

Original Article

Cisplatin inhibits the growth, migration and invasion of cervical cancer cells by down-regulating IL-17E/IL-17RB

Jiao Cheng^{1,2}, Chun-Jie Gu^{1,2}, Bing Zhang¹, Feng Xie², Min-Min Yuan², Ming-Qing Li^{2,3}, Jin-Jin Yu¹

¹Department of Obstetrics and Gynecology, The Affiliated Hospital of Jiangnan University, Wuxi 214062, Jiangsu Province, People's Republic of China; ²Laboratory for Reproductive Immunology, Hospital of Obstetrics and Gynecology, Fudan University, Shanghai 200011, People's Republic of China; ³Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Shanghai 200011, People's Republic of China

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Abstract: Interleukin (IL)-17E mainly produced by immune cells, is a distinct member of the IL-17 cytokine family, which has multifarious immunomodulatory activities. As a potent anticancer drug, cisplatin is commonly used against various types of solid tumors. The present study was performed to investigate whether cisplatin regulates the expression of IL-17E and its receptor IL-17RB, and the role of IL-17E in cervical cancer cells *in vitro*. The expression of IL-17E and IL-17RB in cervical cancer cells was detected by flow cytometry and ELISA. The viability, apoptosis, migration and invasion of cervical cancer cells were analyzed by CCK8, Annexin V-7AAD apoptosis, transwell migration, wound healing, and matrigel invasion assays. Here, we found that cervical cancer cells co-expressed IL-17E and IL-17RB, especially HeLa and SiHa cells. Recombinant human IL-17E protein (rhIL-17E) enhanced the viability, migration and invasion of HeLa and SiHa cells, and blocking IL-17E with anti-human IL-17E neutralizing antibody promoted the apoptosis of HeLa and SiHa cells. Cisplatin significantly down-regulated the expression of IL-17E and IL-17RB, and further reversed the regulatory effects of rhIL-17E on viability, apoptosis, migration and invasion of HeLa and SiHa cells. The results suggest that cisplatin inhibits the viability, migration, invasion, and promotes the apoptosis of cervical cancer cells possibly by down-regulating IL-17E/17RB signaling. Cisplatin may be the first choice for cervical cancer patients with abnormally high IL-17E expression.

Keywords: IL-17E, IL-17RB, cervical cancer cells, cisplatin, viability, invasion

Introduction

Cervical cancer acts as the second most common tumor among women all over the world [1, 2], which poses a great threat to the body and health of women accounting for 10-15% of cancer-related mortalities. Accumulating evidences reveal that human papillomavirus (HPV) plays a critical effect in the induction and development of human cervical cancer [3]. While some people infected with HPV has not been confirmed as cervical cancer, indicating that more potent factors are involved in the pathogenesis of cervical cancer [4, 5]. Recent studies find that immune cells and their secreted cytokines not only eliminate cancer cells, but also provide a special microenvironment for tumor development as well as promote tumor progression [6].

The production of IL-17 was associated with persistent HPV16/18 infection and cervical epi-

thelial neoplasia and transformation. Blocking IL-17 in persistent viral infection may promote antiviral immunity and prevent progression to cancer [7]. This triggered our curiosity to go on a profound knowledge of the role of IL-17E in cervical cancer.

IL-17E, also known as IL-25, is a novel member of IL-17 family [8]. Most of IL-17 family members have pro-inflammatory function, while IL-17E has a unique role towards progression of type 2 immune responses [9]. It is mainly produced by Th2 cells, eosinophils, basophils, macrophages, mast cells, epithelial cells and endothelial cells [9-11]. The receptor for IL-17E is consisted of the heterodimer complex IL-17RA and IL-17RB. The biological effects of IL-17E are reported to be mediated through IL-17RB (also known as IL-17BR or IL-17Rh1 or Evi27), a member of the IL-17R family of cytokine receptors, because IL-17E has a high affinity to IL-17RB and activates signaling pathways through IL-17RB [10].

Compelling researches demonstrated that IL-17E share some similarities with IL-17A in terms of activating NF- κ B and induce the production of the pro-inflammatory chemokine IL-6 and IL-8 [12-15]. IL-17E also play an important role in different inflammatory conditions, IL-17E directly stimulates epithelial cells to produce pro-inflammatory cytokines that contribute to colitis [16].

However, mounting evidences indicated that persistent inflammation would be a driving force in the journey to cancer. As the component of tumor microenvironment, pro-inflammatory mediators including cytokines and chemokines participate in a complex inflammatory signaling that facilitates extravasation of tumor cells through the stroma, thereby promotes tumor promotion and progression [5]. Mombell *et al.*, have reported that IL-17E indirectly enhances tumor progression by recruiting the c-RAF/S6 kinase pathway and the generation of pro-oncogenic LMW-E in breast cancer [17]. However, Benatar *et al.*, have demonstrated that IL-17E has anti-tumor efficacy in several cancer including breast cancer [18-20] and pancreatic cancer [21]. Therefore, IL-17E may act as a double-edged sword with anti-tumor immunity and tumorigenesis [22, 23]. However, little is known about the role of IL-17E in cervical cancer.

Cis-diammine-dichloro-platinum (cisplatin, DDP) is commonly used against various types of solid tumors, including ovarian, testicular, head, breast and uterine cervical carcinoma cancers [24]. It has reported that cisplatin can regulate the level of several cytokines, for example, CCL5 induced by cisplatin is involved in the cisplatin-resistance in ovarian cancer [25]. Therefore, in the present study, we investigated the influence of IL-17E on the viability, apoptosis, migration, and invasion of HeLa and SiHa cells and the regulatory effect of cisplatin on IL-17E/IL-17RB *in vitro*.

Materials and methods

Cell culture

The cervical cancer cell lines (HeLa, SiHa, C33A and CasKi) were obtained from the cell bank of Chinese Academy of Science (Shanghai, China) and grown in RPMI-1640 medium (Hyclone, Logan, UT, USA) supplemented with 1% antibiotic-antimycotic and 10% fetal bovine serum

(FBS; Hyclone). These cells were incubated in a humidified atmosphere which maintained at 37°C in 5% CO₂.

The detection of the expression of IL-17E/IL-17RB by flow cytometry

The cervical cancer cells were cultured in a 24-well plate at a density of 1×10^5 and incubated with or without cisplatin (5 μ g/ml, JIANGSU HANSON PHARMACEUTICAL.CO.LTD, China) for 24 h, then washed with phosphate-buffered saline (PBS) and digested with 0.25% trypsin only for 30-50 s, blocking with 10% FBS, the collected cells in EP tubes were centrifuged at 1500 rpm for 6 min. Then, these cells were incubated with PE-conjugated human IL-17E antibody (R&D system, USA) and APC-conjugated IL-17RB antibody (R&D system) in darkness for 30 min at room temperature. The cells were washed and analyzed immediately by a flow cytometry (Beckman coulter, USA). The statistical analysis was conducted using isotype-matched controls as references.

Enzyme-linked immunosorbent assay for IL-17E determination

HeLa and SiHa cells (1×10^5 cells/well) were seeded in 24-well plates, and then the supernatant was collected after culture for 48 h. The culture supernatant was gathered and centrifuged at 2000 rpm for 15 min to remove cellular debris, and then stored at -80°C until being proceeded by enzyme-linked immunosorbent assay (ELISA).

Cell viability assay

HeLa and SiHa cells were seeded into 96-well plates at a density of 7000 cells per well, and treated with rhIL-17E (0-100 ng/ml, Peprotech, USA) or Anti-human IL-17E neutralizing antibody (Anti-IL-17E, 0-1 μ g/ml, R&D system) for 48 h. Then CCK-8 solution (Dojindo, Japan) was carefully added to plates (100 μ l per well). The plates were incubated for 0.5 to 4 h at 37°C under the darkness. The absorbance was periodically detected at 450 nm by a microplate reader.

Cell apoptosis assay

Cell apoptosis was analyzed by flow cytometry using an Annexin V/7-AAD Apoptosis Detection Kit (BD, USA). HeLa and SiHa cells were collec-

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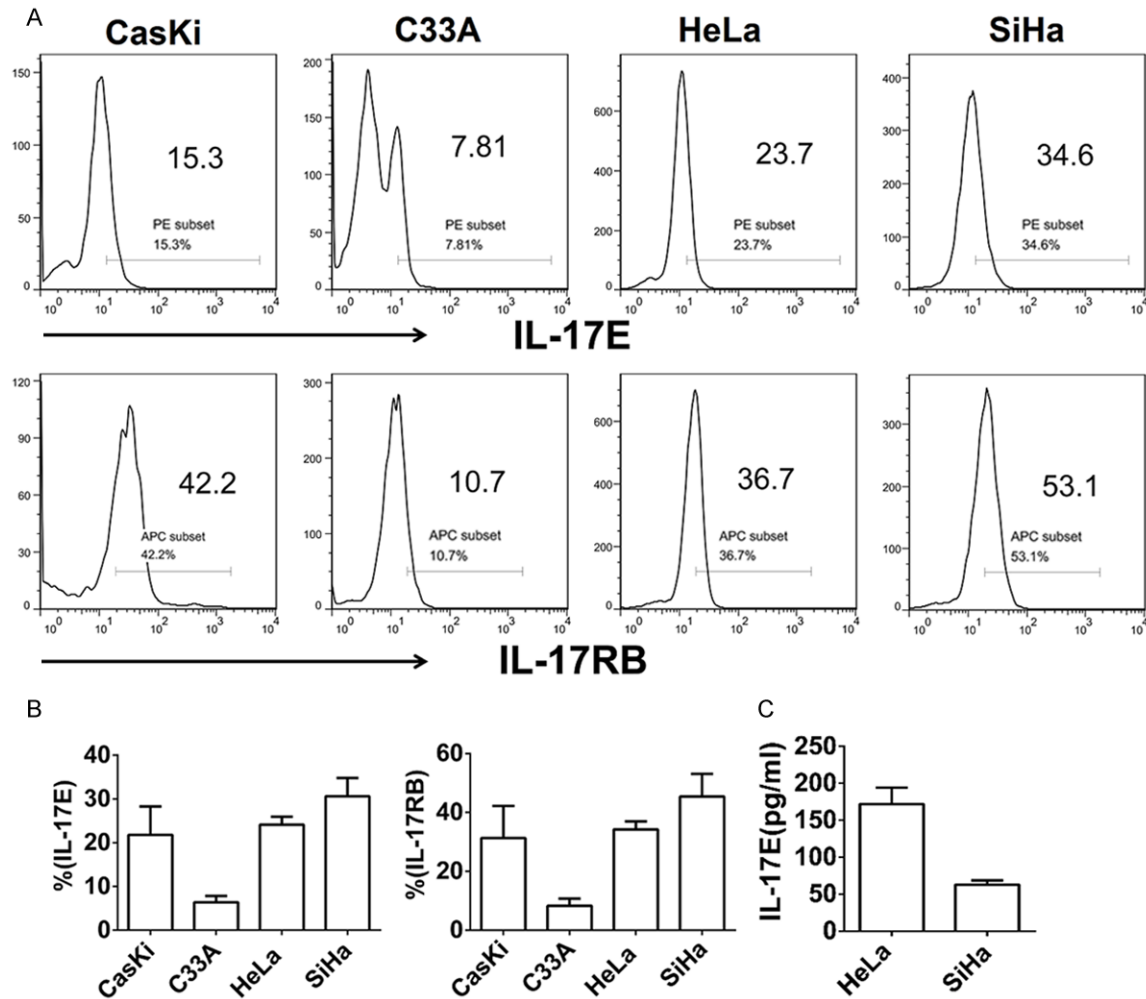


Figure 1. Cervical cancer cell lines co-express IL-17E/IL-17RB. A, B: The expression of IL-17E/IL-17RB in four cervical cancer cells was analyzed by flow cytometry. C: The level of IL-17E in the supernatant of HeLa and SiHa cells at 48 h was measured by ELISA.

ted after treatment with Anti-IL-17E (0.5 $\mu\text{g/ml}$) or rhIL-17E (50 ng/ml), and or cisplatin (5 $\mu\text{g/ml}$) for 48 h, then these cells were digested by 0.25% trypsin without EDTA, then centrifuged at 1200 rpm for 5 min, re-suspended with 200 μl binding buffer, and labeled by Annexin V and 7-AAD according to the protocol. Flow cytometry assay was performed to detect the percentage of early apoptotic cells. The experiment were carried out triplicate, and repeated three times.

Cell migration assay and Matrigel invasion assay

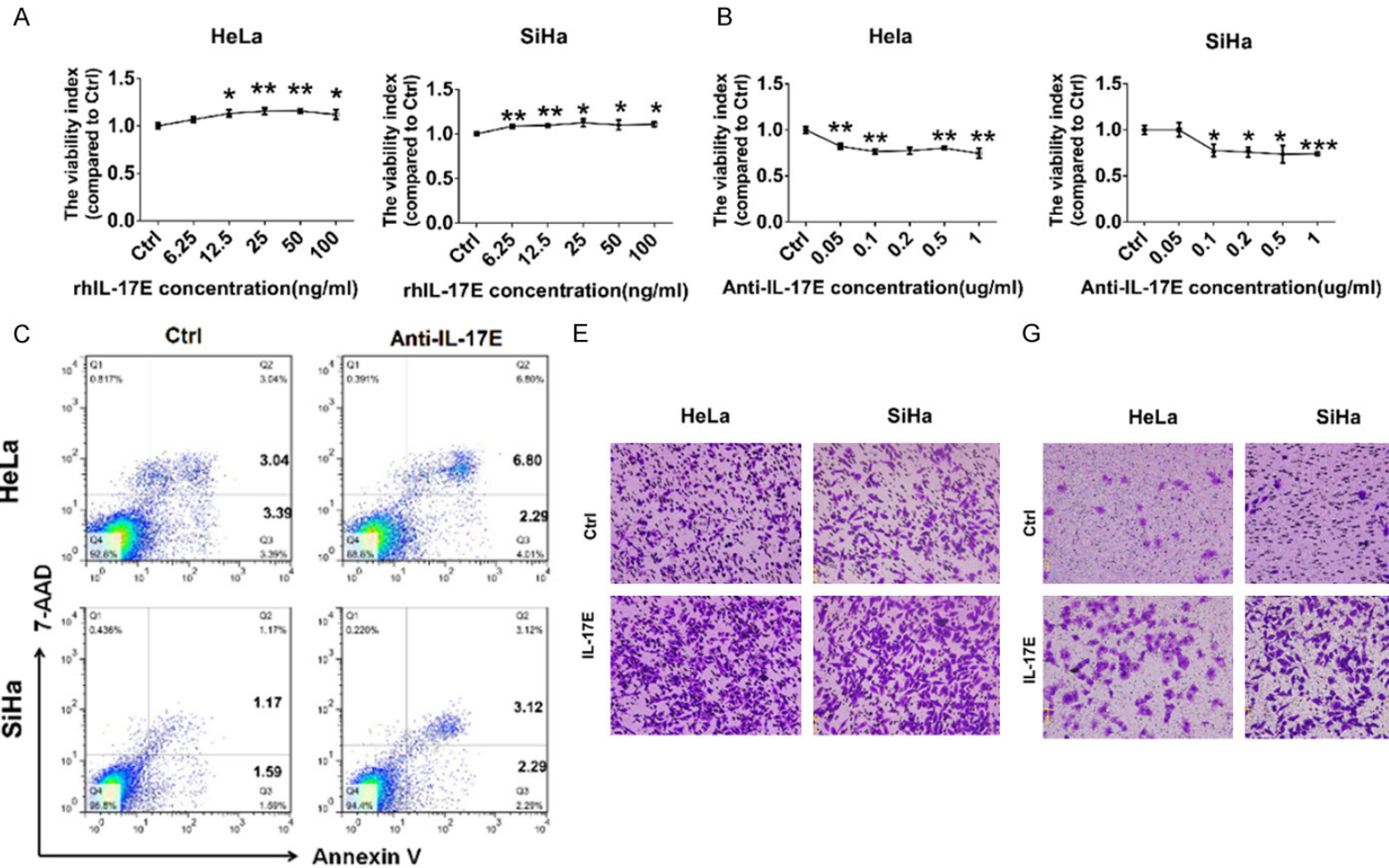
Migration assay was performed by using 24-well transwell (Costar, Cambridge, MA, USA) with polycarbonate filters (8- μm pore size). For matrigel invasion assay, the transwell filters

were pre-coated with Matrigel (BD, USA) which was diluted at 1:8 in serum free medium, and then put it in incubator for 30 minute. After seeding onto the top side of the transwells, HeLa and SiHa cells (1×10^5) were incubated with rhIL-17E (50 ng/ml), and or cisplatin (5 $\mu\text{g/ml}$). The lower chamber was filled with 600 μl complete medium, the upper chamber 200 μl the above medium. After 48 h, the migrated or invasive cells on the lower membrane surface were fixed and stained with 1% crystal violet which was dissolved with methanol. The invasive cells were evaluated in five random fields at $\times 200$ magnification under a light microscope.

Wound healing assay

Wound healing assay was also carried out to evaluate the effect of IL-17E on cell migration,

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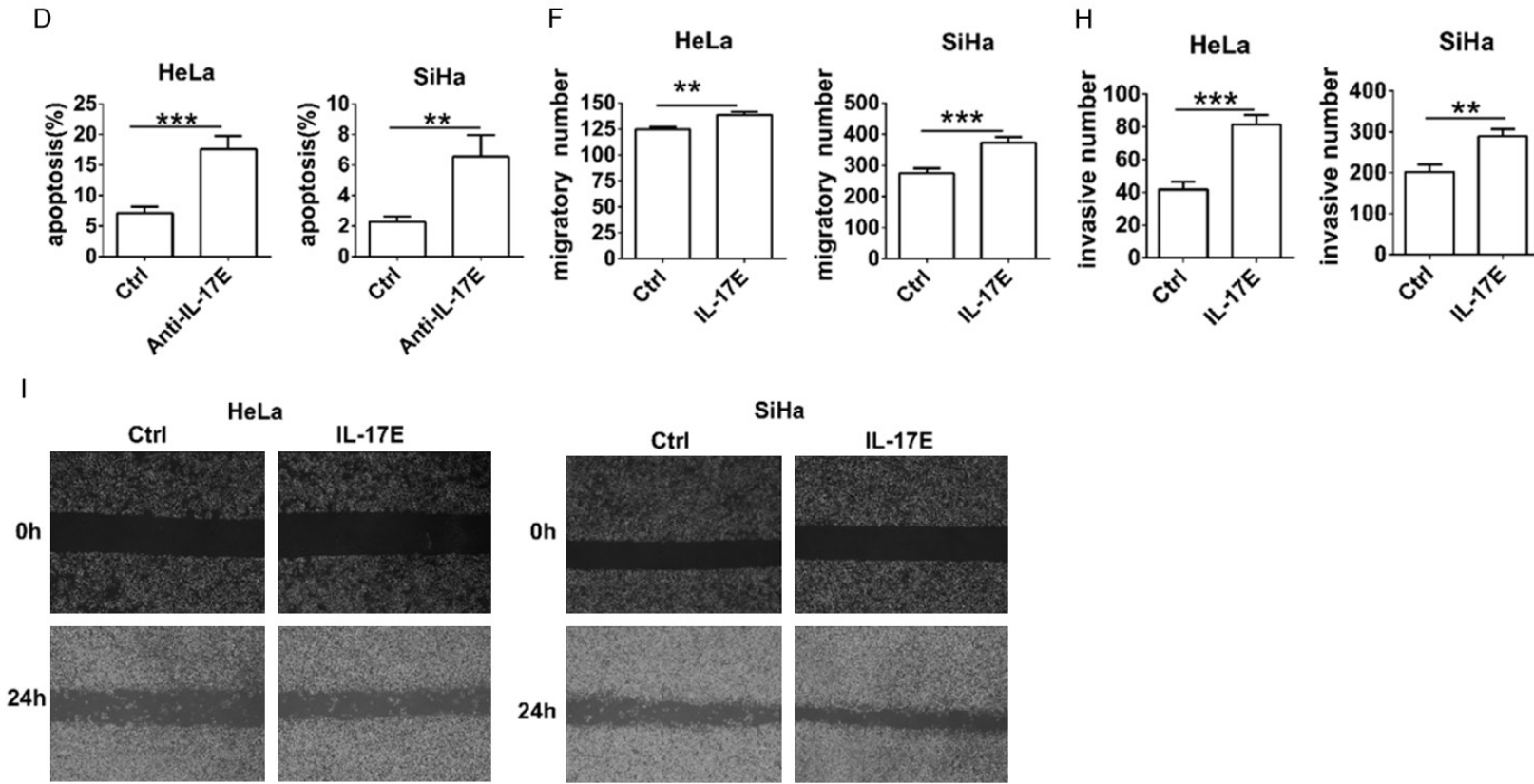


Figure 2. IL-17E promotes proliferation, migration and invasion, and restricts apoptosis of cervical cancer cells. (A, B) HeLa and SiHa cells were treated with rhIL-17E (0-100 ng/ml) or Anti-human IL-17E neutralizing antibody (Anti-IL-17E, 0-1 ug/ml) for 48 h. Then the cell viability was analyzed by CCK-8 assay. (C, D) HeLa and SiHa cells were collected after treatment with Anti-IL-17E (0.5 μ g/ml) for 48 h, and then the apoptosis of these cells was analyzed by apoptosis assay. (E-I) After stimulation with rhIL-17E (50 ng/ml) for 24 h (I) or 48 h (E-H), the ability for migration and invasion of HeLa and SiHa cells was analyzed by cell migration assay (E, F), matrigel invasion assay (G, H) and wound healing assay (I). * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$ (*t*-test or one-way ANOVA).

HeLa and SiHa cells (2×10^5 cells) were seeded a 6-well plate in serum-free medium. After about 12 h, the well reached a confluence of 70%~80%, the cell monolayer was scraped straightly by using a 10 μ l micropipette tip and washed with PBS to remove cell debris. The scraped monolayer was treated with or without rhIL-17E (50 ng/ml) incubated in serum-free medium for another 24 h, and gap distances at indicated time points after wounding were measured under a light microscope. Each experiment was repeated three times.

Statistical analysis

Statistical analysis was performed with the soft of GraphPad Prism 6.01. $P < 0.05$ was considered statistically significant. Differences between groups were analyzed by the Student *t*-test or one-way ANOVA. Data are presented as the mean \pm SEM.

Results

Cervical cancer cell lines co-express IL-17E/IL-17RB

To determine whether the cervical cancer cell lines express IL-17E/IL-17RB, we first analyzed the expression of IL-17E/IL-17RB by flow Cytometry. As observed in **Figure 1**, all cervical cancer cells lines (CasKi, C33A, HeLa and SiHa cells) co-expressed IL-17E and it receptor IL-17RB (**Figure 1A** and **1B**). Further analysis showed that both HeLa and SiHa cells could secrete IL-17E (**Figure 1C**). These data suggested the co-expression of IL-17E and IL-17RB from cervical cancer cells might be associated with regulation role in the biological behavior of cervical cancer cells. According to the relatively high level of IL-17E/IL-17RB in HeLa and SiHa cells, these two cells were chosen to further study the potential function of IL-17E in cervical cancer cells *in vitro*.

IL-17E promotes viability, migration and invasion, and restricts apoptosis of cervical cancer cells

In order to elucidate the role of IL-17E in the progression of cervical cancer, the viability, apoptosis, migration, invasion and wound healing assays were performed. As shown, rhIL-17E could enhance the viability of HeLa and SiHa cells in a dosage-dependent manner ($P < 0.05$ or $P < 0.01$) (**Figure 2A**), especially at the con-

centration of 50 ng/ml. In contrast, blocking IL-17E with Anti-IL-17E significantly decreased the viability of HeLa and SiHa cells ($P < 0.05$, $P < 0.01$ or $P < 0.001$) (**Figure 2B**), and the optimum concentration for inhibition of cell viability was 0.5 μ g/ml. Subsequently, the results of Annexin V/7-AAD apoptosis assay showed that Anti-IL-17E obviously promoted the apoptosis of HeLa and SiHa cells ($P < 0.01$ or $P < 0.001$) (**Figure 2C** and **2D**). Exposure with rhIL-17E led to significant increases of migration and invasion of HeLa and SiHa cells ($P < 0.01$ or $P < 0.001$) (**Figure 2E-H**). In addition, the results of wound healing assay further echoed the finding in cell migration assay (**Figure 2I**). In view of these results above, it can be concluded that IL-17E promotes the growth, migration and invasion of cervical cancer cells, may further play a positive effect in the development of this disease.

Cisplatin down-regulates the expression of IL-17E and IL-17RB in cervical cancer cells

Accumulating studies have demonstrated cisplatin, as the main chemotherapy drug, changes cellular general morphology, makes it difficult to adjust to the local environment thus quickens the pace to death. As shown, cisplatin prominently inhibited the viability of HeLa and SiHa cells in a dosage-dependent manner ($P < 0.01$ or $P < 0.001$) (**Figure 3A**), especially at the concentration of 5 μ g/ml. Further analysis showed that treatment with cisplatin resulted in the obvious down-regulation of IL-17E and IL-17RB in HeLa and SiHa cells ($P < 0.05$, $P < 0.01$ or $P < 0.001$) (**Figure 3B** and **3C**). These data suggest that the anti-tumor activity of cisplatin may be associated with IL-17E/IL-17RB.

Cisplatin restricts the stimulatory effects on growth, migration and invasion of cervical cancer cells induced by IL-17E

As pilot studies reported cytokines (e.g., IL-24) enhanced cisplatin-mediated suppression of tumorous proliferation [26] and elevation of apoptosis [27, 28], which arouse our great interest to explore the effect of the combination of IL-17E and cisplatin on the biological behaviors of cervical cancer cells. In consensus with our hypothesis, cisplatin suppressed the viability, migration and invasion, and promoted the apoptosis of HeLa and SiHa cells ($P < 0.05$, $P < 0.01$ or $P < 0.001$) (**Figure 4A-G**). However, treatment with rhIL-17E led to the

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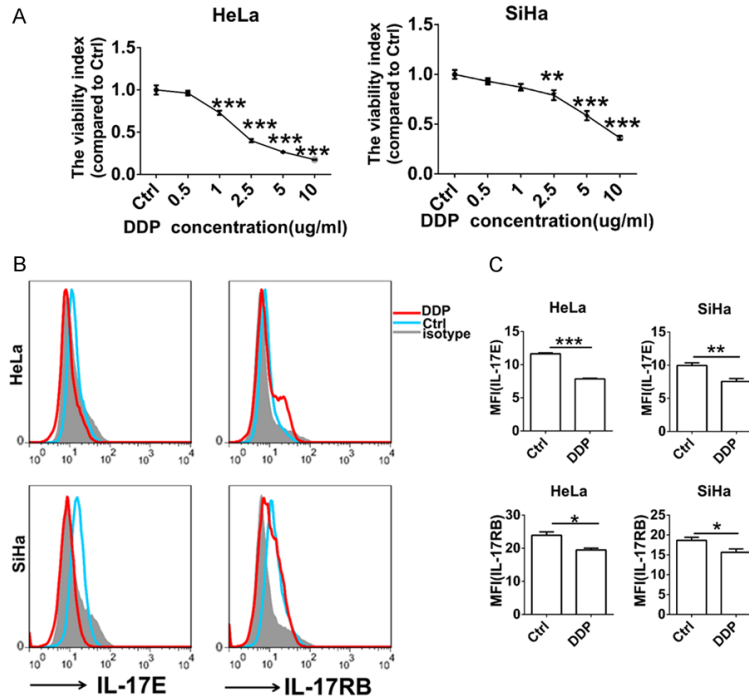


Figure 3. Cisplatin down-regulates the expression of IL-17E and IL-17RB. A: After treatment with cisplatin (0-10 µg/ml) for 24 h, the viability of HeLa and SiHa cells was analyzed by CCK-8 assay. B, C: After treatment with cisplatin (5 µg/ml) for 24 h, the expression of IL-17E and IL-17RB in HeLa and SiHa cells was analyzed by flow cytometry. DDP: cisplatin. * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$ (t -test or one-way ANOVA).

opposite results ($P < 0.05$ or $P < 0.001$) (Figure 4A-G). Interestingly, cisplatin also completely reversed the effects of rhIL-17E on the viability, apoptosis, migration and invasion of HeLa and SiHa cells (Figure 4A-G).

Discussion

Substantial evidences indicate tumor microenvironment play an essential role in tumorigenesis [29, 30]. Pro-inflammatory cytokines are major mediators for linking inflammation and cancer [7, 31]. The IL-17E locus is on chromosome 14, (14q11-12), different from the other IL-17 family members, IL-17E has been shown to play a critical role in the initiation and propagation of the Th2 immune response by major histocompatibility complex (MHC) class II, alongside elevation of IL-4, IL-5 and IL-13 [21]. Here, we found that four cervical cancer cell lines expressed IL-17E, the tendency of high level of IL-17E in cervical cancer cells was consistent with the online database in <http://www.proteinatlas.org/>. According to the high affinity between IL-17E and IL-17RB [32], we further

analyzed the expression of IL-17RB, and found that cervical cancer cells co-expressed IL-17E and IL-17RB.

The IL-17 family has been demonstrated to promote tumorigenicity of cervical tumors in nude mice and is closely related to an increased level of IL-6 at the tumor sites [33]. The up-regulation of IL-6, IL-8, and TNF- α promote the surrounding tissue destruction for assisting in tumor invasion and ultimate metastasis [34]. In most researches, IL-17E exerts an anti-tumor efficacy by several pathways, such as recruitment of eosinophils into tumors [18, 21, 35]. However, it seems to be a contradictory result in breast cancer [17-20, 34, 36], suggesting that the different cell lines maybe the major influence factor. Further analysis in this study showed that IL-17E played a pro-tumor activity possibly through promoting the

viability, migration and invasion of cervical cancer cells, and restricting cell apoptosis in vitro. We have reported previously that cervical cancer is often associated with eosinophils infiltration induced by thymic stromal lymphopoietin (TSLP), and these TSLP-educated eosinophils can further promote the growth of the cervical cancer cells [37]. Therefore, IL-17E may indirectly promote the growth of cervical cancer cells through recruitment of eosinophils, which needs further research.

IL-17E has also been shown to activate the signals of MAPKs like p38 and JNK as well as NF- κ B [15, 38], and transcription factors, such as NF- κ B, NF-ATC1, GATA3, JUNNB and STAT6 [9]. Our previous work has also shown that IL-17E promotes the proliferation of decidual stromal cells by activation of JNK and AKT signal pathways in early pregnancy [16]. However, the downstream signal pathway of IL-17E in cervical cancer cells remains unclear.

It has reported that IL-17E has the potential to promote angiogenesis [39, 40] and initiates the

DDP/IL-17E on cervical cancer cells

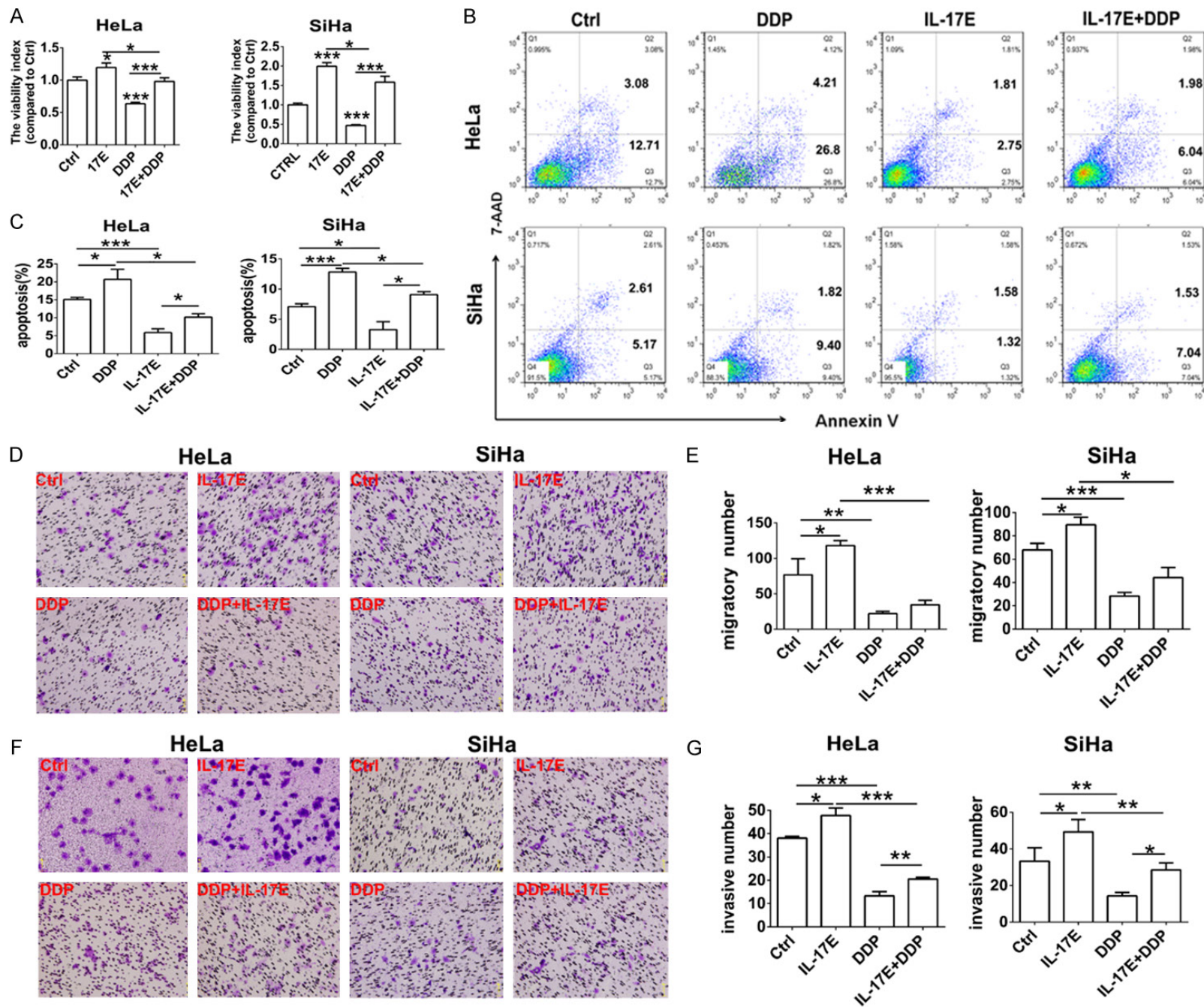


Figure 4. Cisplatin restricts the stimulatory effects on growth, migration and invasion of cervical cancer cells induced by IL-17E. HeLa and SiHa cells were treated with rhIL-17E (50 ng/ml) and or cisplatin (5 μ g/ml) for 48 h, and then the viability, apoptosis, migration and invasion of these cells were analyzed by CCK-8 assay (A), apoptosis assay (B, C), cell migration assay (D, E) and matrigel invasion assay (F, G), respectively. * P <0.05, ** P <0.01 or *** P <0.001 (one-way ANOVA).

Th2 immune response through recruiting or release eosinophils [15, 41] or macrophages [34, 42]. Therefore, in addition to regulating cell growth and invasion, IL-17E may also be involved in promoting angiogenesis and Th2 bias for formation of immune tolerance micro-environment, and further accelerating the progression of cervical cancer cells.

At present, the regulation mechanism of IL-25 expression is still unclear. It has been reported, after being challenged with allergens derived from *Aspergillusoryzae* and ragweed, murine and human lung epithelial cell lines (MLE12 and A549) and murine primary type II alveolar epithelial cells express remarkable IL-17E [43]. So we have the suspicion that high expression of IL-17E in cervical cancer cells may be associated with HPV infection, this doubt needs further study to be answered.

Though cisplatin is a commonly used chemotherapeutic agent, the serious side effect and the development of resistance limit the efficacy of approach [27]. Lymph node metastasis and tumor angiogenesis are important mechanism of cervical cancer invasion [44]. Further researches in current study showed that cisplatin decreased the expression of IL-17E and IL-17RB in cervical cancer cells in vitro. In addition, treatment with cisplatin could completely reverse the effects of rhIL-17E on the viability, apoptosis, migration and invasion of cervical cancer cells, suggesting that cisplatin has the potential to treat cervical cancer patients with aberrantly high level of IL-17E and IL-17RB. However, the underlying mechanism of cisplatin on IL-17E and IL-17RB expression in cervical cancer cells remains to be further investigated. Emerging evidences show cisplatin inhibits angiogenesis and lymphangiogenesis by inhibiting the expression of VEGF [27]. VEGF could be activated by IL-6 and IL-8, and IL-17E regulates the expression of IL-6 and IL-8. Therefore, cisplatin may also suppress angiogenesis by down-regulating IL-17E/VEGF expression.

In conclusion, the high level of IL-17E and IL-17RB not only directly promote the growth

and invasion of cervical cancer cell, but also may indirectly induce Th2 immune response for immunological escape, and further accelerates the development of cervical cancer. IL-17E may be a negative prognostic factor in evaluating cervical cancer patients. The detection of IL-17E and IL-17RB may have a positive significance for drug selection, and cisplatin may be a preferred option for cervical cancer patients with abnormally high expression of IL-17E and IL-17RB.

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Disclosure of conflict of interest

None.

Address correspondence to: Ming-Qing Li, Laboratory for Reproductive Immunology, Hospital of Obstetrics and Gynecology, Fudan University Shanghai Medical College, No.413, Zhaozhou Road, Shanghai 200011, China. Tel: 86-21-63457331; Fax: 86-21-63457331; E-mail: mqli@fudan.edu.cn; Jin-Jin Yu, Department of Obstetrics and Gynecology, The Affiliated Hospital of Jiangnan University, No.200, Huihe Road, Wuxi 214062, Jiangsu Province, China. Tel: 86-510-88682999; Fax: 86-510-85808820; E-mail: yujjwx@126.com

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