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Resistin facilitates metastasis of lung adenocarcinoma through the TLR4/Src/EGFR/PI3K/NF-κB pathway

Wei-Jing Gong[1,2,*] | Jun-Yan Liu[3,*] | Ji-Ye Yin[1] | Jia-Jia Cui[1] | Di Xiao[4] |
Wei Zhuo[1] | Chao Luo[1] | Rui-Jie Liu[5] | Xi Li[1] | Wei Zhang[1] (iD) | Hong-Hao Zhou[1] |
Zhao-Qian Liu[1,*] (iD)

[1]Department of Clinical Pharmacology, Xiangya Hospital, Central South University, Changsha, China

[2]Department of Pharmacy, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

[3]Department of orthopaedics, The First Affiliated Hospital of the University of South China, Hengyang, China

[4]Department of Pharmacy, Xiangya Hospital, Central South University, Changsha, China

[5]Department of Pathology, Xiangya Hospital, Central South University, Changsha, China

Correspondence: Zhao-Qian Liu, Department of Clinical Pharmacology, Xiangya Hospital, Central South University, Changsha 410008, China; and Institute of Clinical Pharmacology, Central South University, Hunan Key Laboratory of Pharmacogenetics, Changsha 410078, China.
(liuzhaoqian63@126.com)

Metastasis is the main cause of lung cancer-related death. The tumor microenvironment greatly contributes to tumor metastasis. Resistin, mainly secreted by tumor-associated macrophages in tumor tissues, is a 12.5-kDa cysteine-rich secretory protein that is found at significantly higher levels in the serum or plasma of cancer patients compared with healthy controls. In this study, we explored the expression and role of resistin in lung adenocarcinoma. Our study showed that resistin was strongly expressed in lung adenocarcinoma tissues and promoted the migration and invasion of lung adenocarcinoma cells in a dose-dependent manner. Toll-like receptor 4 (TLR4) was the functional receptor of resistin for migration and invasion in A549 cells. Src/epidermal growth factor receptor (EGFR) was involved in resistin-induced migration and invasion. Resistin increased the phosphorylation of EGFR through the TLR4/Src pathway. We also found that PI3K/nuclear factor (NF)-κB were the intracellular downstream effectors mediating resistin-induced migration and invasion. Taken together, our results suggested that resistin promoted lung adenocarcinoma metastasis through the TLR4/Src/EGFR/PI3K/NF-κB pathway.

KEYWORDS
invasion, lung adenocarcinoma, migration, resistin, TLR4

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*These authors contributed equally to this work.

1 | INTRODUCTION

Lung cancer is the most frequently diagnosed cancer and contributes to more than one-quarter of cancer-related death. In contrast to the steady increase in the survival rate for most cancers, the 5-year overall survival rate of lung cancer remains approximately 18%.[1] Although significant improvements in diagnosis have been made, most lung cancer patients are diagnosed at a late stage with multiple metastases.[2] Metastasis accounts for more than 90% of lung cancer-related mortality.[3] Adenocarcinoma is the most common histological type of lung cancer.

Cancer is a systemic disease encompassing both tumor cells and host stromal cells. Some of the stromal cells, such as macrophages, dendritic cells, myeloid-derived suppressor cells, and lymphocytes, can secrete cytokines and interact with tumor cells, which plays a vital role in the development and progression of lung cancer.[4] Predominant infiltration of macrophages in tumor tissues is associated with poor prognosis of lung cancer.[5] Human resistin is a 12.5-kDa cysteine-rich secretory protein that is predominantly synthesized in macrophages, dendritic cells, and monocytes.[6] Our previous study showed that circulating resistin levels were significantly higher in cancer patients.[7] Compared with benign prostate hyperplasia, resistin was highly expressed in prostate tumor tissues.[8] The expression of resistin in pancreatic tumors was associated with tumor differentiation and relapse-free survival in patients with pancreatic ductal adenocarcinoma.[9] Resistin showed anti-apoptosis and pro-angiogenesis capabilities in human prostate cancer cell lines.[10] Serum resistin levels were significantly higher in lung cancer patients compared with healthy controls.[11] However, the precise roles and mechanisms of resistin in lung adenocarcinoma have not been fully elucidated.

Toll-like receptor 4 (TLR4) is expressed in both immune cells and cancer cells. The expression of TLR4 in tumor tissues was reported to correlate with malignancy of lung cancer.[12] Hepatocellular carcinoma cells with high TLR4 expression showed enhanced invasion and migration.[13] The activation of TLR4 promoted the migration and invasion of lung cancer cells.[14] Resistin carries out diverse functions through distinct receptors in different cell types. It is postulated that TLR4 is the potential receptor of resistin in lung adenocarcinoma cells. However, this needs to be confirmed. Epidermal growth factor receptor (EGFR) is a predictor of poor prognosis and related to a more aggressive clinical progression in a great variety of cancers, including lung cancer.[15] Toll-like receptor 4 could regulate the activation EGFR pathway by the phosphorylation of the c-Src/EGFR complex.[16] Transcription factor Twist1, a master regulator of embryonic morphogenesis, plays an essential role in metastasis by promoting epithelial–mesenchymal transition.[17] Matrix metalloproteinase 2 (MMP2) is a 72-kDa type IV collagenase that promotes tumor metastasis by degrading the ECM.[18] Both Twist1 and MMP2 are important hallmarks of metastasis and essential for migration and invasion.

In this study, we found that resistin protein expression was upregulated in lung adenocarcinoma tissues compared with corresponding paracarcinoma lung tissues. Resistin promoted the migration and invasion of lung adenocarcinoma cells. Toll-like receptor 4 was the functional receptor of resistin for migration and invasion in lung adenocarcinoma cells. Resistin facilitated metastasis of lung adenocarcinoma through TLR4/Src/EGFR/PI3K/nuclear factor-βK (NF-κB) pathway.

2 | MATERIALS AND METHODS

2.1 | Materials

Antibodies against protein resistin (sc-376336), TLR4 (sc-293072), NF-κB (sc-372), PI3K p-p85 (sc-12929R), EGFR (sc-377229), and phosphorylated (p-)EGFR (sc-12351) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Twist1 (WL0109), PI3K p85 (WL01169), Akt (WL0003b), c-Src (WL01570), p-Src (WL02114), and p-Akt (WL03307) were purchased from Wanleibio (Shenyang, China). Antibodies against MMP2 (#4022S) and GST (#2622) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody against β-actin (A1978) was purchased from Sigma (St. Louis, MO, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG (AP132F) and goat anti-mouse (401215) secondary antibodies were bought from Sigma. Recombinant human resistin and resistin with His-tag were purchased from ProSpec (Rehovot, Israel). Agarose bead-conjugated anti-His mouse clonal antibody (AT0084) was purchased from CMCTAG (San Diego, CA, USA). SB203580 (p38 MAPK inhibitor) and LY294002 (PI3K inhibitor) were purchased from Beyotime (Jiangsu, China). Diphenyliodonium (reactive oxygen species [ROS] inhibitor) was purchased from Sigma. Erlotinib HCl (EGFR inhibitor) was purchased from Selleck Chemicals (Houston, TX, USA). TAK242 (TLR4 inhibitor) was purchased from MedChem Express (Monmouth Junction, NJ, USA).

2.2 | Cell culture

Human lung adenocarcinoma cells A549 and H1299 and human monocytic cell lines U937 were cultured in RPMI-1640 medium supplemented with 10% FBS and maintained at 37°C in humidified 5% CO_2 atmosphere.

2.3 | Patients and specimens

The 32 pairs of lung adenocarcinoma tissues and paracarcinoma lung tissues were collected from Xiangya Hospital, Central South University (Changsha, Hunan, China), between 2012 and 2014. Those specimens were sharply excised, placed in sterile tubes, and immediately frozen in liquid nitrogen before use. The demographic and clinical data were collected. The purpose of this study was informed to all patients or their relatives, and consent forms were signed. The study protocol was approved by the Ethics Committee of Xiangya School of Medicine, Central South University (Registration No. CTXY-110008-3). The study was also registered in the Chinese Clinical Trial Registry (Registration No. ChiCTR-TNC-09000585).[19]

2.4 | Cell proliferation and colony formation

Cell proliferation assay was evaluated using an MTS kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. For colony formation assay, A549 cells were plated into 6-well plates and cultured in RPMI-1640 medium with 10% FBS for 14 days. Cells were then fixed with 4% paraformaldehyde and stained with 0.1% crystal violet.

2.5 | Flow cytometric analyses of apoptosis and cell cycle

The cells were collected by trypsin. For apoptosis analysis, cells were stained with FITC–annexin V and propidium iodide (Beyotime) and analyzed using an FC500 Flow Cytometer (Beckman Coulter, Fullerton, CA, USA) with CXP software (Beckman Coulter). For cell cycle analysis, cells were stained with propidium iodide (Sigma) according to the manufacturer's protocol.

2.6 | Flow cytometric analyses of TLR4 expression on cell surface

After treatment, cells were washed with PBS and blocked with 5% BSA for 1 h. Cells were then incubated with anti-human CD284 (TLR4) Alexa Fluor 488 (E10289-1632; eBioscience, San Diego, CA, USA) or mouse IgG2a k isotype control Alexa Fluor 488 (53472480, eBioscience) in the dark for 30 min on ice and detected by an FC500 Flow Cytometer.

2.7 | Cell migration and invasion assays

The migration and invasion assays were carried out using 24-well Transwell inserts (8.0 μm) (BD Biosciences, San Jose, CA, USA) coated with or without growth factor-reduced Matrigel (BD Biosciences). Cells in serum-free media were seeded into the upper chamber. After incubation, cells inside Transwell inserts were removed by cotton swabs and the migrated or invaded cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The cells on the lower surface were photographed and at least five random fields were counted. At least three independent experiments were carried out.

2.8 | RNA extraction and quantitative RT-PCR analyses

Total RNA was extracted from cultured cells using RNAiso Plus (Takara, Dalian, China). The RT reaction was carried out using the PrimeScript RT reagent kit with gDNA Eraser (Takara). Quantitative real-time PCR was performed on the LightCycler 480 real-time PCR system (Roche Diagnostics, Basel, Switzerland) using SYBR Green PCR Master Mix (Takara). The primers used in the study for real-time PCR assay are listed in Table S1. Relative mRNA expression is presented using the $2^{-\triangle\triangle Ct}$ method. All experiments were performed at least three times.

2.9 | Western blot analyses

Cells or tissues were lysed using mammalian protein extraction reagent RIPA (Beyotime) supplemented with protease inhibitor cocktail (Sigma). The concentration of total protein was measured using a BCA protein assay kit (Beyotime). Samples (30–50 μg protein/lane) were separated by 10% SDS–polyacrylamide gel (Beyotime) and then transferred onto the PVDF membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies at 4°C overnight then incubated with HRP-conjugated secondary antibodies for 1 h. Protein bands were visualized with ECL substrates (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) using the Western-Light chemiluminescent detection system (Bio-Rad, Hercules, CA, USA) according to the protocols.

2.10 | Co-immunoprecipitation assay

To evaluate the binding between resistin and TLR4, A549 cells treated with 500 ng/mL human recombinant resistin with His-tag were lysed and immunoprecipitated with agarose bead-conjugated anti-His mouse clonal antibody. After overnight incubation, immune complexes were collected and immunoblotted using anti-TLR4 antibody.

2.11 | Gluthione S-transferase pull-down assay

The GST fusion plasmids were constructed by inserting DNA fractions encoding TLR4 domains (primers for TLR4 domains are provided in Table S2) into pGEX-4T1 bacterial expression vector. Rosetta (DE3) bacteria transfected with GST-tagged fusion constructs were induced by 0.5 mmol/L isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37°C, resuspended in lysis buffer, and sonicated on ice. The supernatant containing soluble GST-tagged fusion proteins was immobilized with glutathione-Sepharose 4B beads (GE Healthcare). After they were washed three times with binding buffer, the beads were washed with elution buffer to get the fusion proteins. The eluted proteins were visualized by Coomassie blue or immunoblot antibody using anti-GST antibody. To evaluate the binding between TLR4 domains and resistin, the purified GST fusion TLR4 domain proteins were added to U973 cell extracts. After incubation, glutathione-Sepharose 4B beads were adopted for precipitation. The beads were washed and immunoblotted with antiresistin antibody.

2.12 | Immunofluorescence assay

After treatment and incubation, the cells were washed with PBS three times, fixed with 4% paraformaldehyde, blocked with 1% BSA, and then incubated with primary antibody against NF-κB p65. After incubation overnight at 4°C, they were washed and incubated with Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Solarbio, Beijing, China). The cells were counterstained with DAPI (Beyotime) and then visualized under a Leica fluorescence microscope (Leica, Wetzlar, Germany).

2.13 | Statistical analysis

All data Are represented as mean ± SD of at least three independent experiments. Pearson's χ^2-test or Fisher's exact test were applied to analyze difference for qualitative variables. Student's t-test or one-way ANOVA was used for continuous variables. All tests were two-sided, and $P < 0.05$ was considered significant. PASW Statistics version 18.0 (IBM, Armonk, NY, USA) was used for data analysis.
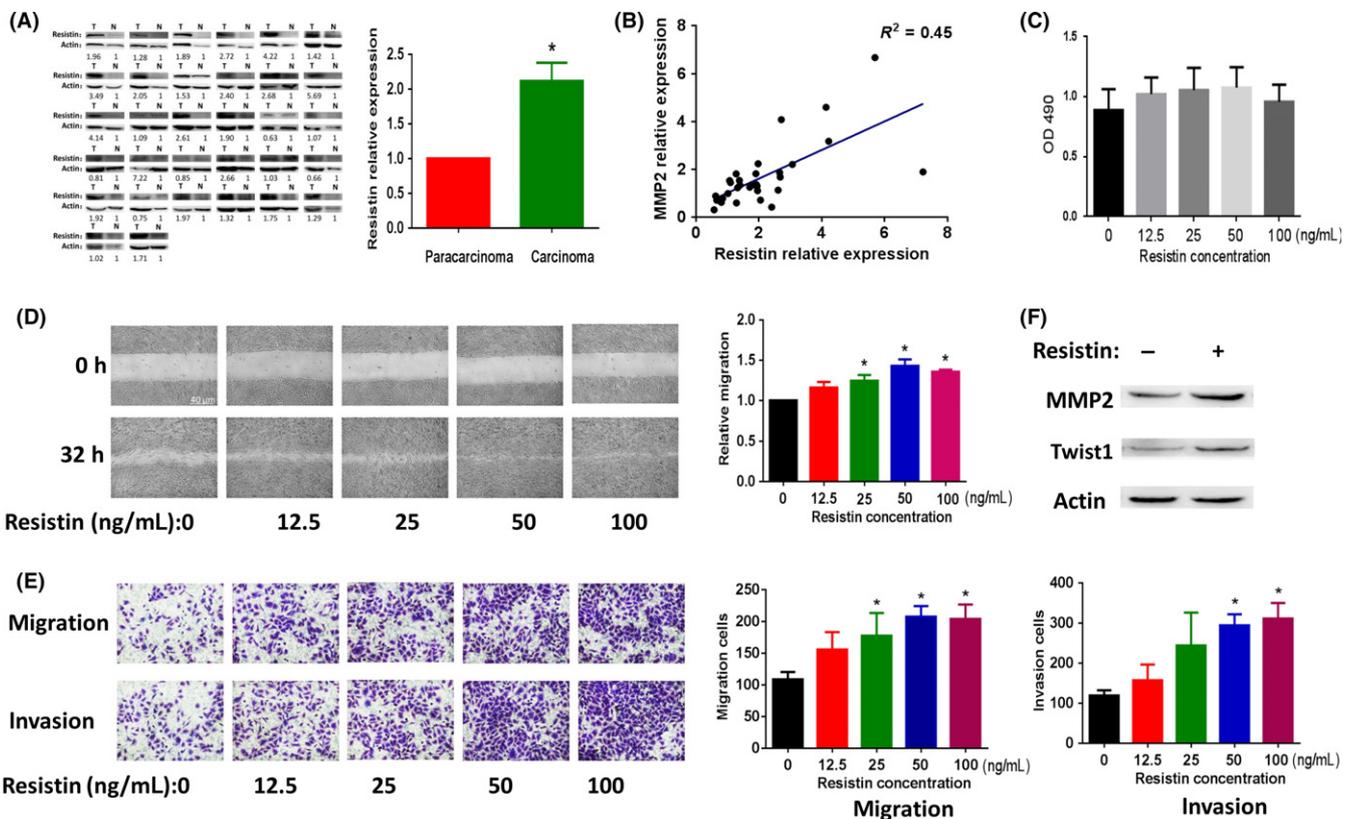
3 | RESULTS

3.1 | Human resistin was upregulated in lung adenocarcinoma tissues and promoted migration and invasion of lung adenocarcinoma cells

Resistin expression in 32 pairs of lung adenocarcinoma tissues and paracarcinoma normal lung tissues was detected by Western blot.

The results revealed a significant elevation of resistin expression in tumor tissues compared with paracarcinoma lung tissues ($P < 0.01$) (Fig. 1A). Moreover, the protein expression of MMP2 and resistin was positively correlated ($R^2 = 0.447$, $P < 0.01$) (Fig. 1B).

The role of resistin in the proliferation of lung adenocarcinoma cells was explored. The results showed that different concentrations of resistin did not significantly affect the proliferation of A549 cells in 48 h (Figs. 1C,S1a–c). However, it showed a trend of increasing proliferation in 48 h when the resistin concentration was 50 ng/mL. Resistin could enhance the proliferation of A549 cells from 6 days (Fig. S1d), which was also confirmed by colony formation assay (Fig. S1e).

The effect of resistin on the migration and invasion of lung adenocarcinoma cells was evaluated. Wound-healing assay revealed resistin could significantly increase the migration of A549 cells in a dose-dependent manner (Fig. 1D). Furthermore, Transwell assays showed that resistin increased the migration and invasion of A549

FIGURE 1. Human resistin is upregulated in lung adenocarcinoma tissues and promotes migration and invasion in A549 cells. (A) Resistin protein expression in lung adenocarcinoma tissues (T) and paracarcinoma tissues (N) was detected by Western blot. (B) Correlation between MMP2 protein expression and resistin protein expression in lung adenocarcinoma tissues. (C) A549 cells were seeded at 2000 cells/well in 96-well plates and treated with different concentrations of resistin (0, 12.5, 25, 50, or 100 ng/mL) for 48 h. Cell proliferation was detected by the MTS assay. OD, optical density. (D) A549 cells were seeded on a 6-well plate and incubated until confluence, then a cell-free space was created by scraping. Migration was induced by treatment with different concentrations of resistin (0, 12.5, 25, 50, or 100 ng/mL) for 32 h. (E) 1×10^4 A549 cells were seeded in the upper chamber in 100 µl serum-free medium with different concentrations of resistin (0, 12.5, 25, 50, 100 ng/ml), and 600 µl 10% FBS RPMI 1640 medium was added to the lower chamber. For invasion assay, the upper insert was pre-coated with growth factor-reduced Matrigel. After 12h incubation, migrated cells were fixed for Transwell migration assay while invaded cells were fixed after 24 h incubation for Transwell invasion assay. (F) A549 cells were treated with or without 50 ng/mL resistin for 24 h. Total protein was isolated and expression of MMP2 and Twist1 was detected by immunoblot assay. β-Actin was used as an internal control. Data represent mean ± SD. $n = 3$; *$P < 0.05$

cells in a dose-dependent manner (Fig. 1E). Resistin could also induce the migration and invasion of H1299 cells (Fig. S2). In A549 cells, resistin increased the protein expression of MMP2 and Twist1 (Fig. 1F), which are essential for cell motility and invasion.

3.2 | Toll-like receptor 4 is the functional receptor of human resistin for migration and invasion

The mechanism of how resistin induced the migration and invasion of lung adenocarcinoma cells was investigated. Resistin is a kind of cysteine-rich secretory protein that typically functions through receptor proteins. However, there is evidence that resistin plays diverse roles through distinct receptors in different cell types. Toll-like receptor 4 is speculated to be the potential receptor of resistin

in lung adenocarcinoma cells. Resistin did not significantly induce the migration or invasion of A549 cells when pretreated with TAK-242 (TLR4 inhibitor) (Fig. 2A). Resistin-induced mRNA and protein expression of MMP2 and Twist1 was inhibited by pretreatment with TAK-242 (Fig. 2B,C). Consistently, knockdown of TLR4 by siRNA transfection reversed resistin-induced migration and invasion (Fig. 2 D). Resistin could not induce the mRNA and protein expression of MMP2 or Twist1 with knockdown of TLR4 (Figs. 2E,S3). These results suggest that resistin might induce migration and invasion of A549 cells through TLR4.

However, resistin did not change the expression of TLR4 or its distribution in the membrane (Fig. 2F,G). Co-immunoprecipitation assay was used to explore the relationship between resistin and TLR4. Results revealed that resistin could bind to TLR4 in A549 cells (Figs. 2H,S4a). Next, the five domains of TLR4 were purified
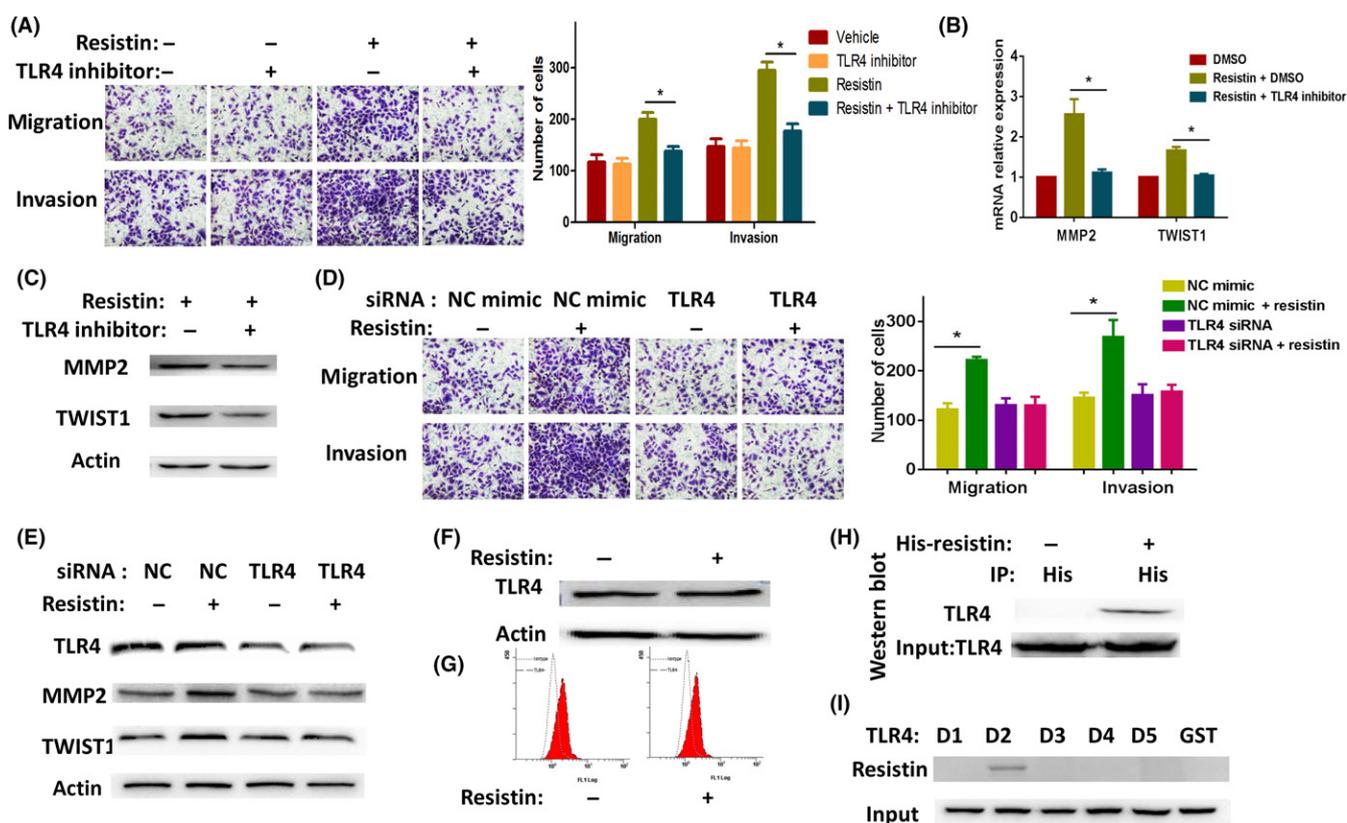
FIGURE 2. Toll-like receptor 4 (TLR4) is the functional receptor of human resistin in A549 lung adenocarcinoma cells for migration and invasion. (A) A549 cells (1×10^4) were seeded in the upper Transwell chamber in serum-free media and incubated with 50 ng/mL resistin after pretreatment with or without 5 μmol/L TAK-242 (TLR4 inhibitor). Migrated or invaded cells were photographed in at least five randomly chosen fields. A549 cells pretreated with or without 5 μmol/L TAK-242 for 1 h were treated with 50 ng/mL resistin for 24 h. The relative mRNA (B) and protein (C) expression of MMP2 and Twist1 was quantitated. (D) A549 cells were seeded in a 6-well plate and transfected with negative control (NC) mimic or TLR4 siRNA at 70% confluence. After 24 h of transfection, cells were seeded in the upper chamber (1×10^4 cells per well) treated with or without 50 ng/mL resistin. Migrated or invaded cells were counted. (E) A549 cells were seeded in a 6-well plate and transfected with NC mimic or TLR4 siRNA at 70% confluence. After 24 h of transfection, cells were treated with or without 50 ng/mL resistin. After 24 h, total protein was isolated and expression of MMP2, TLR4, and Twist1 was detected. (F) A549 cells were treated with or without 50 ng/mL resistin for 24 h. Total protein was isolated and expression of TLR4 was detected. (G) A549 cells were treated with or without 50 ng/mL resistin for 24 h. Expression of TLR4 in A549 membrane was detected by flow cytometry. (H) A549 cells were treated with human recombinant resistin with His-tag (500 ng/mL) for 1 h and then lysed and immunoprecipitated with agarose beads-conjugated anti-His mouse clonal antibody. IP, immunoprecipitation. (I) Five domains of TLR4 and GST were purified and incubated with U937 cell lysates. Immunoprecipitation assay was carried out. Data represent mean ± SD. $n = 3$; *$P < 0.05$

(Fig. S4b–d) and incubated with the lysates of U937 cells. The GST pull-down assay showed that only the second domain of TLR4 could bind to resistin (Fig. 2I). The results revealed that TLR4 might be the functional receptor of resistin in A549 cells for migration and invasion.
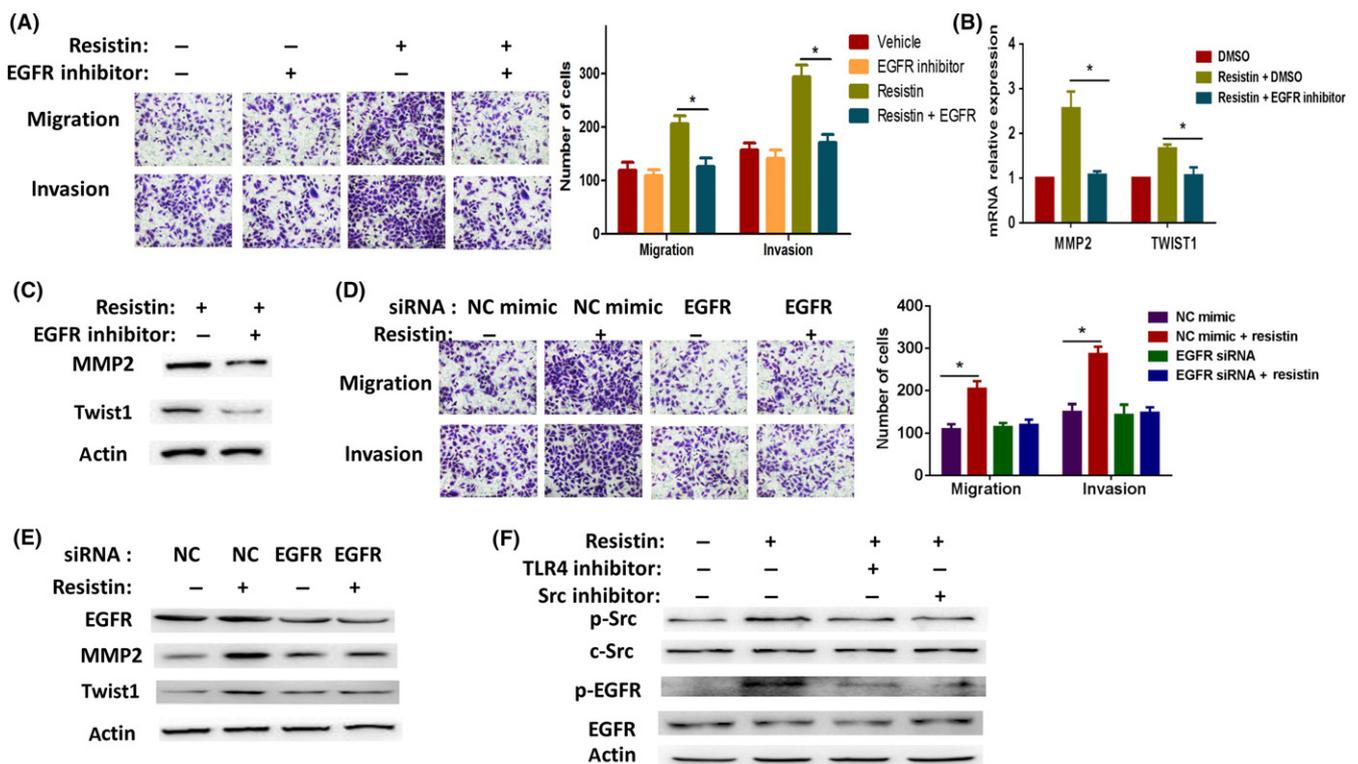
3.3 | Epidermal growth factor receptor involved in resistin-induced migration and invasion

It was reported that overexpression or aberrant activation of EGFR was associated with tumor progression, metastasis, and poor prognosis of lung cancer.[20] Lipopolysaccharide (LPS), a ligand of TLR4, could increase the phosphorylation of EGFR.[21] Epidermal growth factor receptor might be involved in resistin-induced migration and invasion. Pretreatment with erlotinib (EGFR inhibitor) could significantly decrease resistin-induced migration and invasion (Fig. 3A) and the mRNA and protein expression of MMP2 and Twist1 (Fig. 3B,C). Consistently, migration and invasion induced by resistin were inhibited by EGFR siRNA (Fig. 3D). Resistin could not increase either the mRNA or protein expression of MMP2 or Twist1 when EGFR was knocked down (Figs. 3E,S3b). Resistin could induce the phosphorylation of EGFR for 15 min. Resistin could also increase the phosphorylation of Src, but not when TLR4 was inhibited. Furthermore, pretreatment with TAK242 or PP1 (Src inhibitor) inhibited resistin-induced phosphorylation of EGFR (Fig. 3F). Therefore, resistin might increase the migration and invasion of A549 cells through TLR4/Src/EGFR.

3.4 | Intracellular downstream signals PI3K/Akt/NF-κB mediate resistin-induced migration and invasion

It was reported that p38 MAPK, ROS, PI3K/Akt, and NF-κB were downstream signals of EGFR involved in the migration and invasion of various cell types.[22–24] To evaluate the role of intracellular signaling pathways in resistin-mediated effects, the inhibitors specific for regulation of p38 MAPK, ROS, PI3K/Akt, and NF-κB were added to A549 cells 1 h prior to stimulation with resistin. Pretreatment with the inhibitors of PI3K or NF-κB markedly reduced resistin-induced migration and invasion (Fig. 4A). Correspondingly, the protein expression of MMP2 and Twist1 induced by resistin

FIGURE 3. Epidermal growth factor receptor (EGFR) is involved in resistin-induced migration and invasion of lung adenocarcinoma cells. (A) A549 cells (1×10^4) were seeded in the upper chamber in serum-free media and incubated with 50 ng/mL resistin after pretreatment with or without 1 μmol/L erlotinib HCl (EGFR inhibitor). Migrated or invaded cells were counted. (B,C) A549 cells pretreated with or without 1 μmol/L erlotinib HCl for 1 h were treated with 50 ng/mL resistin for 24 h. Relative mRNA (B) and protein (C) expression of MMP2 and Twist1 was quantitated. (D) A549 cells were seeded in a 6-well plate and transfected with negative control (NC) mimic or EGFR siRNA at 70% confluence. After 24 h of transfection, cells were seeded in the upper chamber (1×10^4 cells per well) treated with or without 50 ng/mL resistin. Migrated or invaded cells were counted. (E) A549 cells were seeded in a 6-well plate and transfected with NC mimic or EGFR siRNA at 70% confluence. After 24 h of transfection, cells were treated with or without 50 ng/mL resistin. After 24 h, total protein was isolated and expression of MMP2, EGFR, and Twist1 were detected. (F) A549 cells pretreated with or without 5 μmol/L TAK-242 and/or 10 μmol/L PP1 (Src inhibitor) for 1 h were treated with 50 ng/mL resistin for 15 min. Total protein was isolated and expression of phosphorylated (p-)Src, Src, p-EGFR, and EGFR was detected by Western blot
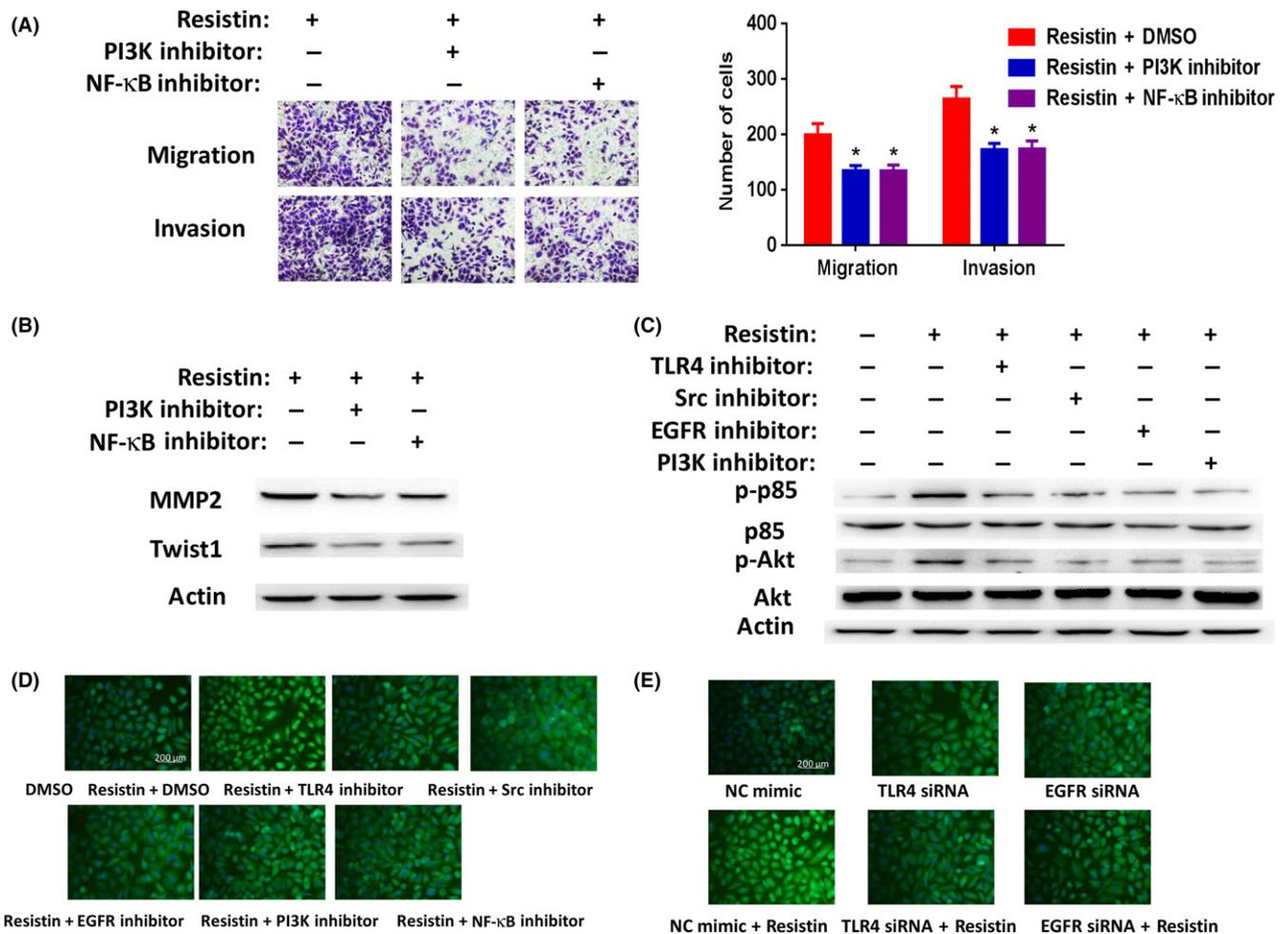
FIGURE 4. Phosphatidylinositol 3-kinase (PI3K)/Akt/nuclear factor-κB (NF-κB) are the downstream signals mediating resistin-induced migration and invasion of lung adenocarcinoma cells. (A) A549 cells (1×10^4) were seeded in the upper Transwell chamber in serum-free media and incubated with 50 ng/mL resistin after pretreatment with or without 10 μmol/L LY294002 (PI3K inhibitor) or 10 μmol/L BAY 11-7082 (NF-κB inhibitor). The migrated or invaded cells were counted. (B) A549 cells pretreated with or without 10 μmol/L LY294002 or 10 μmol/L BAY 11-7082 for 1 h were treated with 50 ng/mL resistin for 24 h. Total protein was isolated and expression of MMP2 and Twist1 was detected by Western blot. (C) A549 cells pretreated with or without 5 μmol/L TAK-242, 10 μmol/L PP1, 1 μmol/L erlotinib HCl, or 10 μmol/L LY294002 for 1 h were treated with 50 ng/mL resistin for 15 min. Total protein was isolated and expression of phosphorylated (p-) p85, p85, p-Akt, and Akt was detected by Western blot. (D) A549 cells pretreated with or without 5 μmol/L TAK-242, 10 μmol/L PP1, 1 μmol/L erlotinib HCl, 10 μmol/L LY294002, or 10 μmol/L BAY 11-7082 for 1 h were treated with 50 ng/mL resistin for 2 h. Cells were fixed and blocked and then incubated with primary antibody against p65 and Alexa Fluor 488-conjugated secondary antibody. Stained cells were photographed with a fluorescence microscope. (E) A549 cells were seeded in a 6-well plate and transfected with negative control mimic, Toll-like receptor 4 (TLR4) siRNA, or epidermal growth factor receptor (EGFR) siRNA at 70% confluence. After 24 h of transfection, cells were treated with or without 50 ng/mL resistin. After 24 h, cells were fixed and blocked and then incubated with primary antibody against p65 and Alexa Fluor 488-conjugated secondary antibody. Stained cells were photographed with a fluorescence microscope

was reduced by pretreatment with PI3K or NF-κB inhibitors (Fig. 4 B). However, neither p38 MAPK nor ROS inhibitors affected resistin-induced migration and invasion (Fig. S5a). Resistin increased the phosphorylation of PI3K p85 and Akt in 15 min. Pretreatment with TLR4, Src, or EGFR inhibitors decreased resistin-induced phosphorylation of PI3K p85 and Akt (Fig. 4C). Because NF-κB activation required nuclear translocation of the p65 subunit of NF-κB, we examined the distribution of the NF-κB p65 subunit by immunofluorescence assay. Resistin could increase the translocation of the NF-κB p65 subunit into the nucleus of A549 cells. However, pretreatment with the inhibitors of TLR4, Src, EGFR,

and PI3K prevented resistin-induced NF-κB nuclear translocation (Fig. 4D). When cells were transfected with TLR4 or EGFR siRNA, the NF-κB nuclear translocation was not induced by resistin (Fig. 4E).

4 | DISCUSSION

The tumor microenvironment is comprised of numbers of tumor cells, infiltrating leukocytes, macrophages, dendritic cells, and endothelial cells. Cytokines secreted by tumor-infiltrating leukocytes,

macrophages, and dendritic cells are essential molecules orchestrating cancer development and invasion.[25] Resistin is a type of cytokine predominantly produced by macrophages, dendritic cells, and monocytes in humans.[26] Here, we found resistin was highly expressed in lung adenocarcinoma tissues and positively correlated with MMP2 expression. Resistin could promote lung adenocarcinoma cell migration and invasion. Toll-like receptor 4, EGFR, PI3K, and NF-κB were key hubs for resistin-induced migration and invasion.

Resistin was originally identified in 2001 by a screening of adipocyte products that were downregulated by rosiglitazone in mice.[27] It might play distinct roles in mice and humans. Resistin was considered a potential link between insulin resistance and diabetes in mice,[27] which was not confirmed in clinical trials. Increasing evidence shows resistin might play an important role in cancer development. Circulating resistin levels were significantly higher in different types of cancer, including cancers of the breast, colorectum, and lung.[7] Our results showed high resistin expression was predominantly observed in lung adenocarcinoma tissues but not in the adjacent normal lung tissues. Resistin could only significantly increase the proliferation of lung adenocarcinoma cells in a relatively long time (6 days), but not in a relatively short time (48 h). These results implied that resistin could facilitate the proliferation of lung adenocarcinoma cells, but quite weakly. Resistin could increase the migration and invasion of lung adenocarcinoma cells, which was consistent with results in breast cancer and chondrosarcoma cells.[28,29]

Human resistin is a kind of cysteine-rich protein that is frequently involved in extracellular protein–protein interactions. It might function through specific receptors. However, recent studies showed resistin could potentially interact with different receptors depending on cellular models. An isoform of decorin (△DCN) was considered the resistin receptor on the surface of adipose progenitor cells and could mediate resistin-dependent proliferation and migration in 3T3-L1 cells.[30] Another study showed the mouse receptor tyrosine kinase-like orphan receptor (ROR1) was the receptor of resistin in 3T3-L1 cells and mediated the function of resistin in glucose uptake and adipogenesis.[31] Adenylyl cyclase-associated protein 1 (CAP1) was also identified as the receptor of resistin in human monocytes, and reportedly modulates inflammatory action induced by resistin.[32] However, both △DCN and ROR1 were only putative receptors for murine resistin, and both △DCN and CAP1 lacked the transmembrane domains. Toll-like receptor 4 is a putative receptor of resistin in the hypothalamus of mice, human myeloid, and epithelial cells.[33,34] It is highly expressed on the surface of human lung cancer cells.[35] Our results showed that when TLR4 activity was inhibited or TLR4 expression was decreased, resistin-induced migration and invasion were prevented, which indicated that TLR4 is involved in resistin-mediated migration and invasion. However, resistin did not affect the expression or distribution of TLR4 in A549 cells. Co-immunoprecipitation assay revealed that resistin could bind to TLR4 in A549 cells. Furthermore, the GST pull-down assay verified that TLR4 interacted with resistin through its own second domain, which suggested that the second domain was the core for the binding between resistin and TLR4. Therefore, TLR4 might be at least the functional receptor of resistin in A549 cells for migration and invasion.

Epidermal growth factor receptor is a receptor tyrosine kinase that is highly expressed in a variety of solid tumors, including lung cancer, and mediates cell migration and invasion.[36] Several studies found that EGFR was required for TLR4-mediated biological activities, such as LPS-induced endotoxicity.[21] Our results revealed when expression or activity of EGFR was inhibited, resistin-induced migration and invasion were prevented, which indicated that EGFR plays a vital role in resistin-induced migration and invasion. Resistin could increase the phosphorylation of EGFR and Src through TLR4. Inhibition of Src could prevent resistin-induced EGFR phosphorylation, so resistin might increase EGFR activity through TLR4/Src. Previous studies showed palmitic acid-induced EGFR activation mediated by TLR4/Src.[37] In addition, TLR4/Src-dependent EGFR signaling was involved in LPS-induced acute lung injury.[38] Heat-shock protein 90 contributed to the migration of glioblastoma cells through the TLR4/Src/EGFR pathway,[39] which is consistent with our study.

It is reported that several signal pathways, such as PI3K/Akt, p38 MAPK, and NF-κB, might be involved in EGFR-mediated effects. The inhibitors were pretreated to evaluate whether the pathways were involved in resistin-mediated migration and invasion. Both PI3K/Akt and NF-κB were found to be downstream signals of resistin-induced migration and invasion. Resistin could increase the production of ROS, which was vital in tumor metastasis.[40] Additionally, p38 MAPK was reported to be involved in migration and invasion.[41] However, our results showed that pretreatment with inhibitors of ROS or p38 MAPK did not affect resistin-induced migration or invasion. Resistin was reported to be an inflammatory marker that promoted the production of pro-inflammatory cytokines interleukin-6 and tumor
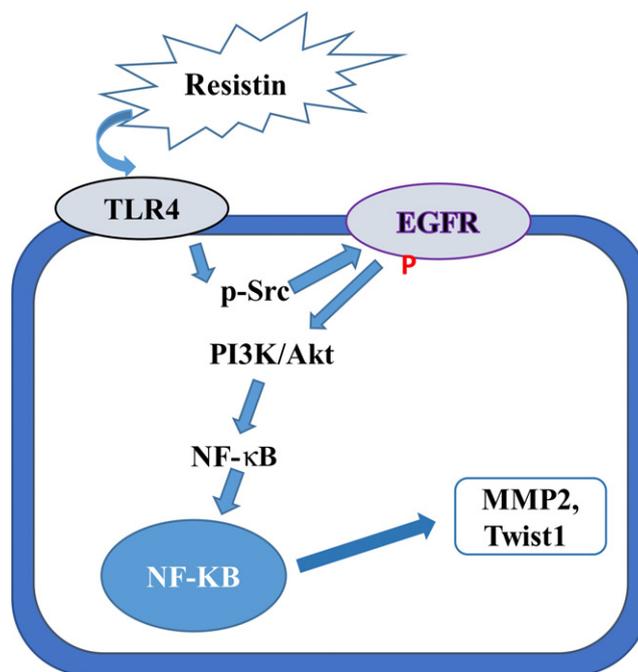
FIG. 5 Schematic illustration of the molecular mechanism underlying the migration and invasion induced by resistin in A549 lung adenocarcinoma cells. EGFR, epidermal growth factor receptor; NF-κB, nuclear factor-κB; TLR4, Toll-like receptor 4

necrosis factor-α, which could promote migration and invasion.[42] However, our results showed resistin did not significantly change the expression of interleukin-6 or tumor necrosis factor-α in A549 cells (Fig. S3c). Resistin increased the phosphorylation of PI3K p85 and Akt through the TLR4/Src/EGFR pathway. The nuclear translocation of NF-κB was repressed by the inhibitors of TLR4, Src, EGFR, and PI3K. The expression of MMP2 and Twist1 was regulated by NF-κB.[43,44] Therefore, we propose a model showing that resistin facilitates lung adenocarcinoma cells migration and invasion through the TLR4/Src/EGFR/PI3K/NF-κB pathway (Fig. 5).

It should not be neglected that the inhibitors or siRNA mimics of the TLR4/Src/EFGR/PI3K/NF-κB pathway might affect the proliferation of lung adenocarcinoma cells. We used relatively low concentrations of inhibitors or siRNA mimics, based on previous reports.[45–47] However, it is difficult to completely eliminate the influence of the inhibitors or siRNA mimics on cancer cell proliferation, which might have an impact on the results of migration and invasion. Therefore, the results should be interpreted cautiously.

In conclusion, resistin was highly expressed in lung adenocarcinoma tissues. It promoted lung adenocarcinoma cells migration and invasion through the TLR4/Src/EGFR/PI3K/NF-κB pathway.

DISCLOSURE STATEMENT

The authors have no conflict of interest.

ORCID

Wei Zhang http://orcid.org/0000-0002-6190-3129
Zhao-Qian Liu http://orcid.org/0000-0003-0428-3928

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SUPPORTING INFORMATION