Supplemental Data

RHOB Influences Lung Adenocarcinoma Metastasis and Resistance in a Host-Sensitive Manner

Luis Ravelo D. et al.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animals and Cell culture

Four week-old athymic nude mice were purchased from Harlan (Barcelona, Spain) and maintained under specific pathogen-free conditions. Murine stromal ST-2 cell line was a kind gift from Dr. Civitelli (Washington University, St. Louis, MO). The A549 cell line was a kind gift from Dr. Gazdar (University of Texas Southwestern, Dallas, TX). The cell line was authenticated by sequencing of several described mutations. A549 cells were obtained from a IIIA stage mixed AC, was derived from tumors T3 or T4 or mediastinal or supraventicular lymphoadenopathy (N2 or N3) lacking clinically detected distant metastasis.

Microarray hybridization and data analysis

We included three different highly metastatic subpopulations (called M1M1, M1M3 and M1M4) (fig. 1e). For each cell line, two independent biological replicates were performed and, for each replicate, a pool of three independent mRNA extractions was used to hybridize each human GeneChip high-density oligonucleotide microarray (Affimetrix HG_U133A_2). As controls we included four parental tumoral cell lines, two of which were transduced with luciferase vector and two other untransduced. Thus, a total set of 10 human expression micorarrays were hybridized: 4 with control samples and 6 metastatic cell lines. Bioinformatic data analysis was performed as detailed previously (1).

In silico experiments

Gene expression and clinical data for 111 cases of primary lung carcinomas were obtained from Bild et al. (2). This cohort of 111 total samples included 58 ACs and 53 squamous cell carcinomas. Using the median as a cut off, samples were stratified as displaying high and low (above and below the median, respectively) RHOB levels, by using a well-annotated probe set 1553962_s_at.

In vitro assays

Chemotaxis assays were performed with conditioned medium from murine bone marrow stromal ST-2 cells as chemoattractant placed in the lower compartment of 8-µm pore Boyden chamber. Cells were maintained in serum free conditions for 24h and seeded (5×10^4) on the upper compartment in serum-free medium per well in 24-well plates (Costar). After 12 hours, cells on the top chamber were wiped with a cotton swab, and cells in the lower compartment were fixed and stained with cristal violet. Number of migratory cells was evaluated with a computerized image analysis system, AnalySIS® (soft imaging system GmbH, Münster, Germany). For the invasion assays, cells (2×10^5) were seeded in each well, in duplicates for each condition. The upper chamber were precoated with 420 ng matrigel per µL (Sigma) dried at room

temperature for 5 hours, or with 50 μg /mL of collagen type I (Inamed, Fremont, CA, USA), dried at 37 ºC overnight. The experiment was performed for 24 h.
Adhesion experiments were performed according to previously published protocols (3). Substrates used were fibronectin, hyaluronic acid, collagen type I, gelatin and as controls plastic and BSA, were used.

Overexpression constructs
Human RHOB cDNA was cloned in pBABE retroviral vector (Addgene, Cambridge, MA, USA) by PCR from lung epithelial cells using primers. Insert cDNA was cloned into pCR2.1 TA, excised with EcoRI and cloned into pBABE-neo and pBABE-hygro (Addgene, Cambridge, MA, USA). The insert was sequenced to check for its integrity. To obtain viral particles, packaging cells were transfected with 8 μg of DNA for each fusion type by LIPOFECTAMINE™ 2000 method according to manufacturer's recommendations. Empty vector was used to obtain mock infective viral particles. Two days after transfection, supernatants were centrifuged for 10 minutes at 600 g and filtered through 0.45 μm pore cellulose acetate filter. For transduction infection, A549 were seeded at $1x10^5$ cells and incubated overnight with viral supernatants in the presence of 4 μg/ml polybrene (Sigma). Forty-eight hours post-infection, cell populations were incubated in medium containing the appropriate antibiotic for two additional weeks. Antibiotic-resistant pools were expanded and frozen at each cell passage.

Knock-down constructs
Lentiviral vectors containing short hairpin for RHOB were obtained from shRNA Mission (Sigma). To obtain viral particles, packaging cells were transfected with 8 μg of plasmid by calcium phosphate method. Empty, and scramble vectors were used to obtain mock and scramble infective viral particles. Two days after transfection, supernatants were centrifuged for 10 min at 600 x g and filtered through 0.45 μm pore cellulose acetate filter. For transduction, cells were seeded at 1×10^5 and incubated overnight with viral supernatants. Forty-eight hours post-infection, cell populations were incubated in medium containing the appropriate antibiotic for two additional weeks. Antibiotic resistant pools were expanded and frozen at each cell passage.

Real-time quantitative PCR
Total RNA was isolated from 70-80% confluent cultures using Trizol RNA (2μg) and DNase I treated and reversed transcribed using Superscript II reverse transcriptase and oligo(dT) primers. Assay-on-Demand™ (Applied Biosystems) was performed using a Gene Amp 7300 sequence detection system (Applied Biosystems) according to manufacturer´s recommendations. The mean cycle threshold value (Ct) from triplicate samples was used to calculate the gene expression. PCR products were normalized to GAPDH levels. Experiment was repeated three times with identical results.

Western blot analysis
Blots were incubated overnight at 4ºC with primary antibodies directed to RHOB diluted 1:600 (119, SantaCruz Biotechnology sc-180), GAPDH at 1:15000 (MA1-81915, ABR) and β-actin at 1:10000 (A5441, Sigma). Blots were washed 3 times with TBS-Tween and incubated with HRP-linked secondary antibodies against rabbit or mouse immunoglobulins (Amersham) for 1 hr at room temperature.

Immunohistochemistry
Specificity of the anti-RHOB antibody was assessed by western blot analysis from protein lysates extracted from paraffin sections, showing correlation for RHOB expression by immunohistochemistry In addition, neutralization using blocking peptide against RHOB antibody confirmed the specificity of staining (Sup. Fig 2 and 3).

Clinical samples
A series of 78 patients with a diagnosis of NSCLC who underwent surgical resection in the Hospital of the University of Navarra (CUN, Pamplona, Spain) from 2000 to 2007 were included in this study. Tissue specimens were examined and classified according to the 2004 WHO classification system. Tumors were further classified according to the revised International System for Staging Lung Cancer. Individuals were separated in two groups selected on the basis of Progressive Disease (PD) compared to a Disease Free (DF) group post-surgery. "PD patients" refers to a history of a completely resected primary lung cancer that developed the disease (recurrence or metastasis) after a 6 months disease free period post-surgery. "DF patients" refers to all other individuals with a total resection of primary lung cancer who continued with any signs of disease. The study protocol was approved by the Local Institutional Medical Ethical Committee. Relapse time was calculated from the date of surgery to the date of detection of recurrence. Survival status was verified and updated as of September 1st, 2009. The mean follow up time was 30 months. None of these patients received neoadjuvant chemotherapy, although 22 patients received only platinum-based adjuvant chemotherapy and 12 received both chemo- and postoperative radiotherapy.
To better assess the role of RHOB in lung cancer, we also evaluated its expression in a validation set of 234 NSCLC cases collected in the MD Anderson Cancer Center (Houston, USA) with the same inclusion criteria as the previous series. Since this series from MD Anderson Cancer Centre includes a longer mean follow up (66 months), we assessed the survival time instead of TP. Detailed clinical and pathologic information of both series is summarized in **Sup. Table S1**.

Immunohistochemical analysis
For this analysis, we selected 78 individuals that were separated in two groups selected on the basis of Progressive Disease (PD) compared to a Disease Free (DF) group post-surgery. PD patients referred to a history of a completely resected primary lung cancer that developed the disease (recurrence or metastasis) after a 6 months disease free period post-surgery. DF patients were all other individuals with a total resection of primary lung cancer who continued with no sign of the disease. Patients having any treatment with radiotherapy or chemotherapy after the surgery were excluded from the analysis. The study protocol was approved by local ethics committee. The inclusion criteria used to enrol the patients were: histology (NSCLC: AC and squamous cell carcinomas), and absence of cancer within the five years previous to the lung cancer surgery, except melanoma or skin cancer. Relapse time was calculated from the date of surgery to the date of detection of recurrence.
Formalin-fixed paraffin-embedded tissue sections were used for the immunohistochemical procedures. Paraffin was removed from the tissues and the sections were hydrated through a graded series of ethanol. Endogenous peroxidase activity was quenched with 3% hydrogen peroxidase for 10 minutes. Microwave antigen retrieval was carried out with EDTA buffer (0,5 mM, pH 8) for 2 x 15 min. Non-specific binding sites were blocked with 5% goat normal serum in TBS-Tween (Wash buffer, Dako) for 30

minutes. Sections were incubated with anti-RHOB antibody (RHOB 119, sc-180) overnight at 4ºC. The working dilution was: 1:75. After rinsing with TBS, sections were incubated with polyclonal Envision complex (Dako). The peroxidase activity was demonstrated by diaminobenzidine. Finally, sections were washed in water, lightly counterstained with haematoxylin, dehydrated and mounted in DPX. Tissues expressing different levels of RHOB were included in each immunohistochemical run to unify the possible intensity discordance. Two observers blinded to the patient status, independently evaluated the extension and intensity of the staining (6). For RHOB, the extension was scored as percentage of positive cells (0-100%) and the intensity of staining was assessed compared with a known external positive control (1+, mild; 2+, moderate and 3+, intense labelling). Discordant independent reading was resolved by simultaneous review by both observers. For anti-GFP staining antigen retrieval was carried out with TE buffer pH 9.0 (95ºC, 30 min). Slides were incubated with an anti-GFP antibody at 1:4000 overnight at 4ºC (ab6556).

Biomolecular interaction analysis by surface plasmon resonance (SPR)

The interaction experiments were performed using surface plasmon resonance (SPR) technology in a BIAcore X Biosensor (BIAcore AB, Uppsala, Sweden). First, the antibodies which were covalently immobilized on the flow channel 2 of a CM5 sensor chip (BIAcore) by amine coupling following the manufacturer's instructions surface density of 4,000 RU, were prepared. To create a reference surface, similar amounts of neutral antibody were immobilized on flow channel 1. Then, recombinant 100 nM of human RHOA, RHOB and RHOC were injected in triplicate in HBS-T buffer [10 mM Hepes, pH 7.4, 150 mM NaCl, 0.005% (v/v) Tween 20] at a flow of 30 µl/min and allowing an association time of 60 s. The antibody surface was regenerated after each analyte injection by a 10-s injection of 100 mM phosphoric acid. Mass transport limitation effects were excluded.

REFERENCES

1. Vicent S, Luis-Ravelo D, Anton I, *et al.* A novel lung cancer signature mediates metastatic bone colonization by a dual mechanism. Cancer Res 2008;68(7):2275-85.
2. Bild AH, Yao G, Chang JT, *et al.* Oncogenic pathway signatures in human cancers as a guide to targeted therapies. Nature 2006;439(7074):353-7.
3. Lai CF, Chaudhary L, Fausto A, *et al.* Erk is essential for growth, differentiation, integrin expression, and cell function in human osteoblastic cells. J Biol Chem 2001;276(17):14443-50.
4. Yin JJ, Selander K, Chirgwin JM, *et al.* TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. J Clin Invest 1999;103(2):197-206.
5. Hernandez I, Moreno JL, Zandueta C, Montuenga L, Lecanda F. Novel alternatively spliced ADAM8 isoforms contribute to the aggressive bone metastatic phenotype of lung cancer. Oncogene 2010;29(26):3758-69.
6. Decaussin M, Sartelet H, Robert C, *et al.* Expression of vascular endothelial growth factor (VEGF) and its two receptors (VEGF-R1-Flt1 and VEGF-R2-Flk1/KDR) in non-small cell lung carcinomas (NSCLCs): correlation with angiogenesis and survival. J Pathol 1999;188(4):369-77.