Downregulation of Rab17 promotes cell proliferation and invasion in non-small cell lung cancer through STAT3/HIF-1α/VEGF signaling

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Abstract

Background: Rab GTPases play a key role in regulating intercellular vesicle trafficking in both exo- and endocytic pathways. Recent studies have reported that Rab small GTPases and the associated regulatory proteins and effectors are involved in many cancers. The purpose of this study was to investigate the biological role of Rab17 in non-small cell lung cancer (NSCLC) and the relative mechanism.

Methods: Rab17 expression in human NSCLC cell lines and tissues was evaluated using real-time PCR (RT-PCR), western blot and immunohistochemical (IHC) staining. NSCLC cell lines with RAB17 stable knockdown were generated to explore its function in vitro and in vivo. Additionally, we investigated the potential mechanism of Rab17 by identifying the expression levels of STAT3/HIF-1α/VEGF pathway using western blot analysis.

Results: Decreased Rab17 expression was correlated with poor overall survival in NSCLC patients. The functional assays showed that knockdown of Rab17 could promote tumorigenic properties of NSCLC cells in vitro and in vivo, including enhanced cell proliferation, colony formation, invasion and migration, angiogenesis and tumor xenograft growth, and suppressed apoptosis. Moreover, Rab17 downregulation decreased epithelial marker E-cadherin and increased mesenchymal markers Vimentin and β-catenin, suggesting knockdown of Rab17 induced epithelial-mesenchymal transition (EMT).

Conclusion: Downregulation of Rab17 promotes cell invasion and enhances tumorigenicity in part through the STAT3/HIF-1α/VEGF pathway, which may represent a novel potential therapeutic target.

Introduction

Lung cancer is the leading cause of cancer-associated deaths around the world.1 Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancer.2 Although diagnostic and therapeutic technologies have made tremendous progress,3,4 the five-year overall survival rate of NSCLC patients is still low.2 Thus, further exploration of the mechanism of the development and metastasis of NSCLC and the provision of therapeutic targets is urgently required.

Loss of epithelial cell polarity is a key event in cancer progression which is generally accepted to occur before the acquisition of invasive and metastatic capabilities of cancer cells.5 For example, in breast cancer, epithelial polarity is progressively lost as tumors progress from premalignant lesions to invasive carcinoma. Moreover, the loss of aspects of epithelial polarity is likely to be driven by alterations to Rab GTPase levels and/or function.7 Rab GTPases play a key role in regulating intercellular vesicle trafficking in both exo- and endocytic pathways.8 Recent research indicates that Rab small GTPases and the associated regulatory proteins and effectors are involved in multiple human diseases, including cancer. The dysregulated expression of RAB25 gene is observed in ovarian cancer,9 prostate cancer,10 and transitional cell carcinoma of the bladder and breast.
cancer.\textsuperscript{11,12} Upregulation of RAB5a and RAB7 occurs in thyroid-associated adenomas,\textsuperscript{13} suggesting the pathologic role of RAB proteins in the progression of tumors in multiple epithelial lineages.

Rab17 was the first identified epithelial-cell-specific small GTPase, and its expression is induced as epithelial cells polarize.\textsuperscript{14,15} A recent report has identified Rab17 as a gene that is suppressed during the acquisition of invasive migration which accompanies upregulation of extracellular regulated protein kinase 2 (ERK2).\textsuperscript{16} Additionally, previous studies have shown that Rab17 levels are suppressed for ERK2 to drive breast cancer invasiveness. Reduced levels of Rab17 have been consistently shown to be associated with increased aggressiveness in hepatocellular carcinoma.\textsuperscript{17,18} Taken together, these studies suggest that Rab17 may inhibit the loss of cell polarity and Rab17 may inhibit cell invasion, which is likely to be mediated by controlling endosomal trafficking of pro- and/or anti-invasive receptor cargoes. However, the function of Rab17 in NSCLC is still unknown. Therefore, the aim of our study was to investigate the biological role of Rab17 in NSCLC and the relative mechanism.

**Methods**

**Cell lines and cell culture**

Non-small cell lung cancer (NSCLC) cell lines H1975 and PC-9 were purchased from American Type Culture Collection (ATCC) and cultured in RPMI medium 1640 containing 10% FBS (fetal bovine serum, Hyclone, Logan, UT, USA) and 100 U/mL penicillin–streptomycin, and incubated at 37°C in an atmosphere of 5% CO₂.

**Patients’ tissue samples**

A cohort of 20 primary NSCLC tissues and adjacent normal tissues were used for immunohistochemical evaluation. All patients were first diagnosed with NSCLC both histologically and clinically. No patients received chemotherapy or radiotherapy before surgery. Written informed consent was obtained from all patients and the study was approved by the Institutional Review Board of Huazhong University of Science and Technology, Wuhan, China.

**Cell transfection**

Lentivirus containing small hairpin (sh) RNA targeting Rab17 (KD) or empty vector (NC) was designed by the GenePharma Company (Shanghai, China). The targeting sequence of Rab17 was 5′-CCACCTCTCTGAAGCTTGA-3′. The lentiviral supernatant was added into H1975 and PC-9 cells and the multiplicity of infection (MOI) was 60.

Puromycin was used to generate stable cell lines. Western blot and qRT-PCR were performed to confirm the silencing of Rab17.

**Quantitative real-time PCR**

Total mRNA of the cells was extracted using Trizol reagent (Invitrogen, USA). Real-time PCR was performed with the SYBR green kit (Takara, China). Expression levels were uniformly normalized to GAPDH gene. The relative expression levels were evaluated using the 2^{-ΔΔCt} method. The primer sequences were as follows: Rab17 sense, 5′-GTGGGCAACAAGACGGACACTCAG-3′; Rab17 antisense, 5′-CTCCCGGGCCCTTGTTCAG-3′; GAPDH sense, 5′-GCACCGTCAAGGCTGAGAAC-3′; and GAPDH antisense, 5′-TGGTGAAGACGCCAGTGGA-3′.

**Cell counting Kit-8 assay**

Cells treated with shRNA targeting Rab17 (KD) and negative control (NC) were plated at a density of 3 × 10⁴ cells/well in 96-well plates. At the indicated time points after transfection, 100 μL culture medium contains 10% Cell Counting Kit-8 reagent (CCK-8, Dojindo, Japan) was added. The cells were subsequently incubated for one hour at 37°C and the optical density was measured at 450 nm. Three independent experiments were performed.

**Colony formation assay**

Briefly, cells were plated in triplicate at 300 cells per well in six-well plates. All the cells were cultured for 14 days. Cell clones were then washed three times with phosphate-buffered saline (PBS), fixed in formaldehyde solution for 30 minutes, and dyed with crystal violet for 20 minutes at room temperature. Afterwards, the dye was washed off and colonies that contained more than 50 cells were counted.

**Flow cytometry analysis**

The cells were seeded in the six-well plates, and then collected at 48 hours after transfection. For the apoptosis assay, cells undergoing apoptosis and necrosis were measured using PE Annexin V Apoptosis Detection Kit I (BD Bioscience, USA). All flow cytometry data were analyzed using FACScan (BD Bioscience).

**Wound healing assay**

Cells were seeded into six-well plates and cultured at a density of 90%. A line wound was then drawn using a sterilized tip with consistent width. After being washed with PBS, the cells were cultured in serum-free RPMI-1640.
medium. Wound closure was visualized in five random fields using a light microscope (Olympus, Japan) after 24 hours of culture.

**Cell invasion and migration assay**

The migration and invasion of cells were examined using 24-well chambers with 8 μM pore size membrane without (migration) or with matrigel (invasion) as previously described. Briefly, 4 × 10^4 cells per well were plated on the inserts and cultured at 37°C in the upper chambers without serum. After 24 hours, the cells on the lower surface of the chamber were fixed, stained and measured under a microscope. The average number of migrated cells from five random optical fields (100× magnification) and triplicate filters was evaluated.

**Western blot analysis**

Cells were harvested and suspended in RIPA lysis buffer. Protein concentrations were detected using BCA protein assay kit (Beyotime, China). Equal amounts of protein was resolved on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to a PVDF membrane (Millipore, USA). This was followed by blocking with 5% BSA, and the PVDF membranes were incubated with primary antibody overnight at 4°C. The primary antibodies included Bcl-2 (#15071), E-cadherin (#14472), Vimentin (#5471), β-catenin (#8480), STAT3 (#9139), p-STAT3 (#9145) (Cell Signaling Technology, MA, USA); Bax (ab32503), HIF-1α (ab51608), VEGFA (ab52917) (Abcam, Cambridge, MA, USA) and GAPDH (G9545, Sigma, St Louis, MO, USA). Membranes were washed with TBST and incubated with HRP-conjugated secondary antibody. Proteins were visualized by ECL Western blot analysis system.

**Subcutaneous animal model**

Four to six week old female BALB/c nude mice were purchased from Beijing Huafukang Bioscience Company (China) and housed under protocols approved by the NLAC Animal Care and Use Committee. Mice were subcutaneously injected with 4 × 10^6 cells/mouse in the right flank (n = 5 each group). Tumors were measured every fourdays using digital calipers and presented as tumor volume (V) using the formula: V = 0.5 × a × b^2, where a and b represent the longer and shorter tumor diameters, respectively.

**TUNEL assay**

Tumor tissues were plated onto cover-slips, and an apoptosis detection kit (Roche, USA) was used to detect DNA fragmentation of individual cells according to the manufacturer’s instructions. The nuclei were stained with DAPI, and TUNEL (terminal deoxynucleotidyl transferase [TdT]-mediated nick-end labeling) staining was assessed. Nuclei that were double-labeled with DAPI and TUNEL were considered positive.

**Immunohistochemical and immunofluorescence staining**

Immunohistochemical and immunofluorescence staining were performed as described previously. The primary antibodies included PCNA and CD34 (Abcam, USA). The sections were examined under a fluorescence microscope (Olympus).

**Phalloidin staining**

Cells were seeded in four-well chambered glass slides and allowed to attach overnight. The cells were then fixed with 4% paraformaldehyde for 30 minutes, and permeabilized with 0.01% Triton X-100 in PBS for three minutes on ice. The cells were blocked with 5% bovine serum albumin (BSA) for 30 minutes. The actin cytoskeleton was stained using Alexa Fluor 488 Phalloidin (Life Technologies, Carlsbad, CA) for 30 minutes. The slides were mounted with Vectashield (Vector Laboratories) containing DAPI (to label cell nuclei), and the images were taken by an inverted fluorescence microscope (Olympus).

**Statistical analysis**

Data were expressed as mean ± standard deviation. Statistical analysis was performed using one-way ANOVA with post hoc tests for comparison between two groups by GraphPad Prism 5.0 software. All experiments were performed at least three times. P < 0.05 was considered as statistically significant.

**Results**

**Rab17 expression correlated with prognosis of patients with NSCLC**

To identify the gene signatures associated with Rab17 in NSCLC, 20 NSCLC tumor samples and paired nontumor tissues were immunohistochemically analyzed. We found that Rab17 expression was downregulated in tumor tissues compared with nontumor tissues (Fig 1a). Furthermore, we searched the Kaplan-Meier plotter database and online data repositories (GSE31210 and GSE13213) to explore the association between the Rab17 expression level and the prognosis of NSCLC patients. Kaplan-Meier survival analysis showed that patients with Rab17 overexpression had significantly longer overall survival, progression-free survival and recurrence-free survival than those with lower levels of Rab17 (P = 0.0031, 0.022, 0.0008, respectively; Fig 1b–d).
Rab17 knockdown promoted NSCLC cells proliferation, colony formation and inhibited apoptosis

Rab17 expression was significantly decreased in all five NSCLC cell lines including A549, H460, HCC827, H1975 and PC-9, compared with that in human lung epithelial cells \((P < 0.001, \text{Fig } 2a,b)\). H1975 and PC-9 cells with relatively high Rab17 expression were further selected as research representatives of NSCLC cells in the following studies. Both H1975 and PC-9 cells were ectopically silenced of Rab17. Immunofluorescent assays, Western blot and qRT-PCR were used to confirm the knockdown efficiency \((P < 0.05, \text{Fig } 2c–e)\).

Knockdown of Rab17 (Rab17-KD) promoted the cell proliferation compared with NC group in H1975 and PC-9 cells.
cells \((P < 0.05, \text{Fig } 3a)\). Colony numbers of Rab17 knockdown H1975 and PC-9 cells were significantly greater than those transfected with NC \((P < 0.05, \text{Fig } 3b)\). Moreover, NSCLC cells knockdown of Rab17 decreased the number of apoptotic cells compared with NC cells by flow cytometry \((\text{Fig } 3c)\). Moreover, knockdown of Rab17 increased Bcl-2 expression, and decreased Bax expression \((P < 0.05, \text{Fig } 3d)\).

**Rab17 knockdown promoted NSCLC migration, invasion, and epithelial-mesenchymal transition**

The wound healing assay showed that the migratory ability in Rab17 knockdown was significantly higher than NC group \((P < 0.05, \text{Fig } 4a)\). Knocking down of Rab17 promoted the migratory \((P < 0.05, \text{Fig } 4b)\) and invasive \((P < 0.01, \text{Fig } 4c)\) abilities of the NSCLC cells as determined by the transwell migration and invasion assays. Furthermore, knockdown of Rab17 in NSCLC cells resulted in decreased formation of actin stress fibers, presenting more spindle shaped appearance, suggesting an increase in invasion of cells \((P < 0.05, \text{Fig } 4d)\). Rab17 knockdown significantly increased Vimentin and \(\beta\)-catenin expression but inhibited E-cadherin expression \((P < 0.05, \text{Fig } 4e)\).

**Rab17 knockdown promoted tumorigenesis in vivo**

Rab17 knockdown promoted tumors growth, and the tumor volume and weight were higher than those in the NC group \((P < 0.01, \text{Fig } 5a, P < 0.001, \text{Fig } 5b)\). The expression of proliferation protein marker PCNA and microvessel marker CD34 were significantly higher in the
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Rab17-KD group compared with the NC group \( (P < 0.05, \text{Fig 5c and Fig S1a,b}) \). In addition, TUNEL assay revealed that Rab17 knockdown decreased the number of apoptotic tumor cells compared with the NC group \( (P < 0.05, \text{Fig 5d and Fig S1c}) \).

Rab17 knockdown promotes NSCLC cells progression through the STAT3/HIF-1α/VEGF pathway

Western blot revealed that Rab17 knockdown significantly increased the phosphorylation of STAT3, and upregulated HIF-1α and VEGFA expression \( (P < 0.05, \text{Fig 6a}) \). To further determine whether Rab17 regulated NSCLC cells migration via STAT3 signaling, we treated H1975 and PC-9 cells knocking down Rab17 with S3I-201 (an selective STAT3 inhibitor) for 24 hours. We found the increased migration was inhibited by S3I-201 in Rab17-KD NSCLC cells \( (P < 0.05, \text{Fig 6b}) \).

Discussion

In this study, we determined that Rab17 expression was downregulated in NSCLC patients and cells, and down-regulation of Rab17 promoted cell invasion and enhanced tumorigenicity. Rab GTPases, driving the membrane...
trafficking events, is likely involved in many aspects of cell biology relevant to carcinogenesis, including loss of cell polarity, cell invasion and metastasis.\textsuperscript{21,22} It is already known that deletion of Rab25 accelerates tumorigenesis in a mouse model of colon cancer.\textsuperscript{23} One interpretation of this is that loss of Rab25 leads to disruption of trafficking pathways that maintain aspects of normal epithelial polarity. It has previously been reported that Rab17 was one of many genes to be upregulated in cancer cell lines exhibiting a more epithelial morphology, whereas Rab17 was downregulated in cells displaying a more mesenchymal morphology.\textsuperscript{24} It indicates that Rab17 was associated with the maintenance of a polarized epithelial morphology, which might explain why metastatic tumor cells migrated with the acquisition of mesenchymal characteristics following the decrease of Rab17 levels.

Previous studies showed that reduced levels of Rab17 were associated with increased aggressiveness in hepatocellular carcinoma. Rab17 downregulation significantly promoted the tumorigenic properties of hepatocellular carcinoma cells in vitro and in vivo, as demonstrated by enhanced cell proliferation, colony formation, invasion and migration, decreased G1 arrest, and increased tumor xenograft growth and angiogenesis.\textsuperscript{18} However, its effect and mechanism in NSCLC has not been studied. In this study, we observed that the Rab17 expression was downregulated in tumor samples compared with paired nontumor tissues. Moreover, low levels of Rab17 mRNA were correlated with poor NSCLC survival, which was consistent with previous research.\textsuperscript{7} Rab17 knockdown could promote NSCLC cells proliferation, invasion and inhibit cells apoptosis in vitro and tumor growth in vivo, which was consistent with IHC results. As an important mechanism of tumor metastasis, EMT has been evaluated in many different types of cancers, including NSCLC.\textsuperscript{25,26} EMT is a conserved cellular process in which epithelial cells temporarily lose their cell polarity. We found that Rab17 knockdown decreased epithelial marker E-cadherin and increased mesenchymal

\begin{figure}[h]
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\caption{Rab17 knockdown promoted tumorigenesis in vivo. (a,b) Tumor sizes and tumor weight were measured, and tumor growth curves were obtained. Data are presented as tumor volume in the mean ± SD. (c) Immunohistochemical staining of PCNA and CD34 in xenograft tumors. (d) Apoptosis in tumor tissues was assessed using the TUNEL assay; the nuclei were counterstained with DAPI. Representative images show apoptotic (fragmented) DNA (green staining) and the corresponding cell nuclei (blue staining). *\(P<0.05\), **\(P<0.01\), #\(P<0.001\).}
\end{figure}
markers Vimentin and β-cadherin, further confirming that Rab17-KD could facilitate EMT.

VEGF-A has been implicated as a major paracrine mediator in the pathogenesis of NSCLC.\textsuperscript{27} STAT3 and HIF-1α, two major transcription factors that regulate VEGF, have been found to be consistently upregulated in various cancers and associated with poor clinical outcomes in patients.\textsuperscript{28,29} STAT3 is a well-characterized oncogene that affects various biological processes including promoting cell proliferation and survival by regulating Cyclin D1 and Bcl-2, inducing tumor invasion and metastasis by regulating E-cadherin, MMP-9, promoting angiogenesis by targeting VEGF and HIF1α expression.\textsuperscript{30,31} Angiogenesis is also reported to be linked with STAT3.\textsuperscript{32} As prominent transcription targets for STAT3, VEGF and HIF-1α are important proangiogenic factors.\textsuperscript{33} Our results showed that silenced expression of Rab7 could increase VEGF level as well as the density of microvessel in xenograft tumors, indicating that Rab17 could inhibit angiogenesis by suppressing VEGF signaling. Furthermore, Rab17 could suppress HIF-1α expression in NSCLC cells via inhibiting the STAT3 signaling pathway. These data were consistent with previous findings in other cell lines,\textsuperscript{34,35} indicating the STAT3/HIF-1α/VEGF may be responsible for the angiogenic effects of Rab17 knockdown. In summary, Rab17 knockdown promoted NSCLC invasion and tumorigenesis in part through the STAT3/HIF-1α/VEGF pathway.

In conclusion, Rab17 expression was downregulated in NSCLC patients and cells; moreover, downregulation of Rab17 promoted EMT and activated STAT3/HIF-1α/VEGF pathway. Our findings not only contribute to better understand the role of Rab17 in NSCLC but also provide strong support that targeting STAT3/HIF-1α/VEGF axis might be a valuable clinical option to treat NSCLC.

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Disclosure
The authors declare that they have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

**Figure S1.** Rab17 knockdown promoted tumorigenesis in vivo. (a–b) Quantitative analysis of immunohistochemical staining of PCNA and CD34 in xenograft tumors. (c) Quantitative analysis of apoptotic cells in tumor tissues.