

ARTICLE



Upregulation of KLHL17 promotes the proliferation and migration of non-small cell lung cancer by activating the Ras/MAPK signaling pathway

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Analysis of the Gene Expression Profiling Interactive Analysis (GEPIA) database revealed that Kelch-like 17 (KLHL17) is overexpressed in non-small cell lung cancer (NSCLC) including adenocarcinoma (ADC) and squamous cell carcinoma (SCC). We therefore explored the role of KLHL17 in the development and progression of NSCLC. Immunohistochemistry and western blotting showed that KLHL17 expression was significantly higher in the tumor tissues from 173 patients with NSCLC, compared with the corresponding non-neoplastic tissue. In addition, upregulated KLHL17 expression was positively correlated with tumor size, lymph node metastasis and tumor node metastasis (TNM) stage, and affected the overall survival (OS) of patients with NSCLC. Consistent with clinical samples, in vitro studies demonstrated that KLHL17 expression was higher in various cell lines of NSCLC (A549, H1299, H460 and SK cells) as compared to normal human bronchial epithelial cells (HBE cells). Overexpression of KLHL17 in the cell lines of NSCLC with *KLHL17*-Flag plasmid promoted the proliferation and migration of tumor cells, which was associated with elevated activation of Ras/MAPK signaling and increased expression of cyclin D1, cyclin D-dependent kinases 4 (CDK4), matrix metalloproteinase 2 (MMP2) and Ras homolog gene family member A (RhoA). In contrast, knockdown of KLHL17 in the cell lines of NSCLC using KLHL17 small interfering RNA suppressed the proliferation and migration of tumor cells, in association with reduced activation of Ras/MAPK signaling and decreased expression of cyclin D1, CDK4, MMP2 and RhoA. Moreover, treatment of tumor cells with Ras inhibitor salirasib prevented KLHL17-induced Ras/MAPK activity as well as tumor proliferation and migration. These results suggest that upregulated KLHL17 in NSCLC promotes the proliferation and migration of tumor by activating Ras/MAPK signaling pathway. Therefore, KLHL17 may be a novel therapeutic target for the treatment of NSCLC.

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INTRODUCTION

Despite enhanced recognition and concerns regarding physical health, the incidence of lung cancer has continuously shown an upward trend^{1,2}, which not only puts a burden on the affected families but also on society. Lung cancer accounts for 13% of all global cancer cases and 23% of all cancer-related deaths³. NSCLC is the most common type of lung cancer that accounts for 85% of all lung cancer^{4,5} and is associated with poor prognosis and limited treatment options^{6,7}. Although standard surgical lobectomy has been and is currently the gold standard for early-stage NSCLC, not all patients are medically fit to undergo surgery. Additionally, a proportion of lung cancer patients develop local recurrence or systemic metastasis, even after curative resection. Over the past decade, advances in molecular targeted therapies and immunotherapies provide hope for patients with lung cancer including NSCLC⁴. Unfortunately, fewer than 25% of patients benefit from current targeted therapies or immunotherapies, and inherited/acquired resistance may eventually develop during treatment⁴. Thus, the identification of specific novel molecular

targets and the development of matched targeted therapies remain urgently needed.

KLHL17 is one of the 42 known members of the Kelch protein family characterized by a broad-complex, tramtrack, bri-a-brac/poxvirus and zinc finger (BTB/POZ) domain, BTB and C-terminal Kelch (BACK) domain, and five Kelch repeats^{8,9}. The BTB/POZ domain has multiple functions, including the maintenance of the cytoskeleton¹⁰, ion channel gating¹¹, transcriptional repression¹² and ubiquitination of targeted proteins via cullin E3 ligase^{13,14}. Kelch repeats or domains are evolutionarily conserved structures from *Drosophila melanogaster* to humans¹⁵. Our team is committed to the study of the mechanism of the Kelch protein family in NSCLC. The previous results showed that Kelch-like 38 (KLHL38) and Kelch-like 18 (KLHL18) had an impact on the proliferation, migration and prognosis of NSCLC, and played a role in promoting or inhibiting the development of NSCLC^{16,17}. What's more, a previous study identified the methylation of *KLHL17* gene in early-stage breast tumors compared with that in adjacent normal samples¹⁸. Intersection analysis indicated that KLHL17 was correlated with both overall

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Table 1. KLHL17 status in patients with non-small cell lung cancer according to clinicopathological characteristics.

| Characteristics | Number of patients | KLHL17 negative or weak expression | KLHL17 overexpression | P value |
|-----------------|--------------------|------------------------------------|-----------------------|---------|
| Age (years) | | | | 0.482 |
| <60 | 90 | 55 | 35 | |
| ≥60 | 83 | 55 | 28 | |
| Gender | | | | 0.656 |
| Female | 53 | 35 | 18 | |
| Male | 120 | 75 | 45 | |
| Smoking status | | | | 0.824 |
| Yes | 124 | 58 | 66 | |
| No | 49 | 22 | 27 | |
| Histopathology | | | | 0.343 |
| ADC | 74 | 36 | 38 | |
| SCC | 99 | 41 | 58 | |
| Differentiation | | | | 0.022 |
| Well-moderate | 83 | 60 | 23 | |
| Poor | 90 | 50 | 40 | |
| Size | | | | <0.001 |
| ≤3 cm | 90 | 72 | 18 | |
| >3 cm | 83 | 38 | 45 | |
| Nodal status | | | | 0.004 |
| N0 | 109 | 78 | 31 | |
| N1, N2, N3 | 64 | 32 | 32 | |
| TNM stage | | | | 0.004 |
| I | 80 | 60 | 20 | |
| II–III | 93 | 50 | 43 | |

survival (OS) and disease free interval (DFI) in prostate adenocarcinoma¹⁹. However, no study to date has reported the role of KLHL17 in lung cancer. In this study, we hypothesized that KLHL17 might play a role in the development and progression of NSCLC and examined whether KLHL17 exerts tumor-promoting function in NSCLC using clinical samples and NSCLC cells.

MATERIALS AND METHODS

Oncomine database analysis

The Gene Expression Profiling Interactive Analysis (GEPIA) database (<http://gepia.cancer-pku.cn/>) was used to analyze the difference between the KLHL17 expression of NSCLC tissue and non-neoplastic tissue. The analysis data originated from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) databases, which reflected the gene expression. Oncomine public cancer microarray database (<https://www.oncomine.org/resource/login.html>) was used to analyze the data information of gene expression array of NSCLC²⁰. The data set was defined as $p < 0.01$ and $\log_2FC > 1.5$, and the mRNA expression level of KLHL17 was analyzed and compared between tumor tissue and non-neoplastic tissue.

Patients and specimens

In this study, tissue samples were obtained from 173 randomly selected patients who were diagnosed with lung cancer and underwent tumor resection surgery between 2010 and 2018. None of the patients had received radiotherapy or chemotherapy before the surgery, which was confirmed by reviewing the patients' medical records. The clinical and pathological characteristics of the cases are summarized in Table 1. This study was approved by the Ethics Committee of China Medical University, and all patients signed an informed consent form.

NSCLC cell culture

All cell lines were purchased from Shanghai Cell Bank (Shanghai, China) and cultured in a medium containing 10% fetal bovine serum (FBS;

FB15015; Clark Biosciences, Richmond, VA, USA). HBE cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Gibco, New York, USA), whereas NSCLC cell lines A549, H1299, H460, and H292 were cultured in Roswell Park Memorial Institute 1640 medium (Gibco, New York, USA). The SK cell line was cultured in minimal essential medium (Gibco, New York, USA).

Plasmid construction and transfection

KLHL17 small interfering RNA (*siKLHL17*) and its negative control (negative control) were purchased from RiboBio Co., Ltd. (Guangzhou, China). KLHL17-Flag and its negative control plasmid pCMV6 were purchased from Origene (Rockville, MD, USA). *siKLHL17* and KLHL17-Flag were transfected into A549 and H1299 lung cancer cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions to downregulate or upregulate KLHL17 expression, respectively.

Tissue immunohistochemistry

NSCLC tissue sections were incubated with anti-KLHL17 antibody (HPA031251, Sigma-Aldrich, St. Louis, MO, USA) at 4 °C overnight at a dilution of 1:100. After tissue sections were developed, the expression of KLHL17 was analyzed based on the color and staining intensity (brown denoted high expression, yellow moderate expression, light yellow low expression, and colorless indicated negative expression), and expression levels were compared between normal and adjacent tissue samples.

Cellular immunohistochemistry

Round glass slides of suitable sizes for each well of a 24-well plate were placed in a cell culture dish, on which 10^3 cells were seeded. After incubation at 37 °C overnight, the glass slides were washed three times with phosphate-buffered saline (PBS), and the cells were fixed with pre-cooled methanol. The slides containing the cells were then incubated overnight at 4 °C with anti-KLHL17 antibody (HPA031251, Sigma-Aldrich) at a 1:100 dilution. The next day, the cells were stained with crystal violet solution, and the expression of KLHL17 was observed.

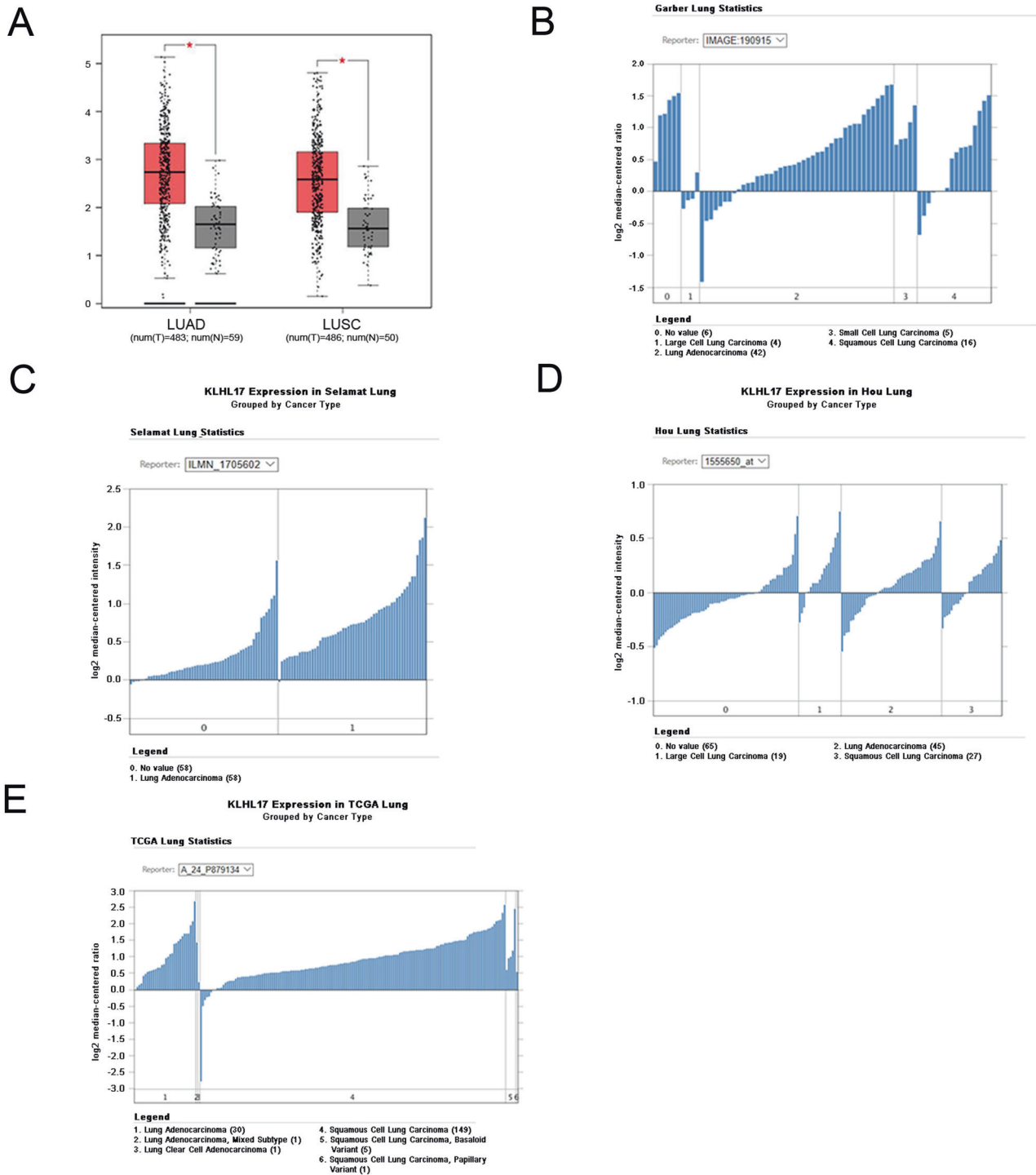


Fig. 1 Database information showing high expression of *KLHL17* in non-small cell lung cancer. A Analysis of the Gene Expression Profiling Interactive Analysis (GEPIA) database showing the expression of *KLHL17* in lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC). Red: the tumor tissue; gray: the adjacent normal tissue. $P < 0.05$. **B–E** Data retrieving from Garber Lung oncomine database, Selamat Lung oncomine database, Hou Lung oncomine database and the Cancer Genome Atlas (TCGA) oncomine database showing high expression of *KLHL17* in most non-small cell lung cancer samples.

Immunofluorescence

Round glass slides of suitable sizes for each well of a 24-well plate were placed in a cell culture dish, on which 10^3 cells were added. After incubating at 37 °C overnight, the cells were fixed with 4% paraformaldehyde followed by treatment with 0.1% Triton X-100 for 10 min. The glass slides were washed with PBS three times followed with 3% bovine serum

albumin treatment (A8010, Solarbio, Beijing, China) to block the cells for 2 h. The cells were then incubated with anti-*KLHL17* antibody and incubated overnight at 4 °C as described above for immunohistochemistry. The next day, glass slides were incubated with a fluorescent secondary antibody (A0562, Beyotime, China) in the dark for 2 h, and the cells were imaged using a confocal microscope (FV3000, Olympus, Fukuoka, Japan).

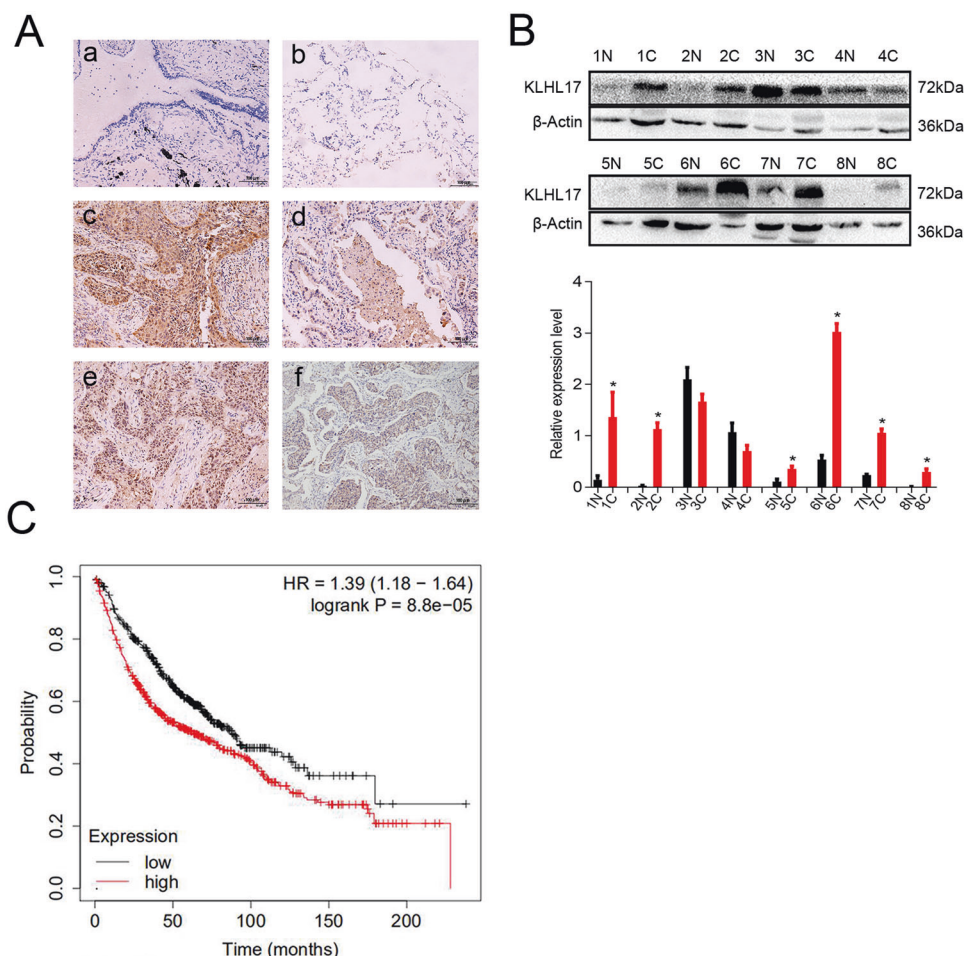


Fig. 2 KLHL17 is associated with poor prognosis of patients with non-small cell lung cancer. **A** Representative immunohistochemistry images of KLHL17 staining in normal bronchial epithelium (a), normal alveolar epithelium (b), highly differentiated lung squamous cell carcinoma (c), highly differentiated lung adenocarcinoma (d), poorly differentiated lung squamous cell carcinoma (e), and poorly differentiated lung adenocarcinoma (f). **B** Western blotting analysis of KLHL17 expression in eight paired non-small cell lung cancer tissues and their corresponding adjacent normal tissues. $^*P < 0.05$ vs corresponding adjacent normal tissues. **C** Kaplan-Meier plotter analysis showing the correlation between KLHL17 expression and the prognosis of patients with lung cancer. Red line: high KLHL17 expression; black line: low KLHL17 expression ($P = 8.8e^{-05}$). Data were from the Kaplan-Meier plotter database (<https://kmplot.com/analysis/index.php?service&cancer=lung>).

Western blotting

Approximately 10^6 cells were placed in a centrifugal tube and centrifuged at 4°C (3000 rpm; 5 min). After obtaining the pellet, four times the volume of the cell lysate was treated with a protease inhibitor (P0013; Beyotime Biosciences, Shanghai, China) and phosphatase inhibitor (B14002 and B15002, respectively; Biotool, Shanghai, China) at a 100:1:1 ratio, and a 35- μg sample was prepared. Next, the proteins were separated using SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA), and blocked with 5% skim milk (232100; BD Co., Franklin Lakes, NJ, USA) for 2 h. Membranes were incubated with primary antibodies at 4°C overnight, followed by incubation with a peroxidase-conjugated secondary antibody (A0208, A0216, Beyotime, China) for 2 h at 37°C . The antibodies used in this experiment were as follows: anti-MMP2 (409904 s, 1:1,000), anti- β -actin (3700 s, 1:2000), anti-CDK4 (12790 s, 1:1,000), anti-Cyclin D1 (55506 s, 1:1,000), anti-phospho-MAPK (4370 s, 1:1,000), and anti-RhoA (2117 s, 1:1,000) were obtained from Cell Signaling Technology (Danvers, MA, USA), and anti-PanRas (60309-1-Ig, 1:1,000) was obtained from Proteintech Group (Chicago, IL, USA). Proteins were visualized using enhanced chemiluminescence (ECL 34080; Thermo Fisher Scientific, Waltham, MA, USA). The ImageJ software was used to quantitatively evaluate the grayscale integral value of each band.

Proliferation assay

Cells (A549, H1299 and SK) transfected with *siKLHL17* or *KLHL17*-Flag were added to a 96-well culture plate at 3,000 cells/well with five replicates and

cultured with 10% FBS for 5 days. One culture plate was removed per day, to which 20 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazole salt (MTS; G3580, Promega, Madison, WI, USA) solution was added to measure cell viability and proliferation. The cells were cultured at 37°C for 1 h in the dark, and cell proliferation was measured at a wavelength of 490 nm.

Colony formation assay

Cells (A549, H1299 and SK) transfected with *siKLHL17* or *KLHL17*-Flag were plated in a 6-well culture plate at 800 cells/well and incubated with 10% FBS at 37°C for 2 weeks. The cell culture plate was then washed with PBS three times and the cells were fixed in pre-chilled methanol. The cells were stained with crystal violet staining solution, and images were acquired to determine the number of colonies.

Migration assay

Cells (A549, H1299 and SK) transfected with *siKLHL17* or *KLHL17*-Flag were added to the upper chamber of a transwell chamber at 5×10^4 cells/100 μL and cultured in presence of 2% FBS; 600 μL of 20% FBS was added to the lower chamber and the cells were incubated at 37°C for 24 h. The transwell chamber was then washed with PBS three times and fixed with pre-chilled methanol followed by staining with crystal violet solution. The number of migrating cells was counted from the images of stained cells.

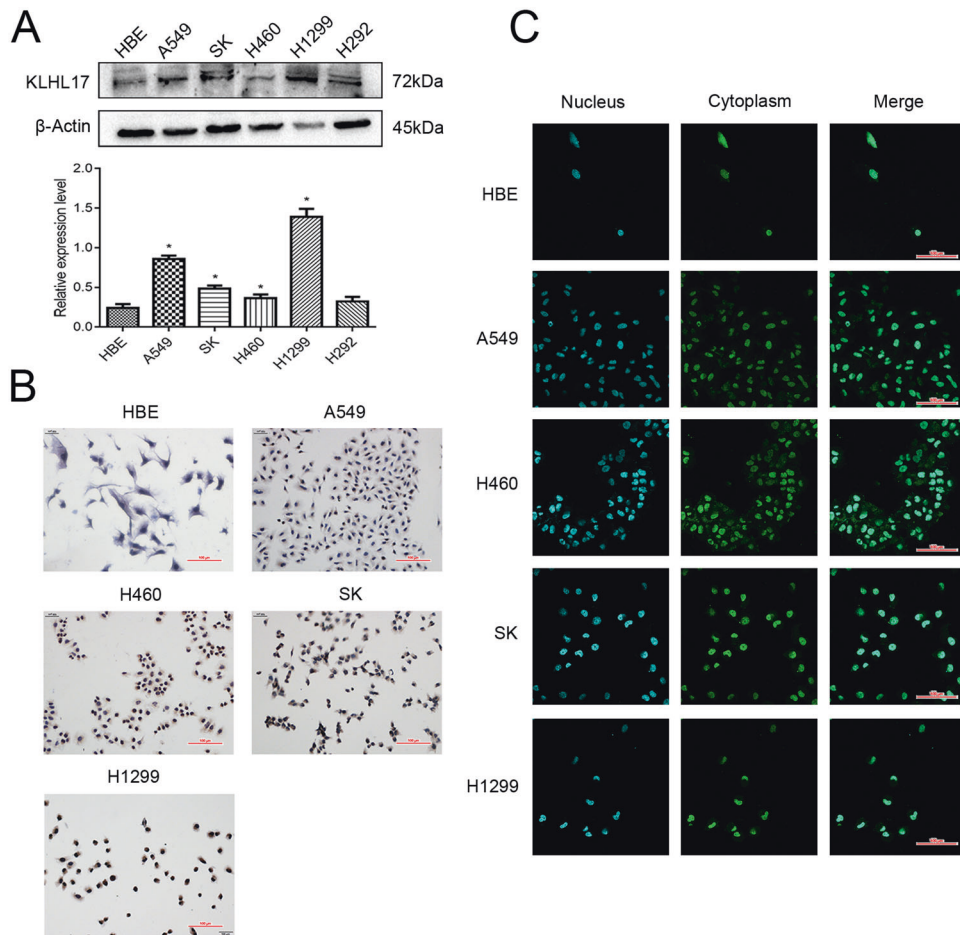


Fig. 3 KLHL17 is highly expressed in the cell lines of non-small cell lung cancer (NSCLC). **A** Western blotting analysis showing expression of KLHL17 in normal human bronchial epithelial (HBE) cells and various NSCLC cell lines. **B** Immunocytochemistry images showing abundant KLHL17 expression in different NSCLC cell lines. **C** Confocal microscopy images showing the localization of KLHL17 in various NSCLC cell lines.

Inhibitor treatments

The cells were treated with *KLHL17*-Flag followed by 20 μ M of the Ras inhibitor salirasib (HY-14754, MCE) for 24 h.

Gene set enrichment analysis

To reveal the unique biological significance and key pathways associated with *KLHL17*, we used Gene Set Enrichment Analysis (GSEA) (version 6.2) for analysis and selected the enrichment items with the absolute value of normalized enrichment score (NES) > 1, $P < 0.05$ and false discovery rate (FDR) < 0.25 as the potential biological function and carcinogenic mechanism of the target gene. Kyoto Encyclopedia of Genes and Genomes (KEGG) information was included in the analysis.

Statistical analysis

All data were analyzed using SPSS version 24.0 (Beijing, China) and GraphPad Prism 5 (La Jolla, CA, USA). χ^2 test was used to assess the correlation between clinicopathological factors and *KLHL17* expression. Differences between two groups were analyzed with Student's *t* test. Overall survival was assessed using Kaplan–Meier survival analysis and the log rank test. All experiments were repeated independently at least three times under the same conditions. The sample size chosen for each experiment was based on a previous study²¹. Results with $P < 0.05$ were considered statistically significant.

RESULTS

Increased *KLHL17* expression in the tumor tissues is associated with poor prognosis of patients with NSCLC

We first evaluated the expression of *KLHL17* by screening the GEPIA database (<http://gepia.cancer-pku.cn/>) and searching the public

cancer microarray database (Oncomine), the results showed that we found that *KLHL17* expression was significantly higher in lung cancer tissues, particularly in lung adenocarcinoma and squamous cell carcinoma, compared with that in normal lung tissues (Fig. 1).

Then we evaluated the expression of *KLHL17* in the tumor tissues from 173 patients with NSCLC using immunohistochemistry. Our results showed that no *KLHL17* expression was observed in either normal bronchial epithelium or normal alveoli of the lung tissues, whereas positive staining for *KLHL17* was detected in both lung squamous cell carcinoma and lung adenocarcinoma tissues (Fig. 2A). To quantitatively analyze protein levels of *KLHL17* in tumor tissues, eight pairs of fresh tissue samples were randomly selected from these 173 tissue samples for western blotting analysis and the results showed that the protein levels of *KLHL17* were markedly higher in the tumor tissues than in the adjacent normal tissues for six pairs of the eight pairs of tissue samples (Fig. 2B). These data are consistent with the observation from immunohistochemistry.

Kaplan–Meier plotter analysis revealed that *KLHL17* expression was significantly associated with the degree of NSCLC differentiation ($P = 0.022$), tumor size ($P < 0.001$), lymph node migration ($P = 0.004$), and TNM stage ($P = 0.004$). Moreover, the survival of patients with lung cancer who had high *KLHL17* expression was significantly shorter than that of those with low *KLHL17* expression (Fig. 2C).

KLHL17 is highly expressed in NSCLC cells

We next evaluated the protein levels of *KLHL17* in five commonly used NSCLC cell lines and normal HBE cell line using western

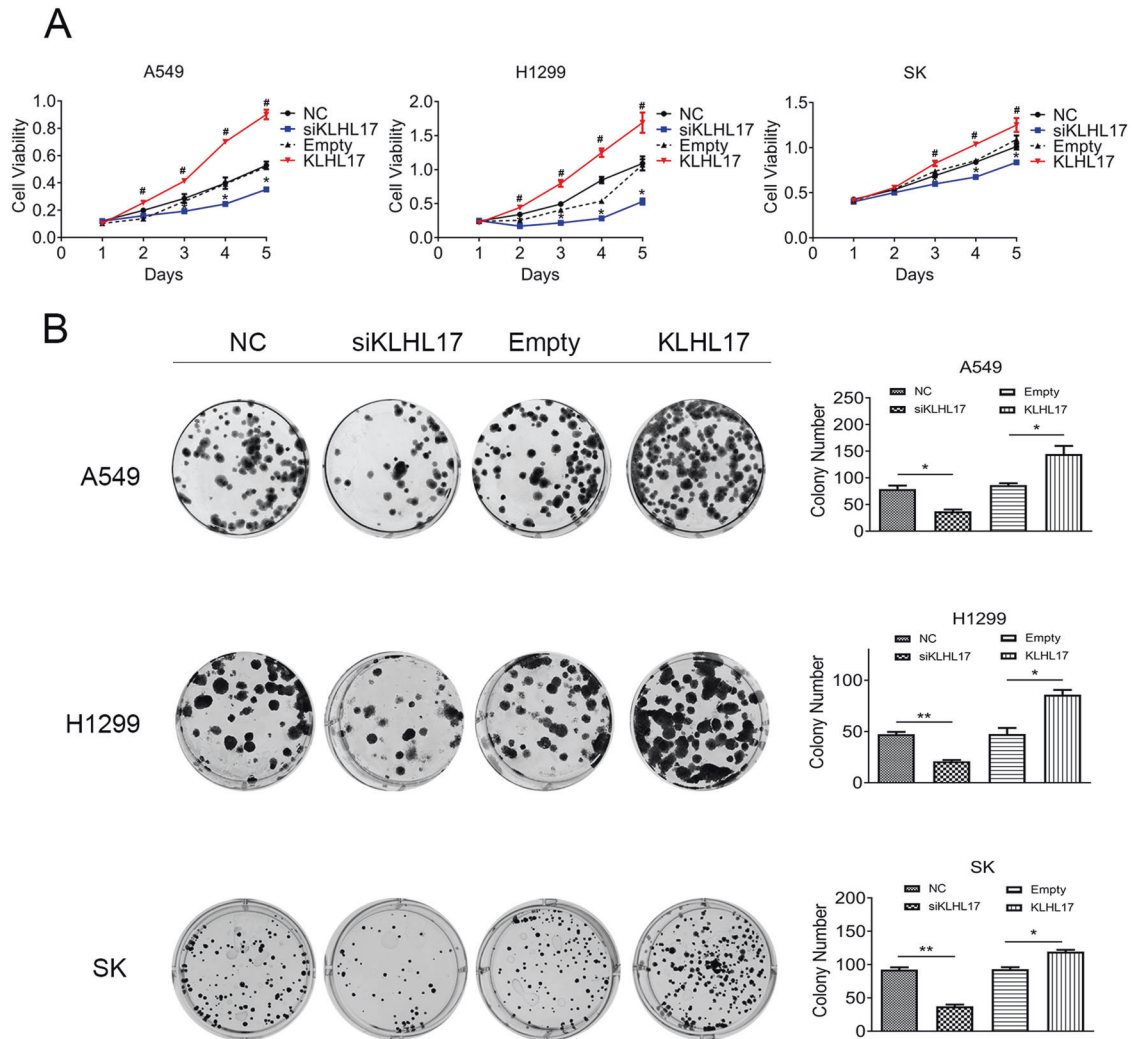


Fig. 4 KLHL17 promotes the proliferation of non-small cell lung cancer cells. **A** MTS assay showing effects of KLHL17 overexpression with *KLHL17*-Flag plasmid (KLHL17) or KLHL17 knockdown with small interfering RNA (siKLHL17) on the proliferation ability of A549, H1299 and SK cells. * $P < 0.05$ vs negative control (NC), # $P < 0.05$ vs Empty. **B** Colony formation assay showing the effects of KLHL17 overexpression or KLHL17 knockdown on colony formation ability of A549, H1299 and SK cells. Quantitative analysis of the numbers of colonies is shown on the right. * $P < 0.05$, ** $P < 0.01$ vs negative control (NC) or Empty.

blotting. As shown in Fig. 3A, KLHL17 protein levels were significantly higher in four NSCLC cell lines as compared to normal HBE cell line. Consistent with western blotting data, subsequent immunohistochemical analysis (Fig. 3B) and confocal imaging (Fig. 3C) showed abundant expression of KLHL17 in four NSCLC cell lines, which was predominantly located in the nucleus of tumor cells. These in vitro results confirmed the findings from clinical samples.

KLHL17 promotes the proliferation and migration of NSCLC cells

Based on the results of western blotting, immunohistochemical and immunofluorescence studies showing that A549, H1299 and SK cell lines had more abundant expression of KLHL17, we selected these cell lines to further examine the role of KLHL17 in the proliferation and migration of NSCLC cells.

MTS assay showed that A549, H1299 and SK cells transfected with *KLHL17*-Flag plasmid to overexpress KLHL17 had significantly enhanced proliferation ability, whereas these cells transfected with *siKLHL17* to knock down KLHL17 expression exhibited decreased cell proliferation than respective control cells (Fig. 4A, $P < 0.05$). Additionally, cells overexpressing KLHL17 with *KLHL17*-Flag plasmid showed increased colony-forming ability, whereas

suppressing KLHL17 using *siRNA* decreased the colony-forming ability of both A549, H1299 and SK cells (Fig. 4B, $P < 0.05$). Similarly, the Transwell assay revealed that high expression of KLHL17 promoted the migration ability but downregulated KLHL17 reduced the migration ability in all three selected NSCLC cell lines (Fig. 5, $P < 0.05$).

KLHL17 exerts its tumor-promoting effect by activating Ras/MAPK signaling pathway

Using GSEA for possible pathways and genes related to NSCLC enrichment, we found that the KLHL17 and Ras/MAPK signaling pathway have a close relationship with NSCLC (Fig. 6A, B). Rho A, cyclins and MMPs play important roles in tumor proliferation and migration^{21,22}. Ras/MAPK signaling has been shown to contribute to tumor proliferation and migration by regulating Rho A, cyclins and MMPs²³. To determine the molecular mechanism by which KLHL17 promotes tumor proliferation and migration of NSCLC, we assessed activation of Ras/MAPK signaling and expression of Rho A, cyclin D1, CDK4 and MMP2 in the cell lines of NSCLC treated with *KLHL17*-Flag plasmid and *siKLHL17*.

Western blotting analysis showed that overexpression of KLHL17 with *KLHL17*-Flag plasmid elevated expression of Ras,

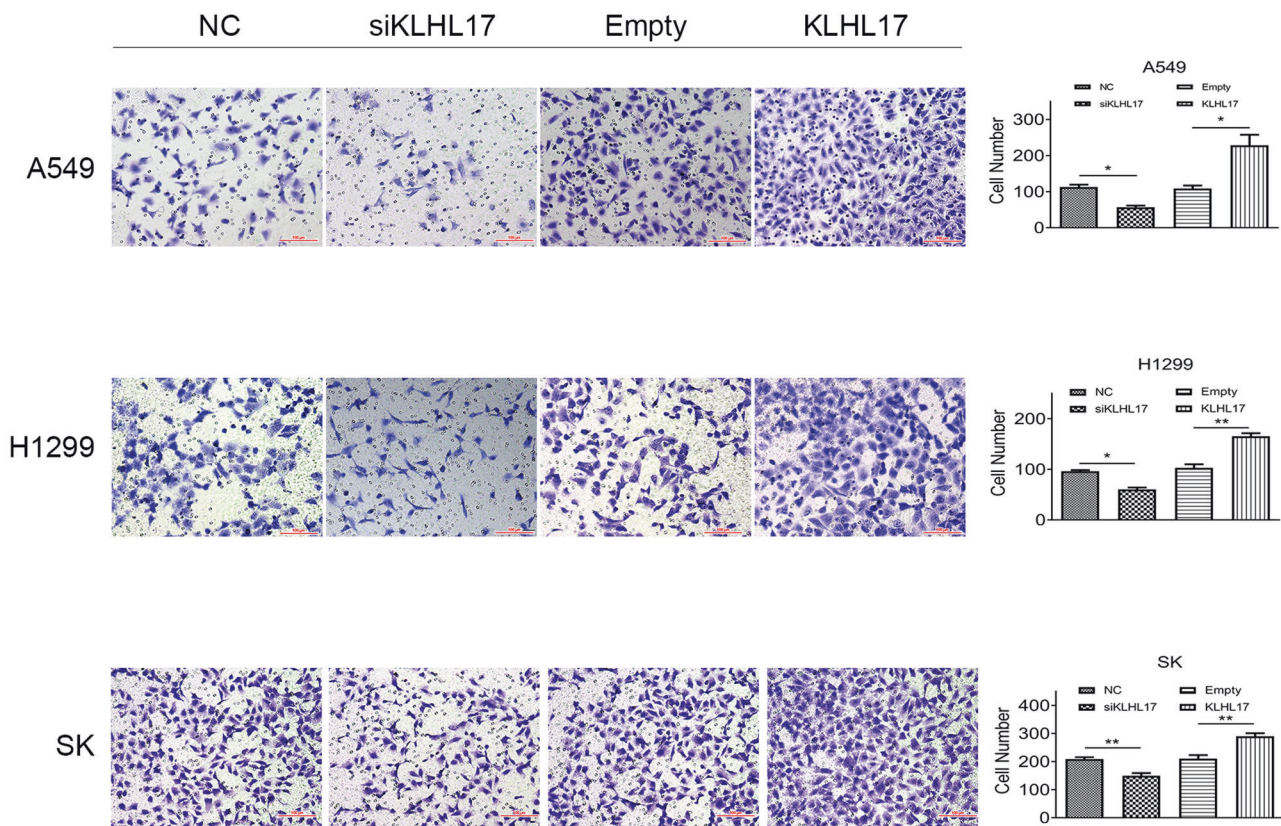


Fig. 5 KLHL17 promotes the migration of non-small cell lung cancer cells. Transwell cell migration assay showing the effects of KLHL17 overexpression or KLHL17 knockdown on migration ability of A549, H1299 and SK cells. Quantitative analysis of the numbers of migrating cells is shown on the right. * $P < 0.05$, ** $P < 0.01$ vs negative control (NC) or Empty.

phosphorylated extracellular regulated protein kinases (ERK) 1/2, Rho A, cyclin D1, CDK4 and MMP2, whereas knockdown of KLHL17 with siKLHL17 reduced expression of Ras, phosphorylated ERK 1/2, Rho A, cyclin D1, CDK4 and MMP2 in A549 (Fig. 6C), H1299 (Fig. 6D) and SK cells (Fig. 6E).

To further confirm the effect of KLHL17 on Ras/MAPK signaling pathway, we treated *KLHL17*-Flag-transfected A549, H1299 and SK cells with salirasib, a specific inhibitor of Ras. As presented in Fig. 7, salirasib treatment prevented KLHL17-induced increase in expression of phosphorylated ERK1/2, which was accompanied by reduction in expression of Rho A, cyclin D1, CDK4 and MMP2 in A549, H1299 and SK cells (Fig. 7A). In addition, salirasib treatment also suppressed the ability of KLHL17 to promote cell proliferation (Fig. 7B), colony formation (Fig. 7C) and cell migration (Fig. 7D) in A549, H1299 and SK cells.

DISCUSSION

The novel findings of this study are: (1) KLHL17 expression is significantly higher in the tumor tissues from patients with NSCLC, compared with normal lung tissues; upregulated KLHL17 expression is positively correlated with tumor size, lymph node metastasis and TNM stage, and affected the overall survival of patients with NSCLC. (2) KLHL17 expression is higher in various cell lines of NSCLC (A549, H1299, H460 and SK cells) as compared to normal human bronchial epithelial cells. (3) Overexpression of KLHL17 in the cell lines of NSCLC promotes the proliferation and migration of tumor cells, which is associated with elevated activation of Ras/MAPK signaling and increased expression of cyclin D1, CDK4, MMP2 and RhoA. In contrast, knockdown of KLHL17 in the cell lines of NSCLC suppresses the proliferation and migration of tumor cells, in association with reduced activation of

Ras/MAPK signaling and decreased expression of cyclin D1, CDK4, MMP2 and RhoA. (4) Treatment of tumor cells with Ras inhibitor salirasib prevents KLHL17-induced Ras/MAPK activity as well as tumor proliferation and migration.

Kelch-like proteins that are encoded by 42 Kelch-like gene family members consist of the prominent class of substrate adaptors of cullin3-RING ligases^{8,9,24}. Cullin3-RING ligases play essential roles in cancer progression by regulating protein hemostasis²⁴. Structurally, substrate binding pockets that are composed by six kelch repeat domain of *KLHLs* differ from each other²⁵. *KLHLs* exert different biological function via specificities of substrate recognizing in many diseases²⁶. Accumulating evidence shows that *KLHLs* display aberrant alterations in the tumor progression including dysregulation of *KLHL* expression^{27–30}, indicating that alteration in *KLHLs* might contribute to cancer progression. For example, increased *KLHL22* expression promotes malignant melanoma growth in vitro and in vivo³¹. *KLHL14* is specially upregulated in ovarian or endometrial cancer and upregulation of *KLHL14* is positively associated with worse outcome in patients with ovarian or endometrial cancer³². However, the role of *KLHL17* in the pathogenesis of cancer remains unclear. In this study, expression of *KLHL17* in the tumor samples from 173 patients with NSCLC was assessed. We found that *KLHL17* expression is significantly higher in the tumor tissues compared with normal lung tissues. In addition, upregulated *KLHL17* expression is positively correlated with tumor size, lymph node metastasis and TNM stage as well as worsened survival among patients with NSCLC. In vitro study showed that various cell lines of NSCLC (A549, H1299, H460 and SK cells) had higher *KLHL17* expression than normal human bronchial epithelial cells, which confirmed the finding from clinical samples. Overexpression of *KLHL17* in the cell lines of NSCLC promoted the

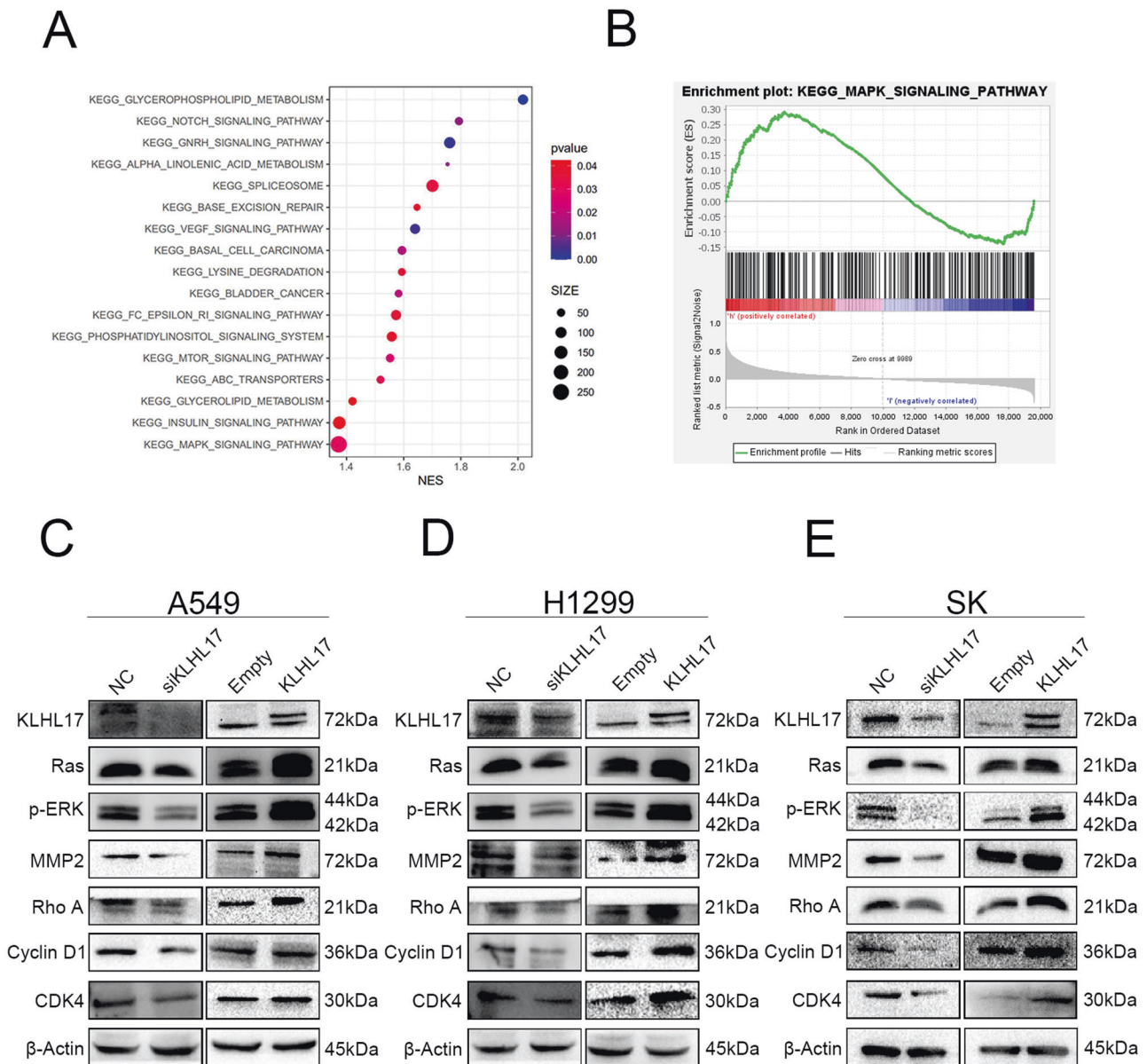


Fig. 6 KLHL17 regulates the Ras/MAPK signaling pathway. **A** and **B** Analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Set Enrichment Analysis (GSEA) databases showing the association between KLHL17 and Ras/MAPK signaling pathway. **C–E** Effects of KLHL17 overexpression with *KLHL17*-Flag plasmid (KLHL17) or KLHL17 knockdown with small interfering RNA (siKLHL17) on Ras/MAPK signaling pathway and expression of cyclin D1, CDK4, MMP2 and RhoA in A549, H1299 and SK cells.

proliferation and migration of tumor cells, whereas knockdown of KLHL17 in the cell lines of NSCLC suppressed the proliferation and migration of tumor cells. These results provide direct evidence that KLHL17 is overexpressed in the NSCLC and that overexpression of KLHL17 contributes to proliferation, migration and invasion of NSCLC. These findings also suggest that KLHL17 expression in the tumor tissues predicts the poor prognosis in patients with NSCLC.

The Ras/MAPK pathway is known to play an important role in cell cycle progression, protein synthesis, differentiation, migration, survival and senescence³³. Additionally, the Ras/MAPK pathway has been considered as a major driver of oncogenesis and is dysregulated in nearly 30% of human cancers, primarily by mutation in the *Ras* genes³⁴. The direct inhibition of Ras/MAPK ERK1/2 pathway has been suggested to provide valuable therapeutic options for cancers driven by *Ras* mutation³⁴. An early study has

demonstrated that ERK1/2 is activated in NSCLC and that ERK1/2 activation is positively correlated with tumor stage and lymph node metastases³⁵. Our present data showed that overexpression of KLHL17 elevated expression of Ras and phosphorylated ERK1/2 in cells of NSCLC, which was associated with increased expression of Rho A, cyclin D1, CDK4 and MMP2, the major factors for tumor proliferation and migration^{21,22}. In contrast, knockdown of KLHL17 reduced expression of Ras and phosphorylated ERK 1/2 in cells of NSCLC, leading to decreased expression of Rho A, cyclin D1, CDK4 and MMP2. More importantly, we found that inhibition of Ras/MAPK pathway with a Ras inhibitor suppressed the ability of KLHL17 to promote cell proliferation, colony formation and cell migration in cells of NSCLC. These results suggest that upregulated KLHL17 in NSCLC promotes tumor proliferation and migration by activating Ras/MAPK pathway to increase expression of Rho A, cyclin D1, CDK4 and MMP2.

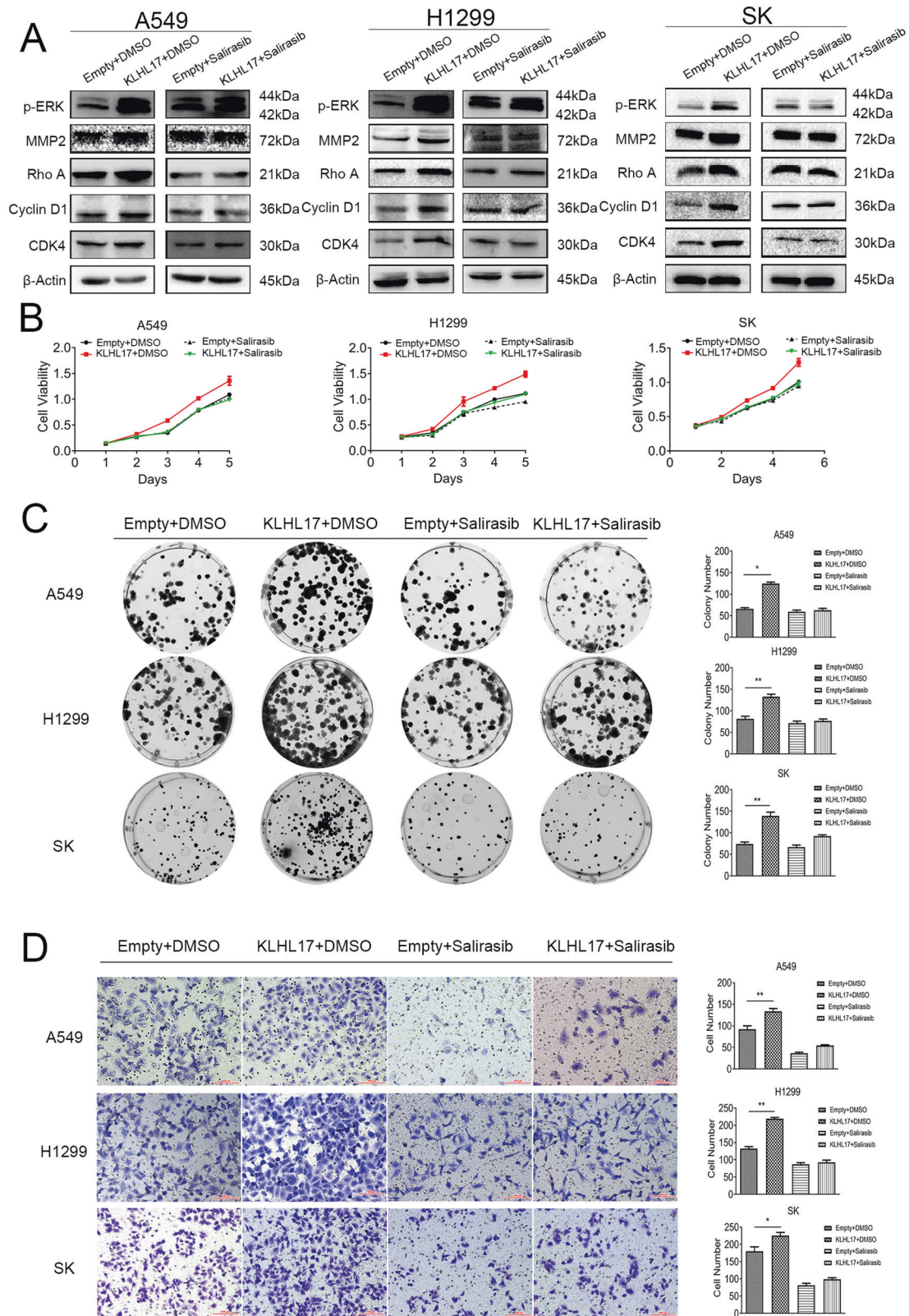


Fig. 7 Inhibition of Ras/MAPK signaling pathway with Ras inhibitor salirasib prevents KLHL17-induced Ras/MAPK activity as well as tumor proliferation and migration. **A** Effect of Ras inhibitor salirasib treatment on KLHL17-induced expression of phosphorylated ERK1/2, cyclin D1, CDK4, MMP2 and RhoA in A549, H1299 and SK cells. **B–D** Effect of Ras inhibitor salirasib treatment on KLHL17-induced proliferation ability, colony formation ability and migration ability of A549, H1299 and SK cells. * $P < 0.05$, ** $P < 0.01$ vs E + D. E + D: Empty+DMSO; K17 + D: KLHL17 + DMSO; E + S: Empty+salirasib; K17 + S: KLHL17 + salirasib.

In conclusion, the present study demonstrates for the first time that KLHL17 is upregulated in NSCLC and that upregulated KLHL17 is associated with poor prognosis in patients with NSCLC. KLHL17 promotes tumor proliferation and migration in NSCLC by activating Ras/MAPK signaling pathway. The findings suggest that KLHL17 may be a novel therapeutic target for the treatment of NSCLC.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

ZL designed the study, conducted experiments, acquired and analyzed data, and wrote the manuscript. MZ, XJ, YZ, SZ, YX, HR, HS, and HW conducted the experiments and acquired data. XQ was responsible for the conception and supervision of the study and wrote the manuscript. All authors corrected drafts and approved the final version of the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

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