

## Interleukin 21 promotes the migration and invasion of breast cancer cells through regulating PI3K/AKT signaling pathway

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**Abstract:** Breast cancer is a heterogeneous disease and is the leading cause of cancer-related death among females. Interleukin 21 (IL-21), which belongs to the common  $\gamma$ -chain ( $\gamma_c$ ) family, is a novel tumor suppressor. In the present study, we aim to investigate the impacts of IL-21 on cell migration and invasion in breast cancer. The expressions of IL-21 in breast cancer tissue on mRNA and protein levels were evaluated by qRT-PCR and western blot methods. At the same time, scratch and transwell assays were conducted to examine the effect of IL-21 on the migration and invasion of breast cancer cell line MCF-7. Finally, the expression of PI3K and AKT, as well as migration and invasion related molecules were examined by western blot methods. We found that IL-21 was up-regulated in breast cancer tissues; moreover, treatment of IL-21 significantly increased the migration and invasion of MCF-7 cells in a dose-dependent manner. Meanwhile, IL-21 also increased the expressions of PI3K, p-AKT, MMP-3, MMP-9 and ICAM-1. To sum up, IL-21 might regulate the cell migration and invasion ability of breast cancer through activating the PI3K/AKT signaling pathway.

### 1. Introduction

Breast cancer is a heterogeneous and is the leading cause of cancer-related death with the highest recurrence rate [1,2]. Despite the great progress in the diagnosis and treatment methods for breast cancer and a significant decrease in the mortality rate [2], there were still approximately 252,710 new cases diagnosed with breast cancer and about 40,610 breast-cancer-related deaths among women in the United States in 2017 [3]. Surgery, chemotherapy, radiation therapy, hormone therapy and targeted therapy are the main methods for breast cancer treatment [4]. However, the patients with breast cancer are still confronted with many side effects including pain, infection, tenderness, bleeding and temporary swelling from surgical treatment, weight changes, nausea, hair loss, fatigue, vomiting from chemotherapy, soreness, skin changes and swelling [5]. Moreover, the potential molecular mechanisms in breast cancer has not been fully elucidated. Therefore, to find a novel biomarker for breast cancer is in urgent need.

Interleukin 21 (IL-21), which belongs to the common  $\gamma$ -chain ( $\gamma_c$ ) family, is a novel cytokine affecting the proliferation, survival and function of T-cells [6]. In the case of cancer studies, the roles of IL-21, either as a tumor suppressor or oncogene, have been discussed in many previous studies. For example, IL-21 could induce the anti-tumor effects of NK cells via the NKG2D pathway [7].

Additionally, IL-21 also enhanced NK cell lytic activity against Ab-coated tumor cells [8]. In breast cancer, it was observed that IL-21 may serve as an oncogene[9]. However, the potential mechanism of IL-21 in regulating the migration and invasion of breast cancer is still unclear.

In the present study, we will explore the mechanisms of IL-21 in regulating cell migration and invasion in breast cancer via regulating the PI3K/AKT signaling pathway. This may provide a new aspect for the treatment of breast cancer.

## 2. Materials & methods

*Patients and tissues* All of 30 patients diagnosed with breast cancer was recruited from Jiangyin Hospital of Traditional Chinese Medicine during August, 2016 to October, 2017. Additionally, no radiotherapy or chemotherapy were performed prior the surgery. In brief, tumor tissues and adjacent normal tissues ( $\geq 5$  cm away from tumor edge) were taken from patients and fixed in 10% formalin for storage. Furthermore, the experiments were proved by the Ethics Committee of Jiangyin Hospital of Traditional Chinese Medicine and all patients had signed and handed over the informed consent.

*Cell lines and treatment.* Breast cancer cell line MCF-7 was purchased from Chinese Academy of Sciences (Shanghai, China). Meanwhile, the cells were incubated with RPMI-1640 medium (Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS, Bovogen) at 37 °C in an atmosphere with 5% CO<sub>2</sub>.

Cells were treated with IL-21 (10 or 20 ng/ml) and incubated with RPMI-1640 medium in a humidified incubator with 5% CO<sub>2</sub> at 37 °C for 6 h.

*Quantitative real-time PCR (qRT-PCR).* qRT-PCR was employed for the expression profile of mRNAs in tissues and cells. The total RNAs were extracted using TRIzol Reagents (Sigma, USA) and the RNAs concentration were determined by Nanodrop 2000 (Molecular Devices). Then, the DNA Polymerase (APExBIO, USA) with M-MLV RT (Promega, USA) were applied for cDNA synthesis. qRT-PCR was conducted with miScript SYBR Green PCR Kit (QIAGEN, Germany) in accordance with the manufacturer's protocols. The thermocycling condition was set by 96 °C for 15 min and the PCR reaction was set as 96 °C for 5 min (initiation), 94 °C for 30 sec (denaturation), 65 °C for 30 sec (annealing) and 70 °C for 1 min (extension) for 40 cycles respectively. The GAPDH was used as internal control. Then, the relative expression of mRNAs was quantified by 2<sup>(-Delta Delta C(T))</sup> Method [10].

*Western blot.* The proteins were isolated from cells using RIPA lysis buffer (Thermo Fisher Scientific, USA) and BCA Kit (Thermo Fisher Scientific, USA) were adopted for evaluating the concentrations of proteins. Subsequently, the protein samples with 2 µl/per lane were separated by 15% SDS-PAGE. Then, the proteins were transferred to PVDF membrane (Bio-Rad) blocked with 5% skimmed milk for 2 h prior to the antibodies block. After that, the membrane were cultured with primary antibodies including anti-IL-21 (ab154767, 1:2000, Abcam, USA), -PI3K (ab151549, 1:1000, Abcam, USA), -AKT (ab8805, 1:1000, Abcam, USA), -p-AKT (ab133458, 1:2000, Abcam, USA), -MMP-3 (ab53015, 1:1000, Abcam, USA), MMP-9 (ab73734, 1:1000, Abcam, USA), ICAM-1 (ab25375, 1:1000, Abcam, USA) and -GAPDH (ab181603, 1:10000, Abcam, USA) antibodies at room temperature for 1 h. Additionally, cells were incubated with HRP-conjugated secondary antibodies (ab6721, 1:1000, Abcam, USA) for 45 min at room temperature. ECL kit ((Thermo Fisher Scientific, USA) was used for visualization and the image was analyzed by ImageJ (WCIF, Canada).

*Scratch and transwell assay.* The scratch and transwell assay were performed for the evaluation of cell migration and invasion ability. For scratch assay, cells were placed into 6-well plates (2×10<sup>5</sup> cells/well) until the cells reached 90%. To obtain a monolayer cell culture to generate a scratch across the center of the wells. Then a 200 µl pipette tip was applied to scratch cells. Then, the cells were incubated with RPMI-1640 medium at 37 °C with 5% CO<sub>2</sub> for 48 h. Besides, the migrated cells were captured by microscope (ZEISS, Germany).

Cells were collected, suspended in non-serum DMEM, and moved onto the upper chamber with

8-μm pore size (Falcon; BD Biosciences, US). Furthermore, the upper chamber pre-coated with Matrigel (BD Biosciences, US) was incubated at 37 °C for 1 h for gel formation. The lower chamber was added with DMEM containing 10 FBS. Then cells were fastened with 4% paraformaldehyde and stained with 0.4% Trypan Blue. After 48 h incubation, the number of invading cells were quantified with microscope (Olympus, Japan, magnification, ×200).

### 3. Results

*Up-regulated IL-21 in breast cancer tissues.* In order to evaluate the expression profile of IL-21 in breast cancer tissues, the mRNA and protein of IL-21 was isolated and measured by qRT-PCR and western blot. As shown in Figure 1A, the mRNA level of the IL-21 was significantly up-regulated in breast cancer tissues compared with control group ( $p<0.01$ ). Furthermore, the protein level of IL-21 breast cancer tissues was significantly higher than that of normal cells (Figure 1B).

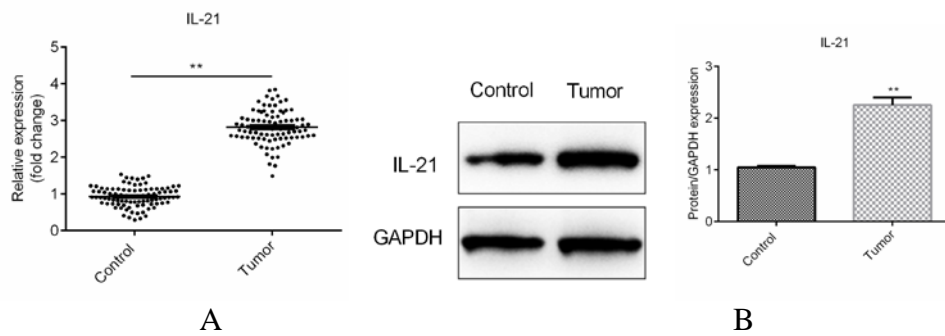


Figure 1 Upregulated IL-21 in breast cancer tissues

The expression of IL-21 in breast cancer tissues was evaluated at mRNA and protein levels by qRT-PCR and western blot. (A) Expression of mRNA of IL-21 in tissues; (B) Expression of IL-21 at protein level in tissues from one representative patient. Control: paracarcinoma tissues; Tumor: breast cancer tissues. \*\* $p<0.01$ .

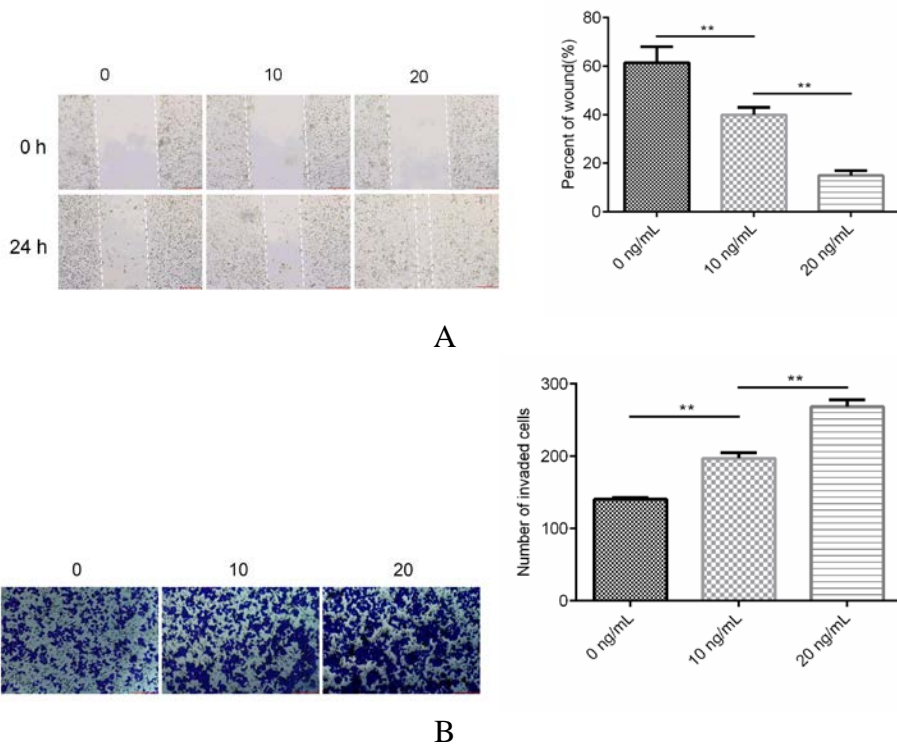


Figure 2 IL-21 can promote the migration and invasion of MCF-7 cells *in vitro*

The scratch and transwell assay were performed to investigate the impact of IL-21 on cell

migration and invasion. (A) Results of the scratch assay; (B) Results of the transwell assay. \*\* $p < 0.01$ .

*IL-21 promoted the migration and invasion of MCF-7 cells in vitro.* To further explore the effect of IL-21 on the migration and invasion of breast cells, MCF-7 cells were treated with IL-21, and the migration and invasion ability was determined by scratch and transwell assay respectively. It was observed that the migration rate of MCF-7 in IL-21 treated cells was significantly increased compared with the control group in a dose-dependent manner (Figure 2A). Moreover, treatment of IL-21 also markedly inhibited the invasion of MCF-7 cells *in vitro* (Figure 2B).

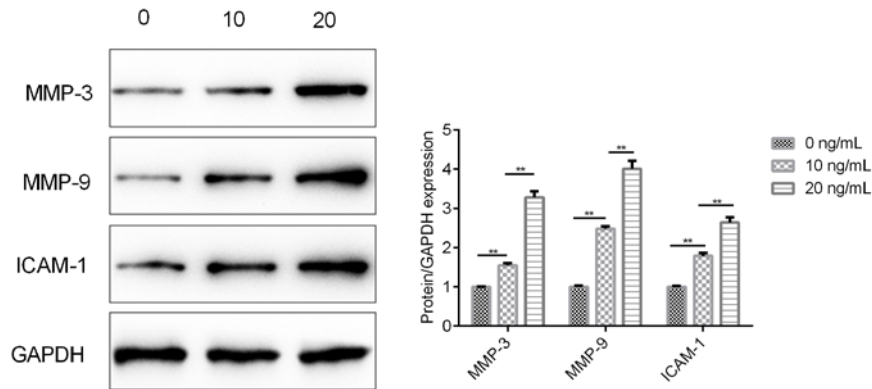


Figure 3 Effect of IL-21 on the expressions of migration and invasion related molecules in MCF-7 cells *in vitro*

The expressions of MMP-3, MMP-9, ICAM-1 and  $\beta$ -catenin were examined by western blot. GAPDH was regarded as the internal control. \*\* $p < 0.01$ .

*IL-21 can affect the expression of migration and invasion related proteins in MCF-7 cells in vitro*

Next, western blot assay was performed to measure the expression of proteins that might be associated with cell migration, invasion and growth. It was observed that IL-21 increased the expressions of MMP-3, MMP-9, ICAM-1 in a dose-dependent manner (Figure 3,  $p < 0.01$ ).

*IL-21 can affect the migration and invasion of MCF-7 cells via inhibiting the PI3K/AKT signaling pathway in vitro*

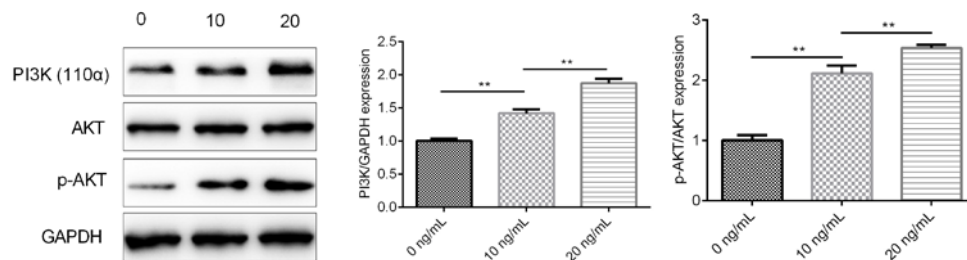


Figure 4 IL-21 can promote the migration and invasion of MCF-7 cells via regulating PI3K/AKT signaling pathway *in vitro*

The expressions of PI3K and AKT were examined by western blot. GAPDH was regarded as the internal control. \*\* $p < 0.01$ .

Finally, the effect of IL-21 on PI3K/AKT signaling pathway in MCF-7 cells was determined by western blot assay. It was observed that the expressions of PI3K and p-Akt were significantly increased compared with control group (Figure 4,  $p < 0.01$ ).

#### 4. Discussion

In the present study, we found that IL-21 was upregulated in breast cancer. IL-21 may function as an oncogene in breast cancer, which was consistent with the studies of Mittal D et al and Wang et al [9,11]. In our study, the results showed the IL-21 increased the expressions of PI3K, p-AKT, MMP-3, MMP-9, ICAM-1, which were associated with the migration and invasion of breast cancer.

Breast cancer is a multi-faceted disease involving complex interactions between neoplastic and normal cells. Previous studies defined the molecular, cellular and environmental contributions in the process of tumor development[12]. Recent studies highlighted the roles of IL-21 in different types of cancers, either as tumor suppressor or oncogene[6,8,13-17]. In the present study, we observed that IL-21 was up-regulated in breast cancer, which was consistent with previous findings; moreover, treatment of IL-21 can lead to increased migration and invasion ability of breast cancer cell line MCF-7, suggesting that IL-21 may exert carcinogenic effects via promoting the metathesis of the tumor.

MMP-3 and MMP-9 belong to a large family of zinc-dependent endopeptidases that is capable of degrading extracellular matrix components. MMP-3 and MMP-9 play vital roles in cell migration and invasion[18-20]. Meanwhile, the MMPs induced ECM degradation could not only eliminate the barriers to migration and invasion but also activate the molecular signaling pathways for breast cancer cells[19,21,22]. Intercellular adhesion molecule 1 (ICAM-1) is a surface glycoprotein, and it was reported that reported increased ICAM-1 expression was positively correlated with the metastatic and invasive of breast cancer. In the present study, the expressions of MMP-3, MMP-9 and ICAM-1 were significantly increased in IL-21 treated breast cancer cells. Therefore, IL-21 may inhibit the migration and invasion of MCF-7 cells via regulating the expression of MMP-3, MMP-9 and ICAM-1.

Additionally, PI3K/AKT signaling pathway has been closely correlated with the proliferation, differentiation and metastasis of breast cancer. However, it remains unclear whether IL-21 exerts its oncogenic role via activating the PI3K/Akt signaling pathway. In the present study, we found that IL-21 can increase the expression of PI3K and p-AKT, suggesting that IL-21 may promote the migration and invasion of MCF-7 cells via activating the PI3K/AKT signaling pathway.

Collectively, we investigated the effects of IL-21 on cell migration and invasion ability of breast cancer. We found that IL-21 may increase the migration and invasion of breast cancer cell via regulating PI3K/AKT signaling pathway. Therefore, inhibiting the expression of IL-21 in breast cancer would be an effective method to control the development of this disease. This may provide a clinical value to the treatment of breast cancer.

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