithelial to mesenchymal phenotype is involved in such a process. RhoB is a potential candidate due to its role in the acquisition of epithelial to mesenchymal features. We report in this study that RhoB expression was directly correlated with the proportion of lepidic features in all adenocarcinomas. Moreover in the murine model of lung cancer used in this study, RhoB loss led to the appearance of higher grade lesions with invasive features, consolidating the hypothesis of a protective role of RhoB in the acquisition of invasive and aggressive properties in this particular type of cancer. We have recently observed an association between the loss of RhoB expression and the acquisition of a migratory and invasive phenotype of bronchial epithelial cells (18). The involvement of RhoB in cell migration has been also documented in several developmental studies (42, 43). In agreement with our findings, RhoB has been shown to inhibit migration and invasion of Ras-transformed murine fibroblasts (13), as well as macrophage migration on the fibronectin substratum (44). Recently, Vega and colleagues (45) also showed that RhoB-depleted cells migrate faster by regulating β1-integrin surface levels. In synthesis, we propose that loss of RhoB increases cell invasiveness and motility properties, thus leading to shorter PFS and worse OS in patients. Moreover, RhoB might be considered as a surrogate biomarker due to its association with EGFR mutations that are frequently found in the histologic subtype.

In summary, RhoB appears to play a crucial role in the acquisition of an aggressive phenotype in adenocarcinomas with lepidic component by controlling both proliferation and invasion. The RhoB status may be considered a useful biomarker in lung adenocarcinoma and might help us

Published OnlineFirst October 15, 2014; DOI: 10.1158/1078-0432.CCR-14-0506

define the prognosis of adenocarcinoma that has lepidic features, and particularly the switch from an aerogenous progression toward invasive development.

Disclosure of Potential Conflicts of Interest

J. Cadranel is a consultant/advisory board member for AstraZeneca and Roche. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): O. Calvayrac, A. Pradines, I. Raymond-Letron, V. Lauwers-Cances, T. Filleron, J. Cadranel, M. Beau-Faller, G. Favre, J. Mazières
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Acknowledgments

The authors thank Georges C. Prendergast (Lankenau Institute for Medical Research, Philadelphia, Pennsylvania) and K. Politi (Yale Cancer Center, New Haven, Connecticut) for kindly providing us the RhoB null mice and the EGFR mice model of lung oncogenesis (developed at Sloan Kettering Institute, New York, NY). The authors thank Magali Farella for mice genotyping, Laure Lazorthes, and Céline Bleuart for technical expertise in IHC and Yann Figuères for his help in graphics.

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Received March 12, 2014; revised August 9, 2014; accepted September 13, 2014; published OnlineFirst October 15, 2014.

References

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010;127:2893–917.
2. Goldstraw P, Ball D, Jett J, Le Chevalier T, Lim E, Nicholson A, et al. Non–small cell lung cancer. Lancet 2011;378:1727–40.
3. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
4. Kratz JR, He J, Van Den Eeden SK, Zhu ZH, Gao W, Pham PT, et al. A practical molecular assay to predict survival in resected non-squamous, non–small cell lung cancer: development and international validation studies. Lancet 2012;379:823–32.
5. Vannini I, Fanini F, Fabbri M. microRNAs as lung cancer biomarkers and key players in lung carcinogenesis. Clin Biochem 2013;46:918–25.
6. Mazieres J, Antonia T, Daste G, Muro-Cacho C, Berchery D, Tillement V, et al. Loss of RhoB expression in human lung cancer progression. Clin Cancer Res 2004;10:2742–50.
7. Sato N, Fukui T, Taniguchi T, Yokoyama T, Kondo M, Nagasaka T, et al. RhoB is frequently downregulated in non–small cell lung cancer and resides in the 2p24 homozygous deletion region of a lung cancer cell line. Int J Cancer 2007;120:543–51.
8. Huang M, Prendergast GC. RhoB in cancer suppression. Histol Histopathol 2006;21:213–8.
9. Karlsson R, Pedersen ED, Wang Z, Brakebusch C. Rho GTPase function in tumorigenesis. Biochim Biophys Acta 2009;1796:91–8.
10. Ridley AJ. RhoA, RhoB and RhoC have different roles in cancer cell migration. J Microsc 2013;251:242–9.
11. Chen Z, Sun J, Pradines A, Favre G, Adnane J, Sebti SM. Both farnesylated and geranylgeranylated RhoB inhibit malignant transformation, induce apoptosis and suppress human tumor growth in nude mice. J Biol Chem 2000;275:17974–8.
12. Mazieres J, Tillement V, Allal C, Clanet C, Bobin L, Chen Z, et al. Geranylgeranylated, but not farnesylated, RhoB suppresses Ras transformation of NIH-3T3 cells. Exp Cell Res 2005;304:354–64.
13. Jiang K, Delarue FL, Sebti SM. EGFR, ErbB2 and Ras but not Src suppress RhoB expression while ectopic expression of RhoB antagonizes oncogene-mediated transformation. Oncogene 2004;23:1136–45.
14. Liu AX, Rane N, Liu JP, Prendergast GC. RhoB is dispensable for mouse development, but it modifies susceptibility to tumor formation as well as cell adhesion and growth factor signaling in transformed cells. Mol Cell Biol 2001;21:6906–12.
15. Forget MA, Desrosiers RR, Del M, Moumdjian R, Shedid D, Berthelet F, et al. The expression of rho proteins decreases with human brain tumor progression: potential tumor markers. Clin Exp Metastasis 2002;19:9–15.
16. Adnane J, Muro-Cacho C, Mathews L, Sebti SM, Munoz-Antonia T. Suppression of rho B expression in invasive carcinoma from head and neck cancer patients. Clin Cancer Res 2002;8:2225–32.
17. Couderc B, Pradines A, Rafii A, Golzio M, Deviers A, Allal C, et al. *In vivo* restoration of RhoB expression leads to ovarian tumor regression. Cancer Gene Ther 2008;15:456–64.
18. Bousquet E, Mazieres J, Privat M, Rizzati V, Casanova A, Ledoux A, et al. Loss of RhoB expression promotes migration and invasion of human bronchial cells via activation of AKT1. Cancer Res 2009;69:6092–9.
19. Wislez M, Beer DG, Wistuba I, Cadranel J, Saijo N, Johnson BE. Molecular biology, genomics, and proteomics in bronchioloalveolar carcinoma. J Thorac Oncol 2006;1:S8–12.
20. Politi K, Zakowski MF, Fan PD, Schonfeld EA, Pao W, Varmus HE. Lung adenocarcinomas induced in mice by mutant EGF receptors found in human lung cancers respond to a tyrosine kinase inhibitor or to down-regulation of the receptors. Genes Dev 2006;20:1496–510.
21. Rouquette I, Lauwers-Cances V, Allera C, Brouchet L, Milia J, Nicaise Y, et al. Characteristics of lung cancer in women: importance of hormonal and growth factors. Lung Cancer 2012;76:280–5.
22. Cadranel J, Quoix E, Baudrin L, Mourlanette P, Moro-Sibilot D, Morere JF, et al. IFCT-0401 trial: a phase II study of gefitinib administered as first-line treatment in advanced adenocarcinoma with bronchioloalveolar carcinoma subtype. J Thorac Oncol 2009;4:1126–35.
23. Travis WD, Brambilla E, Noguchi M, Nicholson AG, Geisinger KR, Yatabe Y, et al. International association for the study of lung cancer/american thoracic society/european respiratory society international multidisciplinary classification of lung adenocarcinoma. J Thorac Oncol 2011;6:244–85.
24. Goldstraw P, Crowley J, Chansky K, Giroux DJ, Groome PA, Rami-Porta R, et al. The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM Classification of malignant tumours. J Thorac Oncol 2007;2:706–14.
25. Groome PA, Bolejack V, Crowley JJ, Kennedy C, Krasnik M, Sobin LH, et al. The IASLC Lung Cancer Staging Project: validation of the proposals for revision of the T, N, and M descriptors and consequent stage groupings in the forthcoming (seventh) edition of the TNM classification of malignant tumours. J Thorac Oncol 2007;2:694–705.
26. Nikitin AY, Alcaraz A, Anver MR, Bronson RT, Cardiff RD, Dixon D, et al. Classification of proliferative pulmonary lesions of the mouse:

Published OnlineFirst October 15, 2014; DOI: 10.1158/1078-0432.CCR-14-0506

recommendations of the mouse models of human cancers consortium. Cancer Res 2004;64:2307–16.
27. Lajoie-Mazenc I, Tovar D, Penary M, Lortal B, Allart S, Favard C, et al. MAP1A light chain-2 interacts with GTP-RhoB to control epidermal growth factor (EGF)-dependent EGF receptor signaling. J Biol Chem 2008;283:4155–64.
28. Garfield DH, Cadranel J, West HL. Bronchioloalveolar carcinoma: the case for two diseases. Clin Lung Cancer 2008;9:24–9.
29. Gerald D, Adini I, Shechter S, Perruzzi C, Varnau J, Hopkins B, et al. RhoB controls coordination of adult angiogenesis and lymphangiogenesis following injury by regulating VEZF1-mediated transcription. Nat Commun 2013;4:2824.
30. Zalcman G, Closson V, Linarescruz G, Lerebours F, Honore N, Tavitian A, et al. Regulation of ras-related RhoB protein expression during the cell cycle. Oncogene 1995;10:1935–45.
31. Fritz G, Kaina B, Aktories K. The ras-related small GTP-binding protein RhoB is immediate-early inducible by DNA damaging treatments. J Biol Chem 1995;270:25172–7.
32. Liu A, Cerniglia GJ, Bernhard EJ, Prendergast GC. RhoB is required to mediate apoptosis in neoplastically transformed cells after DNA damage. Proc Natl Acad Sci U S A 2001;98:6192–7.
33. Luis-Ravelo D, Anton I, Zandueta C, Valencia K, Pajares MJ, Agorreta J, et al. RHOB influences lung adenocarcinoma metastasis and resistance in a host-sensitive manner. Mol Oncol 2014;8:196–206.
34. Fero ML, Randel E, Gurley KE, Roberts JM, Kemp CJ. The murine gene p27Kip1 is haplo-insufficient for tumour suppression. Nature 1998;396:177–80.
35. Kwabi-Addo B, Giri D, Schmidt K, Podsypanina K, Parsons R, Greenberg N, et al. Haploinsufficiency of the Pten tumor suppressor gene promotes prostate cancer progression. Proc Natl Acad Sci U S A 2001;98:11563–8.
36. Meyer N, Peyret-Lacombe A, Canguilhem B, Medale-Giamarchi C, Mamouni K, Cristini A, et al. RhoB promotes cancer initiation by protecting keratinocytes from UVB-induced apoptosis but limits tumor aggressiveness. J Invest Dermatol 2014;134:203–12.
37. Maeda Y, Tsuchiya T, Hao H, Tompkins DH, Xu Y, Mucenski ML, et al. Kras(G12D) and Nkx2–1 haploinsufficiency induce mucinous adenocarcinoma of the lung. J Clin Invest 2012;122:4388–400.
38. Kerr KM, MacKenzie SJ, Ramasami S, Murray GI, Fyfe N, Chapman AD, et al. Expression of Fhit, cell adhesion molecules and matrix metalloproteinases in atypical adenomatous hyperplasia and pulmonary adenocarcinoma. J Pathol 2004;203:638–44.
39. Awaya H, Takeshima Y, Amatya VJ, Ishida H, Yamasaki M, Kohno N, et al. Loss of expression of E-cadherin and beta-catenin is associated with progression of pulmonary adenocarcinoma. Pathol Int 2005;55:14–8.
40. Borczuk AC. Assessment of invasion in lung adenocarcinoma classification, including adenocarcinoma in situ and minimally invasive adenocarcinoma. Mod Pathol 2012;25:S1–10.
41. Garfield DH, Cadranel JL, Wislez M, Franklin WA, Hirsch FR. The bronchioloalveolar carcinoma and peripheral adenocarcinoma spectrum of diseases. J Thorac Oncol 2006;1:344–59.
42. Henderson DJ, Ybot-Gonzalez P, Copp AJ. RhoB is expressed in migrating neural crest and endocardial cushions of the developing mouse embryo. Mech Dev 2000;95:211–4.
43. Vignal E, de Santa Barbara P, Guemar L, Donnay JM, Fort P, Faure S. Expression of RhoB in the developing Xenopus laevis embryo. Gene Expr Patterns 2007;7:282–8.
44. Wheeler AP, Ridley AJ. RhoB affects macrophage adhesion, integrin expression and migration. Exp Cell Res 2007;313:3505–16.
45. Vega FM, Colomba A, Reymond N, Thomas M, Ridley AJ. RhoB regulates cell migration through altered focal adhesion dynamics. Open Biol 2012;2:120076.

Published OnlineFirst October 15, 2014; DOI: 10.1158/1078-0432.CCR-14-0506

Clinical Cancer Research

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Clin Cancer Res 2014;20:6541-6550. Published OnlineFirst October 15, 2014.