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RYBP Inhibits Progression and Metastasis of Lung Cancer by Suppressing EGFR Signaling and Epithelial-Mesenchymal Transition[1,2]

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Abstract

Lung cancer (LC) is a common lethal malignancy with rapid progression and metastasis, and Ring1 and YY1 binding protein (RYBP) has been shown to suppress cell growth in human cancers. This study aimed to investigate the role of RYBP in LC progression and metastasis. In this study, a total of 149 LC patients were recruited, and the clinical stage of their tumors, metastasis status, survival time, presence of epidermal growth factor receptor (EGFR) mutation, and RYBP expression levels were measured. RYBP silencing and overexpression were experimentally performed in LC cell lines and in nude mice, and the expressions of genes in EGFR-related signaling pathways and epithelial-mesenchymal transition (EMT) were detected. The results showed that RYBP was downregulated in LC compared with adjacent normal tissues, and low RYBP expression was associated with a more severe clinical stage, high mortality, high metastasis risk, and poor survival. Cell proliferation and xenograft growth were inhibited by RYBP overexpression, whereas proliferation and xenograft growth were accelerated by RYBP silencing. EGFR and phosphorylated-EGFR levels were upregulated when RYBP was silenced, whereas EGFR, p-EGFR, p-AKT, and p-ERK were downregulated when RYBP was overexpressed. Low RYBP expression was related to a high metastasis risk, and metastasized tumors showed low RYBP levels. Cell migration and invasion were promoted by silencing RYBP but were inhibited by overexpressed RYBP. In addition, the EMT marker vimentin showed diminished expression, and E-cadherin was promoted by the overexpression of RYBP. In conclusion, our data suggest that RYBP suppresses cell proliferation and LC progression by impeding the EGFR-ERK and EGFR-AKT signaling pathways and thereby inhibiting cell migration and invasion and LC metastasis through the suppression of EMT.

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Introduction

Lung cancer, the most common cause of cancer death [1,2], was estimated to have caused 733,300 new cases and 610,200 new deaths in China in 2015 [3]. Approximately 10% to 15% of LCs are small cell lung cancers [4], and about 80% are non–small cell lung cancers (NSCLCs) [5], including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, among others. The LC survival rate is very low, as it tends to progress and metastasize quickly.

A huge amount of effort has been devoted over the past decades to investigating the molecular mechanisms of LC progression and metastasis. The roles of biomarkers such as epidermal growth factor receptor (EGFR), ALK, MET, ROS-1, and KRAS have been illustrated in lung adenocarcinoma, and therapies targeting these biomarkers have been utilized in clinical settings [6]. EGFR in particular is a tyrosine kinase receptor and accelerates the autophosphorylation of tyrosine kinase, participating in cell differentiation, growth, and proliferation. EGFR mutations are often found in LC patients, mediating the response of LC to tyrosine kinase inhibitors (EGFR-TKIs, e.g., afatinib, erlotinib, and gefitinib) [7–9]. Phosphorylated EGFR mainly activates intracellular signal transduction pathways like RAS-RAF-MEK-ERK (extracellular signal–regulated kinase)-MAPK and PI3K-AKT-mTOR and thereby regulates cell survival and proliferation [10,11]. Reportedly, LC metastasis is related to epithelial-mesenchymal transition (EMT) [12], a pivotal mechanism underlying cancer metastasis and invasion [13]. EMT is characterized by loss of the epithelial phenotype, acquisition of the mesenchymal phenotype, as well as the loss of cell-cell polarity and adhesion; its hallmarks include functional loss of E-cadherin and upregulation of vimentin [14].

As a polycomb group protein, Ring 1 and YY1-binding protein (RYBP) represses gene transcription via epigenetic chromatin modification [15]. RYBP induces cell-cycle arrest, accelerates p53-mediated apoptosis via interaction with murine double minute2 (MDM2) and regulating the MDM2-p53 loop [16], and enhances Hippi-mediated apoptosis by modulating the interaction between caspase 8 and Hippi [17]. Infection with adenovirus-RYBP promotes apoptosis and inhibits tumor cell proliferation [18]. RYBP is a tumor suppressor in cancers like hepatocellular carcinoma (HCC) [19] and NSCLC [20] and has been found to be downregulated in HCC tissues compared with the matched noncancerous liver tissues. In addition, adenovirus-mediated overexpression of RYBP induces apoptosis in HCC cells and inhibits cell growth, invasion, and HCC tumor growth [19]. In a very recent report, Voruganti et al. found that RYBP was downregulated in NSCLC tissues and that low levels of RYBP were correlated with poor survival [20]. Also, RYBP overexpression has been shown to induce apoptosis in NSCLC cells and inhibit cell viability and tumor xenograft growth [20]. However, little is known about the role of RYBP in LC metastasis or the association of RYBP with EMT or EGFR-ERK/AKT signaling pathways.

In this study, RYBP level and EGFR mutation in LC were detected, and their associations with LC metastasis and survival were analyzed. Moreover, RYBP silencing and overexpression were performed to investigate the roles of RYBP in LC progression and metastasis, along with the corresponding mechanisms. Results of this study might provide a novel target, RYBP, for blocking LC progression and metastasis.

Methods

Patients and Tissue Specimens

A total of 149 LC patients who had undergone surgery by the same surgical team were recruited from the First Affiliated Hospital of Guangzhou Medical University between 2010 and 2015. Diagnoses of LC were pathologically confirmed by at least two pathologists, and LC stages were determined based on the International Union Against Cancer guidelines [21]. Survival time was defined as the interval between initial surgery and death. The clinical features of the patients are summarized in Table 1. According to the protocols approved by the Institutional Review Boards of the First Affiliated Hospital of Guangzhou Medical University, paired LC tissues and adjacent normal tissues were obtained from patients before any surgery, radiation therapy, or chemotherapy was administered. The First Affiliated Hospital of Guangzhou Medical University ethics committee approved this study, and all patients signed written informed consent forms before sampling and enrollment.

Cell Lines and Cell Culture

Human cell lines BEAS-2B, HCC827, PC9, NCI-H358, NCI-H1965, NCI-H1975, and A549 were purchased from ATCC (Manassas, VA). Cells were cultured in a medium composed of Dulbecco's modified eagle medium, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum (FBS) in a 37°C and 5% CO_2 incubator.

Transient Transfection

For the LC cell line with highest RYBP level, RYBP silencing was performed, mediated by shRNA. In contrast, RYBP overexpression was conducted in the LC cell line with lowest RYBP level. For the silencing and overexpression experiments, RYBP-vectors, RYBP-shRNAs, and the corresponding scrambled controls were designed and constructed. Transient transfection was then performed using Lipofectamine 2000 (Invitrogen, CA), and only the RYBP-vector and RYBP-shRNA with most significant effects were shown.

RNA Extraction and Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

According to manufacturer's instructions, total RNA was extracted from samples using the TRIzol kit (Invitrogen, USA), and the complementary DNA was synthesized using the PrimeScript RT reagent Kit (TaKaRa, Dalian, China). Next, qRT-PCR was

Table 1. Correlation of RYBP Expression in Tissue with Patients' Clinicopathological Variables in 149 Cases of Lung Cancer

Characteristics	RYBP Expression (%)		
	High (n = 74)	Low (n = 75)	P Value
Gender			.92
Male	39 (50)	39 (50)	
Female	35 (49.3)	36 (50.7)	
Age			
≥60	31 (46.3)	36 (53.7)	.556
<60	43 (52.4)	39 (47.6)	
TNM stage			<.001
I	57 (82.6)	12 (17.4)	
II	11 (16.9)	54 (83.1)	
III	6 (40)	9 (60)	
Clinical stage			.0036
I	38 (65.5)	20 (34.5)	
II	23 (34.8)	43 (65.2)	
III	13 (52)	12 (48)	
Distant metastasis			.0048
M0	34 (66.7)	17 (33.3)	
M1	40 (40.8)	58 (59.2)	
EGFR status			.0004
No mutation	36 (70.6)	15 (29.4)	
mutation	38 (38.8)	60 (61.2)	

performed in triplicate using the following primers: sense: 5′-TTTGCCCAGAAAGACAGCTT-3′ and antisense: 5′-GTCGT GCACATGCCAGTAAC-3′ for RYBP and sense: 5′-GAGTCAA CGGATTTGGTCGT-3′ and antisense: 5′-GACAAGCTT CCCGTTCTCAG-3′ for GAPDH, which was the internal control for all samples. Real-time PCR was performed using LightCycler Real-Time PCR System (Roche Diagnostics, Switzerland). The change of RYBP expression was calculated between samples using the $2^{-\Delta\Delta Ct}$ method.

Western Blot

Total proteins were extracted from the samples and quantified using ice-cold radioimmunoprecipitation assay lysis buffer and the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Equal quantities of protein were separated on 10% sodium dodecyl sulfate polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech), which were then blocked at room temperature for 1 hour in Tris-buffered saline with 0.2% Tween 20 containing 5% skim milk and probed with primary antibodies overnight at 4°C. Secondary antibodies included horseradish peroxidase label and were incubated for 1 hour at room temperature. The diluted concentrations of the primary antibodies were as follows: anti-human RYBP, GAPDH, EGFR, and phosphorylated-EGFR antibodies were from Sigma (St. Louis, MO); anti-human AKT, phospho-AKT, ERK, and phospho-ERK were from CST; anti-human E-cadherin and vimentin were from Santa Cruz Biotechnology.

Cell Proliferation and Colony Formation

Proliferation of LC cells was determined using the colony counting method. Cells were inoculated in six-well plates (1000 cells per well) and incubated for 1 week. Thereafter, cells were fixed with 4% paraformaldehyde for 15 minutes and stained by Giemsa for 30 minutes before colony counting.

Migration and invasion

In the migration assay, 1×10^5 cells were suspended in 200 μl FBS-deleted medium and then placed into the upper Matrigel-free chamber of a Transwell (Millipore, MA), whereas 600 μl of medium plus 10% FBS was placed in the bottom chamber. Instead, 1×10^5 cells were plated in the upper Matrigel (BD, CA)-coated Transwell chamber in the invasion assay. After a 24-hour incubation at 37°C, cells in the bottom chamber were stained with 1% crystal violet and counted under an optical microscope.

LC Model in Nude Mice

LC cells carrying either the RYBP-vector or RYBP-shRNA were subcutaneously inoculated in nude mice to create transplantation tumor models of human LC, and the models inoculated with LC cells carrying scrambled-vector or scrambled-shRNA were set as the negative controls. Tumor volume and weight were detected, and metastasized tissues from nude mice models were stained with hematoxylin and eosin. Animal experiments were approved by the animal research ethical committee of the First Affiliated Hospital of Guangzhou Medical University.

Statistical Analysis

Statistical analyses were conducted using SPSS 19.0 statistical software (SPSS, Chicago, IL), and results were shown as mean ± standard deviation (SD) based on at least three independent experiments. Differences of RYBP mRNA level between LC tissues and paired normal tissues were analyzed using the paired *t* test. The Kaplan-Meier method was used to calculate cumulative survival rates, and the log-rank test was utilized to determine the differences between survival curves. Univariate and multivariate cox regression analyses were performed for death and metastasis risks. For all analyses, a *P* value < .05 was set as the cutoff criterion for statistical significance.

Results

Low RYBP Expression in LC Cell Lines and Tissues

RYBP levels in BEAS-2B, HCC827, PC9, NCI-H358, NCI-H1965, NCI-H1975, and A549 cells were detected and were found to be significantly lower in LC cell lines than in normal human lung epithelial cells. (Figure 1, *A* and *B*) Additionally, all six LC cell lines predictably showed different RYBP levels because their origins also had different differentiation levels. Among these LC cell lines, A549 showed the highest RYBP level, so RYBP silencing was performed in A549. PC9 showed the lowest RYBP level, so the RYBP overexpression experiment was conducted in PC9. Expression levels of RYBP were also shown to be remarkably lower in LC tissues in comparison with the corresponding adjacent normal tissues (Figure 2, *A–C*). In addition, 75 of the 149 patients displayed low RYBP expression in their tumor tissues, whereas the remaining 74 patients had high RYBP expression (Table 1). EGFR mutation was found in 65.8% (98/149) of patients, 80% (60/75) of patients had low RYBP expression, and 48.6% (36/74) of patients showed high RYBP expression (Table 1). Distribution of TNM stage ($P < .001$), clinical stage ($P = .0036$), distant metastasis ($P = .0048$), and EGFR mutation status ($P = .0004$) in the low-RYBP group was significantly different from that in the high-RYBP group (Table 1), indicating the association between high clinical stage and low RYBP expression level.

RYBP Inhibits Death and Metastasis of LC

Multivariable Cox regression analyses were performed to evaluate whether EGFR mutation status and RYBP expression could be independent prognostic factors to predict hazard. Our data showed that EGFR mutation status, together with RYBP expression, acts as a potential predictive factor (Table 2). These results might be caused by EGFR-TKI therapy targeting the EGFR mutation.

In contrast, LC patients with low RYBP expression had a higher death risk in comparison with those with high RYBP expression ($P < .0001$, Figure 3*A*), suggesting a protective role of RYBP against LC death. Similarly, LC patients with low RYBP expression had a higher metastasis risk in comparison with those with high RYBP expression ($P = .04$, Table 2), and tumors without metastasis showed significantly higher RYBP mRNA levels than those with metastasis ($P < .01$, Figure 3*B*). These results suggest that RYBP plays a protective role against LC metastasis.

RYBP Represses the Proliferation, Migration, and Invasion of LC Cells

Transfection of A549 cells with RYBP-special shRNA resulted in a marked downregulation of RYBP protein levels compared with the negative control group. In addition, transfection of PC9 cells with the RYBP vector resulted in a marked upregulation of RYBP protein levels compared with the negative control. (Figure 4*A*) Cell proliferation was seen to significantly increase in A549 cells with silenced RYBP (*P* value < .05, Figure 4*B*) and was suppressed in PC9 cells with overexpressed RYBP (*P* value < .05, Figure 4*C*) in

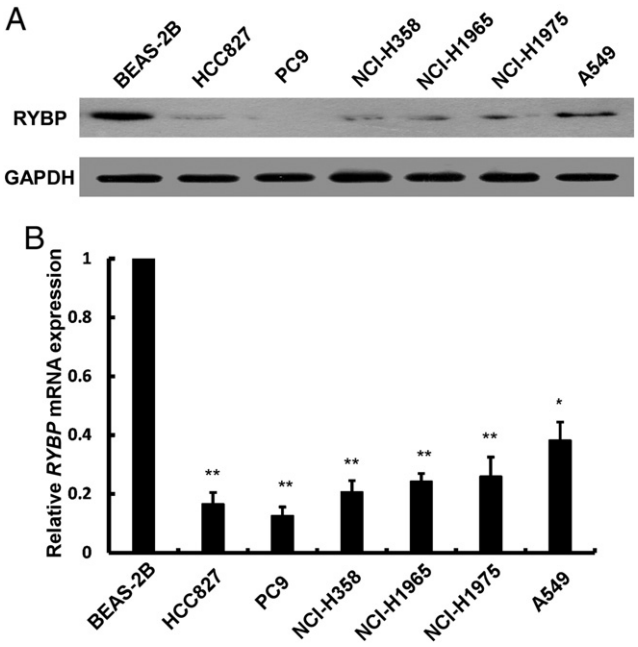

Figure 1. RYBP is downregulated in LC cell lines. (A) RYBP was downregulated in LC cell lines, as detected by Western blot and GAPDH as the loaded control. (B) RYBP was downregulated in LC cell lines, as measured by qRT-PCR and normalized by GAPDH. *P < .05 versus BEAS-2B.

comparison with the control group. In the Transwell assay, many more cells were found in the Matrigel-free and Matrigel-coated chambers of the RYBP-silencing group compared with the negative control (P value < .01, Figure 4*D*), indicating that RYBP silencing significantly promoted the migration and invasion capabilities of LC cells. On the contrary, overexpression of RYBP in PC9 cells obviously suppressed cell migration and invasion (P value < .01, Figure 4*E*).

RYBP Impedes the Growth of LC Xenograft

In nude mice models, the volume and weight (P value < .05) of tumors injected with RYBP-silence-A549 increased significantly in comparison with models injected with the negative control A549 (Figure 5). In contrast, the volume and weight (P value < .05) of tumors injected with RYBP-overexpress-PC9 decreased remarkably (Figure 5). These results suggest that RYBP might inhibit the growth of LC xenografts in nude mice models.

Effects of RYBP on EGFR-Related Signaling Pathways and EMT

Expression levels of EGFR, p-EGFR, AKT, p-AKT, ERK, p-ERK, E-cadherin, and vimentin were detected in the A549 cell line with or without RYBP silencing, as well as in PC9 cells with or without RYBP overexpression. Briefly, levels of EGFR, p-EGFR, and vimentin were elevated, whereas the E-cadherin level decreased in A549 cells after silencing RYBP (Figure 6*A*). Levels of EGFR, p-EGFR, p-AKT, p-ERK, and vimentin decreased, whereas the E-cadherin level increased in PC9 cells after RYBP overexpression (Figure 6*B*).

Discussion

LC is a common malignancy with rapid progression and metastasis. To investigate the roles of RYBP in LC progression and metastasis, as well as the corresponding mechanisms in the EGFR-ERK/AKT signaling pathway and EMT, we detected RYBP expression levels and EGFR mutation status in LC and performed RYBP silencing and overexpression studies both *in vitro* and *in vivo*.

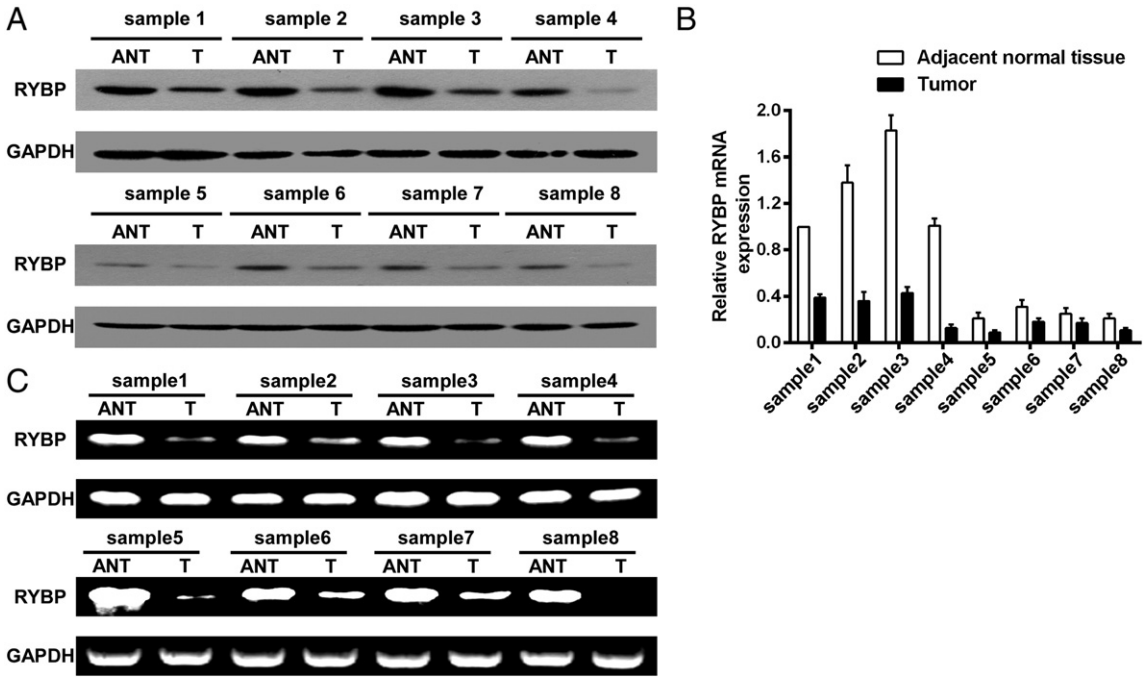

Figure 2. RYBP is downregulated in LC tissues. (A) RYBP expression levels were measured in eight pairs of LC and ANT samples with Western blot. GAPDH was loaded as the control. (B) RYBP was downregulated in LC in comparison with ANT (qRT-PCR). Relative RYBP levels were normalized by GAPDH. (C) RYBP expression levels were detected with qRT-PCR in eight pairs of LC and ANT samples. GAPDH was loaded as the control.

Table 2. Multivariate Analysis of Factors Associated with Metastasis Survival of Patients with Lung Cancer

Characters	HR	(95% CI)	*P* Value
Gender	1.8	(0.8-3.7)	.1
Age	1.0	(0.8-1.3)	.96
TNM stage			
Clinical stage	2.6	(0.8-7.5)	.27
Distant metastasis	1.8	(0.9-3.7)	.04
EGFR status	0.1	(0.1-0.3)	<.0001
RYBP expression	3.6	(1.7-8.1)	.03

In the present study, RYBP was obviously downregulated in LC tissues in comparison with the adjacent normal tissues. More severe clinical stage, high death hazard, and poor survival were strongly associated with the low expression levels of RYBP. A549 proliferation was significantly enhanced after silencing RYBP, whereas PC9 proliferation was remarkably inhibited after being exposed to RYBP overexpression. In nude mice models, growth of the LC xenograft was significantly accelerated by RYBP silencing and accordingly impeded by RYBP overexpression. These results were consistent with previous studies about the role of RYBP in NSCLC [20] and HCC [19]. Taken together, these findings suggest that RYBP is a crucial tumor suppressor in LC, impeding its formation and progression.

EGFR mutations occur in 79.7% (59/74) of patients with high RYBP expression, indicating that RYBP expression might be associated with EGFR mutations. In addition, both EGFR and p-EGFR levels were elevated in A549 cells after silencing RYBP, whereas EGFR, p-EGFR, p-AKT, and p-ERK were shown to be lower in PC9 cells after RYBP overexpression. These results indicate that RYBP might affect the EGFR-ERK and EGFR-AKT signaling pathways. Reportedly, overactivation of the ERK pathway is present in 30% to 50% of NSCLC cases, particularly NSCLC with EGFR mutation, and plays an oncogenic role in LC by regulating cell proliferation and survival [11]. AKT has also been shown to be overexpressed in LC [10], and its phosphorylation has been observed in at least 60% of NSCLCs [22]. Once activated by phosphorylation, AKT inactivates proapoptotic proteins and cell cycle–regulatory molecules, thus modulating the balance between cell apoptosis and survival [10,23]. Taken together, RYBP might inhibit cell proliferation and LC progression via repression of the EGFR-ERK and EGFR-AKT signaling pathways.

LC patients with low RYBP expression had a high metastasis risk, and metastasized tumors had low RYBP levels. RYBP silencing promoted cell migration and invasion, whereas RYBP overexpression suppressed the migration and invasion of LC cells. These results indicate that RYBP might inhibit LC metastasis. Moreover, vimentin was upregulated and E-cadherin was downregulated after silencing RYBP. In contrast, vimentin was downregulated and E-cadherin was upregulated after the overexpression of RYBP. Reportedly, upregulation of vimentin and E-cadherin loss are the hallmarks of EMT [14]. Therefore, RYBP might inhibit cell migration and invasion and LC metastasis by impeding EMT.

EGFR is a 170-kD transmembrane glycoprotein and can activate tyrosine kinases in the cell mainly by homo- or heterodimers produced by its autophosphorylation [24]. Activation of the EGFR

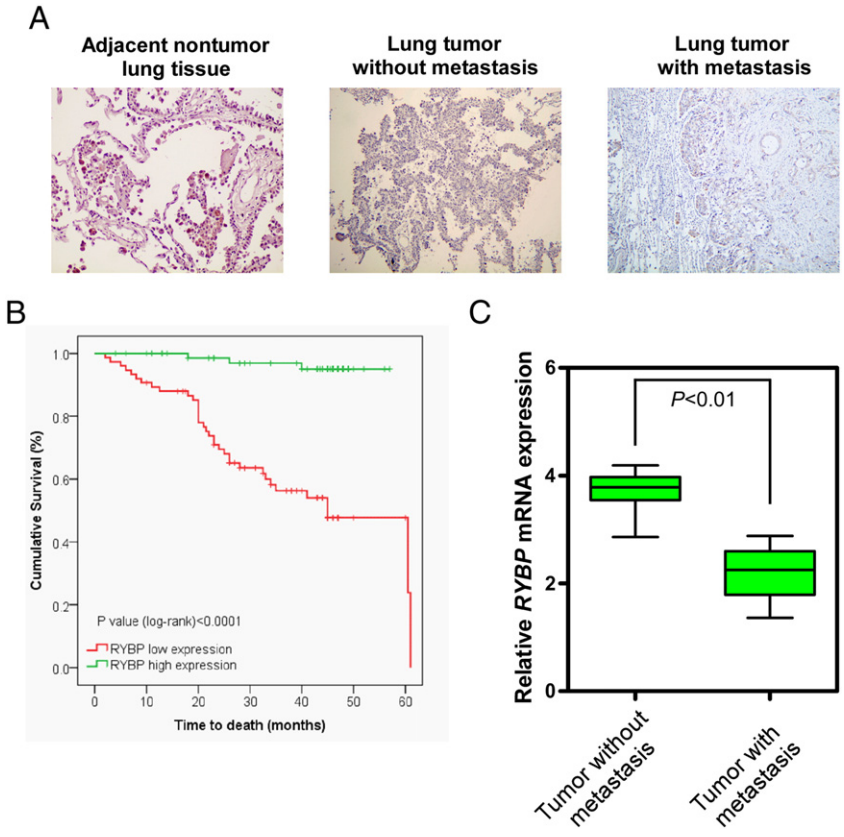

Figure 3. Low RYBP expression was associated with aggressive phenotype and poor prognosis in LC. (A) Downregulation of RYBP is associated with poor survival as determined by log-rank Kaplan-Meier survival analysis. (B) Low RYBP expression was associated with aggressive metastasis in LC.

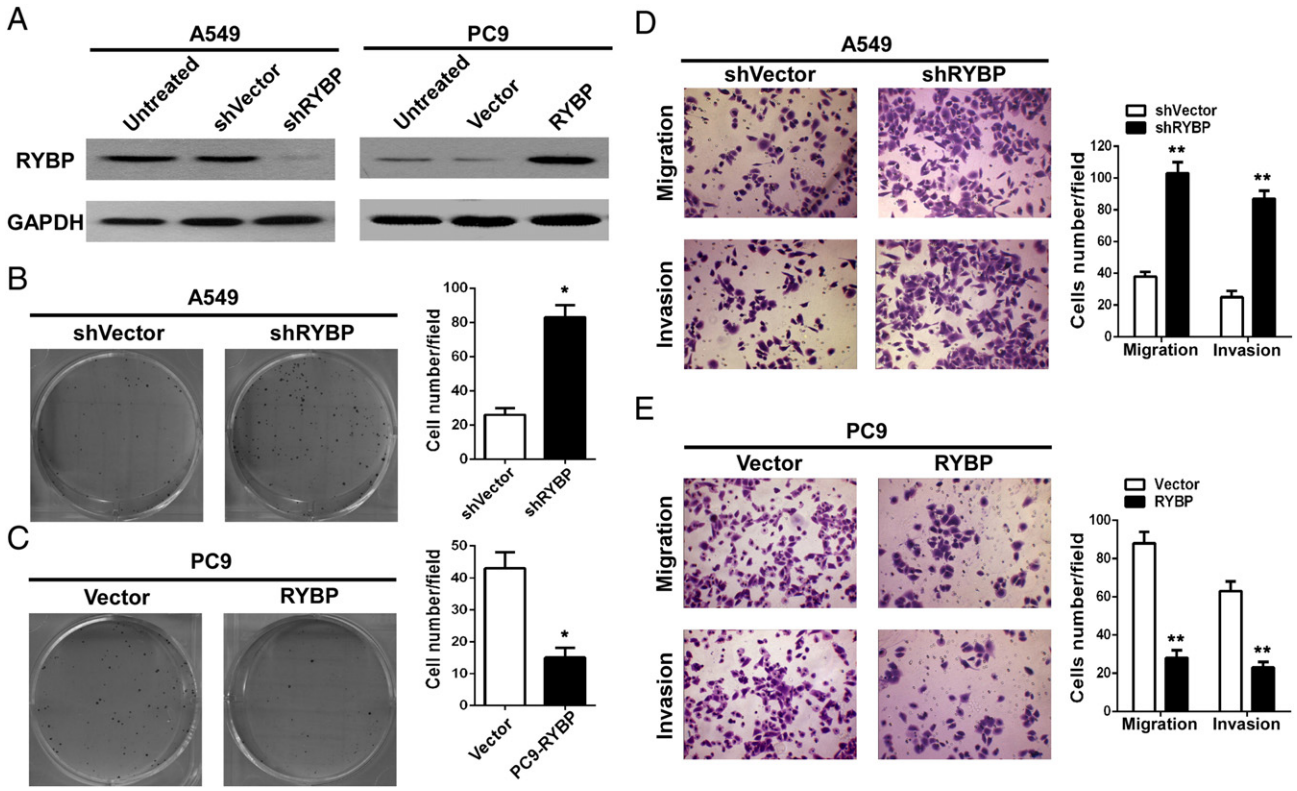

Figure 4. RYBP represses the proliferation, migration, and invasion of LC cells. (A) The knockdown and overexpression efficiency of RYBP were measured in A549 and PC9 cells. (B) Silencing of RYBP promoted A549 cell proliferation. (C) Upregulation of RYBP inhibited PC9 cell proliferation. (D) RYBP knockdown promoted A549 cell migration and invasion. (E) Upregulation of RYBP inhibited PC9 cell migration and invasion. *$P < .05$ versus shVector or Vector; **$P < .01$ versus shVector or Vector.

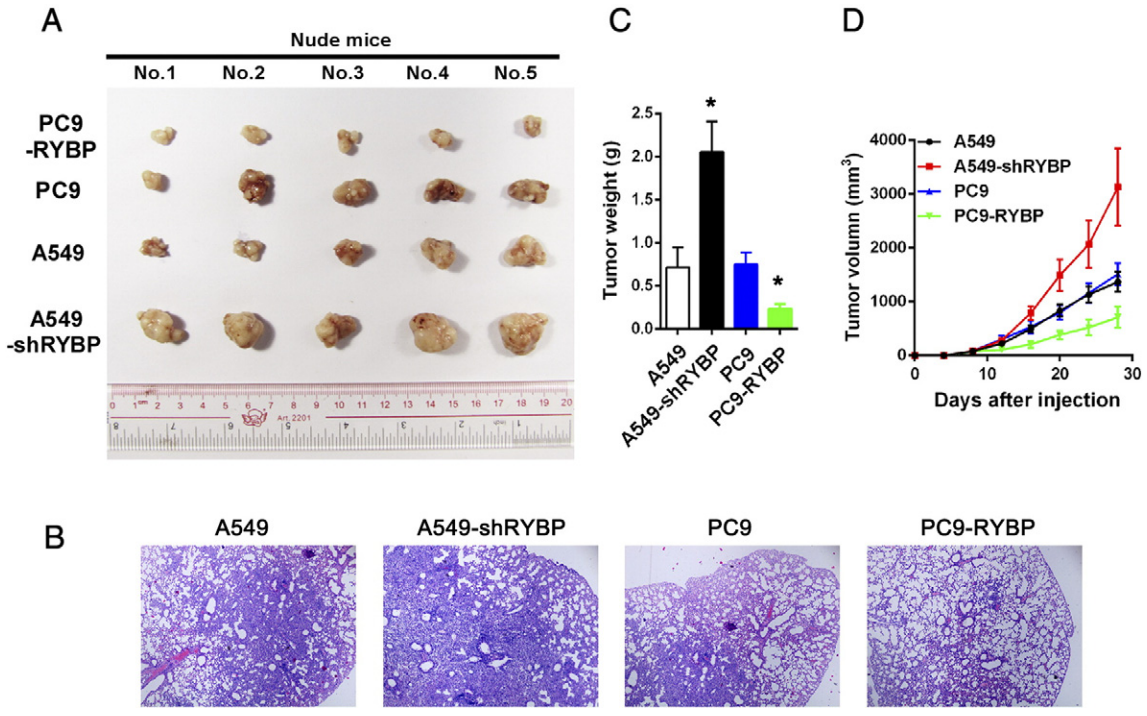

Figure 5. RYBP impedes LC xenograft growth and metastasis. Xenograft model in nude mice. A549, A549-shRYBP, PC9, and PC9-RYBP cells were inoculated in the fat pat of nude mice ($n = 5$/group). (A) Images of the tumors from all mice in each group. (B) The lung metastasis staining in hematoxylin and eosin, (C) tumor volume, and (D) mean tumor weight were analyzed. *$P < .05$ versus A549 or PC9.

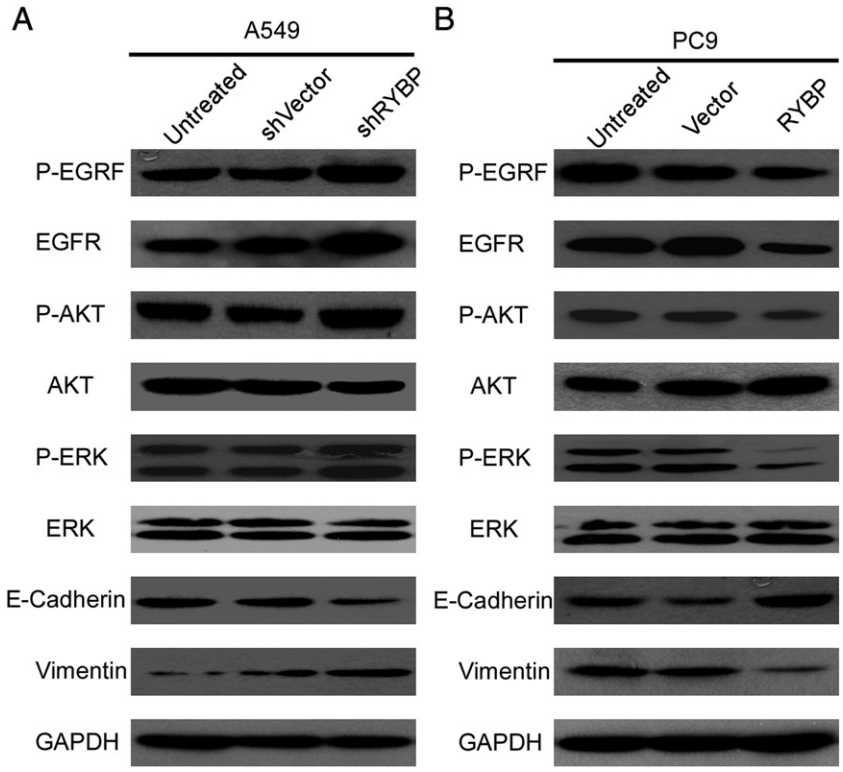

Figure 6. RYBP regulated LC progression via the EGFR/AKT/ERK/EMT signaling pathway. (A) Knockdown of RYBP expression upregulated EGFR/AKT/ERK expression and EMT signaling pathways in A549 cells. (B) Overexpression of RYBP expression attenuated EGFR/AKT/ERK expression and the EMT signal pathway in PC9 cells. GAPDH was loaded as the control.

signaling pathway is closely related to the development and progression of lung cancer [25]. RAS-RAF-MEK-ERK (extracellular signal–regulated kinase)-MAPK and PI3K-AKT-mTOR are the most extensively studied pathways that transduce the activated signals into the cell [10,11]. AKT and ERK are the key proteins in these two pathways, which then go on to activate downstream signaling factors and thereby regulate a variety of cell functions. They are therefore potential targets for lung cancer therapy [26]. Studies have shown that blocking the ERK pathway can prevent lung cancer from entering EMT and confer on them a sensitivity to EGFR inhibitors [27,28]. We know that activation of AKT is the key feature of EMT. Larue et al. suggest that AKT can inhibit the expression of E-cadherin through the repression of transcription and then induction of EMT [29]. In addition, one study showed that silencing E-cadherin improves the phosphorylation rate of EGFR, which suggests that EGFR plays an important role in EMT [30]. Taken together, we posit that RYBP inhibits cell migration/invasion and LC metastasis through EGFR-ERK/AKT-EMT. However, the present study needs more data to confirm that RYBP itself has a role in these pathways, which will be our next research goal.

In summary, the results of this study suggest that RYBP suppresses LC progression via repressing the EGFR-ERK and EGFR-AKT signaling pathway, and that RYBP inhibits LC metastasis by impeding EMT. In our future research, therapeutic studies will be performed in animal models of LC/metastasis using siRNAs that target RYBP. This may provide new directions for the development of RYBP-based therapeutic strategies for the treatment of LC.

Conclusions

Our data suggest that RYBP suppresses cell proliferation and LC progression by impeding the EGFR-ERK and EGFR-AKT signaling pathways and inhibits cell migration and invasion, and thereby LC metastasis, via the suppression of EMT.

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