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RASAL2 promotes lung cancer metastasis through epithelial–mesenchymal transition

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ABSTRACT

It was reported that genetically-engineered RASAL2 knockout mice are prone to development of several sporadic tumor, including lung adenocarcinoma. However, a causative relationship between RASAL2 deficiency and lung adenocarcinoma development still remains unknown. In the present study, RASAL2 level was determined in patients with lung adenocarcinoma and control subjects in an attempt to explore its potential clinical diagnostic and prognostic value. Low RASAL2 expression levels were found in 71% (37 of 52) of lung adenocarcinoma, which were correlated with lymph node metastasis in lung adenocarcinoma. Moreover, Low RASAL2 expression levels were correlated with reduced overall survival (OS) in lung adenocarcinoma. We find that inactivation of RASAL2 promotes lung cancer cell migration through the induction of epithelial mesenchymal transition (EMT) and promoted lung metastasis in nude mice. Our results suggest that the down-regulation of RASAL2 promotes metastatic progression of lung adenocarcinoma, hence it could serve as a potential target for the development of lung cancer therapies.

1. Introduction

Lung cancer is a leading cause of cancer mortality worldwide; it accounts for over a million deaths annually and still has a poor prognosis [1]. Non-small cell lung cancer (NSCLC) is the predominant form of lung cancer and consists of 2 major histological subtypes: squamous cell carcinoma and adenocarcinoma [2]. Lung adenocarcinoma is the most common type of lung cancer and accounts for almost half of primary lung tumors [3]. Searching and validating the molecular mechanisms involved in the pathogenesis of lung adenocarcinoma will be helpful for the development of better prognostic markers and novel therapeutic targets to improve clinical treatment of patients with lung adenocarcinoma. Oncogenic Ras represents a common molecular change in human lung adenocarcinomas [4,5].

The Ras function as binary switches cycling between an inactive GDP-bound form and active GTP-bound form [6]. The conversion from the inactive to active state is catalyzed by guanine nucleotide exchange factors (GEF), and the return to the inactive form is aided by GTPase-activating proteins (GAPs) [7,8]. RASAL2, which is a Ras-GAP, functions as a tumor suppressor in breast cancer [9]. RASAL2 inactivation plays a causal role in breast cancer development and metastasis. Rasal2/Trp53 mutant animals developed highly metastatic mammary adenocarcinomas, hepatocellular carcinomas, lung adenocarcinomas, and various sarcomas [9]. In addition, RASAL2 is identified as a tumor suppressor in ovarian cancer, and down-regulation of RASAL2 promotes ovarian cancer progression [10]. RASAL2 was also identified as important candidate involved in the progression of thyroid cancer [11]. However, the roles of RASAL2 in lung cancer metastasis and the related mechanisms are still unclear. In this study, we demonstrated that RASAL2 knockdown was important for lung cancer metastasis. Mechanistically, RASAL2 knockdown promoted lung cancer metastasis through induction of epithelial mesenchymal transition (EMT) potentially via ERK activation. Taken together, our data have uncovered the important role of RASAL2 and related mechanism in lung cancer metastasis as well as identified a potential target for future metastatic lung cancer therapy.

2. Material and methods

2.1. Tissue specimens and immunohistochemistry analysis

A total of 52 human lung cancer specimens were collected with patient consents in the First Affiliated Hospital of Henan University of Traditional Chinese Medicine from January 2006 to June 2009 with the approval from the Institute Research Ethics Committee. The lymph node status was pathologically evaluated using

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resected specimens. The patients' clinical records and histopathologic diagnosis were documented, including follow-up data as of December, 2011. IHC staining of RASAL2 was performed using a rabbit anti-RASAL2 polyclonal antibody (Abcam) at 1:500 dilution. Five-micron tissue slides from tumor and adjacent normal lung tissue were deparaffinized using xylene. Heat-mediated antigen retrieval was performed using citrate buffer.

2.2. Transfection and RNA Interference

The RASAL2-shRNA1 target sequence was 5′-CCCTCGTGTTCTTG CTGATAT-3′. The RASAL2-shRNA2 target sequence was 5′-GCCTTC-CACC TCTTCATAGTA-3′ [10]. Cell lines were plated in six-well plates with fresh media without antibiotics for 24 h before transfection. Transfection was performed using Lipofectamine2000 (Life Technologies) according to manufacturer's protocol. Transfected cells were plated in 10 cm dishes for selection with G418 (500 mg/ml; Life Technologies). Stable transfectants were maintained in regular medium with G418 (300 mg/ml) for further analysis.

2.3. qRT-PCR and Western blotting

Expression of RASAL2 mRNA was determined by quantitative reverse-transcription (qRT-PCR). Actin was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. The primers used for PCR were as follows: RASAL2: sense 5′-AGCAGAAAGGTCCCC TCGTAG-3′; antisense 5′-AGGGT-GAGGTATTTGCAGTGT-3′; Actin: sense 5′-CG TGGACATCCGTAAA-GACC-3′; antisense 5′-ACATCTGCTGGAAGGTG GAC-3′ [10]. For Western blot, the cells were lysed in RIPA buffer. Proteins at the same amount were separated by 12% SDS polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. After probing with antibodies, the signals were visualized by Supersignal enhanced chemiluminescence reagent (Pierce, Rockford, IL). The antibodies used were anti-RASAL2 (Santa Cruz Biotechnology), E-cadherin, Vimentin and p-EKR (Cell Signaling Technology).

2.4. Wound healing assay

Cell migration was assessed by measuring the movement of cells into a scraped, acellular area created by a 200 ml pipette tube, and the spread of wound closure was observed after 48 h and photographed under a microscope. We measured the fraction of cell coverage across the line for migration rate.

2.5. Transwell assay

Cells were treated with PD98059 at a confluence of 70% for 24 h. 1×105 cells were suspended in media and added on top of the Boyden chamber membrane (Corning). Culture medium with 10% FCS was added on the bottom of the membrane. Chambers were incubated at 37 °C, 5% CO_2 for 4 h. Migrated cells were fixed and stained with crystal violet/methanol. Cells not migrated were removed with a q-tip. Pictures (10 magnifications) were taken and migrated cells were counted and normalized to control cells (100% migration).

2.6. Animal experiments

Female BALB/c nude mice and female severe combined immunodeficient/beige mice at 6 weeks old, were purchased from the Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, P.R. China). For tumor metastasis analysis, nude mice (for) were inoculated with 1×106 viable A549 cells in 200 ml of phosphate buffered saline via tail vein injection. Four weeks later, mice were sacrificed and examined for development of pulmonary metastasis under microscopy.

2.7. Statistical analysis

Differences between variables were assessed by the χ^2 test. The Kaplan–Meier method was used to estimate overall survival. P value less than 0.05 was considered statistically significant.

3. Results

3.1. RASAL2 expression was associated with improved survival in lung adenocarcinoma

RASAL2 expression was analyzed in 52 paired lung adenocarcinomas and adjacent non-tumor tissues. Results were compared with the clinicopathologic features of the patients from whom the tissues were taken. Expression of RASAL2 in non-tumor tissues and carcinomas detected by immunohistochemistry were semi-quantitated. Overall, expression of RASAL2 was absent in 10 of 52 in non-tumor tissues (19%) and 37 of 52 (71%) carcinomas. Representative examples of RASAL2 protein expression in lung adenocarcinoma samples are shown in Fig. 1. To characterize the correlation between downregulation of RASAL2 and clinical features of lung adenocarcinomas, several clinicopathological characteristics including cell differentiation, tumor size and smoking status were compared between patients with or without expression of RASAL2 (Table 1). The result found that expression of RASAL2 was not associated with tumor size ($P = 0.233$), poor differentiation ($P = 0.346$), smoking status ($P = 0.219$), patient's age and gender (data not shown). However, expression of RASAL2 was significantly correlated with lymph node metastasis ($P = 0.016$).

Kaplan–Meier analysis using Cox proportional hazards modeling also demonstrated strong associations between RASAL2 expression and survival. Overall survival was significantly better in patients with high RASAL2 expression group than in those with low RASAL2 expression group in all patients with lung adenocarcinomas (Fig. 2).

3.2. RASAL2 knockdown promoted Migration of lung cancer cells

To investigate the role of RASAL2 in lung cancer metastasis, we first used shRNAs to knockdown RASAL2 expression in A549 cells (Fig 3A and B). We found that decreased RASAL2 expression dramatically promoted the migration in wound-healing assay in A549 cells (Fig. 3C). The effect of RASAL2 on migration capacity of A549 cells was also assessed by the transwell assays. The results showed that knockdown of RASAL2 caused an increasement of cell migration in the A549 cell lines using a transwell assay (Fig. 3D). We further examined the impact of RASAL2 knockdown upon lung cancer metastasis using lung seeding assays in vivo. A549 cells with or without RASAL2 knockdown were injected intravenously into nude mice (five to six mice each group) via tail vein. Many large lung tumor nodules were visible in RASAL2 knockdown group but not the control group (Fig. 4A), suggesting that RASAL2 knockdown significantly promoted the lung metastasis ability of A549 cells.

3.3. RASAL2 knockdown promotes migration capability of lung cancer cells via EMT through ERK regulation

The mechanism by which decreased RASAL2 expression promoted migration in lung adenocarcinoma cells has yet to be elucidated. EMT is an important event during cancer progression and

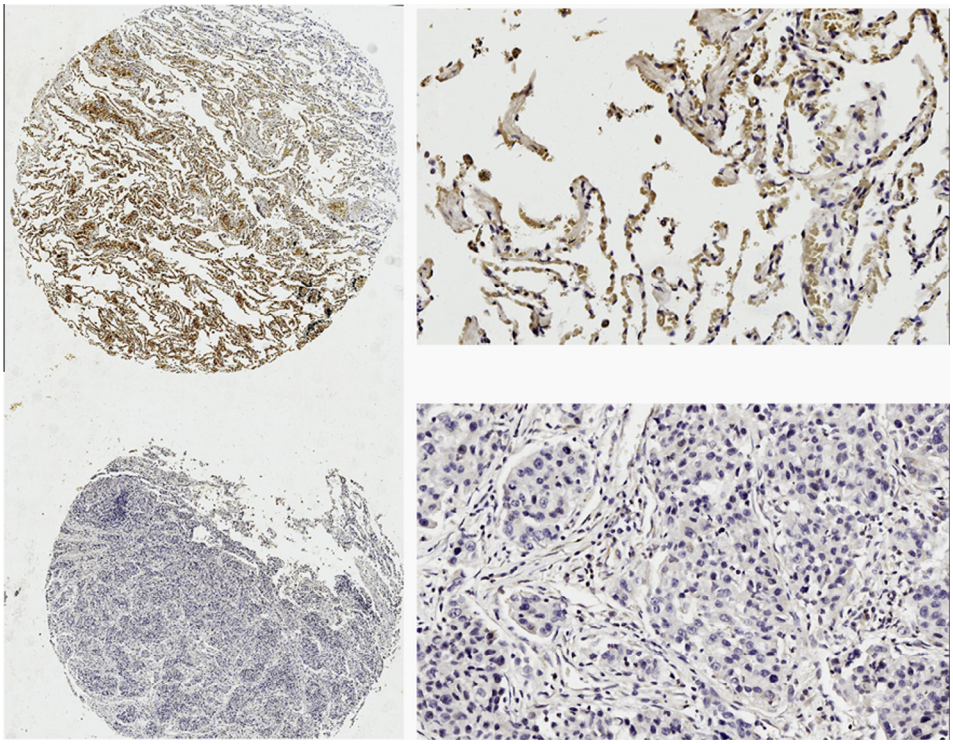

Fig. 1. RASAL2 expression in lung adenocarcinoma and its adjacent alveolar epithelial cells. RASAL2 expression was assessed by immunohistochemical staining in a human alveolar epithelial cells (upper panel) and lung adenocarcinoma cells (lower panel). Original magnification: left panel, ×50; right panel, ×400.

Table 1
RASAL2 expression and clinical characteristics of lung adenocarcinoma patients.

Group		RASAL2 expression		*P* value
		Low	High	
Cancer tissues		37	15	
Differentiation	Well	4	4	
	Moderate	23	6	
	Poor	10	5	$P = 0.346$
LN metastasis	Yes	27	4	
	No	10	11	$P = 0.016$
Size (cm)	<3	20	8	
	⩾3	17	7	$P = 0.233$
Smoking status	Yes	22	9	
	No	15	6	$P = 0.219$

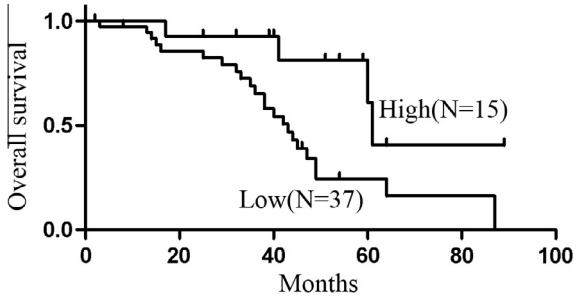

Fig. 2. Overall survival curves according to RASAL2 gene expression. Patients with low RASAL2 expression showed a significantly poorer prognosis than those with high RASAL2 expression.

metastasis, which improves cell intrinsic capabilities for local invasion and distant organ metastasis. We found RASAL2 knockdown in A549 cells increased protein level of the mesenchymal marker Vimentin but decreased the epithelial marker E-cadherin by Western blot analyses.

RASAL2 regulation of EMT and cell invasion is at least partially ERK-dependent in breast cancer and ovarian cancer. We then asked whether ERK was involved in the EMT process induced by decreased RASAL2 expression in lung cancer cells. To test this hypothesis, A549 cells was treated with PD98059, an ERK pathway inhibitor. The RASAL2-silencing A549 cells showed a significant increase in the p-ERK (Fig. 4B). Our results also showed that PD98059 increased Vimentin expression but inhibited E-cadherin expression regulated by shRASAL2 in A549 cells (Fig. 4B). Moreover, we found that decreased RASAL2 expression dramatically promoted the migration in transwell assay, the converse phenomena were observed when ERK was inactivated by PD08059, suggesting that RASAL2 regulation of EMT and cell migration is at least partially ERK-dependent (Fig. 4C).

4. Discussion

EMT has been considered to be a critical biological process in tumor invasion, progression and metastasis [12,13]. However, the underlying mechanisms remain to be fully understood. It was reported that genetically-engineered RASAL2 knockout mice are prone to development of highly metastatic lung adenocarcinomas [9]. However, the expression of RASAL2 in lung adenocarcinomas and the molecular mechanisms by which RASAL2 exerts its functions and modulates the malignant phenotypes of lung adenocarcinoma cells remain largely unknown. In this study, we demonstrate that decreased RASAL2 expression is an important player in promoting lung cancer metastasis via regulation of the EMT process potentially through ERK activation.

First, RASAL2 expression was analyzed in 52 paired lung cancers and adjacent non-tumor tissues. We found that RASAL2 expression was down-regulated in lung adenocarcinomas tissues compared with normal tissue, which was kept in line with its role of tumor suppressor gene. We found that downregulation of RASAL2 was not associated with tumor size, poor differentiation, patient's age

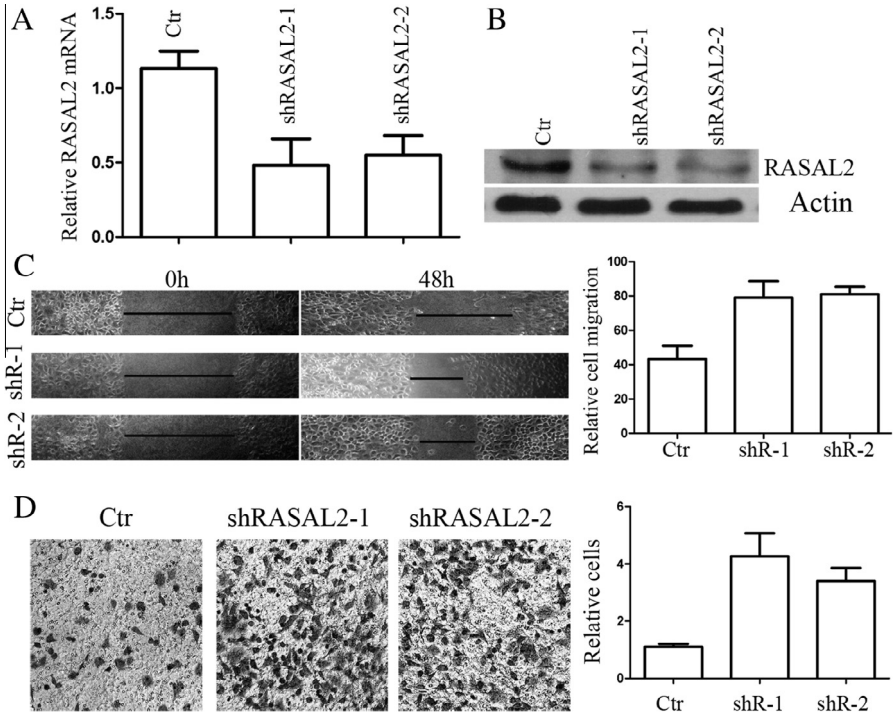

Fig. 3. RASAL2 is important for motility and metastasis of A549 cells. (A) Real-time RT-PCR showed the efficiency of RASAL2 knockdown in A549 cells. Data were shown as mean ± SD. (B) Western blot showed the efficiency of RASAL2 knockdown in A549 cells. Values were normalized to Actin. (C) The wound-healing assay showed knockdown of RASAL2 obviously promoted the migration of A549 cells. Data were shown as mean ± SD. (D) Knockdown of RASAL2 significantly promoted migration of A549 cells assessed by transwell assay. Data were shown as mean ± SD.

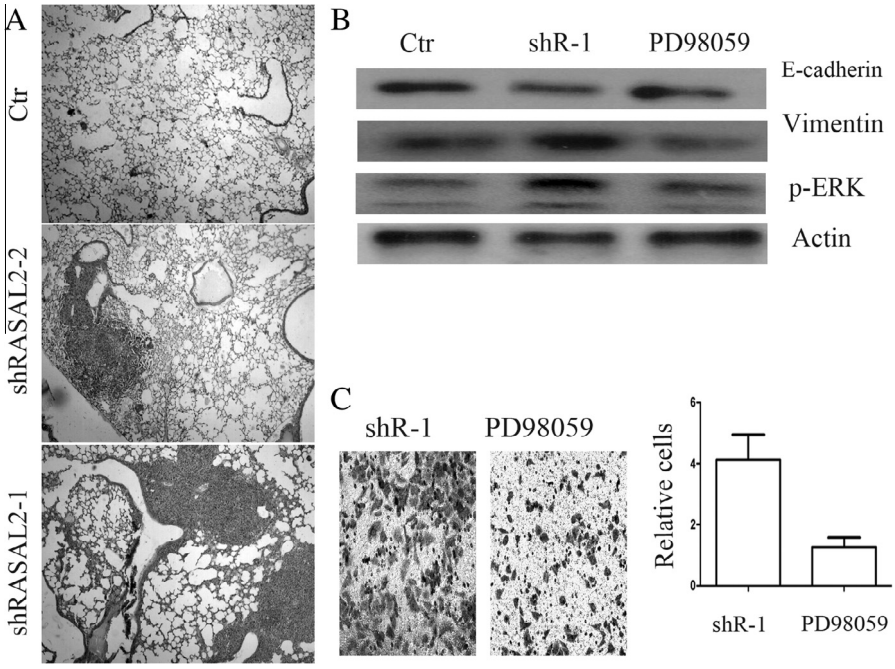

Fig. 4. ERK potentially contributes to EMT induced by knockdown of RASAL2 in lung cancer cells. (A) Pathology analyses showed that knockdown of RASAL2 significantly increased lung metastasis of A549 cells in nude mice lung seeding assay. (B) Knockdown of RASAL2 decreased E-cadherin and p-ERK expression of but increased vimentin expression in A549 cells assessed by Western blot. Inhibition of EKR by PD98059 partially reversed the modulation of E-cadherin or vimentin expression by knockdown of RASAL2 in A549 cells. Actin was used as control. (C) Inhibition of EKR by PD98059 partially reversed the modulation of cell migration by knockdown of RASAL2 in A549 cells.

and gender. However, downregulation of RASAL2 was significantly correlated with lymph node metastasis. Similarly, Down-regulation of RASAL2 expression is observed in primary human breast cancer and ovarian cancer, and is associated with metastasis. These reports are consistent with our results. We also found that overall survival was significantly better in patients with high RASAL2 expression group than in those with low RASAL2 expression group in all patients with lung adenocarcinomas.

Previous studies have shown that knockdown of RASAL2 dramatically decreases the motility and metastasis of breast cancer cells and increase spontaneous lung cancer metastasis in vivo [9]. On the other hand, it was reported that depletion of RASAL2 specifically inhibited cancer cell growth and cell invasive capacities in liver cancer. However, there is no effect on normal cell growth, suggesting a ubiquitous causal role for RASAL2 in driving cancer growth and metastasis. RASAL2 depletion interferes with AKT, WNT, and MAPK signaling pathways as well as regulation of epigenetic proteins that were previously demonstrated to drive cancer growth and metastasis [14]. To investigate the role of RASAL2 in lung cancer metastasis, we first used shRNAs to knockdown RASAL2 expression in A549 cells. The effect of RASAL2 on migration capacity of A549 cells was assessed by the transwell and wound-healing assay assays. We found that decreased RASAL2 expression dramatically promoted the migration ability of A549 cells. We further examined the impact of RASAL2 knockdown upon lung cancer metastasis using lung seeding assays in vivo. Pathology analyses showed that knockdown of RASAL2 significantly increased lung metastasis of A549 cells in nude mice lung seeding assay. The mechanism by which decreased RASAL2 expression promoted migration in lung adenocarcinoma cells has yet to be elucidated. E-cadherin is essential for epithelial cell adhesion, loss of E-cadherin expression would likely promote cancer metastasis [15]. We found that knockdown RASAL2 expression in A549 cells increased protein levels of the mesenchymal marker Vimentin but decreased the epithelial marker E-cadherin, suggesting that low expression of RASAL2 in vitro could significantly promote the EMT process. It was reported that RASAL2 regulation of EMT and cell invasion is at least partially ERK-dependent in ovarian carcinoma and breast cancer [10]. Consistently, we found that inactivation of ERK by an ERK pathway inhibitor reverse E-cadherin downregulation and inhibited the migration ability of lung cancer cells caused by shRASAL2 in A549 cells.

RASAL2 was also identified as an ECT2-interacting protein that regulates RHO activity in astrocytoma cells [16]. RASAL2 knockdown leads to a conversion to an amoeboid phenotype. Increase in ECT2 expression or knockdown of RASAL2 expression both increased invasive capability of astrocytoma cells [16]. Amoeboid invasion is a rapid single-cell type of invasion that does not rely on degradation of the extracellular matrix, as opposed to mesenchymal invasion, which is slow and requires degradation of the extracellular matrix before cellular movement [16,17].

ECT2 amplification and overexpression was detected only in invasive lung adenocarcinoma, and not in *in situ* carcinoma [18]. ECT2 overexpression was an independent prognostic factor in lung adenocarcinoma [19]. We examined whether RASAL2 expression inversely correlated with ECT2 expression in lung adenocarcinoma. As expected, an inverse correlation between RASAL2 and ECT2 expression was observed lung adenocarcinoma ($r = -0.312$, $P = 0.005$) (Table S1). However, it remains unknown whether RASAL2 can regulate ECT2 activity and whether ECT2 is implicated in EMT induced by RASAL2 knockdown. We are currently undertaking studies to examine whether RASAL2 physically interacts with and modulates ECT2 activity in lung adenocarcinoma.

In summary, in this study, we investigated the potential role of RASAL2 in lung adenocarcinoma metastasis and its underlying mechanisms. Our data suggest that downregulation of RASAL2 plays an important role in lung adenocarcinoma cell metastasis, and that RASAL2 could be used as a new prognostic marker and as an effective therapeutic target for lung adenocarcinoma.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.020.

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