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A Positive Feedback Loop of IL-17B-IL-17RB Activates ERK/β-catenin to Promote Lung Cancer Metastasis

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Abstract

Inflammation contributes to the development and progression of cancer. Interleukin-17 (IL-17) is an inflammatory cytokine that functions in inflammation and cancer, as well as several other cellular processes. In this study, we investigated the roles and the prognostic value of IL-17 and the IL-17 receptor (IL-17R) in lung cancer. Gene expression microarray analysis followed by Kaplan-Meier survival curve showed that IL-17B was associated with poor patient survival, and IL-17B receptor (IL-17RB) was up-regulated in lung cancer tissue compared with normal tissue. Expression of IL-17RB was associated with lymph node metastasis and distant metastasis, as well as poor patient survival. IL-17RB overexpression significantly increased cancer cell invasion/migration and metastasis *in vitro* and *in vivo*. IL-17RB induced ERK phosphorylation, resulting in GSK3β inactivation and leading to β-catenin up-regulation. IL-17RB also participated in IL-17B synthesis via the ERK pathway. IL-17RB activation is required for IL-17B-mediated ERK phosphorylation. Taken together, IL-17B-IL-17RB signaling and ERK participate in a positive feedback loop that enhances invasion/migration ability in lung cancer cell lines. IL-17RB may therefore serve as an independent prognostic factor and a therapeutic target for lung cancer.

Keywords

IL-17RB; IL-17B; ERK; lung cancer; metastasis

Abbreviations

IL-17RB- IL-17B receptor, IL-17B- Interleukin 17B, ERK- Mitogen-Activated Protein Kinase 1, GSK3β-Glycogen Synthase Kinase 3 Beta, NSCLCs-non-small cell lung cancers, COPD-chronic obstructive pulmonary disease, EMT-Epithelial-Mesenchymal Transition, shRNA-short hairpin RNA

Introduction

Lung cancer is associated with high mortality worldwide, and more than 80% of lung cancer cases are non-small cell lung cancers (NSCLCs) [15, 35]. Therefore, it is critical for researchers to identify prognostic factors and therapeutic targets for lung cancer. Accumulating evidence shows that a strong association exists between chronic inflammation and tumorigenesis in different types of cancers, including lung cancer [2, 7, 10]. Moreover, lung diseases, including chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis, tobacco smoke, air pollutants, pulmonary infections and occupational dust, are all associated with lung cancer, suggesting chronic inflammation plays an important role in the pathogenesis of lung cancer [7, 10, 27, 28].

The interleukin receptor family is distinct from other single-transmembrane domain protein families and, upon interleukin binding, functions in multiple cellular processes, including inflammation, immunity and cancer [24]. In particular, the interleukin 17 (IL-17) family (IL-17A to IL-17F) and its distinct cytokine receptor family (IL-17Rs, including IL-17RA to IL-17RE) are important for normal host immune responses and the pathogenesis of human diseases, including airway inflammation and cancer [18, 19, 23, 38, 46].

High IL-17 levels have been observed in multiple cancers, including colorectal cancer [22], hepatocellular carcinoma [41], breast cancer [4, 49], ovarian cancer [21], bladder cancer [37], prostate cancer [31], and lung cancer [5]. Furthermore, IL-17 promotes tumor development by inducing the suitable microenvironments at tumor sites by recruiting myeloid-derived suppressor cells [14]. Loss of IL-17 in mice is associated with reduced Stat3 signaling and reduced tumorigenesis [37].

The interactions between IL-17 ligands and receptors are complicated. IL-17A and IL-17B are the ligands for IL-17RA and IL-17RB, respectively [32, 45]. Furthermore, IL-17A binds to IL-17RA/IL-17RC with relatively higher affinity than IL-17F, and IL-17E (IL-25) may have a higher affinity for IL-17RB than IL-17B [23, 24, 39, 45]. IL-17R expression is up-regulated in the stimulated, inflamed tissues of patients with bowel diseases and various cancers [13, 32, 43], while IL-17E is secreted by non-malignant mammary epithelial cells and induces breast cancer apoptosis through IL-25R, an IL-17RB/IL-17RA heterodimer [9, 30]. In addition, high IL-17RB expression is correlated with a poor prognosis in patients with breast cancer [9, 16]. Accumulating evidence suggests that IL-17 plays important roles in cancer progression; however, the role of its receptor in lung cancer remains unclear.

In this study, we investigated whether IL-17 ligands and receptors regulated malignant phenotypes in lung cancer. We evaluated the relationship between IL-17 and patient survival by kaplan-merier plotter and also examined the expression levels of IL-17 receptors by cDNA microarray. According to the bioinformatical analysis, we identified the targets and then provided biological studies for verification. We focused on IL-17RB, the receptor responsible for the effects of IL-17B signaling on cancer progression, since a previous study showed that blocking IL-17RB inhibited the invasion and metastatic ability in cancer cells [40].

Materials and Methods

Kaplan-Meier analysis of IL-17 family

Kaplan-Meier analysis of overall survival was performed by using publicly available lung cancer microarray datasets (sample from 866 patients with adenocarcinoma and 675 patients with squamous cell carcinoma), stratified according to the expression of *IL-17A*, *IL-17B*, *IL-17C*, *IL-17D*, *IL-17E* and *IL-17F*. The publicly available online lung cancer microarray datasets were used for real-time validation of biomarkers related to patients survival (http://kmplot.com/analysis/index.php?p=service& cancer=lung) [12].

Tissue sample collection

Paired cancer tissue samples and adjacent non-cancerous lung tissue samples were collected from 139 newly diagnosed lung cancer patients who were surgically treated at Kaohsiung Medical University Hospital from 2007 to 2013 (sample from 105 patients with adenocarcinoma and 34 patients with squamous cell carcinoma). This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital (KMUHIRB-E(II)-20170140), and written informed consent was obtained from each patient who participated in the study. Staging was performed according

to the American Joint Committee on Cancer (AJCC) tumor-node-metastasis

(TNM) staging system [29]. Disease-free survival (DFS) was defined as the

period between the date of surgery and the date of recurrence, and overall

survival (OS) was defined as the period between the date of surgery and the

date of the patient's death from cancer.

Immunohistochemistry

Immunohistochemistry (IHC) was performed with a fully automated

Bond-Max System (Leica Microsystems, Wetzlar, Germany). All staining steps

were performed by the automated instrument, according to the manufacturer's

instructions (Leica Microsystems). The primary antibodies used for this

experiment are shown in Supplementary Table 7.

Evaluation of immunohistochemical staining

IL-17RB expression was scored on a scale ranging from 0 to 4, according

to the percentage of positively stained cells (0, negative; 1, ≤25% of cells

stained positive; 2, 26–50% of cells stained positive; 3, 51–75% of cells

stained positive; and 4, ≥76% of cells stained positive). The score for each

specimen was determined by two independent experts simultaneously and

under the same conditions. Snail expression was scored on a scale ranging from 0-3, according to the percentage of positively stained cells (0, negative; 1, ≤30% of cells stained positive; 2, 31–50% of cells stained positive; and 3, ≥51% of cells stained positive). Similarly, Twist expression was scored on a scale ranging from 0-3, according to the percentage of positively stained cells (0, negative; 1, ≤50% of cells stained positive; 2, 51–75% of cells stained positive; and 3, ≥76 of cells stained positive).

Cell lines and cell culture conditions

The lung cancer cell lines CL1-0, CL1-5, H441, H460 and H520 were cultured in RPMI-1640 medium (containing 10% FBS and 1% PSG). The A549 and H1299 cell lines were maintained in DMEM-F12 and DMEM with 10% FBS and 1% PSG, respectively.

Reagents

PD98059 (mitogen-activated protein kinase inhibitor, #P215) was purchased from Sigma (St. Louis, MO, USA). U-46619 (activator of ERK1 and ERK2, #sc-201242) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The Proteome Profiler™ Human Phospho-kinase Array Kit

(ARY003B) and recombinant human IL-17B (1248-IB-025/CF) were purchased from R&D Systems.

Lentiviral infection

The negative control lentiviral expression vector (LPP-NEG-Lv-105-025) and the lentiviral expression vector containing IL-17RB (LPP-Z1990-Lv105-200-S) were purchased from GeneCopoeia[TM]. The pLKO-.1-shLuc967, shIL-17RB (TRC58814, TRC58816) and shIL-17B (TRC8595, TRC8596) lentiviral shRNA supernatants were purchased from National RNAi Core Facility (Taipei, Taiwan).

Western blot analysis

Total protein (50 μg) was extracted from the above cell lines and loaded onto a 10% SDS-PAGE gel. After electrophoresis, the proteins were transferred to a PVDF membrane, which was blocked with 5% noon-fat milk in TBST. Western blotting was performed using the appropriate antibodies (Supplementary Table 7).

Growth curve assay

Cells (2000 cells/well) were seeded in a 96-well plate and incubated at 37°C with 5% CO_2 for 24 to 72 h. The cell growth curve was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Cell migration and invasion assays

Cell migration and invasion assays were performed with a Falcon[TM] HTS Multiwell Insert System (8-µm pores, BD Biosciences, San Jose, CA). For invasion assay, the upper chamber of each multiwell insert (transwell) was precoated with Matrigel (BD Biosciences, San Jose, CA), and medium containing 10% FBS was added to each 24-well plate. The cells were then resuspended in serum-free medium ($2x10^5$/mL) and loaded into the upper chamber of the transwell in 100 µL aliquots. After 18-24 h, the cells that had migrated to the lower chamber were stained by crystal violet and counted under a light microscope (entire well). All experiments were performed in quadruplicate. The procedure for the migration assay was similar to that for the invasion assay; however, the upper chamber of the transwell was not coated with Matrigel for the migration assay. To block IL-17B, we incubated IL-17RB-overexpressing cells with an IL-17B-neutralizing antibody (4 µg/mL) for 48 h. Specifically, the cells were trypsinized for the transwell assay after

incubation, and an IL-17B-neutralizing antibody was added to the upper chamber of the transwell.

Animal studies

The animal experiments were conducted in accordance with a protocol approved by the Use Committee of Kaohsiung Medical University. Six-week-old male immunodeficient mice (BALB/cAnN.Cg-Foxn1nu/CrlNarl) were purchased from the National Laboratory Animal Center (NLAC, Taiwan). For *in vivo* metastasis assay, cells were resuspended in PBS (1×10^6 cells/100 μL PBS/mouse) before being injected into each mouse via the tail vein. Five weeks after tail vein injection, all mice were sacrificed and the numbers of lung nodules were quantified by a dissecting microscope after the appropriate tissues were stained with hematoxylin and eosin (H&E).

Enzyme-linked Immunosorbent Assay (ELISA)

The cells were plated in 6-well plates and incubated at 37°C for 24 h, and then the medium was replaced with fresh medium, and the cells were incubated at 37°C for an additional 24 h. The conditioned medium was then collected, and IL-17B expression was determined by a human IL-17B duo-set

ELISA kit (DY1248, R&D Systems).

Real time-PCR

The stable cell lines CL1-0/vector control and CL1-0/IL-17RB (3×10^5 cells/dish) were separately plated in 6-cm culture plates and incubated overnight at 37°C with 5% CO_2. The CL1-0/IL-17RB cells were then treated with a MEK/ERK inhibitor for 24 h, followed by total RNA extraction for real-time PCR analysis (SYBR system). The following primers were used for this experiment: *IL-17B*, forward: 5'TCAACTTGCAGCTGTGGATGTC-3' and reverse: 5'GGTCCTCCTGCATGGTGAAG-3'; and *GAPDH*, forward: 5'GCACCACCAACTGCTTAGCA-3' and reverse: 5'TCTTCTGGGTGGCA GTGATG-3'.

Statistical analysis

All statistical analyses were performed using SPSS (Statistical Package for the Social Sciences, version 19) software. Tissues with IL-17RB expression scores of 1 and 2 were categorized as having low IL-17RB expression, and tissues with scores of 3 and 4 were categorized as having high IL-17RB expression. Chi-square tests were performed to determine if differences in age

at diagnosis, sex, tumor grade, tumor size (T stage), the extent of lymph node metastasis (N stage), the extent of distant metastasis (M stage), patient performance and smoking status existed between the high IL-17RB expression group and the low IL-17RB expression group. Survival curves were generated by the Kaplan-Meier method, and the log rank test was used to evaulate the significance of differences between curves. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated by univariate and multivariable Cox regression models, which were used to investigate the associations between clinicopathological characteristics and patient survival. $P<0.05$ was considered statistically significant. Spearman's correlation test was performed to evaluate the significance of correlations between paramters. Difference between two groups was compared by Student *t* test. Multiple group settings were compared by Tukey's *post-hoc* test. Other materials and methods provided in the online supplement.

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Results

In silico mRNA profiles of _IL-17_ and the _IL-17R_ in lung cancer

We evaluated the expression of the IL-17 family in lung cancer with online survival analysis software [12]. High _IL17B_, _IL17C_ and _IL17E_ expression was significantly associated with worse overall survival. However, _IL17A_, _IL17D_ and _IL17F_ expression was not significantly associated with worse overall survival (Fig. 1A). Cytokines act on target cells by binding to specific receptors. For example, IL-17B binds to IL-17RB, IL-17C binds to a heterodimer comprising IL-17RA/IL-17RE, and IL-17E binds to a heterodimer comprising IL-17RA/IL-17RB [34]. We subsequently examined the expression profiles of _IL-17RA_, _IL-17RB_ and _IL-17RE_ in other microarray datasets. Expression of _IL-17RA_, _IL-17RB_ and _IL-17RE_ in 20 normal lung tissues and the tissues from 226 patients with lung adenocarcinoma (AdC) was compared in the GSE31210 datasets. _IL-17RB_ and _IL-17RE_ expression levels were significantly up-regulated in patients with lung AdC compared with the normal lung tissue specimens (Fig. 1B). We also analyzed _IL-17R_ expression in normal lung tissues and their paired tumor tissues from 5 patients with squamous cell carcinoma (SCC) in the GSE3268 datasets (Supplementary Fig. 1). _IL-17RA_ expression was down-regulated in patients with lung AdC and SCC compared

with the normal lung tissue specimens. Notably, IL-17RA/IL-17RE

heterodimers and IL-17RA/IL-17RB heterodimers are required to facilitate

IL-17C- and IL-17E-mediated signaling, respectively [34]. These results

suggested that IL-17B and IL-17RB may play an important role in lung cancer

progression.

**IL-17RB up-regulation is associated with poor clinical outcomes in lung
cancer**

To study the expression of IL-17RB protein in lung cancer patients, 40 sets

of primary lung tumor specimens and paired pulmonary alveolar tissue

specimens were analyzed by IHC. IL-17RB was significantly up-regulated in

21 of the 40 lung tumor specimens compared with the pulmonary alveolar

tissue specimens from the same patients (Fig. 2A; Supplementary Table 1

and Table 2). To further evaluate IL-17RB protein in lung cancer, we

examined its expression in primary lung tumors from 139 patients with lung

cancer by IHC and correlated IL-17RB expression with the clinicopathological

characteristics of these patients. As shown in Fig. 2B, IL-17RB protein

expression in cancer tissues was classified into four quartiles (scores).

Tissues with scores of 1 and 2 were categorized as having low IL-17RB

expression, and tissues with scores of 3 and 4 were categorized as having high IL-17RB expression. We found that high IL-17RB expression levels in lung cancer tissues were significantly associated with positive lymph nodes metastasis (N status; P=0.002) and positive distant metastasis (M status; P=0.03) (Table 1).

Survival analysis by the log-rank test demonstrated the decreased disease-free survival (DFS) and overall survival (OS) rates in the high IL-17RB expression group compared with the low IL-17RB expression group (P<0.001)(Fig. 2C). We also evaluated the risk factors associated with lung cancer. Hazard ratios (HRs) were estimated by univariate and multivariable Cox regression (Fig. 2D; Supplementary Table 3 and Table 4). The univariate and multivariate analyses of DFS and OS showed that IL-17RB expression was a significant predictor of patient outcome (Fig. 2D; Supplementary Table 3 and Table 4).

Up-regulation of IL-17RB expression enhances lung cancer cell invasion/migration and metastasis

From clinical data analysis, we found that IL-17RB may play an important role in lung cancer metastasis (Table 1). We then evaluated the effect of

IL-17RB on lung cancer cell invasion and migration. The western blotting results showed that the levels of endogenous IL-17RB were up-regulated in highly invasive CL1-5 and A549 cells, and IL-17RB was positively correlated with invasion ability in 6 human lung cancer cell lines (Fig. 3A-B). Knocking down IL-17RB expression in the highly invasive CL1-5 and A549 lung cancer cell lines significantly reduced invasion/migration ability in these cell lines compared with the shluc control cell line (Fig. 3C; Supplementary Fig. 2A). Conversely, overexpressing IL-17RB in the poorly invasive H441 and CL1-0 cell lines significantly enhanced their invasion/migration ability compared with the control cell lines (Fig. 3D; Supplementary Fig. 2B). Neither knockdown nor overexpression of IL-17RB in lung cancer cells affected the proliferation potential of the cells (Supplementary Fig. 2C).

We next investigated the effect of IL-17RB on metastasis *in vivo*. We stably overexpressed IL-17RB in H441 cells and intravenously injected the cells into the tail veins of mice. IL-17RB overexpression resulted in a significant increase in the number of metastatic nodules in the lungs of the indicated mice compared with vector control (Fig. 3E). We also quantified the degree of lymph node invasion by analyzing the expression of cytokeratin 18 (CK18), a lymph node metastasis marker [42, 47]. IL-17RB overexpression

resulted in an increase of CK18 expression in mouse lymph nodes compared

with vector control (Fig. 3F).

IL-17RB promotes invasion/migration through activation of ERK

signaling pathway

To determine the molecular mechanisms by which IL-17RB regulates

cancer cell invasion/migration, we used human phosphokinase arrays to

examine control cells (H441/vector) and IL17RB-overexpressing H441 cells

(Fig. 4A). The analysis showed that p-ERK1/2, p-GSK3β(Ser9), β-catenin,

p-AMPK and p-Chk2 expression levels were increased in

IL17RB-overexpressing H441 cells compared with control cells (Fig. 4A). We

confirmed these results using western blotting, which showed that p-ERK1/2,

p-GSK3β (Ser9), β-catenin and p-Chk2 expression levels were significantly

increased in IL-17RB-overexpressing H441 cells compared with control cells

(Fig. 4B). We focused on ERK1/2 in subsequent experiments because it

phosphorylates the Ser9 residue of GSK3β, leading to β-catenin up-regulation

[8]. We investigated whether IL-17RB promotes invasion/migration via ERK

activation. Transwell assay demonstrated that blocking ERK1/2

phosphorylation with an MEK/ERK inhibitor (PD98059) inhibited

invasion/migration in IL-17RB-overexpressing cells (Fig. 4C).

We next investigated whether IL-17RB expression inactivates GSK3β and up-regulates β-catenin via the ERK1/2 pathway. As shown in Fig. 4D, IL-17RB-induced phosphorylation of ERK1/2 and GSK3β (Ser9) and expression of β-catenin were abolished by the MEK/ERK inhibitor PD98059. Similarly, IL-17RB knockdown resulted in reduced phosphorylation of ERK1/2 and GSK3β (Ser9) and expression of β-catenin (Fig. 4E). These data suggested that IL-17RB enhanced lung cancer cell invasion through the ERK/GSKβ/β-catenin signaling pathway.

IL-17RB up-regulation correlates with Snail/Twist expression in human lung cancer

Activation of ERK pathway has been shown to be associated with epithelial-mesenchymal transition (EMT) [6, 26, 33]. We examined whether IL-17RB expression up-regulates EMT marker expression through the ERK1/2 pathway. The results showed that the expression of Snail and Twist was significantly up-regulated in IL-17RB-overexpressing cells but was decreased in both MEK/ERK inhibitor-treated and IL-17RB-knockdown cells (Fig. 5A-B). We next examined whether IL-17RB expression was correlated with Snail and

Twist expression in lung cancer tissues. IHC staining for IL-17RB and Snail showed that IL-17RB expression was positively correlated with Snail expression in lung cancer tissues (Spearman nonparametric correlation test; correlation coefficient=0.247; *P*=0.014; n=79) (Fig. 5C and 5E; Supplementary Table 5). Similar result was observed between IL-17RB and Twist, as IL-17RB expression was positively correlated with Twist expression in lung cancer tissues (Spearman nonparametric correlation test; correlation coefficient=0.234; *P*=0.007; n=109) (Fig. 5D and 5F; Supplementary Table 6). Moreover, we analyzed the GSE3141 datasets, which contained data for 111 patients with lung cancer, and obtained results similar to our study (Fig. 5G).

IL-17RB up-regulation mediated IL-17B secretion through the ERK pathway

In our study, the MEK/ERK inhibitor PD98059 decreased IL-17RB expression in IL-17RB-overexpressing cells (Fig. 4D and 5A). Furthermore, it has been reported that IL-17RB expression is induced by IL-17B in other cell types [1, 16]. We therefore explored the possibility that IL-17B induces IL-17RB expression in lung cancer cells. IL-17B treatment caused significant IL-17RB up-regulation in CL1-0 and H441 lung cancer cells (Fig. 6A). Also,

IL-17RB overexpression resulted in the up-regulation of IL-17B expression levels, whereas treatment with the MEK/ERK inhibitor PD98059 down-regulated IL-17B levels in IL-17RB-overexpressing cells (Fig. 6B; Supplementary Fig 3). These results indicated that IL-17RB-ERK signaling may be involved in IL-17B synthesis.

We also investigated whether IL-17B mediated invasion/migration in IL-17RB-overexpressing cells. IL-17RB overexpression-induced migration and invasion activity was suppressed by an IL-17B-neutralizing antibody (Fig. 6C). In addition, IL-17B knockdown resulted in IL-17RB and ERK pathway down-regulation in IL-17RB-overexpressing CL1-0 cells (Fig. 6D). Conversely, treatment with the ERK activator U46619 caused a significant increase in ERK phosphorylation in CL1-0 cells, leading to IL-17B and IL-17RB up-regulation, whereas treatment with the MEK inhibitor caused a decrease in ERK phosphorylation in CL-1-0 cells (Fig. 6E). We subsequently evaluated IL-17B levels in the serum of mice with lung metastasis of IL-17RB-overexpressing cancer cells and found that IL-17B levels were significantly higher in these mice than in vector control-treated mice (Fig. 6F). Furthermore, correlation analysis of *IL-17B* and *IL-17RB*, which was performed using GSE3141 datasets (lung cancer microarray datasets), showed that *IL-17B* expression

was positively correlated with *IL-17RB* expression (Fig. 6G). Taken together,

our results suggest that IL-17RB activation is required for IL-17B-mediated

ERK phosphorylation, which leads to up-regulation of β-catenin, Snail and

Twist and contributes to lung cancer metastasis (Fig. 6H).

Discussion

In this study, the IL-17 family was assessed in lung cancer patients, with high IL-17B and its receptor IL-17RB identified as poor prognostic factors. IL-17B and IL-17RB were associated with poor survival in patients with lung cancer, with high IL-17RB expression associated with shorter DFS and OS times, indicating that they play an important role in lung cancer progression. Although other members of the IL-17 family have been implicated in tumor metastasis, such as IL-17A mediated endothelial breach promoting lung metastasis [20], we have demonstrated that these ligands are not associated with a poorer prognosis and are not significantly expressed in lung cancer tissues.

IL-17RB overexpression increased migration/invasion activity in noninvasive lung cancer cell lines, whereas IL-17RB knockdown inhibited migration/invasion ability in highly invasive cell lines. Our data demonstrated that increases in IL-17RB-mediated phosphorylation of ERK led to phosphorylation of GSK3β, which induced the up-regulation of β-catenin, a transcription factor in the Wnt pathway [25]. Moreover, the IL-17RB-ERK pathway mediated IL-17B up-regulation in lung cancer cell lines, whereas treatment with IL-17B increased the effects of IL-17RB expression via the ERK

pathway in lung cancer cells. These findings suggest that IL-17B-IL-17RB signaling and ERK participate in a positive feedback loop to promote lung cancer metastasis (Fig. 6H).

Furthermore, we showed that IL-17RB overexpression promoted invasion/migration activity and increased IL-17B synthesis via the ERK pathway in lung cancer cells. The invasion/migration assay showed that IL-17B blockade inhibited invasion/migration ability in IL-17RB-overexpressing cells (Fig. 6C). Knocking down IL-17B significantly reduced IL-17RB expression and ERK phosphorylation in IL-17RB-overexpressing cells compared with IL-17RB-overexpressing shluc cells (Fig. 6D). Moreover, treatment with IL-17B induced IL-17RB up-regulation in CL1-0 and H441 lung cancer cells, whereas knockdown of IL-17B inhibited IL-17RB expression in IL-17RB-overexpressing cells (Fig. 6A and 6D). Based on these findings, we surmised that IL-17RB is required for IL-17B activation, which results in enhanced invasion/migration in lung cancer cells.

Other mechanistic pathways have been suggested for IL-17, including IL-17B/IL-17RB pro-survival effects in breast cancer cells via TRAF6, NF-kB and BCL-2 mediated anti-apoptosis [16]. IL-17 has also been shown to promote invasion and metastasis via MMP7 induced EMT in prostate cancer

[48]. One study relevant to lung cancer suggests that IL-17 may promotes lung cancer cell migration/invasion activity by inducing EMT and up-regulating the NF-kB/ZEB1 pathway, but this study fails to distinguish between IL-17 subtypes, and the effects may stem from the less clinically relevant IL-17A form [11]. In agreement with our result (Fig. 1A), Jungnickel et al. also showed that the expression of IL-17C correlates with poor disease outcome in lung cancer, but they did not investigative the role of its receptor in lung cancer [17]. In contrast, our data using the clinically relevant IL-17B subtype demonstrated that EMT in lung cancer may be mediated via Snail and Twist, as IL-17RB overexpression resulted in their up-regulation, whereas IL-17RB knockdown inhibited their expression in lung cancer cell lines.

An early study reported that T-helper type 17 (Th17) cells, a CD4^{+} T cell subset, generated IL-17 in response to the cytokines IL-6, TGF-β, IL-21, IL-1, and IL-23 in autoimmune diseases [36]. Targeting ERK/MAPK signaling with a MEK inhibitor effectively reduces IL-17 synthesis in Th17 cells, whereas treatment with IL-23 and IL-1β increases IL-17 levels in Th17 cells [3, 36]. Furthermore, treatment with the ERK activator U49919 significantly up-regulated IL-1β and IL-6 in the microglia cell line BV2 [44]. Here, we showed that U49916 treatment increased IL-17B and IL-17RB expression in

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CL1-0 lung cancer cells (Fig. 6E). Moreover, ERK phosphorylation induces IL-17B synthesis in lung cancer cells, a change inhibited by the MEK inhibitor (Fig. 6E). Recent studies also showed that IL-17B treatment or IL-17RB overexpression led to ERK phosphorylation and promoted metastasis whereas treatment with a neutralizing antibody against IL-17RB inhibits metastasis in pancreatic cancer [40]. We could not exclude the possibility that IL-17B is secreted by other cell types; however, this is the first study to demonstrate that IL-17RB mediated IL-17B synthesis via the ERK pathway, providing the basis for a therapeutic target that may reduce lung cancer cell metastasis.

In conclusion, this is the first study to identify clinically relevant subtypes of the IL-17 family through analysis of lung cancer patient tissues – IL-17B and IL-17RB - and delineate their mechanism of action in lung cancer invasion and metastasis. As clear evidence emerges of the role that inflammation plays in cancer progression, IL-17B-IL-17RB has been identified as both a key prognostic marker and ideal target for lung cancer treatment.

Acknowledgments

Author contributions statement

The authors contributed in the following way: design and write the manuscripts: Y.-F.Y, Y.-C.L and S.-S.F.Y; provide materials: S.-S.F.Y; perform experiments: Y.-F.Y, Y.-N.C and W.-C.C; interpret data: Y.-F.Y, Y.-C.L, S.L, Y.-C.H and S.-S.F.Y.

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Figure Legends

Fig1. Identification of IL-17RB as a potential target for the prevention of lung cancer progression. (A) Kaplan-Meier analysis of OS, according to the expression of *IL17A*, *IL17B*, *IL17C*, *IL17D*, *IL17E* and *IL17F*, using publicly available lung cancer online microarray datasets software. HR=hazard ratio. (B) Relative *IL17RA* (228685_at, 220101_at and 229295_at), *IL17RB* (219255_x_at and 224156_x_at) and *IL17RE* (236186_x_at) mRNA levels in patients with lung cancer adenocarcinoma in the GSE31210 datasets. The significance is determined by Student *t* test. **, *P*<0.01.

Fig2. IL-17RB is overexpressed in lung cancer and is correlated with poor survival. (A) Quantification of IL-17RB expression in 40 paired lung cancer specimens and significance determined by Student *t* test. *, *P*<0.05. (B) IL-17RB expression in lung cancer tissues. (C) Kaplan-Meier survival curves for disease-free survival (DFS) and overall survival (OS) in lung cancer patients according to IL-17RB expression, as determined by IHC. (D) HRs for the risk of death, as determined by multivariable Cox regression, in lung cancer patients.

Fig3. IL-17RB enhances migration/invasion and metastasis in lung cancer. (A) Top, Western blot analysis of IL-17RB expression in lung cancer cell lines. Bottom, invasive abilities of lung cancer cell lines. (B) Correlation between IL-17RB expression and invasion ability in lung cancer cell lines. (C) IL-17RB knockdown on migration/invasion potential of A549 and CL1-5 lung cancer cells. Top, Western blot analysis of IL-17RB expression in IL-17RB-knockdown cells vs. shluc control cells. Bottom, A549 and CL1-5 migration and invasion ability after IL-17RB knockdown. The data are presented as the mean ± SD and the significance is determined by one way ANOVA followed by Tukey's *post hoc* test. *, $P<0.05$; **, $P<0.01$. (D) IL-17RB overexpression on migration/invasion potential of CL1-0 and H441 lung cancer cells. Top, Western blot analysis of IL-17RB expression after IL-17RB overexpression vs. vector control treatment. Bottom, CL1-0 and H441 migration and invasion ability after IL-17RB overexpression. The data are presented as the mean ± SD and the significance is determined by Student *t* test. **, $P<0.01$. (E) Representative image of H&E-stained lung tissues from mice intravenously injected with H441-vector or H441-IL17RB (n=6). The data are presented as the mean ± SD and the significance is determined by Student *t* test. **, $P<0.01$. (F) Representative images of CK18 expression in the lymph

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nodes of vector control mice and IL-17RB-overexpressing mice. The data are presented as the mean ± SD and the significance is determined by Student t test. **, $P<0.01$.

Fig4. IL-17RB promotes invasion/migration through the ERK/β-catenin pathway. (A) Top, phosphorylation kinase array assay in IL-17RB-overexpressing H441 cells. Bottom, phosphorylation levels of candidate proteins relative to the control. (B) Western blot analysis of candidate kinases in IL-17RB-overexpressing H441 cells. (C) Migration and invasion ability in CL1-0/IL-17RB and H441/IL-17RB cells after treatment with the MEK/ERK inhibitor PD98059 (25 μM). The data are presented as the mean ± SD and the significance is determined by one way ANOVA followed by Tukey's *post hoc* test. *, $P<0.05$; **, $P<0.01$. (D) Western blot analysis of IL-17RB, ERK, GSK3β and β-catenin expression in CL1-0/IL-17RB and H441/IL-17RB cells after treatment with the MEK/ERK inhibitor PD98059 (25 μM). (E) Comparison of IL-17RB, ERK, GSK3β and β-catenin expression between IL-17RB-knockdown A549 and CL1-5 cells and shluc control cells.

Fig5. IL-17RB expression is correlated with Snail/Twist expression in

lung cancer tissues. (A) Western blot analysis of IL-17RB and EMT markers in CL1-0/IL-17RB and H441/IL-17RB cells after treatment with the MEK/ERK inhibitor. (B) The expression of EMT markers in A549 and CL1-5 lung cancer cells after IL-17RB knockdown. (C) Representative images of IL-17RB and Snail IHC staining in lung cancer tissues. (D) Representative images of IHC analysis for IL-17RB and Twist in lung cancer tissues. (E and F) Quantification of IL-17RB and Snail (E)/Twist (F) expression by IHC analysis of lung cancer tissues. The number (n) of specimens is shown at the top of each column. *, $P<0.05$. (G) Analysis of the correlation between *IL-17RB* expression and *Snail/Twist* mRNA expression using GSE3141 datasets.

Fig6. IL-17RB enhanced lung cancer migration/invasion in a IL-17B-mediated manner. (A) Top, Western blot analysis of IL-17RB expression following treatment with IL-17B (12.5 ng/mL). Bottom, relative expression of IL-17RB in IL-17B treated cells relative to untreated cells and the significance is determined by Student *t* test. *, $P<0.05$. (B) IL-17B levels in IL-17RB-overexpressing cells after treatment with the MEK/ERK inhibitor PD98059. IL-17B concentrations in conditioned medium were quantified by ELISA. The data are shown as the mean ± SD and the significance is

determined by one way ANOVA followed by Tukey's *post hoc* test. *, $P < 0.05$.

(C) Migration/invasion ability in CL1-0/IL-17RB cells and H441/IL-17RB cells after pretreatment with an IL-17B-neutralizing antibody (4 µg/mL) or control IgG (4 µg/mL) for 48 hours. The data are shown as the mean ± SD and the significance is determined by one way ANOVA followed by Tukey's *post hoc* test. *, $P < 0.05$, **, $P < 0.01$. (D) IL-17B levels in IL-17RB-overexpressing CL1-0 cells after IL-17B knockdown. Left, IL-17B levels were measured by ELISA. The data are shown as the mean ± SD and the significance is determined by one way ANOVA followed by Tukey's *post hoc* test. **, $P < 0.01$. Right, Western blot analysis of IL-17RB, p-ERK1/2 and ERK expression following IL-17B knockdown. (E) Western blot analysis of p-ERK1/2, ERK, IL-17B and IL-17RB expression following treatment with the ERK activator U46619 (10 µM) or the MEK inhibitor PD98059 (25 µM). (F) IL17B levels, determined by ELISA, in the serum of mice with lung metastasis of IL-17RB-overexpressing cancer cells and vector control-treated mice (n=5). The data are shown as the mean ± SD and the significance is determined by Student *t*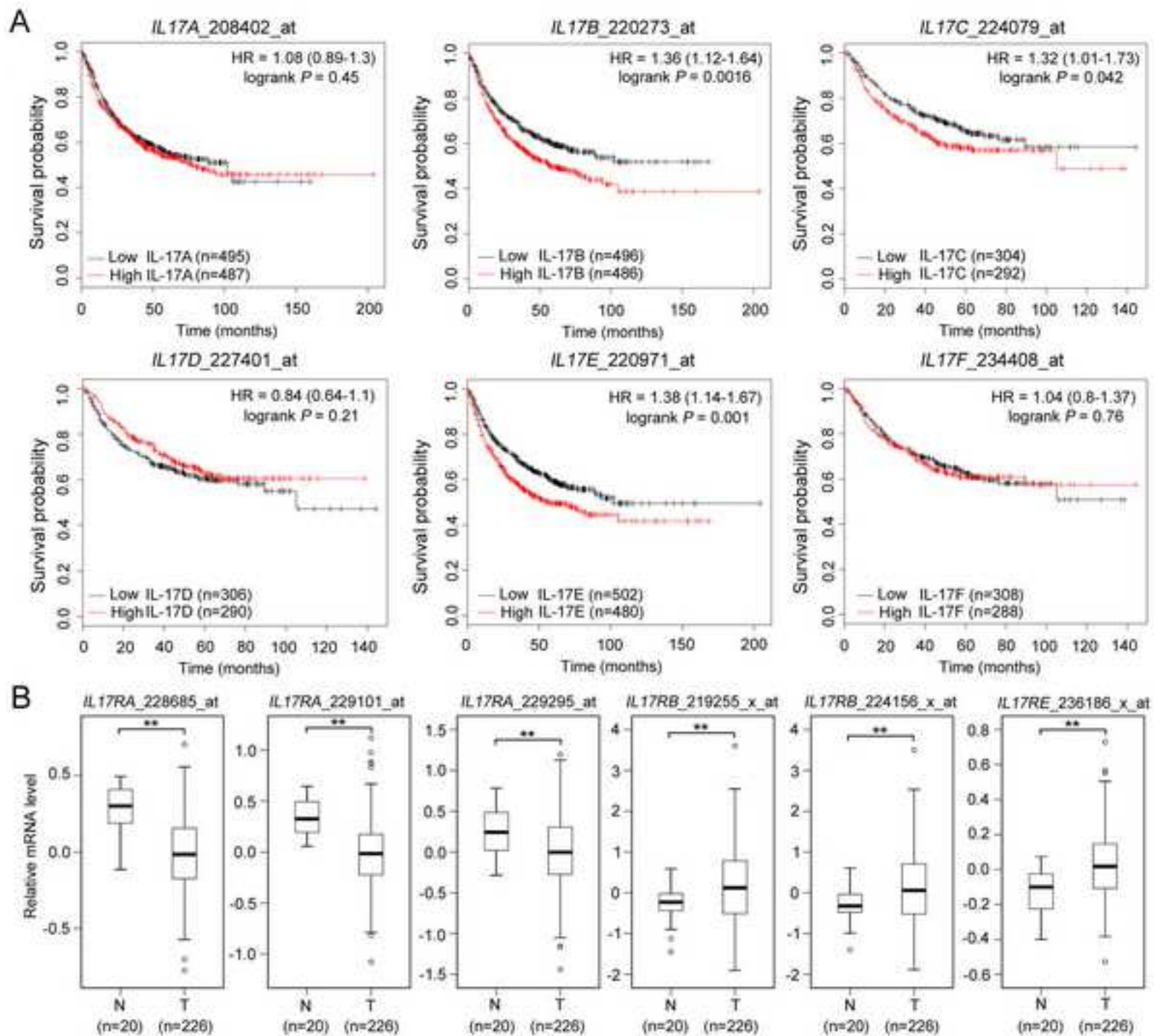 test. **, $P < 0.01$. (G) *IL-17B* and *IL-17RB* mRNA expression correlation analysis was performed using GSE3141 datasets. (H) Model showing that IL-17B-IL-17RB signaling induced ERK phosphorylation, leading to up-regulation of β-catenin, Snail, Twist, and

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cell invasion and metastasis in lung cancer.
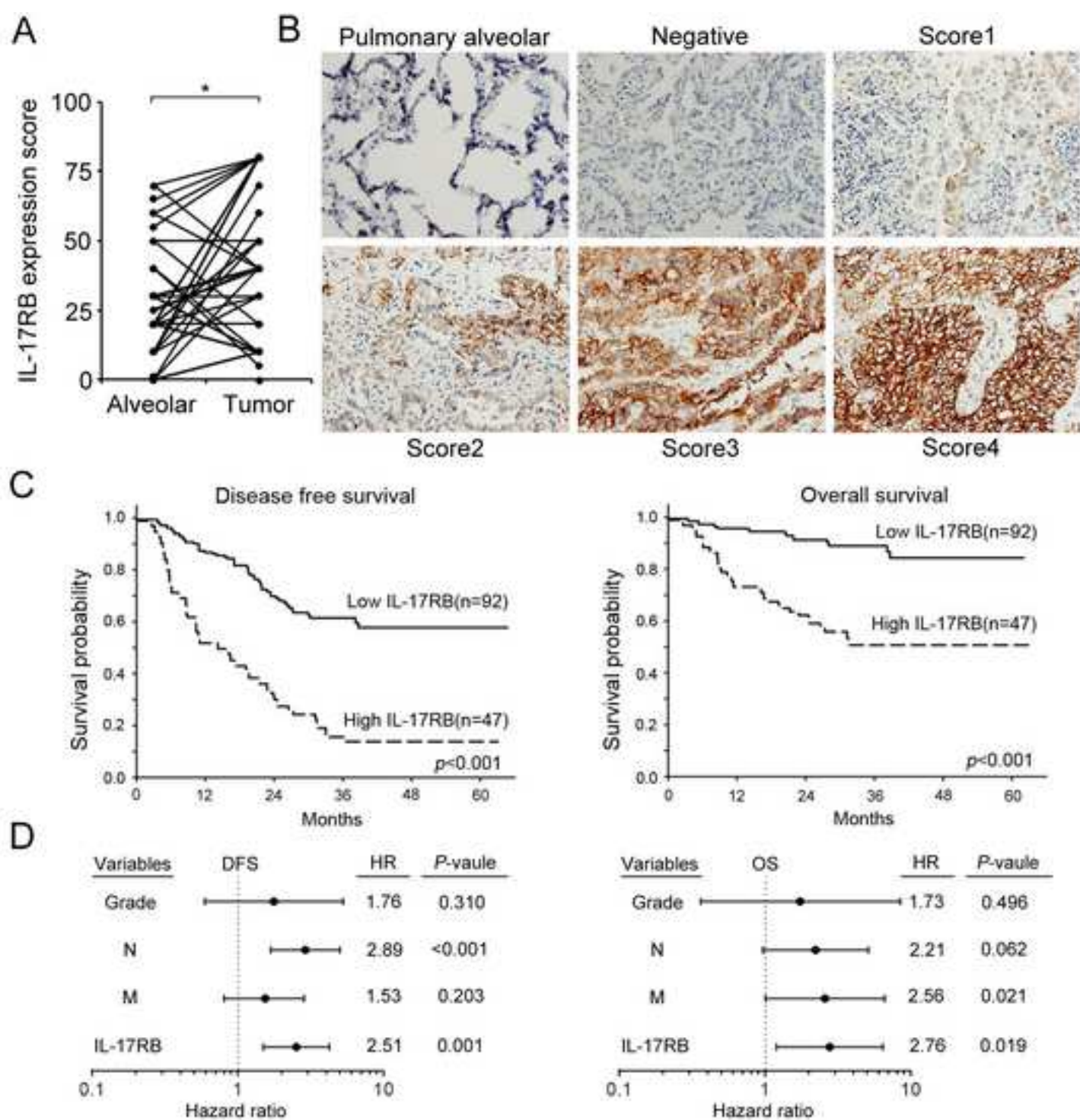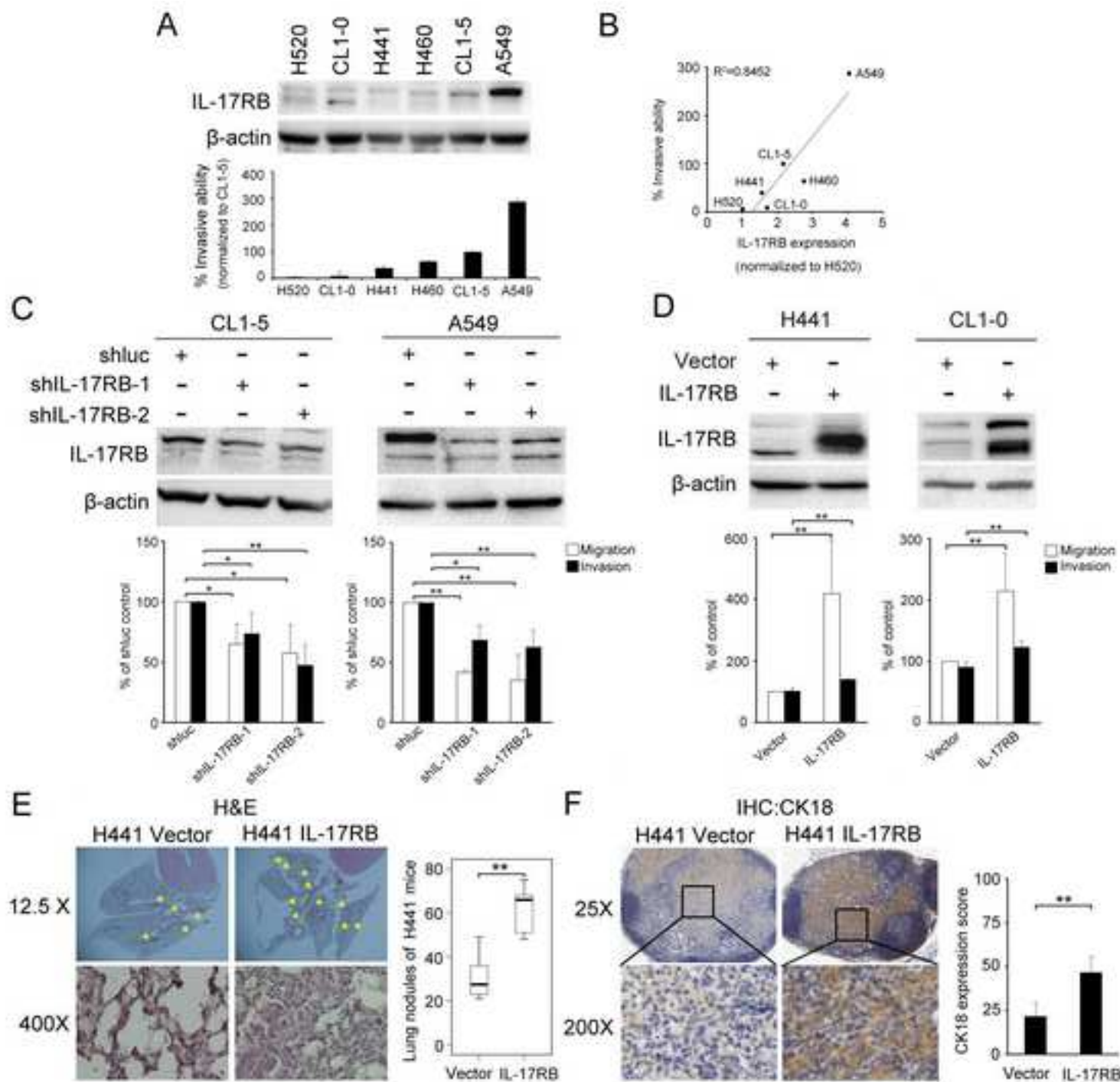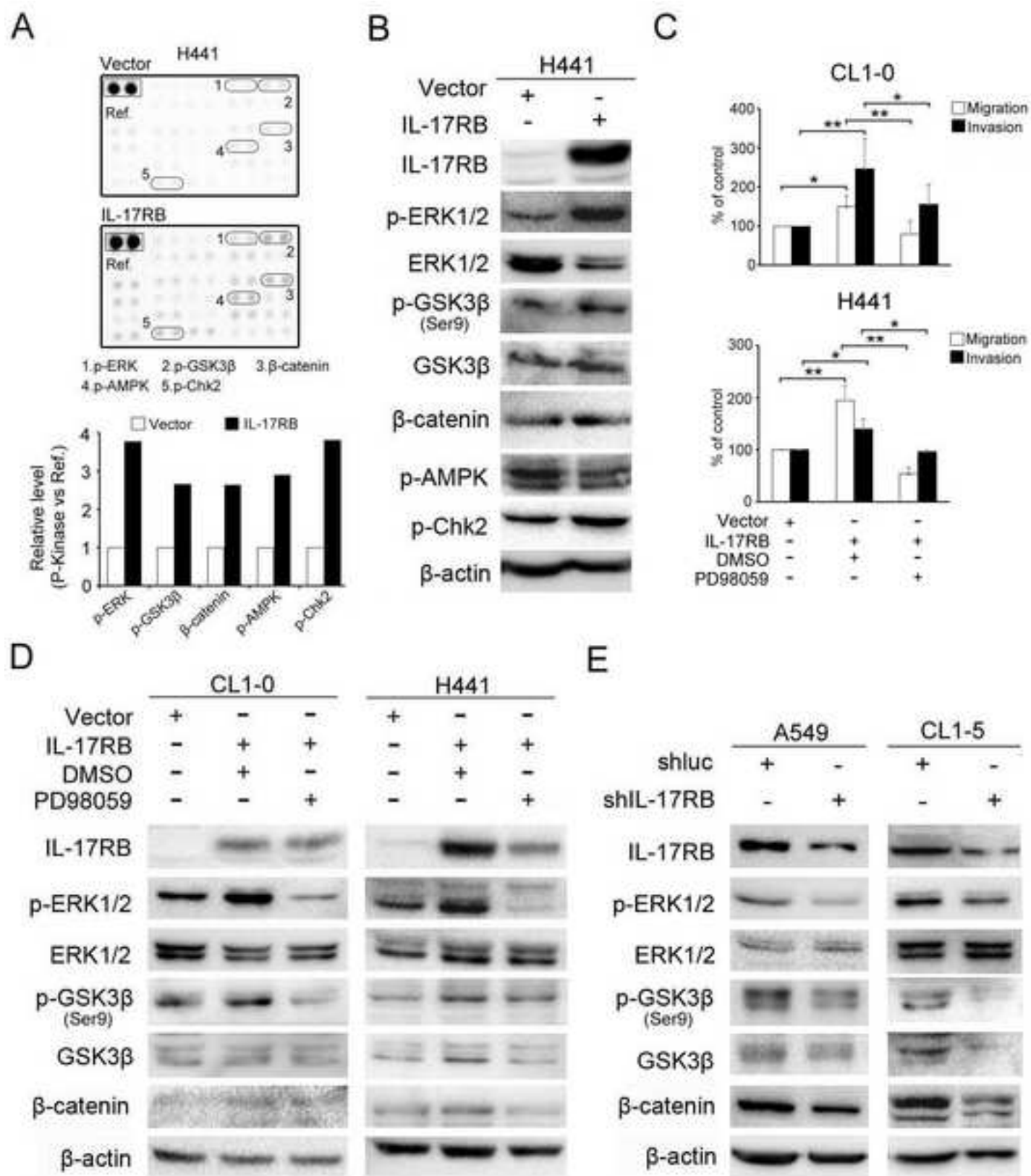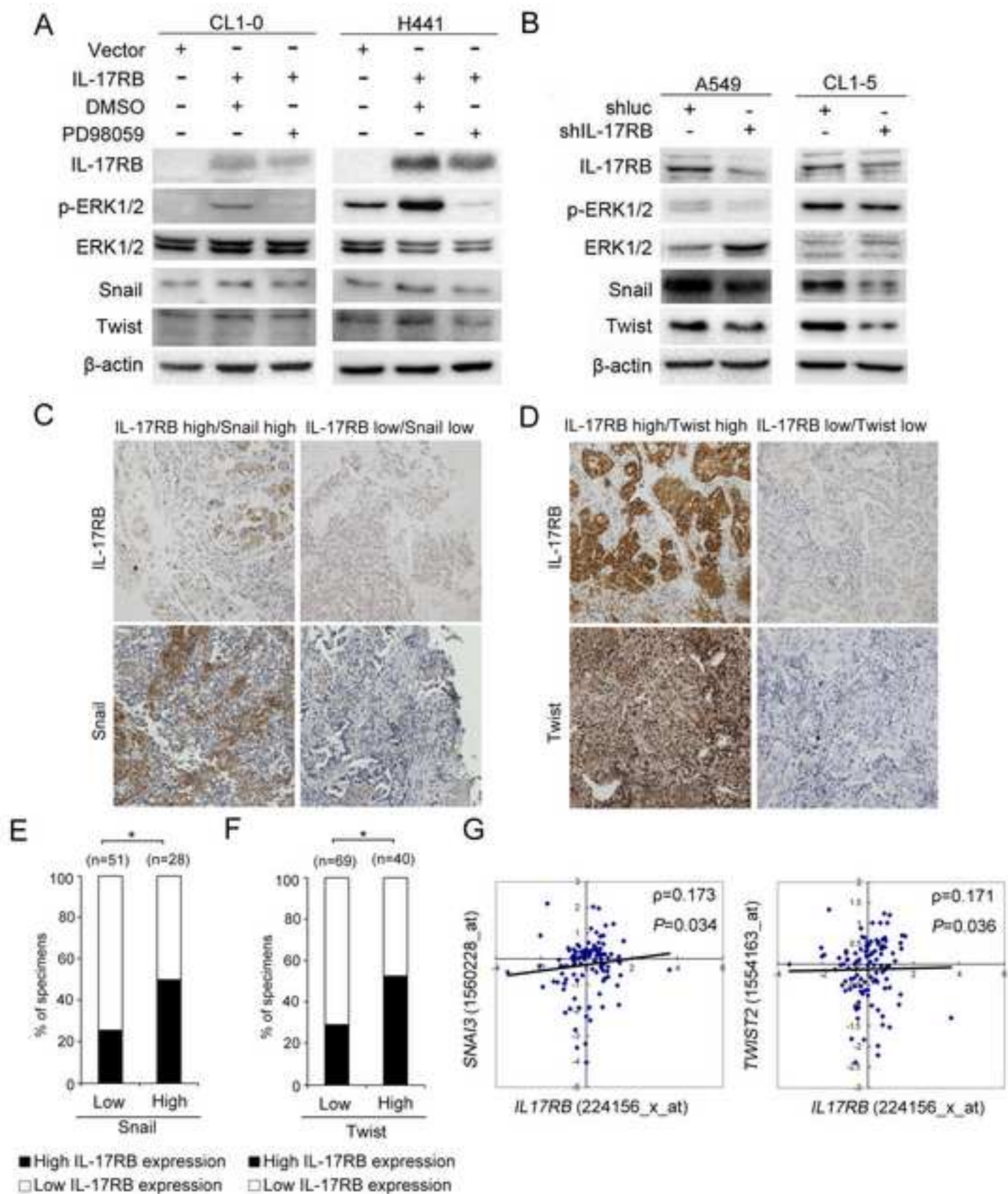
Figure1 Identification of IL-17RB

Figure2_IL-17RB is overexpressed IHC

Figure3_3.IL-17RB enhances migration

Figure4_ERK/ß-catenin

A

H441

Vector

Ref.

1

2

4

3

5

IL-17RB

Ref.

1

2

4

3

5

1.p-ERK 2.p-GSK3β 3.β-catenin
4.p-AMPK 5.p-Chk2

Relative level (P-Kinase vs Ref.)

☐ Vector ■ IL-17RB

p-ERK p-GSK3β β-catenin p-AMPK p-Chk2

B

H441

| | Vector | + | − |
| | IL-17RB | − | + |

IL-17RB
p-ERK1/2
ERK1/2
p-GSK3β (Ser9)
GSK3β
β-catenin
p-AMPK
p-Chk2
β-actin

C

CL1-0

☐ Migration ■ Invasion

% of control

H441

☐ Migration ■ Invasion

% of control

Vector	+	−	−
IL-17RB	−	+	+
DMSO	−	+	−
PD98059	−	−	+

D

	CL1-0			H441		
Vector	+	−	−	+	−	−
IL-17RB	−	+	+	−	+	+
DMSO	−	+	−	−	+	−
PD98059	−	−	+	−	−	+

IL-17RB
p-ERK1/2
ERK1/2
p-GSK3β (Ser9)
GSK3β
β-catenin
β-actin

E

	A549		CL1-5	
shluc	+	−	+	−
shIL-17RB	−	+	−	+

IL-17RB
p-ERK1/2
ERK1/2
p-GSK3β (Ser9)
GSK3β
β-catenin
β-actin

Figure5_Snail/Twist expression
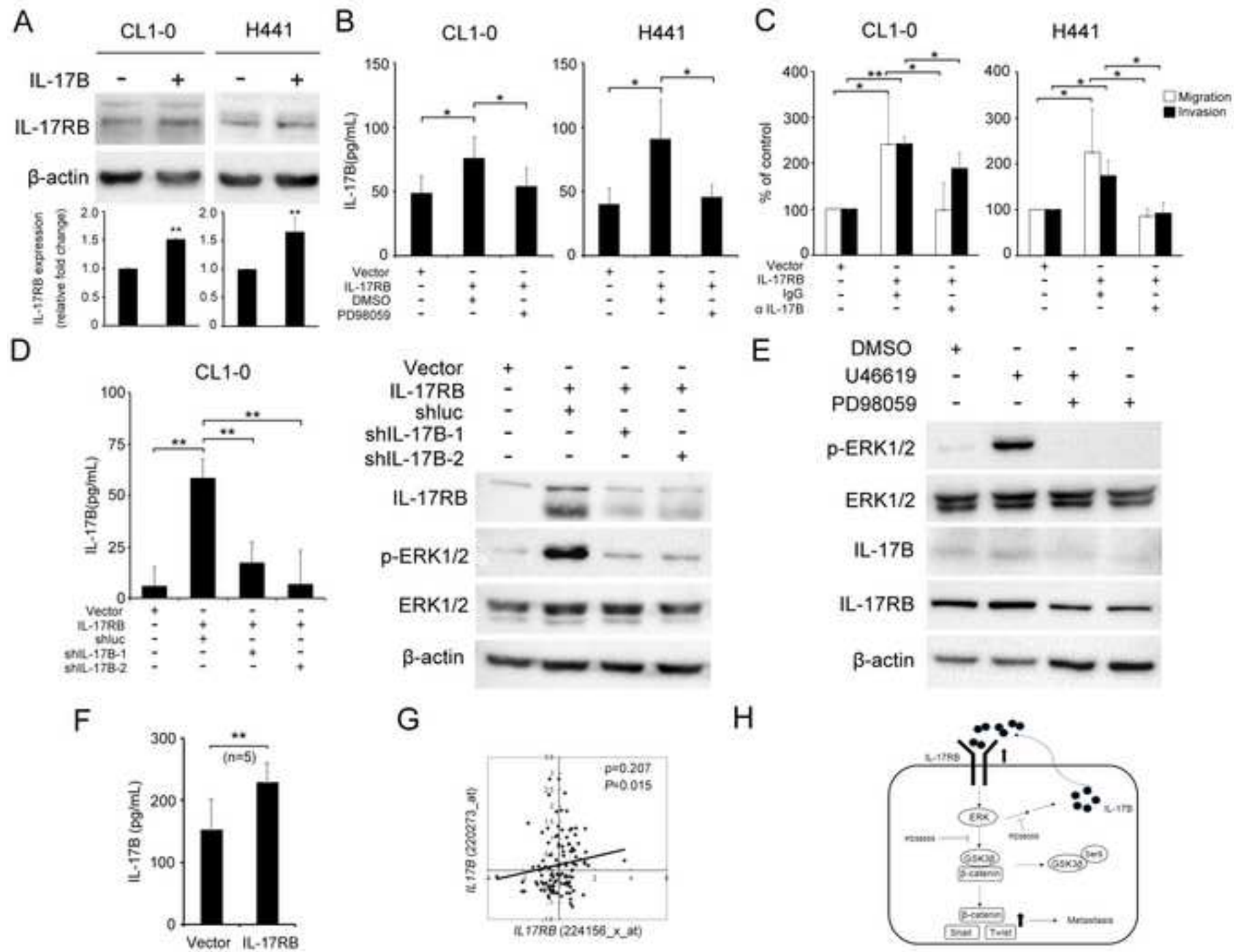
Figure6_IL-17B-IL-17RB

Table1

Table 1. Association between IL-17RB expression and clinicopathological characteristics in lung cancer.

Variables	Item	Patient No.	IL17-RB		p value*
			Low (1,2)	High (3,4)	
			No.	No.	
		139	92	47	
Age (y)	≤70	93	61	32	0.833
	>70	46	31	15	
Sex	Female	47	34	13	0.273
	Male	92	58	34	
Grade	I	26	20	6	0.093
	II	99	66	33	
	III	14	6	8	
T status	T1/T2	113	74	39	0.716
	T3/T4	26	18	8	
N status	Negative (N0)	84	64	20	0.002*
	Positive (N1/N2/N3)	55	28	27	
M status	Negative	119	83	36	0.030*
	Positive	20	9	11	
Performance	0	105	73	32	0.144
	1	34	19	15	
Smoking status	Never	85	62	23	0.094
	Former	25	13	12	
	Current	29	17	12	

*P values <0.05 were considered statistically significant (chi-square test for categorical variables).