

Interleukin-17A promotes esophageal adenocarcinoma cell invasiveness through ROS-dependent, NF- κ B-mediated MMP-2/9 activation

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Abstract. Interleukin-17A (IL-17A), a pro-inflammatory cytokine secreted primarily by Th17 cells, has been proved to be involved in the microenvironment of certain inflammation-related tumors. However, the role of IL-17A in cancer development has always been controversial. In this study, we investigated the effect of IL-17A on the regulation of esophageal adenocarcinoma (EAC) cell invasiveness and related molecular mechanism. Surface IL-17 receptor (IL-17R) expression on human EAC cell line OE19 was examined using flow cytometry. The effect of IL-17A on cell proliferation was measured by MTT assay. Cell migration and invasive ability *in vitro* were assessed by wound-healing and Matrigel-coated Transwell invasion assay. Intracellular reactive oxygen species (ROS) levels were determined by flow cytometry and fluorescence microscope. The protein expression levels of MMP-2, MMP-9, NF- κ B and p-I κ B- α were detected by western blotting. Our results showed that IL-17A promoted migration and invasion of OE19 cells in a dose-dependent manner, however it had less effect on OE19 cell proliferation. Furthermore, IL-17A treatment significantly upregulated the expression of MMP-2 and MMP-9, stimulated intracellular ROS production, increased I κ B- α phosphorylation and NF- κ B nuclear translocation. Nevertheless, IL-17A-induced expression of MMP-2/9 and OE19 cell invasiveness were both

inhibited by pretreatment with N-acetyl-L-cysteine (NAC, a ROS scavenger) or pyrrolidine dithiocarbamate (PDTC, a NF- κ B inhibitor). In conclusion, these findings demonstrate that IL-17A can promote the migration and invasiveness of EAC cells through ROS/NF- κ B/MMP-2/9 signaling pathway activation, indicating that IL-17A may be a potential therapeutic target for EAC.

Introduction

Esophageal cancer is the eighth most common malignant disease worldwide and also ranks as the sixth most common cause of cancer mortality (1). As one of the main histological types of esophageal cancer, the incidence rate of esophageal adenocarcinoma (EAC) was reported to be rapidly increasing among the past three decades (2). In comparison to esophageal squamous cell carcinoma (ESCC), EAC often shows less response to chemotherapy and radiotherapy, which leads to a relative poor prognosis of EAC patients. In addition, EAC is often diagnosed at advanced stages due to its early metastasis which is also considered as the most frequent cause of death in EAC patients (3). As a consequence, it has always been meaningful to explore the molecular mechanism underlying EAC metastasis and identify novel potent chemotherapeutic targets which may be applied to suppress cancer metastasis and in turn improve the prognosis.

The development of EAC is mostly associated with Barrett's esophagus, a precancerous lesion of esophagus, which is considered as a complication of gastroesophageal reflux disease (4). Accordingly, there has been increasing scientific evidence suggesting that chronic inflammation induced by duodenogastroesophageal refluxate plays a vital role in the pathogenesis and development of EAC (5,6). In the course of the carcinogenic process, the inflammatory cells and pro-inflammatory cytokines provide a proper microenvironment for tumor growth, invasion, and metastasis.

Interleukin-17A (IL-17A), a pro-inflammatory cytokine mainly secreted by Th17 cells, is proved to play an important role in many inflammatory and autoimmune diseases (7).

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Abbreviations: IL-17A, interleukin-17A; EAC, esophageal adenocarcinoma; ROS, reactive oxygen species; NAC, N-acetyl-L-cysteine; PDTC, pyrrolidine dithiocarbamate

Key words: interleukin-17A, esophageal adenocarcinoma, migration, invasiveness, reactive oxygen species, NF- κ B

Some evidence has revealed that IL-17A could cause an increase in generation of ROS, leading to a proinflammatory activation (8,9). Recent studies have shown that IL-17A or IL-17-producing cells were present in the microenvironment of certain inflammation-related tumors such as ovarian, prostatic and gastric cancer (10-12). However, the role of IL-17A in cancer development remains controversial. Some studies revealed that IL-17A promoted tumor growth through inducing IL-6 production, which in turn upregulated pro-survival and pro-angiogenic genes (13,14). Moreover, increased intratumoral IL-17-producing cells were found to be correlated with poor survival in hepatocellular carcinoma patients (15). On the contrary, some studies revealed that IL-17A could induce ESCC tumor cells to produce inflammatory chemokines, which are connected with the migration of T cells, NK cells, DCs, and B cells (16,17). Clinical evidence also proved that tumor infiltrating IL-17A-producing cells correlated with better overall survival of ESCC patients and might serve as a potential prognostic marker for ESCC (18,19).

To date, little is known about the role of IL-17A in EAC development. Therefore, the goal of the present study was to investigate whether IL-17A is involved in the regulation of EAC invasiveness, and if so, to explore the effect of ROS/NF- κ B/MMP-2/9 signaling pathway underlying IL-17A-regulated EAC invasiveness.

Materials and methods

Cell line and cell culture. The human esophagus adenocarcinoma cell line OE19 was a gift from the Gastroenterology Department of Southwest Hospital of Third Military Medical University. OE19 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C, 5% CO₂ air atmosphere.

Flow cytometry. Surface IL-17 receptor (IL-17R) expression on OE19 cells was examined using flow cytometry as described previously (20). In brief, OE19 cells were washed three times, harvested, and resuspended in cold PBS. Then the cells were incubated with PE-labeled mouse anti-human IL-17RA antibody (eBioscience, CA, USA) in the dark for 1 h at 4°C. Isotype-matched antibody was used as a negative control. After washing, at least 1x10⁵ stained cells were analyzed using flow cytometry (BD Biosciences). Fluorescence-positive cells were quantified.

Cell proliferation assay. The effect of IL-17A on OE19 cell proliferation was measured by MTT assay. The cells were seeded in a 96-well plate at a density of 5,000 monolayer cells per well. After 24 h, the cells were incubated with IL-17A (rhIL-17A, R&D System, Minneapolis, MN, USA) in increasing concentrations (0, 1, 5, 10, 50 and 100 ng/ml) for 24, 48 and 72 h, respectively. Subsequently, the cells were washed with PBS and incubated with 20 μ l MTT solution (5 g/l) for 4 h. After that, 150 μ l DMSO was added to each well to dissolve the crystals and then the plates were shaken for 10 min in the dark. Finally, the optical density (OD) was measured at 490 nm using multifunctional fluorescence microplate reader (Polarstar Otima, BMG, Australia).

Cell migration assay. Cell migration ability *in vitro* was assessed by wound-healing assay. OE19 cells were seeded in 12-well flat bottomed plates and grown to ~80-90% monolayer confluence. A sterile pipette tip was used to carefully scratch a straight strip in the center of the well. After washing the cell debris, a wide range of doses of IL-17A (0, 1, 10 and 100 ng/ml) was added to cells. After 24 h incubation, the wound edges of different treatment groups were observed and captured. The migration rate was expressed as the percentage of average migration distance to average starting (0 h) wound distance, respectively.

Cell invasion assay. The *in vitro* invasive assay was performed with 24-well Transwell (8- μ m pore size; Corning, NY, USA) precoated with Matrigel (BD Biosciences, San Jose, CA, USA). Cells (2x10⁵), suspended in 200 μ l serum-free medium in the absence or presence of IL-17A (1, 10 and 100 ng/ml), were seeded into the upper chamber, and 500 μ l medium supplemented with 10% FBS was placed into the lower chamber. After 24 h incubation, the cells on the upper surface of the membrane were removed by cotton swabs. Subsequently, the cells on the lower surface were fixed with paraformaldehyde, and stained with crystal violet. Finally, invasive cells in five microscopic fields were counted and captured.

Measurement of intracellular ROS levels. Intracellular ROS levels were measured by oxidation-sensitive fluorescent dye DCFH-DA. OE19 cells were preconditioned with NAC (5 mM, Beyotime, Shanghai, China) or vehicle for 2 h, then stimulated with IL-17A (100 ng/ml) for another 6 h. After treatment, cells were washed twice and incubated in 10 μ M DCFH-DA (Invitrogen, Carlsbad, CA, USA) for 30 min. Then the cells were washed three times and collected for further analysis. Fluorescence was detected by flow cytometry and fluorescence microscopy, respectively.

Western blot analysis. OE19 cells grown in 6-well plates were pretreated with NAC or PDTC (100 μ M, Sigma-Aldrich, St. Louis, MO, USA) for 2 h, then stimulated with IL-17A (100 ng/ml) for 6 h (for the measurement of NF- κ B p65, p50, p-I κ B- α), or 24 h (for the measurement of MMP-2/9). After incubation, whole cell proteins were extracted using cell lysis buffer. Nuclear lysates were harvested using NucBuster™ Protein Extraction kit (Novagen, Darmstadt, Germany) according to the manufacturer's instructions. Proteins were boiled for 5 min, separated by 8 or 10% SDS-PAGE, transferred to PVDF membranes, and incubated overnight at 4°C with primary antibodies against NF- κ B p65 (1:1,000, Millipore), NF- κ B p50 (1:1,000, Millipore), p-I κ B- α (1:500, Cell Signaling Technology), MMP-2 and MMP-9 (1:1,000, Abcam), then followed by a secondary antibody for 2 h at room temperature. Histone H1 and GAPDH (1:2,000, Santa Cruz Biotechnology) were used as loading control.

Statistical analysis. All data are represented as mean \pm SD from at least three independent experiments performed in triplicate. One-way ANOVA and Student's t-test were conducted to analyze the difference between groups using SPSS 17.0 software. P<0.05 was considered statistically significant.

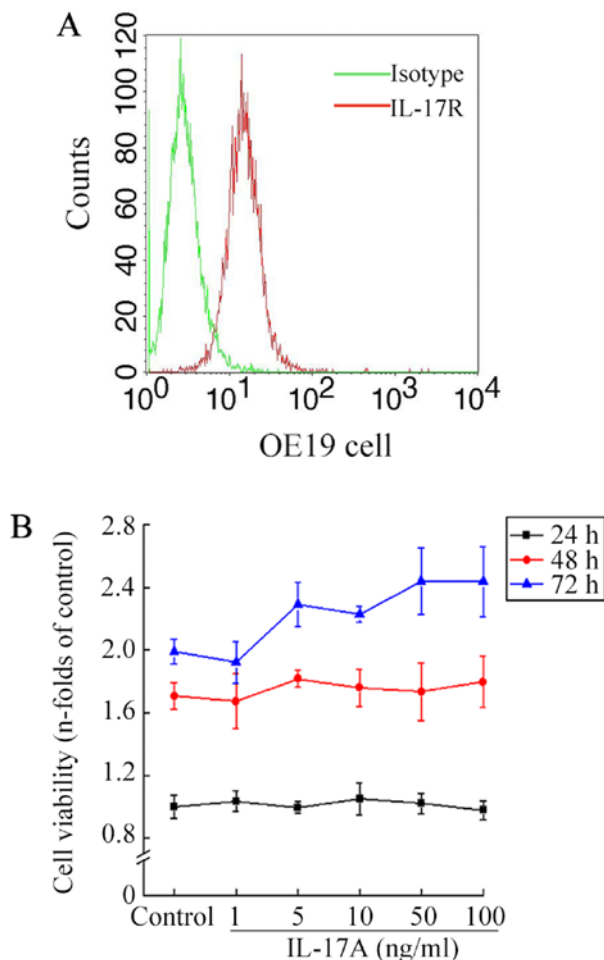


Figure 1. IL-17R expression on OE19 cells and the effect of IL-17A on cell proliferation. (A) OE19 cells were incubated with PE-labeled mouse anti-human IL-17RA antibody (red line) or isotype control antibody (green line). The expression of IL-17R on the surface of OE19 cells was easily detectable by flow cytometry. (B) A wide range of concentrations of IL-17A had no direct effect on cell proliferation *in vitro* for 24, 48 and 72 h, respectively (all $P > 0.05$). Data are presented as mean \pm SD from at least three independent experiments performed in triplicate.

Results

IL-17A has no direct effect on OE19 cell proliferation. In order to assess the biological effects of IL-17A on OE19 cells, firstly we examined the expression of IL-17R on OE19 cells. Flow cytometry confirmed that the cells expressed IL-17RA on the surface at the protein level (Fig. 1A). This result is in accordance with the ubiquitous expression of IL-17R (21). Subsequently, MTT assay was used to test the possibility that IL-17A might modulate OE19 cell proliferation *in vitro*. Cells were treated with IL-17A at concentrations ranging from 1 to 100 ng/ml. The result showed that the cell viability did not differ significantly among groups with different IL-17A concentrations at the exposure times of 24, 48 or 72 h, respectively (all $P > 0.05$, Fig. 1B).

IL-17A promotes OE19 cell migration and invasion as well as upregulates MMP-2 and MMP-9 expression. To determine whether IL-17A could promote OE19 cell migration and invasion, wound healing and Matrigel-coated Transwell invasion

assay were performed. The wound healing assay showed that the migration rate of OE19 cells reached $\sim 26\%$ in the absence of IL-17A, however, IL-17A treatment remarkably promoted cell migration rate in a dose-dependent manner (Fig. 2A and B). The following Transwell invasion assay indicated that the number of invasive cells was significantly higher in IL-17A-treated group than the control group, and similarly the number markedly increased along with the increasing concentrations of IL-17A (Fig. 2C and D).

MMPs are known to play crucial roles in cancer metastasis by degrading the extra cellular matrix (22), we next investigated the effect of IL-17A on MMP-2 and MMP-9 expression in OE19 cells. Western blotting showed that the protein levels of MMP-2 and MMP-9 were significantly upregulated in IL-17A-treated cells compared with the untreated control, respectively (Fig. 2E and F). These results suggested that IL-17A promoted the migration and invasion of OE19 cells possibly by upregulating expression of MMP-2 and MMP-9.

IL-17A increases intracellular ROS levels. There is accumulating evidence that ROS perform an important function in tumor development (23,24), and IL-17A was also reported to cause an increase in intracellular ROS levels (8,9), so we next examined the effect of IL-17A on ROS production in OE19 cells. Fluorescence microscopy showed that IL-17A at the concentration of 100 ng/ml caused ~ 2 -fold increase in intracellular ROS levels (Fig. 3A and C). The promoting effect of IL-17A on ROS production was further confirmed by flow cytometry (Fig. 3B), whereas it was strikingly attenuated by the pretreatment with NAC (Fig. 3).

IL-17A activates NF- κ B in a ROS-dependent manner. It has been well-documented that NF- κ B is a major transcription factor that mediates migration and invasion of cancer cells, which is also downstream of oxidative stress (25). Therefore, we next detected whether IL-17A could induce NF- κ B activation in a ROS-dependent manner, which in turn promotes cell invasion. As shown in Fig. 4A and C, the protein levels of both p65 and p50 in the whole cell did not differ between groups with or without IL-17A, however, they were remarkably elevated in the nucleus in IL-17A-treated cells compared with the untreated cells (Fig. 4B and D). The nuclear/overall ratio of p65 and p50 were also increased by IL-17A (Fig. 4E). Furthermore, the protein level of p-I κ B- α was markedly upregulated in IL-17A-treated cells (Fig. 4A and C). Whereas, pretreatment with NAC or PDTC significantly reversed these changes, which indicated that IL-17A could induce NF- κ B activation in a ROS-dependent manner.

IL-17A promotes cell invasiveness through ROS/NF- κ B/MMP-2/9 signaling pathway. To address the effect of ROS/NF- κ B signaling pathway on IL-17A-induced MMP-2/9 expression and cell invasion, OE19 cells were pretreated with NAC or PDTC for 2 h and then incubated with IL-17A (100 ng/ml) for 24 h. Western blotting showed that the effect of IL-17A on the upregulation of MMP-2/9 expression was both significantly inhibited (Fig. 5A and B). Furthermore, Transwell invasion assay indicated that IL-17A-induced cell invasiveness was also markedly attenuated by NAC or PDTC *in vitro* (Fig. 5C and D).

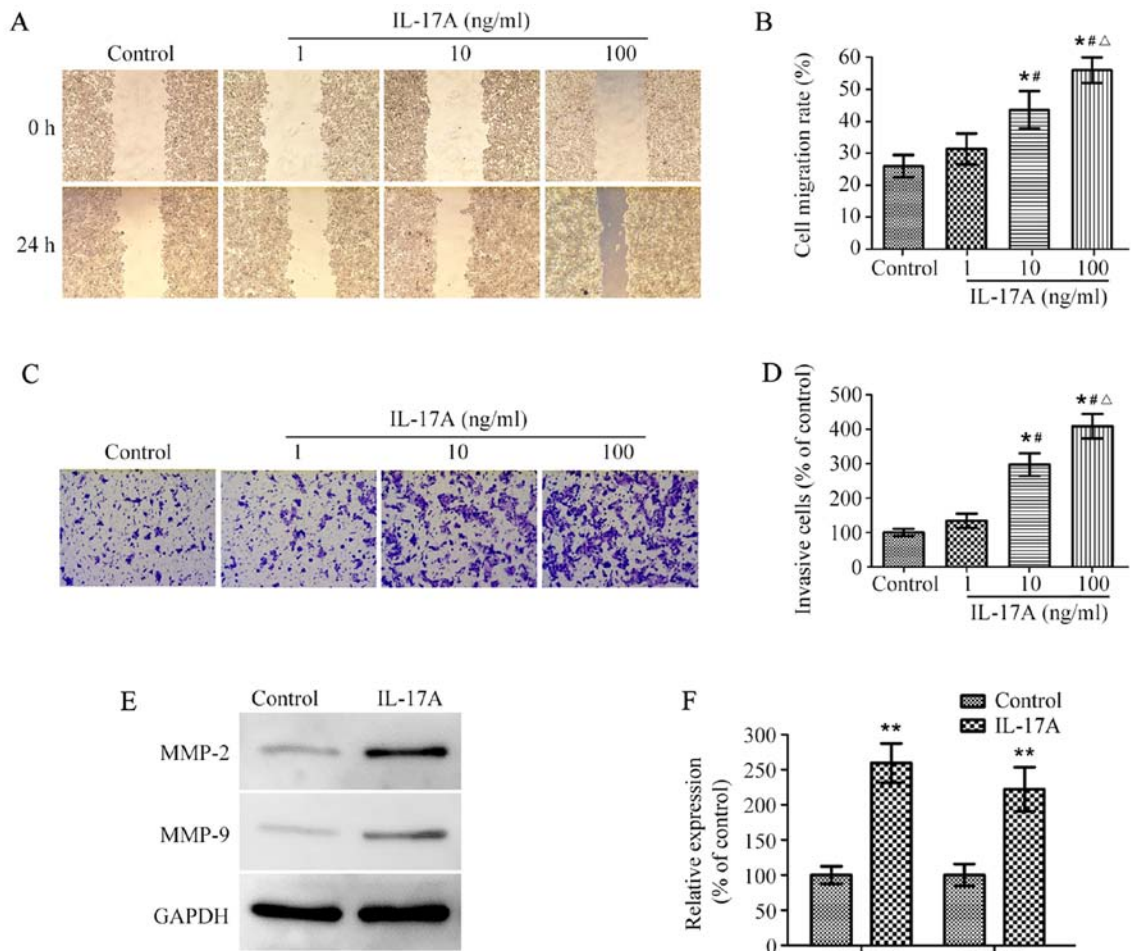


Figure 2. IL-17A promotes OE19 cell migration and invasion as well as upregulates MMP-2/9 expression. (A) Wound-healing assay showed that the cell migration ability was significantly enhanced in a dose-dependent manner after IL-17A treatment for 24 h. (B) The migration rate was quantified as a percentage of average migration distance to average wound distance. (C) Matrigel-coated Transwell invasion assay revealed that the number of invasive cells was remarkably increased in a dose-dependent manner after IL-17A treatment for 24 h. (D) The relative number of invasive cells was counted and expressed as a percentage of control. (E) Western blotting was used to detect the protein expression levels of MMP-2/9 in OE19 cells treated with or without IL-17A (100 ng/ml) for 24 h. (F) The relative protein levels were quantified and expressed as a ratio to control. Data are presented as mean \pm SD from at least three independent experiments performed in triplicate. * P <0.05 and ** P <0.01 compared with the control group; $^{\Delta}P$ <0.05 compared with 1 ng/ml IL-17A group; $^{\Delta}P$ <0.05 compared with 10 ng/ml IL-17A group.

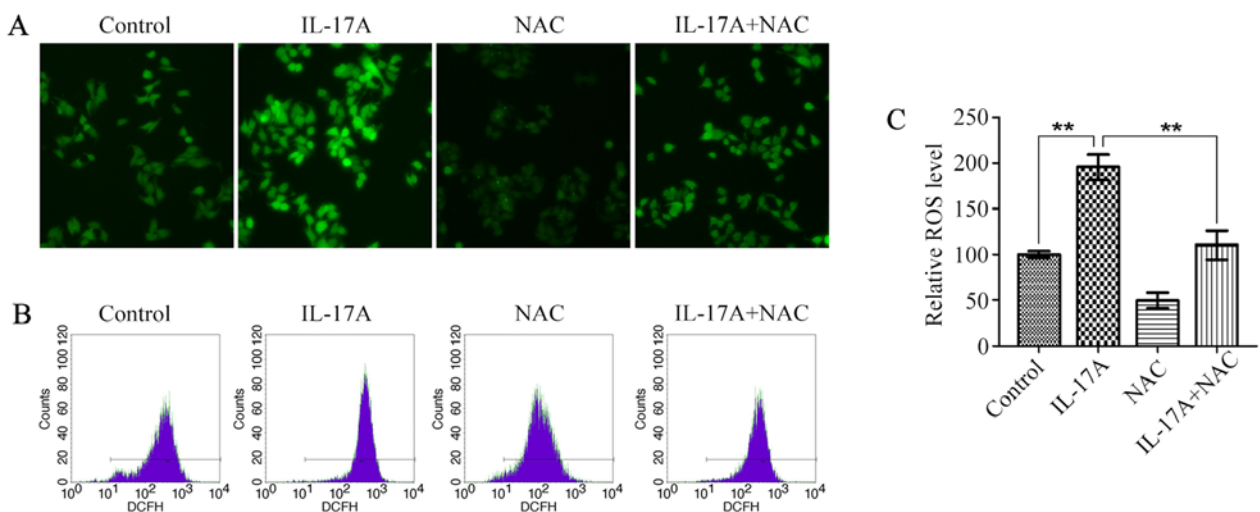


Figure 3. IL-17A increases intracellular ROS levels. (A) Representative images of OE19 cells showed the intracellular ROS production which was labeled by a fluorescence probe and detected using a fluorescence microscope. (B) Flow cytometry was performed to further assess the intracellular ROS levels. (C) The relative ROS levels were quantified and expressed as percentage of control. Data are presented as mean \pm SD from at least three independent experiments performed in triplicate. ** P <0.01.

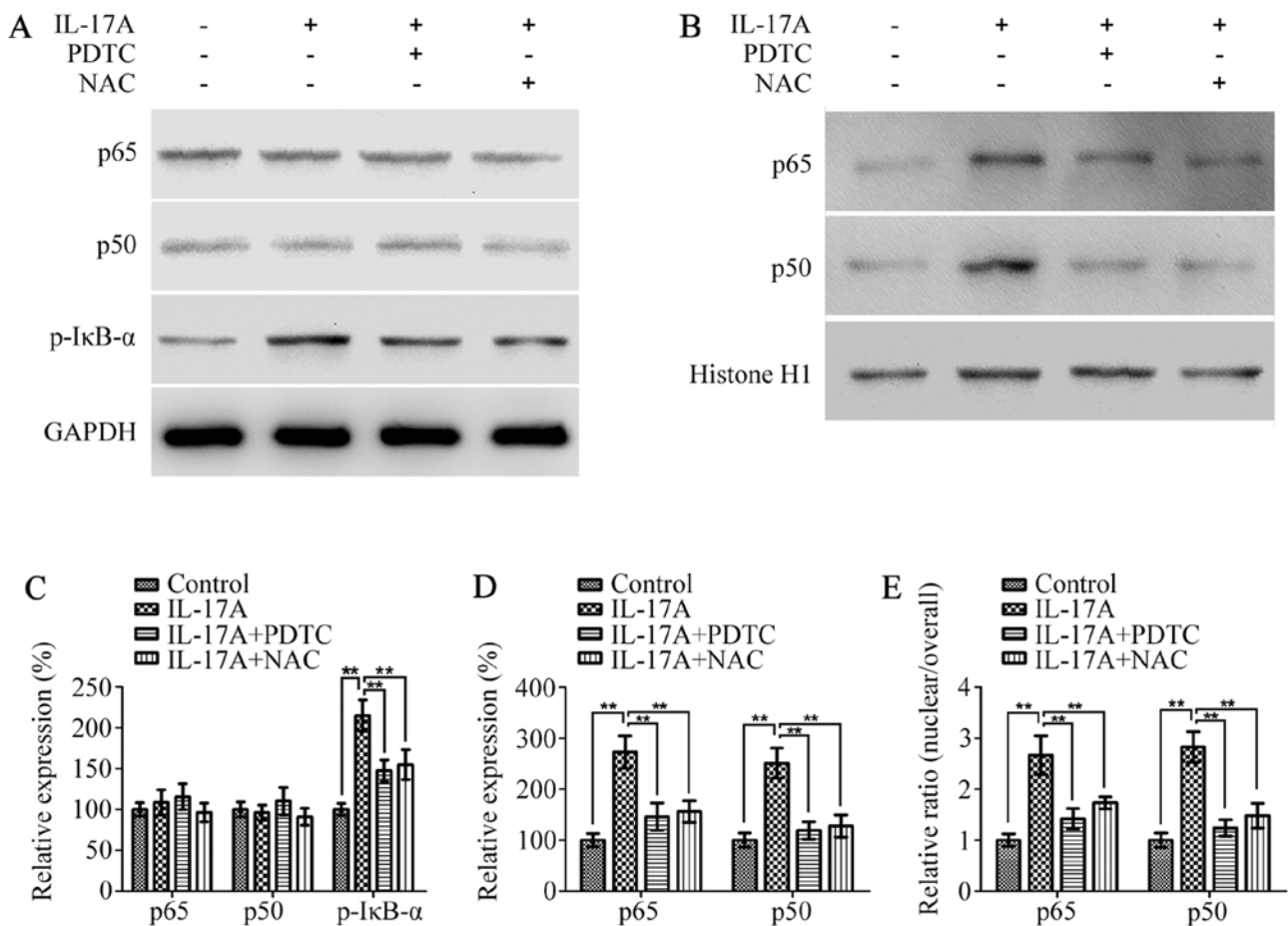


Figure 4. IL-17A induces NF- κ B activation in a ROS-dependent manner. Western blotting was used to detect the protein expression levels of overall p65, p50 and p-I κ B- α (A) and nuclear p65 and p50 (B) in IL-17A-treated OE19 cells with or without pretreatment of PDTC or NAC. (C and D) The relative protein levels were quantified and expressed as percentage of control. (E) Nuclear translocation of p65 or p50 was expressed as the relative ratio of nuclear to overall p65 or p50. Data are presented as mean \pm SD from at least three independent experiments performed in triplicate. ** P <0.01.

Discussion

It has been well documented that chronic inflammation plays a pivotal role in the pathogenesis and development of malignant diseases, however, the role of IL-17A in cancer is currently under debate. In this study, we aimed to address whether IL-17A is involved in the regulation of EAC metastasis, and the results suggested that IL-17A could promote EAC cell invasiveness through ROS-dependent, NF- κ B-mediated MMP-2 and MMP-9 activation.

Since proinflammatory cytokine was reported to be closely linked with the inflammation-intestinal metaplasia-dysplasia-adenocarcinoma sequence in the lower esophagus, our research group has been engaged in clarifying the role of proinflammatory cytokine in the pathogenesis and development of EAC (26,27). Growing scientific evidence showed that IL-17A is a vital inflammatory cytokine in many inflammatory diseases, and Th17 cells increased in a variety of human malignant diseases (10,12). Previous study suggested that high levels of intratumoral IL-17A-producing cells were correlated with overall survival and disease-free survival in hepatocellular cancer patients (15). Also, higher proportion of circulating Th17 cells was detected in patients with advanced esophageal cancer, and the proportion of Th17 cells in patients

with lymph node metastasis was higher than those with non-lymph node metastasis (28). Whereas, further studies revealed that IL-17A probably enhanced the tumor killing capability via stimulating expression of Granzyme B and FasL (17), and also induced ESCC tumor cells to produce more chemokines, which subsequently promote the migration of T cells, NK cells, and dendritic cells (16). However, the role of IL-17A in EAC development is still unknown, and the related molecular mechanisms remain to be elucidated. In this study, we found that IL-17A had less effect on EAC cell proliferation, whereas wound-healing and Transwell invasion assays showed that IL-17A significantly promoted the migration and invasion of EAC cells. These results indicated that IL-17A had no biological action on promoting or suppressing EAC cell growth, however it could enhance cell motility *in vitro*.

Cancer cell metastasis relies on the degradation of the extra cellular matrix, which is mainly catalyzed by MMPs (29). Thus, we subsequently investigated whether MMPs were involved in IL-17A-induced EAC cell invasion. Our study revealed that IL-17A treatment could markedly increase the protein levels of both MMP-2 and MMP-9, indicating that upregulation of MMP-2 and MMP-9 expression might be responsible for this pro-invasive behavior. Similarly, previous study also demonstrated that IL-17A increased cell motility

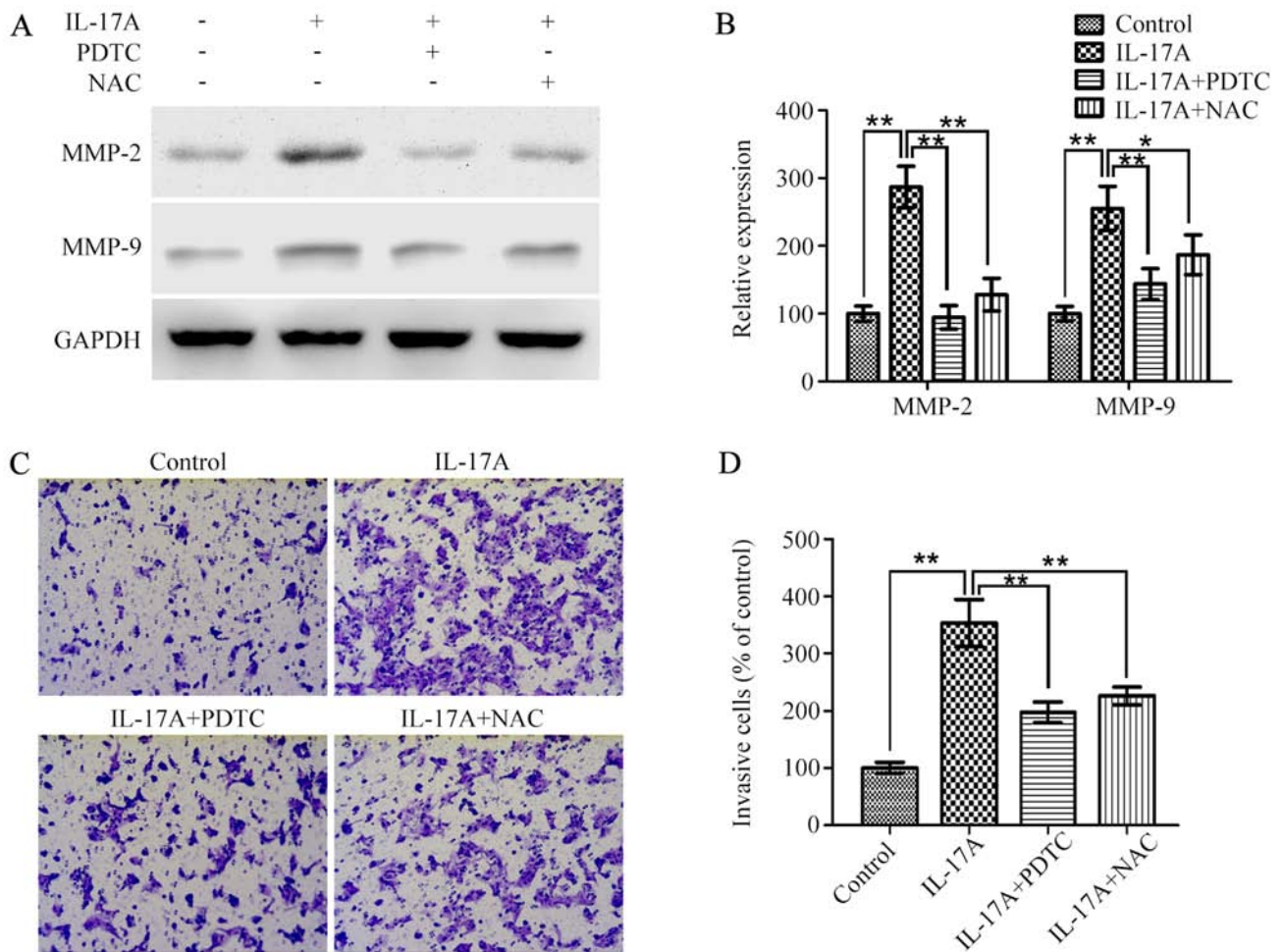


Figure 5. Blockage of ROS/NF- κ B signaling pathway inhibits IL-17A-induced MMP-2/9 expression and OE19 cell invasion. (A) Western blotting was used to analyze the protein expression of MMP-2/9 in IL-17A-treated OE19 cells with or without pretreatment of PDTC or NAC. (B) The relative protein levels were quantified and expressed as percentage of control. (C) Matrigel-coated Transwell invasion assay was performed to detect cell invasiveness in IL-17A-treated OE19 cells with or without pretreatment of PDTC or NAC. (D) The relative number of invasive cells was counted and expressed as percentage of control. Data are presented as mean \pm SD from at least three independent experiments performed in triplicate. * P <0.05; ** P <0.01.

in lung cancer by activating MMPs (30). However, it differs between the mechanisms underlying IL-17A-induced expression of MMPs.

Compelling evidence has been found to show that ROS and NF- κ B are downstream of IL-17A (31,32) and perform an important function in proinflammatory signaling and EAC development (33,34). Therefore, we next investigated whether the pro-invasion effect of IL-17A was through activating ROS/NF- κ B signaling pathway. In our study, the intracellular ROS levels, I κ B- α phosphorylation as well as NF- κ B nuclear translocation were all enhanced in IL-17A-treated OE19 cells. ROS scavenger could remarkably inhibit IL-17A-induced NF- κ B activation, which further demonstrated that the activation of NF- κ B was ROS-dependent. These findings are supported by studies of others describing oxidative stress as an important regulator of NF- κ B activation (35,36). Furthermore, the following experiments showed that ROS-scavenging and NF- κ B inhibition both remarkably diminished the promoting effect of IL-17A on MMP-2/9 expression as well as cell invasiveness. Taken together, these results suggested that IL-17A could activate ROS/NF- κ B signaling pathway, subsequently upregulate MMP-2/9 expression and thereby promote OE19

cell invasiveness. As previously reported, IL-17A was proved to promote gastric and colorectal cancer invasiveness via NF- κ B-mediated MMP expression (37,38). In addition, IL-17A also induced the migration and invasion of cervical cancer cells by activating the p38/NF- κ B signaling pathway (39). Our results are consistent with these studies, which imply that IL-17A might play a crucial role in tumor migration and invasion.

In conclusion, this study provided evidence that IL-17A could promote the migration and invasion of EAC cells. Moreover, we revealed the molecular mechanism that IL-17A induced EAC cell invasiveness through ROS-dependent, NF- κ B-mediated MMP-2/9 activation. Considering that tumor metastasis is often associated with poor prognosis and high mortality among EAC patients, our findings may contribute a new molecular target for EAC therapy.

Acknowledgements

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