The effect of IL-22 on the migration and invasion of cervical cancer through Wnt/β-catenin pathway

Jia Li¹*, Wei You²

¹Department of Obstetrics and Gynecology, Zigong Third People's Hospital, Zigong 300052, Sichuan Province, China
²Department of Obstetrics and Gynecology, Zigong Fourth People's Hospital, Zigong 300052, Sichuan Province, China

* Corresponding author
E-mail: 330559448@qq.com

Abstract: The present study was aimed to detect the effect of IL-22 on the migration and invasion of cervical cancer. Carcinoma-associated fibroblasts (CAFs) and normal human fibroblasts (NFs) were obtained from patients. The cell morphology observation was conducted using a microscope, the expressions of vimentin and alpha smooth muscle actin (α-SMA) were examined to identity CAFs and NFs. The IL-22 concentration in supernatant was determined by Enzyme Linked Immunosorbent Assay (ELISA). The Hela cells were treated with normal medium, NF supernatant, CAF supernatant, CAF supernatant + IL-22 antibody, or IL-22, respectively. Thereafter, the transwell assay and wound healing assay were performed. The expression of β-catenin was observed by immunofluorescence analysis. The expressions of E-cadherin, Cytokeratin, N-cadherin, Vimentin, β-catenin and Dishevelled-2 (DVL2) were measured by western blot. As a result, the CAFs and NFs were successfully isolated. The IL-22 content in CAF supernatant was higher than that in NF supernatant. With the treatment of CAF supernatant and IL-22, the migrative and invasive property of Hela cell was increased, which was moderated by CAF supernatant + IL-22 antibody. Whereas the NF supernatant could inhibit the cellular migration and invasion. The administration of CAF supernatant and IL-22 downregulated the expressions of E-cadherin, Cytokeratin, and upregulated the
expressions of N-cadherin, Vimentin, DVL2. Nonetheless, the NF supernatant markedly reversed these alterations. The CAF supernatant + IL-22 antibody treatment scarcely influenced the protein levels. In conclusion, IL-22-induced epithelial-mesenchymal transition (EMT) could promote migration, metastasis and invasion in cervical carcinoma possibly through the Wnt/β-catenin signaling pathway.

**Key words:** cervical carcinoma, IL-22, migration and invasion, Wnt/β-catenin pathway
Introductions

In recent years, cervical cancer has been defined as the third most prevalent gynecological malignancy around the world. Unfortunately, there has been no target drugs for the intervention of cervical cancer in clinical (1). With the limited therapeutic strategy, over 85% mortality occurred in developing countries (2). The metastatic tumor in cervix uteri is of chemotherapy resistant in most clinical cases. Thus, it is urgent to detect the underlying mechanism and develop the corresponding candidate molecular drugs.

The chronic infection with genital high-risk human papillomaviruses (HPV) remains the leading cause of cervical cancer. Other risk factors including excessive sexual history, less inflammatory response and weakened immune activity also contribute to the pathogenesis of cervical cancer (3). Of note, The high-risk HPV oncoproteins interfere with inflammatory signaling and conduct to the immune-suppressing activities (4).

The infection with HPV initiates the immune response which is featured by the infiltration of regulatory T cell and differentiation. Accumulating evidence confirmed that T helper cells regulated the inflammatory reaction and malignant tumors (5). Currently, the identified inflammatory Th subset Th22 has been elicited to be involved in the etiology of gynecological tumorigenesis. Th22 secrets IL-22 and TNF-α, but not IL-4, IL-17A or IFN-γ (6). IL-22, the member of IL-10 cytokine family which produced by T cells, is the mainly effector cytokine of Th22 cells. It is suggested that IL-22 play critical roles in the development of inflammatory and autoimmune dysfunctions including multiple sclerosis, psoriasis and rheumatoid arthritis (7-9). IL-22 mediates the tumor proliferation, invasion, metastasis, dysplasia and apoptosis (10). Previous literature has displayed the essential role of IL-22 in invasive cervical carcinoma (11). However, the underlying mechanism of IL-22 in the invasion and migration of cervical cancer remain not fully elucidated.

The Wnt/β-catenin signaling pathway contains a variety of molecules in the mediation of carcinogenesis. Upon aberrant activation, the β-catenin accumulates in
cytoplasm and translocates into nucleus. Mounting evidence demonstrated that the Wnt/β-catenin governed epithelial-mesenchymal transition (EMT). The suppression of E-cadherin results in the release of β-catenin, which consequently accelerates the invasion of cervical cancer (12). The recruitment with β-catenin leads to the progressive inhibition of E-cadherin and the engagement of mesenchymal indicator including vimentin and N-cadherin. It was hypothesized that Wnt/β-catenin signaling-related EMT might drive the IL-22-induced metastasis and invasion in cervical cancer. Herein, the present research was carried out to evaluate the effect of IL-22-induced EMT on the migration and invasion in cervical carcinoma-related fibroblast. The mechanism investigation was also performed to detect its potential pathogenesis via the Wnt/β-catenin pathway.

Methods

Reagents

IL-22 enzyme-linked immunosorbent assay (ELISA) kit was supplied by Elabscience Biotech. Co. Ltd. (Wuhan, Hubei, China). Dulbecco’s modified Eagle’s medium (DMEM) was produced by Life Technologies (Carlsbad, CA, USA). All primary antibodies and horseradish peroxidase-conjugated anti-rabbit antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA).

Isolation and cultivation of CAFs and NF

Briefly, the uterine specimens from cervical cancer tissues and Ovarian cancer tissue after hysterectomy were isolated to obtain CAFs and normal human fibroblasts (NFs) following the instructions described in former literature, respectively (13). After washing twice using phosphate-buffered saline (PBS), the residual connective tissues were minced and subjected to collagenase for 2 h at 37°C. The samples were cultured around Dulbecco’s modified Eagle’s medium (DMEM) medium containing 10% fetal bovine serum (Gibco, USA), 100 U/ml penicillin and 100 U/ml streptomycin. When the confluent monolayers were observed, the non-adherent cells were discarded. 2-3 passages later, the retained purified fibroblasts confirmed by western blot analysis were applied for the experiments. The cellular morphology was identified under
microscope by two experienced investigator who were blinded to this study.

**Enzyme Linked Immunosorbent Assay (ELISA)**

The release of IL-22 into the culture medium was determined using IL-22 commercial ELISA kit(Elabscience, Wuhan, China). The procedure was conducted in accordance with the instruction. The absorbance values were recorded using an automatic microplate spectrophotometer. The corresponding concentration of IL-22 was calculated by a standard curve.

**Cell cultivation and treatment**

The Hela human cervical cancer cell line was purchased from the American Type Culture Collection(Manassas, VA, USA). The cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin in a humidified 5% CO₂ atmosphere at 37°C. The cells were randomly divided into 5 groups: control group, CAF supernatant group, NF supernatant group, CAF supernatant + IL-22 antibody group, positive control(IL-22) group. The Hela cells were treated with the corresponding supernatant for 24 h and then harvested for wound healing assay, transwell detection, western blot analysis and immunofluorescence observation. Simultaneously, the control cells were incubated with normal medium.

**Transwell assay**

The cellular metastatic ability was evaluated using transwell assays. In general, the NFs (1 × 10⁵ cells/well) suspended in DMEM medium with 0.1% fetal bovine serum were plated onto the upper chambers. Whereas the lower chamber was filled with the cells around DMEM supplemented with 10% fetal bovine serum. After incubating for 24 h, the cells were washed by PBS. The cells in upper chamber were fixed in 95% ethanol and stained with crystal violet in gluteraldehyde. Under microscope observation, the transferred distance of migrated cells through the filter membrane was quantified.

**Wound healing assay**

The scratch wound healing assay was carried out to examine the cell migration of
CAFs and NFs. The CAFs and NFs were seeded onto six-well culture plates until confluency of monolayer reaching 80%. A sterile 200 μl pipette tip was used to scrape the bottom of culture plates. After wounding, the non-adherent cells and debris around cell-free area were carefully removed with PBS. The cells were treated with serum-free medium for 24 h. The cell migration was visualized and photographed in a specific region using microscope. Finally, the invasive cell number was counted.

**Immunofluorescence**

The Hela cells were seeded onto 6-well plate and washed with PBS. After fixing with 3.7% (w/v) paraformaldehyde for 0.5 h at room temperature, the samples were rehydrated and permeabilized with 0.2% Triton X-100 and 5% serum. Then the samples were treated with 2% BSA and antibodies at the dark atmosphere overnight. After washing, the sections were exposure to propidium iodide conjugated secondary antibodies(1:100) visualized under a fluorescence microscope.

**Western blot analysis**

The cells were washed twice with ice-cold PBS and lysed in RIPA buffer(Beyotime, Nanjing, China), followed by centrifugation. The BCA assay was performed to measure the protein content. The samples were fractioned by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and electro-blotted onto polyvinylidenedifluoride (PVDF) membranes. Then the blots were blocked with 5% fat-free milk powder dissolved in TBST. After an incubation with corresponding primary antibodies at 4 ° C overnight, the membranes were washed and detected by the incubation with HRP-conjugated second antibodies at room temperature for 1 h. The blots were developed using the enhanced chemiluminescence method(KeyGEN Biotechnology, Nanjing, China). Consequently, the Clinx ChemiScope chemiluminescence imaging system was applied for visualization.

**Statistical analysis**

The statistics in the current study were presented as mean value ± SD. Comparisons between groups were analyzed by one-way analysis of variance or
ANOVA with Tukey multiple comparison test. \( p<0.05 \) was considered to indicate a statistically significant difference.

**Results**

The identification of NF and CAF cells

The cellular morphology of CAF and NF cells was visualized under microscope. As revealed in Fig 1A, it was observed that the CAF cells were larger than NFs. The fibroblasts were identified based on a combination of positive indicators. Vimentin was considered as a key defining feature of CAFs and NFs, while \( \alpha \)-SMA served as the notable biomarker for NFs. As shown in Fig 1C, the vimentin expressions in NFs and CAFs were both enhanced, whereas the higher protein level of \( \alpha \)-SMA was only observed in CAFs. The analytical data proved that the isolation of CAF and NFs were successful.

Additionally, the concentrations of inflammatory cytokine IL-22 in the supernatant of CAF and NFs were also detected. As illustrated in Fig 1C, the content of IL-22 in CAF supernatant was evidently more than that in NF supernatant. It was displayed that CAF generated and secreted more IL-22 than NF (Fig 1B).

**Fig 1.** The identification of NF and CAF cells.

The cell morphology of NF and CAF cells (A). The contents of IL-22 in the supernatant of
NF and CAF(B). The expressions of Vimentin and \(\alpha\)-SMA in NF and CAF cells (C).

\*\(p<0.05\) compared with NF cells.

Fig 2. The effect of IL-22 on scratch wound healing assay.

The Hela cells were randomly divided into 5 groups: control group(1), CAF supernatant group(2), NF supernatant group(3), CAF supernatant + IL-22 antibody group(4), positive control(IL-22) group(5). The Hela cells were treated with the corresponding supernatant and then harvested 24 h later. \*\(p<0.05\) compared with NF cells.

The effect of IL-22 on cell metastasis of cervical cancer cells

To evaluate the effect of IL-22 on migration ability of Hela cell, we performed the scratch wound healing assay in vitro. As depicted in Fig 2, the results showed that NF supernatants group presented less invasive cell number and weaker wound healing ability than those in control group\((p<0.05)\), while CAF supernatants group conduced to the opposite effect than NF supernatants group\((p<0.05)\). The population of invasive cell in CAFs supernatants + IL-22 antibodies group was similar with that in control group. Of note, the treatment of IL-22(200 ng/ml) dramatically increased invasive cell number \((p<0.05)\). Our experimental data verified the critical role of
IL-22 in invasion and migration of Hela cells.

The effects that IL-22 produced on invasion and migration of Hela cells were also assessed by transwell measurement. As revealed in Fig 3, the migrated cells counting indicated that the cells migrate through transwell in control group were remarkably more than that in NFs supernatant group($p< 0.05$), and less than that in CAFs supernatants group. The treatment with CAFs supernatants+ IL-22 antibodies scarcely influenced the invasive cell numbers. As expected, the images of Hela cells stained with crystal violet demonstrated that the incubation with IL-22(200 ng/ml) led to the promotion of invasion through the microporous membrane($p< 0.05$). The results suggested that IL-22 secreted by CAF could accelerate the property of invasion and migration in Hela cells.

The Hela cells were randomly divided into 5 groups: control group(1), CAF supernatant group(2), NF supernatant group(3), CAF supernatant + IL-22 antibody group(4), positive control(IL-22) group(5). The Hela cells were treated with the corresponding supernatant and then harvested 24 h later. *$p< 0.05$ compared with NF cells.
The effect of IL-22 on the expression of β-catenin in cervical cancer cells

The immunofluorescence observation was carried out to detect the β-catenin expression of Hela cells in the present research. As presented in Fig 4, more β-catenin immunofluorescence activity was located in cytoplasm and nucleus in CAFs supernatant and IL-22 treated groups. Whereas the location of β-catenin was found at the cell membrane in NFs supernatant and CAFs supernatants + IL-22 antibodies group. The immunofluorescence staining results demonstrated that the effect of IL-22 on Hela cells might be associated with β-catenin.

Fig 4. The effect of IL-22 on the expression of β-catenin by immunofluorescence staining.

The Hela cells were randomly divided into 5 groups: control group(1), CAF supernatant group(2), NF supernatant group(3), CAF supernatant + IL-22 antibody group(4), positive control(IL-22) group(5). The Hela cells were treated with the corresponding supernatant and then harvested 24 h later. *p < 0.05 compared with NF cells.

The effects of IL-22 on the expression of E-cadherin, N-cadherin, Cytokeratin, Vimentin, β-catenin and DVL2

Next, we performed the western blot experiment to verify the effects of IL-22 on epithelial biomarker N-cadherin, mesenchymal index E-cadherin, cervical cancer indicator Cytokeratin and fibroblasts characteristic Vimentin. As illustrated in Fig 5, the treatments with NFs supernatants or CAFs supernatants + IL-22 antibodies seldom influenced the protein levels of E-cadherin and Cytokeratin(p < 0.05 or p < 0.01). By
contrast, the incubations with CAFs supernatants and IL-22(200 ng/ml) markedly decreased the levels of E-cadherin and Cytokeratin\((p < 0.05 \text{ or } p < 0.01)\). Moreover, the expressions of N-cadherin and Vimentin were down-regulated in NFs supernatants group\((p < 0.05)\), but up-regulated in CAFs supernatants group compared with those in control group \((p < 0.05)\). The levels of N-cadherin and Vimentin in IL-22 group were remarkably elevated compared with those in control group. The treatment with CAFs supernatants + IL-22 antibodies group did not significantly affect the concentrations of N-cadherin and Vimentin. The analytical results indicated that IL-22 could accelerate the Epithelial-mesenchymal transition (EMT).

Furthermore, the Wnt/\(\beta\)-catenin pathway-related \(\beta\)-catenin and DVL2 were detected. The \(\beta\)-catenin\((p < 0.01)\) and DVL2\((p < 0.05)\) expressions were inhibited by NFs supernatants treatment, and promoted by CAFs supernatants treatment. Nevertheless, the exposure to IL-22(200 ng/ml) effectively reduced the \(\beta\)-catenin and DVL2 contents \((p < 0.01)\). Our data revealed that the effect of IL-22 on Epithelial-mesenchymal transition was related to the Wnt/\(\beta\)-catenin signaling cascade.
Fig 5. The effect of IL-22 on the expression of E-cadherin, N-cadherin, Cytokeratin, Vimentin, β-catenin and DVL2 in Hela cells by Western blot analysis.

The Hela cells were randomly divided into 5 groups: control group(1), CAF supernatant group(2), NF supernatant group(3), CAF supernatant + IL-22 antibody group(4), positive control(IL-22) group(5). The Hela cells were treated with the corresponding supernatant and then harvested 24 h later. *p< 0.05 compared with NF cells.

Discussion

The acceleration of tumor proliferation is implicated with the surrounding stroma. As the special kind of fibroblasts, CAFs can be identified by its specific morphological feature and biological behavior. Interstitial NF is important for maintaining epithelial homeostasis (14). The differentiation from different type cells to CAFs is attributed to the inflammatory cytokines secretion by tumor tissues. The CAFs produce strong contractility alpha-SMA which is applied to be the reliable
indicator for CAFs (15). Vimentin is a pivotal mesenchymal index frequently correlated with EMT and is upregulated in the metastasis of cancer (16). Both CAFs and NFs express Vimentin, whereas α-SMA is the major characteristic of CAFs, but not NFs. Therefore, the specific marker α-SMA has been widely applied to identify the NFs and CAFs (17). Our results demonstrated that the isolation of CAF and NFs were successful. It was also found that CAFs generated more IL-22 concentration than NFs.

The cell mediated immunity may be a spontaneous clearance for HPV incidence. Th cells, the subgroup of lymphocytes is universally considered to manipulate a series of gynecological malignant tumors (18). The inflammatory cytokines, including IL-22, are the distinct characteristic of Th22 compared with other Th cells. In the past few decades, the Th22 cells and IL-22 have been reported to play prominent roles in chronic immunity and tumorigenesis (19). IL-22 was previously defined as IL-10-related-T-cell-derived inducible factor (IL-TIF). It contains a heterodimeric transmembrane receptor complex namely IL-22R1 and IL-10R2 chains. IL-22 initially combines with the IL-22R1 subunit which subsequently alters conformationally to allow the binding with IL-10R2, triggering the downstream cascade pathway. IL-22 is a critical component of immune-epithelial cell network (20). Th22 cells-generating IL-22 has been illustrated to regulate various malignancies including liver, gastric, lung and pancreatic tumors (21-23). Growing evidence demonstrated that IL-22 functioned as a prominent inflammatory mediator governing the initiation and progression of epithelial ovarian cancer (24).

EMT is highly related to the tumor proliferation, metastasis and invasion. The repression for the migration and progression of EMT is a long-standing tumor therapeutic strategy (25). The major feature of EMT is the shift from E-cadherin expression to N-cadherin expression, which is the common change happening during the metastasis of human gynecological cancer (26). As the proteins of keratin-containing intermediate filaments, Cytokeratin locates in the cytoskeleton of epithelial cells and serves as epithelial markers (27). Additionally, it was observed an increase in cytokeratin, which was also implicated as an epithelial stem cell marker.
This work displayed that the treatments with NFs supernatants or CAFs supernatants + IL-22 antibodies seldom influenced the protein levels of E-cadherin and Cytokeratin, while the incubations with CAFs supernatants and IL-22(200 ng/ml) markedly decreased the levels of E-cadherin and Cytokeratin. Interestingly, the treatment exerted reversed effect on N-cadherin and Vimentin. It was noteworthy that the treatment with CAFs supernatants + IL-22 antibodies group did not significantly affect the concentration of E-cadherin, Cytokeratin, N-cadherin nor Vimentin. The analytical results indicated that the IL-22 could participate in the modulation of the Epithelial-mesenchymal transition.

It is widely acknowledged that Wnt/β-catenin signaling functions as a pivotal target molecule for the exploration of therapeutical strategy for various tumorigenesis. The Wnt/β-catenin signaling modulates substantial cellular process, such as proliferation, differentiation, invasion and metastasis (29,30). The activation of Wnt/β-catenin pathway leads to the transcriptional initiation of downstream specific target component, which promotes cellular proliferation and suppresses cell apoptosis in clinical gynecological cancer (31). The aberrant modulation of the canonical Wnt/β-catenin cascade may be the candidate controlling target for the intervention of cervical cancer (32). It was previously indicated that Wnt/β-catenin pathway was related to IL-22-induced keratinocyte proliferation (33). Thacker et al proved that the regulation of inflammatory cytokines through Wnt/β-catenin was beneficial for the treatment of cervical cancer. DVL2 is a critical adaptor of Wnt/β-catenin pathway and serves as a scaffold protein between canonical Wnt receptors and downstream signaling event. DVL2 degradation plays an important role in the Wnt/β-catenin mediated autophagy pathway in the proliferation of MCF7 cells (34). Numerous evidence proved the crucial role of Wnt/β-catenin signaling in epithelial-mesenchymal transition (35). The suppression of E-cadherin modulates the EMT in epithelial cells via the β-catenin release. The upregulation of target downstream genes in Wnt/β-catenin is associated with the invasiveness of cancer cells (36). The western blot results demonstrated that the β-catenin and DVL2 expressions
were obviously enhanced in CAFs supernatants and IL-22 treated group, while were weakened in NF supernatants treated group. The exposure to CAFs supernatants + IL-22 antibodies did not affect the β-catenin and DVL2 levels. The immunofluorescence staining also showed that similar alteration of β-catenin. The data suggested that Wnt/β-catenin pathway was correlated with the IL-22-induced Hela cells.

In conclusion, the present investigation elucidated that IL-22 could induce migration, metastasis and invasion through Wnt/β-catenin signaling cascade in cervical carcinoma. Further researches are necessary to evaluate its pathogenesis in the future.

References:


