RhoB determines tumor aggressiveness in a murine EGFR\textsuperscript{L858R}-induced adenocarcinoma model and is a potential prognostic biomarker for lepidic lung cancer.

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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. Based on 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 knock-out mice. We conclude that RhoB status may help define the prognosis of adenocarcinoma with lepidic features.
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 immunohistochemistry 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\textsuperscript{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. Lastly we showed that EGFR\textsuperscript{L858R}/rhob\textsuperscript{+/+} mice developed mainly diffuse lung tumors with a lepidic pattern while EGFR\textsuperscript{L858R}/rhob\textsuperscript{+/−} and EGFR\textsuperscript{L858R}/rhob\textsuperscript{−/−} 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.
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 stage-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 histopathological 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 post-operative 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, RRM1), cell cycle (CDK4, CDK6, cyclin D1), apoptosis (caspase-3, Bcl-2, p53), and major oncogenes (KRas, 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 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 downregulated in cancer tumors (6, 7, 15, 16) and \textit{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 while 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...
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) 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) (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, for subsequent immunohistochemistry 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 tumor-node-metastasis 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. 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 immunohistochemistry procedures for patients from series 1 and 3. After rehydratation, deparaffinized sections were pretreated by microwave epitope retrieval. Endogenous peroxidase activity was quenched before a blocking step of non-specific 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 Biotechnologies, Inc., 1:75) overnight at 4ºC. After washes, sections were then incubated with 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), blinded to the patients' status, independently evaluated the extension and intensity of the staining. For RhoB, the extension was scored as 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'-TTGTGCCTGTCTTAGAAGTG-3'; reverse, 5'-CAAAGTGCAGGTCAGAATGCTAC-3') and PPIA
(Hs_PPIA_3_SG QuantiTect primer assay, Qiagen). The relative RhoB mRNA expression was calculated according to the $2^{-\Delta\Delta Cq}$ 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 (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 six-week-old mice were fed *ad libitum* with food pellets that contained doxycycline (1g/kg) for 8 weeks, and were then sacrificed by cervical dislocation. The lungs were excised and inflated via intra-tracheal infusion with 4% buffered formaldehyde, and immersion-fixed for 24 h at room temperature before dehydration and paraffin-embedding. Four-micrometer paraffin sections were used for H&E staining and immunohistochemistry using standard procedures. The proliferating index was determined by Ki67 staining (SP6, Thermoscientific), and the lung
vessels were stained with CD34 antibody (MEC 14.7 AbD, Serotec). Transgene expression was evaluated with an anti-EGFR\textsuperscript{L858R} antibody (Cell Signaling). Digital slides were blind evaluated by two operators of whom a veterinary pathologist, according to reference papers (20, 26). Detailed methods for tumor grading and quantification of the tumor to lung-area ratio are described in Supplemental material.

Statistics

Continuous variables are presented as their means (standard deviations [SD]) or their medians (interquartile range [IQR]) according to their distributions. The chi-squared or Fisher’s exact test was used to compare categorical variables, and Student’s t-test, variance analysis or a non-parametric 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's models. To address confounding, a multivariate Cox regression model was performed. The initial model included all variables which 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 overall survival, was used to determine the cut-off 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 immunohistochemistry 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 short hairpin RNA (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 Figure S1A and B).

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 Figure S1C).

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 to 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 histological features. We did not find any difference in RhoB expression between the various histological 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 other showed that RhoB is able to regulate of many growth factor receptors, among which EGFR (27) and to interact with KRas signaling pathway (12, 18). EGFR and KRas are crucial markers in bronchioloalveolar carcinoma development (28). We thus analysed 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 biological 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 overall (OS) and progressive free survival (PFS) while 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 post-operative 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 vs. 53% for patients with negative-to-mild RhoB expression (Figure 1A).
multivariate analyses, low RhoB expression remained associated with shorter survival times (adjusted HR 8.13 CI95% [1.91-34.55]; p = 0.005). PFS was also strongly influenced by RhoB expression. In univariate and multivariate analyses (HR 5.24, CI95% [2.23-12.28]; p <0.0001 and adjusted HR 5.58 CI95% [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 (Figure 1B).

We further analyzed a second series of 48 NSCLC patients 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) (Figure 1C, D).

**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 (Figure 2). Twenty nine % 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 (Figure 2A).

We then analyzed a third independent series of patients harboring advanced adenocarcinoma with lepidic features enrolled in a clinical trial (IFCT-0401) (22). Interestingly, loss of RhoB expression was associated with shorter survival time (p = 0.009), as also observed in the two other series (Figure 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 wild-type, 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 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 Figure S2 A and B). 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 Figure S2Ca), multifocal lung adenomas (supplementary Figure S2Cb) and, in some cases, adenocarcinomas (supplementary Figure S2Cc-d). Tumors were EGFR^{L858R}-positive (Supplementary Figure S2A and S2Dg-h), mucin-negative as determined by PAS-diastase staining (Supplementary Figure S3), and the presence of intra-alveolar 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 (Figure 3A). This was correlated with a significant increase in both number and size of tumor nodules (Figure 3B and C) and with a greater proliferating index, as determined by Ki-67 staining (Figure 3D, Supplementary Figure S2Dk-l).
We further evaluated the aggressiveness of these tumors by establishing a 4-grade classification modified from reference histopathological criteria (26). We observed according to this classification a slight but not significant increase in the number of grade I (Figure 4Aa-b) and II (Figure 4Ac-d) tumors in \(\text{EGFR}^{L858R/rhon^-/^-}\) and \(\text{EGFR}^{L858R/rhon^+/+}\) mice compared with \(\text{EGFR}^{L858R/rhon^+/+}\) mice (Figure 4B). In contrast, the number of lesions with grade III (Figure 4Ae-f) increased dramatically in \(\text{EGFR}^{L858R/rhon^-/^-}\) and \(\text{EGFR}^{L858R/rhon^+/+}\) mice, while tumors with the highest grade IV (Figure 4Ag-h), corresponding to adenocarcinomas in our classification, were exclusively detected in \(\text{EGFR}^{L858R/rhon^-/^-}\) and \(\text{EGFR}^{L858R/rhon^+/+}\) mice (Figure 4B).

Because recent findings related RhoB to angiogenesis (29), we investigated whether RhoB could influence neo-vessel formation in this model. CD34 immunostaining highlighted the tumor vascular supply but was consistent with the pre-existing alveolar vascular network and did not suggest a significant neoangiogenesis (Supplementary Figure S4). Moreover, no difference in vessel content was found between the three \(\text{rhon}\) genotypes (Supplementary figure S4).

We also analyzed downstream signaling pathways of EGFR by western blot 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 \(\text{EGFR}^{L858R}\)-driven tumors, although increase of phospho-Erk was almost but not quite statistically significant in \(\text{EGFR}^{L858R/rhon^+/+}\) 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 Figure S5).
Discussion

Herein we have identified RhoB as a potential prognostic factor in patients operated from lung cancer. In the first series of stage I to III NSCLC we observed by immunohistochemistry that RhoB loss of expression was associated with a shorter progression-free and overall survival 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 study's monocentric design. 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 chemonaive patients. Since it is known that RhoB expression can be influenced by chemotherapy, (32) that may explain the discrepancies with another study in which RhoB was rather associated with a worse outcome, but the survival data were reported in a small subgroup of patients (treated adenocarcinoma, n=38) (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 wild-type, heterozygous or null mice (14). Of interest, we report that loss of RhoB, even partially (as no significant differences were observed between heterozygous and knock-out 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 Ki-67 index, which is fully consistent with our previous observations in human invasive carcinomas (6), and with susceptibility to carcinogens in knock-out 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) while the in vivo restoration of RhoB 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 p27Kip (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 since others 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\textsuperscript{G12D} context, but not in combination with oncogenic EGFR\textsuperscript{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 towards “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 et al. also showed that RhoB-depleted cells migrate faster by regulating β1-integrin surface levels (45). In synthesis, we propose that loss of RhoB increases cell invasiveness and motility properties, thus leading to shorter progression-free survival and worse overall survival in patients. Moreover, RhoB might be considered as a surrogate biomarker due to its association with EGFR mutations which are frequently found in the histological 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. RhoB status may be considered a useful biomarker in lung adenocarcinoma and might help us define the prognosis of adenocarcinoma that has lepidic features, and particularly the switch from an aerogenous progression towards invasive development.
Acknowledgments

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References

**Table 1:** RhoB expression according to clinical and pathological characteristics.

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</tr>
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<td>ADC/SqCC (%)</td>
<td>80/20</td>
<td>90/10</td>
<td>92/8</td>
<td>100/0</td>
<td>0.556</td>
</tr>
<tr>
<td>Emboli (%)</td>
<td>65</td>
<td>50</td>
<td>44</td>
<td>29</td>
<td>0.343</td>
</tr>
<tr>
<td>Necrosis (%)</td>
<td>50</td>
<td>69</td>
<td>40</td>
<td>29</td>
<td>0.04</td>
</tr>
<tr>
<td>Lepidic component</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>70</td>
<td>75</td>
<td>36</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>0&lt;%&lt;10</td>
<td>15</td>
<td>10</td>
<td>40</td>
<td>0</td>
<td>0.0003</td>
</tr>
<tr>
<td>10&lt;%&lt;70</td>
<td>15</td>
<td>13</td>
<td>20</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>&gt;70%</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>29</td>
<td></td>
</tr>
</tbody>
</table>

*M/F: male/female; packs/y: packs year; ADC/SqCC: adenocarcinoma/squamous-cell carcinoma.*
**Table 2:** Prognostic value of clinical and biological characteristics.

<table>
<thead>
<tr>
<th>Item</th>
<th>Overall survival</th>
<th></th>
<th>Progression-free survival</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p</td>
<td>HR [CI95%]</td>
<td>p</td>
<td>HR [CI95%]</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>0.486</td>
<td>0.771 [0.371-1.605]</td>
<td>0.822</td>
<td>0.934 [0.544-1.621]</td>
</tr>
<tr>
<td>Age</td>
<td>0.922</td>
<td>1.037 [0.500-2.151]</td>
<td>0.898</td>
<td>0.965 [0.560-1.664]</td>
</tr>
<tr>
<td>Tobacco use</td>
<td>0.604</td>
<td>1.322 [0.459-3.814]</td>
<td>0.5811</td>
<td>1.289 [0.603-2.754]</td>
</tr>
<tr>
<td>Adjuvant CT</td>
<td>0.869</td>
<td>0.938 [0.439-2.006]</td>
<td>0.402</td>
<td>1.268 [0.726-2.215]</td>
</tr>
<tr>
<td>No. of tumors</td>
<td>0.009</td>
<td>2.823 [1.246-6.395]</td>
<td>0.016</td>
<td>2.232 [1.139-4.371]</td>
</tr>
<tr>
<td>Necrosis</td>
<td>0.49</td>
<td>1.297 [0.619-2.718]</td>
<td>0.249</td>
<td>1.384 [0.794-2.413]</td>
</tr>
<tr>
<td>Emboli</td>
<td>0.0021</td>
<td>2.318 [1.113-4.824]</td>
<td>0.005</td>
<td>2.179 [1.253-3.787]</td>
</tr>
<tr>
<td>TNM Stage 0</td>
<td>0.512</td>
<td>1.0</td>
<td>0.011</td>
<td>1.0</td>
</tr>
<tr>
<td>Stage IA-IB</td>
<td></td>
<td>1.039 [0.330-3.277]</td>
<td>0.011</td>
<td>2.463 [0.881-6.886]</td>
</tr>
<tr>
<td>Stage IIA-IIB</td>
<td></td>
<td>1.546 [0.536-4.456]</td>
<td></td>
<td>4.265 [1.620-11.226]</td>
</tr>
<tr>
<td>Stage IIIA-IIIB</td>
<td></td>
<td>2.088 [0.636-6.853]</td>
<td></td>
<td>3.793 [1.268-11.342]</td>
</tr>
<tr>
<td>At least 10% of lepidic component</td>
<td>0.503</td>
<td>0.770 [0.358-1.657]</td>
<td>0.0156</td>
<td>0.659[0.369-1.177]</td>
</tr>
<tr>
<td>None to mild RhoB expression</td>
<td>0.0008</td>
<td>7.923 [1.882-33.349]</td>
<td>&lt; 0.0001</td>
<td>5.236 [2.233-12.281]</td>
</tr>
</tbody>
</table>

**TNM:** Tumor-Node Metastasis stage. **CT:** chemotherapy
Figure Legends

**Figure 1:** Prognostic value of RhoB in non-small-cell lung cancer. Overall survival (A, C) and progression-free survival (B, D) of operated patients according to RhoB expression assessed by immunohistochemistry from series 1 (0 and + vs. ++ and +++) (A, B), or by RhoB mRNA expression (RT-qPCR) from series 2 (C, D).

**Figure 2:** RhoB expression and prognostic value in adenocarcinoma with lepidic component. A. Percentage of lepidic component in adenocarcinomas according to RhoB expression. B. Overall survival according to RhoB expression in series 3: i.e., 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. Percentage of Ki67-positive cells in tumor nodules. Histograms represent 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).

**Figure 4:** Impact of RhoB expression on lung tumor grade in a murine model of adenocarcinoma with lepidic features. A. Histological grades of lung tumors, highlighting the different stages of tumor progression. (a-b) Grade 1 lesion, consisting of small size focal condensed nodules of atypical adenomatous hyperplasia. (c-d) Grade 2 tumor (low grade adenoma) composed of well-differentiated, more “plump” cuboidal cells. (e-f) Grade 3 tumor (high grade adenoma)
characterized by an increase in cell density, cell size and nuclear to cytoplasmic ratio. (g-h)

Grade 4 tumor (adenocarcinoma) of larger size, showing greater cytological 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 x200 and x400 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 represent means ± SEMs. ($EGFR^{L858R}/rhob^{+/+}$, n=6; $EGFR^{L858R}/rhob^{+/-}$, n=7; $EGFR^{L858R}/rhob^{-/-}$, n=6).
Figure 1

A) Overall survival

B) Progression free survival

C) Series 2 (

D) p = 0.02

p = 0.001

p = 0.12

p = 0.05

Research.

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Figure 2

A

Lepidic (%):  
- 0-10  
- 10-70  
- > 70

Percentage of tumors

RhoB: 0 + ++ +++

B

% survival

High RhoB

Low RhoB

p = 0.009

months

0  5  10  15  20  25  30  35  40
Figure 3

A

Tumor/total lung (%)

rhob: +/+  +/-  -/-

p = 0.005
p = 0.003

B

Number of tumor nodules/cm^2

rhob: +/+  +/-  -/-

p = 0.03
p = 0.016

C

Tumor nodules size (mm^2/cm^2)

rhob: +/+  +/-  -/-

p = 0.0022
p = 0.0012

D

Ki67 positive cells in tumor nodules (%)

rhob: +/+  +/-  -/-

p = 0.0004
p < 0.0001
Figure 4

A

B

Number of tumors / cm²

grade I
grade II
grade III
grade IV

rhob^{+/-}  rhob^{+/-}  rhob^{-/-}
RhoB determines tumor aggressiveness in a murine EGFRL858R-induced adenocarcinoma model and is a potential prognostic biomarker for lepidic lung cancer

Olivier Calvayrac, Anne Pradines, Isabelle Raymond-Letron, et al.

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