Upregulated IL-19 in breast cancer promotes tumor progression and affects clinical outcome

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Statement of Clinical Relevance

Evidence suggests that inflammation is associated with a poor prognosis in breast cancer. Inflammatory mediators, including cytokines and chemokines, in tumor microenvironments affect the progression of breast cancer. IL-19 expression in breast cancer tissue is not only associated with higher mitotic rate, advanced tumor stage and metastasis, but also predicted worse DSS and MFS with more than 3 fold increased risk. The IL-19 in breast cancer cells has an autocrine effect and provides a microenvironment for tumor progression. IL-19 not only directly promoted the proliferation and migration of cancer cells, but was also indirectly involved in tumor progression mediated through MMP2, MMP9, IL-1β, IL-6, TGF-β, CXCR4, and fibronectin. Therefore, antagonizing IL-19 could have therapeutic potentials in breast cancer.
Abstract

Purpose: IL-19 was expressed in invasive ductal carcinoma (IDC) of the breast tissue but not in healthy breast tissue. We explored the effects of IL-19 on the pathogenesis of breast cancer and its clinical outcome.

Experimental Design: Tumor expression of IL-19 was assessed by immunohistochemistry and/or real-time quantitative PCR among two groups of breast IDC patients ($n = 60$ and $143$, respectively) with available clinical and survival data. We examined the effects of IL-19 on cytokines and chemokines production as well as proliferation and migration in breast cancer cells. Mice were injected with IL-19-overexpressing- or vector-control-67NR-cells, the tumor growth and lung metastatic micronodules were measured.

Results: Of the IDC specimens, high IL-19 expression was associated with advanced tumor stage, high tumor metastasis, and worse survival. In vitro, IL-19 induced transcripts of IL-1β, IL-6, TGF-β, MMP2, MMP9, and CXCR4 in 4T1 breast cancer cells; induced fibronectin expression and assembly; and promoted cancer cell proliferation and migration, which were inhibited by anti-IL-19 monoclonal antibody (mAb). Endogenous fibronectin expression and cancer cell migration were lower in IL-19-knockdown 4T1 cells. In 4T1 cells, hypoxia induced IL-19 and CXCR4 expression, which was inhibited by anti-IL-19 mAb. IL-19 overexpression in non-invasive 67NR cancer cells increased cell proliferation and migration. In vivo, mice injected with IL-19-overexpressing-67NR-cell clones showed larger tumors and more metastatic micronodules in the lung.

Conclusions: High IL-19 expression in breast cancer tissue is associated with a poor clinical outcome. IL-19 is pivotal in the pathogenesis of breast cancer.
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Introduction

Breast cancer continues to be one of the leading causes of cancer-related mortality in women. Evidence suggests that inflammation is associated with a poor prognosis in breast cancer (1-2). Inflammatory mediators, including cytokines and chemokines, in tumor microenvironments affect the progression of breast cancer (3-6). Some cytokines, such as interleukin (IL)-1, IL-6, IL-11, and transforming growth factor beta (TGF-β), stimulate cancer cell proliferation and invasion (3). Cytokine receptor activation and intracellular signaling, by nuclear factor kappa B (NF-κB), for example, promote tumor progression (7). On the other hand, interferons, IL-2, and IL-12, all of which induce hormone sensitivity or stimulate cellular immunity, have been used for anti-tumor treatment of advanced breast cancer (8-9). Exploring which cytokines are involved in the pathogenesis of breast cancer thus may help improve therapeutic strategies.

IL-19 is a cytokine in the IL-10 family with multiple roles in immune regulation and disease (10-15). One important cellular source of IL-19 is monocytes, in which lipopolysaccharide (LPS), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, and tumor necrosis factor alpha (TNF-α) upregulate IL-19 expression (16-17). IL-19 binds to IL-20R1/IL-20R2, a heteroreceptor complex that activates STAT 3 (signal transducer and activator of transcription) and induces cell proliferation (15, 18-19). IL-19 induces apoptosis in lung epithelial cells, stimulates liver cells to produce reactive oxygen species (ROS), promotes neutrophil chemotaxis (19), and is involved in inflammatory diseases such as rheumatoid arthritis (20-21). Acutely induced IL-19 in systemic inflammation may promote lung and other tissue injury in mice undergoing endotoxic shock (22). IL-19 alters the balance of Th1 and Th2 cells in favor of Th2 cells (23). The chronic expression of IL-19 is correlated with Th2 cytokine production in patients with uremia and is associated with the pathogenesis of asthma and psoriasis (24-27). IL-19 also can induce the angiogenic potential
of endothelial cells (28).

We previously showed (29) that IL-19 was expressed in some malignant tumors, especially oral squamous cell carcinoma, renal cell carcinoma, invasive ductal carcinoma (IDC) of the breast, and hepatocellular carcinoma. We also showed that IL-19 was expressed more in breast cancer tissue than in healthy breast tissue, which suggests that IL-19 may be involved in the pathogenesis of breast cancer. Little is known about the function of IL-19 in breast cancer. Therefore, we examined the correlation between IL-19 expression in breast tumors and clinical outcome. We overexpressed or deleted IL-19 in cancer cells to investigate the effects of IL-19 on the tumorigenesis and metastasis of breast cancer in vitro and in vivo.
Materials and Methods

Patients and tissue specimens

This retrospective study was done in accordance with the guidelines of the Chi-Mei Medical Center Institutional Review Board (IRB9705-003). Primary localized IDC tissue samples of sixty patients obtained from the Chi-Mei Medical Center Tumor and Serum Bank between January 1999 and December 2001 were used for immunostaining and real-time quantitative (q)PCR analysis. Healthy tissue samples were from non-pathological areas distant from tumors in surgical specimens (confirmed by histology examination). Non-tumor tissue samples with signs of inflammation were excluded (30). The IDC samples for qPCR analysis included >70% tumor cells qualified by pathologist. The clinicopathologic variables evaluated are listed in Table 1. Another group of breast IDC samples of 143 patients obtained from the Pathology Archive of Chi-Mei Medical Center between January 1999 and December 2003 were used for immunostaining. The clinicopathologic variables evaluated are listed in the Supplementary Table S1.

Cell lines

Human (MCF7 and Hs578T) and mouse (67NR and 4T1) breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal bovine serum (Life Technologies, Inc., Rockville, MD), 2 mmol/L of L-glutamine (Life Technologies), 100 μg/mL of streptomycin, and 100 units/mL of penicillin in a humidified 5% CO2 atmosphere at 37°C.

Expressing and purifying IL-19 recombinant protein

A cDNA clone coded for the human (h)IL-19 sequence from leucine to leucine (amino acids 25 to 176) was inserted into the expression vector of *Pichia pastoris* (pPICZ-α; Invitrogen, San Diego, CA). We used affinity chromatography to express and purify hIL-19.
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from the culture medium of the yeast cells (24).

**Generating mouse monoclonal antibody (mAb) against hIL-19**

Mouse mAb against hIL-19 (1BB1) was generated followed the standard protocols (24, 31). We previously reported (22, 29) that 1BB1 neutralized hIL-19. To determine the specificity of IL-19 monoclonal antibody (1BB1), the human IL-10 family including IL-10, -19, -20, -22, -24, and -26 were coated on the plate with various concentrations and analyzed for their binding with 1BB1 (1 μg/ml) using direct ELISA (Supplement Figure S1).

**Immunohistochemistry**

Paraffin-embedded-tissue samples were used for immunohistochemical staining with purified 1BB1 (diluted 1:50) at 4°C overnight (26, 29). Incubating paraffin tissue sections with mouse IgG1 isotype (clone 11711; R&D Systems, Minneapolis, MN) instead of primary antibody was the negative control. Two investigators trained in breast pathology and blinded to the sample sources analyzed the histology and the IL-19 expression levels of at least five sections from each patient. The scoring of immunohistochemical stains in each specimen was determined using a histological (H) score (32). The IL-19 immunostaining was labeled low-grade (H < 200) or high-grade (H ≥ 200).

**Knockdown of IL-19 gene expression**

A short hairpin (sh) mIL-19 RNA constructed with the pGIPZ lentiviral vector was purchased from Thermo Fisher Scientific (Waltham, MA). We silenced mIL-19 gene expression in 4T1 cells using a reagent (Lipofectamine2000; Invitrogen) and the silencing vector (60 pmol/L) and then puromycin, according to the manufacturer’s protocols. A pGIPZ non-silencing vector was used as a negative control.

**IL-19 overexpression in breast cancer cell lines**

Human and mouse IL-19 coding sequences were constructed in PcDNA3.1 and PCEP4 vectors, respectively. MCF-7 and 67NR cells were transfected with the above vector
constructs or vector alone using Lipofectamine 2000 and then neomycin or hygromycin, according to the manufacturer’s protocols.

**Animal experiments**

Female BALB/c mice (age: 6-8 weeks) were used in all experiments. The mice were housed at 20°C in individually ventilated sterile cages. All cage accessories were sterilized and autoclaved before they were placed in the cage. The left mammary fat pad of each mouse was injected with 67NR-IL-19 or 67NR-vector \((2 \times 10^6 \text{ cells})\), as indicated. The growth of the mammary tumor was evaluated by measuring its size every day. Fifteen days after the tumor cells had been injected, the tumors were surgically removed and measured. Thirty days after the removal of tumors, the mice were killed and the lungs were then embedded in paraffin and three non-sequential serial sections per animal were obtained. The presence of metastases was analyzed by light microscopy with hematoxylin/eosin staining. Total number of metastatic micronodules per lung section was counted and averaged among the animals.

**Immunocytochemistry and immunofluorescence**

Anti-hIL-19 (1BB1), anti-hIL-20R1, rat anti-mIL-19, anti-mIL-20R1 mAb (from R&D systems, Minneapolis, MN), and anti-hIL-20R2 and anti-mIL-20R2 mAb (from Abcam, Cambridge, Massachusetts) were used to perform immunocytochemical staining. The immunocytochemical staining was performed as described previously (33). Immunocytochemical and immunofluorescence staining of fibronectin were done using anti-fibronectin mAb (R&D Systems) following the manufacturer's instructions.

**Western blotting**

Western blotting was done using antibodies specific for phosphor-STAT3, -JNK, -ErK, -Akt, and -NFκB (Cell Signaling Technology, Inc., Beverly, MA) or mIL-19, fibronectin, and CXCR4 (R&D Systems) or HIF-1α and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) following the manufacturer's instructions.
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Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using a reagent (RNA-Bee; Tel-Test Inc., Friendswood, TX), and then the total RNA underwent reverse transcription according to the manufacturer’s instructions. IL-19 and CXCR4 were amplified using PCR with gene-specific primers (Supplemental Table S1). PCR products were visualized on 2% agarose gels containing ethidium bromide. β-Actin amplification was used as an internal control.

Real time PCR analysis

Real time quantitative PCR was done using the LightCycler-Fast Start DNA Master SYBR Green I kit (Roche, Indianapolis, IN) according to the manufacturer’s instructions (29). IL-1β, IL-6, IL-19, TGF-β, MMP2, MMP9, and CXCR4 were amplified using PCR with gene-specific primers (Supplemental Table S1).

Flow Cytometry

For cell cycle analysis, synchronized cells were cultured without FBS for 16 h. The cells were treated with IL-19 (200 ng/mL) for 12 h and then fixed in 95% ice-cold ethanol, and stored at −20°C overnight. After PBS washing, the cells were stained for DNA in a solution containing 0.5 mL of RNase (Sigma-Aldrich; 1 mg/mL) and 0.5 mL of propidium iodide (500 μg/mL) for 30 min in the dark and then were flow cytometrically analyzed (FACScan; Becton Dickinson, San Jose, CA). The fraction of cells present in each cell-cycle phase (G1, S, and G2-M) was determined using a software (WinMDI; The Scripps Research Institute, La Jolla, CA) analysis of DNA histograms.

Cell proliferation assay

To determine the effect of IL-19 on cell proliferation, MCF-7 and 4T1 cells were exposed to various concentrations of hIL-19 or mIL-19, as indicated, for 24 h. Cell proliferation was assessed by BrdU incorporation using a BrdU ELISA colorimetric assay (Roche, Indianapolis, IN). To determine the proliferation of IL-19-knockdown cells and
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IL-19-overexpressing cells, the cells were initially plated at a density of $2 \times 10^5$ per 60-mm dish. After the cells had been incubated, they were counted using a hemocytometer and then plotted.

**Cell migration assay**

Cell migration was measured using a modified Boyden chamber with a polycarbonate filter with 8-μm pores (Nucleopore, Inc., Cabin John, MD) (22). The cells were treated with hIL-19 or mIL-19 (400 ng/mL), as indicated, and run over for 6 h.

**Wound Healing Assay**

A "wound healing" assay was used to study alterations in cell motility and migration (34). Microphotographs were taken at 0 h and 24 h or 36 h, as indicated. The percentage of wound healing was quantitatively analyzed by measuring the distances across the wound (N = 20) at the indicated times, and then dividing that by the distance measured at 0 h for each cell line.

**Follow-up and statistical analysis**

Statistical analysis was done using SPSS 14.0 (SPSS Inc., Chicago, IL). Associations and comparisons of IL-19 expression with various parameters were evaluated using Student’s t tests or Spearman correlation analysis. In this cohort, 60 patients had available follow-up data in August 2009 (median, 82.5 mo; mean, 59.6 mo; range, 1-127 mo); 143 patients from another achieve had available follow-up data in August 2010 (median, 103.6 mo; mean, 89.2 mo; range, 5.6-143.6 mo). The endpoint analyzed was the disease-specific survival (DSS) and metastasis-free survival (MFS) of patients with low- and high-grade IL-19 expression, assessed using Kaplan-Meier methods and compared using a log-rank test. A χ² test, Fisher’s exact test, Student’s t test, or Kruskal-Wallis one-way analysis of variance (ANOVA) test, as indicated, and then Dunn’s test, were used. Significance was set at P < 0.05.
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Results

**IL-19 expression in tumor tissue was correlated with tumor metastasis and clinical outcome**

Sixty IDC of the breast tissue samples were immunohistochemically (IHC) stained with 1BB1. Staining intensity was high-grade in 38 samples (Figure 1Aa) and low-grade in 22 (Figure 1Ab). Healthy breast tissue samples were not stained (Figure 1Ac). RT-qPCR showed that transcript levels of IL-19 in healthy breast tissue, high-grade IL-19-stained tissue (IL-19-H), and low-grade IL-19-stained tissue (IL-19-L) were compatible with IHC staining results (Figure 1Ad, Table 1). IL-19 expression levels in breast tumors were related to human epidermal growth factor receptor (HER)-2 status (Table 1). High IL-19 expression was associated with advanced tumor stage and a high incidence of lymph-node metastasis and distant metastasis on follow-ups (Table 1). There were no significant differences in serum levels of IL-19 between the IL-19-H and IL-19-L groups (data not shown). Mitotic figures are an important characteristic of malignant tumor progression. The number of mitotic figures was correlated with the IL-19 expression level in tumor tissue (Figure 1B). DSS for IL-19-L patients was higher than for IL-19-H patients (Figure 1C). To test using IL-19 expression as a prognosticator in breast IDC, we determined clinical outcome in the second achieve including 143 IDC cases using IHC staining. Of the 143 breast IDC samples, 75 were IL-19-H and 68 were IL-19-L (Supplementary Table S1). A strong correlation was substantiated between IL-19 expression with primary tumor status (T) (P = 0.03), nodal status (P = 0.001), advanced stage (P < 0.001), and HER-2 expression (P = 0.009) (Supplementary Table S1). The findings of strong associations between IL-19 expression and several adverse clinicopathologic prognosticators suggested its crucial role in tumor progression of breast cancer. In survival analysis, IL-19 expression was significantly associated with inferior MFS (P < 0.0001) (Supplementary Table S2, Figure 1D). In multivariate analysis, IL-19 expression remained a
IL-19 upregulated in breast tumors with a HR 3.322 (Supplementary Table S3).

**IL-19 induced proliferation and migration in breast cancer cells**

We analyzed the expression of IL-19 and its cognate receptors IL-20R1/IL-20R2 in human and mouse breast cancer cell lines using RT-PCR and immunocytochemical staining. IL-19 mRNA expressed in Hs578T and 4T1 cells but little in MCF-7 and 67NR cells, and all four lines expressed IL-20R1/IL-20R2 mRNA (Figure 2A). Immunocytochemical staining showed that IL-19, IL-20R1, and IL-20R2 were expressed in 4T1 (Figure 2B) and Hs578T cells (Supplement Figure S2A). 67NR and MCF-7 cells were also stained positively for IL-20R1 and IL-20R2 (data not shown). We treated breast cancer cells with IL-19 to determine whether IL-19 directly activated intracellular signals. mIL-19 induced STAT as well as JNK, Erk, AKT and NF-κB phosphorylation in 4T1 cells (Figure 2C). hIL-19 also induced JNK, Erk, and AKT phosphorylation in Hs578T (Supplement Figure S2B) and MCF-7 cells (data not shown). We treated MCF-7 and Hs578T cells with recombinant hIL-19 and then evaluated cell proliferation using a BrdU incorporation assay. hIL-19 promoted the proliferation of both types of cells and anti-hIL-19 mAb, 1BB1, inhibited that proliferation (Figure 2D). Recombinant mIL-19 also induced cell proliferation in 67NR and 4T1 cells (Supplement Figure S3A). Propidium iodide staining with flow cytometry showed that hIL-19 increased the G2/M stage of the cell cycle of MCF-7 cells (Figure 2E), which is consistent with increased cell proliferation. hIL-19 and mIL-19 also induced migration of Hs578T (Figure 2F) and 4T1 cells (Supplement Figure S3B), respectively. Anti-hIL-19- and anti-mIL-19 mAb inhibited the migration of Hs578T and 4T1 cells, respectively. Thus, IL-19 activated intracellular signaling and promoted proliferation and migration in breast cancer cells.

**IL-19 induced fibronectin expression and assembly**

Fibronectin expression and assembly, which are correlated with tumor progression and
promote pulmonary metastasis in breast cancer cells (35), were higher in MCF-7 cells, 4T1
cells, and Hs578T cells (Figure 3 A, B, C) after hIL-19 treatment. Furthermore, we used
shRNA to knock down endogenous IL-19 in tumor cells and analyzed whether it affected
fibronectin expression and assembly. IL-19-shRNA-4T1 (4T1 IL-19 knockdown) cells
showed lower fibronectin expression (Figure 3D) and assembly (Figure 3E) than did
control-shRNA-4T1 cells. Migration (Figure 3F) and proliferation (Figure 3G) in
IL-19-shRNA-4T1 cells were also lower than in control-shRNA-4T1 cells. However, mIL-19
protein treatment increased migration in both control-shRNA-4T1 and IL-19-shRNA-4T1
cells.

**IL-19 upregulated CXCR4, MMP2, MMP9, TGF-β, IL-1β, and IL-6 expression in
breast cancer cells**

Chemokine receptor CXCR4 is critical in breast cancer metastasis (36). To investigate
whether IL-19 induces CXCR4 expression, 4T1 cells were treated with mIL-19 and then
CXCR4 mRNA as well as protein levels were determined using RT-qPCR and Western
blotting, respectively. mIL-19 induced CXCR4 mRNA (Figure 4Aa) and protein (Figure 4Ab)
expression in 4T1 cells, and hIL-19 induced CXCR4 expression in MCF7 cells (data not
shown).

Hypoxia in breast cancer affects tumor metastasis and is associated with a poor clinical
outcome (37-38). Hypoxia upregulates CXCR4 expression in breast cancer (39). We
hypothesized that hypoxia-induced CXCR4 expression in breast cancer was mediated through
IL-19 expression. To test this possibility, we treated MCF-7 cells with CoCl2 (300 ng/mL) to
determine the mRNA levels of hIL-19 and CXCR4 using RT-qPCR. Both IL-19 and CXCR4
were upregulated in response to hypoxia treatment (Figure 4Ba). We next treated MCF-7
cells with CoCl2 or CoCl2 combined with 1BB1 for 24 h to determine whether 1BB1 affected
hypoxia-induced CXCR4 expression. CoCl2 induced CXCR4 protein expression in MCF-7
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cells, which was attenuated by 1BB1 treatment (Figure 4Bb). These data indicated that hypoxia-induced CXCR4 expression is mediated at least in part by IL-19. We also treated MCF-7 cells in a hypoxia chamber for 24 h with anti-hIL-19 mAb (1BB1) or control IgG. CXCR4 mRNA expression levels were significantly higher in hypoxia-treated cells than in control (normoxia) cells, but significantly lower in 1BB1-treated cells than in PBS control (data not shown). This result is consistent with CoCl2 treatment. Both MMP2 and IL-6 are involved in the interaction between tumor cells and endothelial cells and important in metastasis (40-43). IL-1β, IL-6, and TGF-β are also involved in tumor progression (3). RT-qPCR showed that IL-1β, IL-6, TGF-β, MMP2, and MMP9 were upregulated in IL-19-treated 4T1 cells (Figure 4C).

**IL-19 overexpression promoted 67NR cell tumor growth and lung metastasis**

67NR cells are a relatively non-invasive mouse breast cancer cell line with little endogenous IL-19 expression (Figure 2A). To investigate the effect of IL-19 overexpression on 67NR cells and to determine whether IL-19 causes the proliferation and metastasis of these cells in vitro and in vivo, we constructed IL-19-overexpressing-67NR-cell clones (67NR/IL-19). IL-19 overexpression in 67NR cells promoted proliferation and migration (Figure 5Aa and 5Ba, respectively). Similar results were found in IL-19-overexpressing MCF-7 cells (Figure 5Ab and 5Bb). We injected 67NR cells into the left mammary fat pads of BALB/c mice. The growth of tumors was larger in mice injected with 67NR/IL-19 cells than in those injected with control vector-67NR cells (Figure 5Ca). We removed the tumors at 15 days after 67NR cells injection. The tumor sizes were 0.19±0.02 g and 0.10±0.02 g (P=0.036) in 67NR/IL-19 and 67NR/vector cells injected mice, respectively (Figure 5Cb). IL-19 mRNA levels in 67NR/IL-19 tumors were higher than in control vector-67NR tumors (Figure 5Cc). Immunohistochemical staining showed strong IL-19 and fibronectin stained in 67NR/IL-19 but not 67NR/vector tumors (Figure 5Cd). Thirty days after removal of tumors,
the mice were sacrificed and lung metastasis was determined using histology analysis. More lung metastatic micronodules were observed in 67NR/IL-19 injected mice than in control mice (Figure 5D).
Discussion

We showed that IL-19 is involved in the pathogenesis of breast cancer. IL-19 expression in breast cancer tissue is not only associated with higher mitotic rate, advanced tumor stage and metastasis, but also predicted worse DSS and MFS with more than 3 fold increased risk. IL-19 directly affected breast cancer cell proliferation and migration and indirectly affected tumor progression by inducing the expression of cytokines and chemokines. Because no difference was observed in the serum level of IL-19 between patients with high and low IL-19 expression in tumors, it is possible that IL-19 acted primarily as a local mediator in the microenvironment that affects breast cancer cells. In addition, breast cancer cells also expressed IL-19 receptors, which indicated that IL-19 may act in an autocrine manner in breast tumors.

In this study, exogenous IL-19 significantly induced proliferation and migration, which were antagonized by anti-IL-19 mAb. However, the anti-IL-19 mAb did not inhibit the migration of Hs578T cells in PBS control group. We speculated that the concentration of endogenous IL-19 in cultured medium produced by untreated Hs578T cells might be too low to have detectable biological function as exogenous IL-19 did. The little migration activity observed in untreated cells might be attributed to some factors other than IL-19 or it was a nonspecific migration activity.

Hypoxia, which is common in solid tumors, may induce tumor progression by increasing angiogenesis and the expression of chemokines and chemokine receptors, such as CXCR4, which are associated with tumor cell migration (44-45). We showed that hypoxia induced IL-19 as well as CXCR4 expression in breast cancer cell lines. Our preliminary study also identified a functional hypoxia-response element on IL-19 promoter (data not shown) that may explain the upregulation of IL-19 in breast tumor samples. Because 1BB1 reduced hypoxia-mediated CXCR4 expression, we conclude that hypoxia-induced CXCR4 is
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mediated at least in part by IL-19 expression. Therefore, IL-19 may be an upstream molecule that drives CXCR4 expression and tumor metastasis in hypoxia. HER-2 expression is also associated with breast cancer cell progression. HER2 may induce CXCR4 expression, which is important in breast cancer metastasis (46). We found a significant correlation between IL-19 and HER-2 expression. Our in vitro experiments also showed that IL-19 induced CXCR4 expression in MCF-7, which suggested an IL-19/CXCR4 pathway affects breast cancer metastasis. However, the association between IL-19 and HER-2 gene expression and their crosstalk on CXCR4 production await further investigation.

Breast cancer patients frequently develop lung metastasis. Fibronectin assembly is critical for lung metastasis (35), presumably because it facilitates pulmonary vascular arrest via endothelial dipeptidyl peptidase IV (CD26) (47). We showed that IL-19 induced fibronectin expression and assembly in breast cancer cells. Thus, our data further support the relevance of IL-19 expression to lung metastasis in breast cancer.

IL-19 not only directly affected cancer cells by promoting their proliferation and migration, but also induced the expression of IL-1β, IL-6, TGF-β, MMP2, MMP9, CXCR4, and fibronectin, all of which are involved in cancer cell proliferation (IL-1β, IL-6, TGF-β), migration or metastasis (MMP2, MMP9, CXCR4, and fibronectin), or angiogenesis (MMP2 and MMP9) (3, 41). A variety of direct cell-cell, cell-matrix, and paracrine interactions are involved in metastasis. MMP and IL-6 are also involved in endothelial cell injury when tumor cells cross the endothelial barrier (41). Cytokines are intercellular mediators that regulate survival, growth, differentiation, and the effector functions of cells (8, 48). They also represent a network with a large variety of different members that may promote tumor growth. Thus, we consider that IL-19 produced by breast cancer cells promotes tumor progression not only by its autocrine effect, but also by providing a microenvironment for tumor growth and metastasis. Furthermore, cytokines are mediators of the effector response from innate and
acquired cellular immunities (49-50); they are probably involved in the mechanism for tumor cell evasion in the immunosurveillance system. This might also be one of the mechanisms by which overexpression of IL-19 in breast cancer cells promoted tumor growth and metastasis in vivo.

In this study, the ability of tumor growth and metastasis of 67NR cells was significantly promoted after the cells transfected with IL-19 gene. We noted that the IL-19 transfected tumors were almost twice the size of the control ones and therefore this could easily be the reason for the increased metastases. More evidence of direct effect of IL-19 on tumor metastasis in vivo remains to be investigated. Nevertheless, IL-19 indeed has a tremendous contribution to make a non-invasive cell to have more invasive biological behavior. These findings support the effects of IL-19 on breast cancer. Thus, antagonists against IL-19 might have therapeutic potential in breast cancer therapy. More informative in vivo models such as orthotopic implantation model and use of fluorescent protein to visualize cancer in real time (51-52) may confirm the therapeutic role of anti-IL-19 antibody in breast cancer treatment.

In summary, our findings provide evidence that IL-19 is an important mediator in breast cancer. IL-19 expression in breast cancer is correlated with lymph node metastasis and distant metastasis. The DSS and MFS in patients with low IL-19 expression were higher than that in patients with high IL-19 expression. Our study demonstrated that IL-19 not only promoted the proliferation and migration of cancer cells, but was also involved in tumor progression mediated through MMP2, MMP9, IL-1β, IL-6, TGF-β, CXCR4, and fibronectin. We conclude that the IL-19 in breast cancer cells has an autocrine effect and provides a microenvironment for tumor progression. Therefore, antagonizing IL-19 could have therapeutic potentials in breast cancer.

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**Figure Legends**

**Figure 1. IL-19 expression in breast tumors was correlated with clinical outcome.**

(A) Immunohistochemical staining (IHC), showed that IL-19 was strongly (a) or weakly (b) stained in breast invasive duct carcinoma (IDC) cells (arrows) but not stained in healthy breast cells (c) (magnification, ×400). Mitotic figures (a, arrowhead) are commonly found in breast cancer cells strongly stained with IL-19. (d) A real time-quantitative polymerase chain reaction (RT-qPCR) confirmed that IL-19 mRNA expression levels in healthy breast tissue (n = 10), and that high-grade (IL-19-H, H ≥ 200, n = 38) and low-grade (IL-19-L, H < 200, n = 22) IL-19 mRNA expression levels in IDC tissue were compatible with levels detected using IHC. **P < 0.01 vs. healthy controls; *P < 0.05 vs. IL-19-H group. (B) Mitotic figures were correlated with IL-19 expression levels in breast cancer cells. The expression levels of IL-19 in 60 IDC tissue samples were analyzed using H scoring. HPFs, high power fields. (C) Kaplan-Meier plots were used to predict the disease-specific survival of 60 patients based on IL-19 expression levels. (D) Of the 143 patients from another achieve, Kaplan-Meier plots were used to predict the metastasis-free survival base on IL-19 expression levels.

**Figure 2. IL-19 activated intracellular signaling and promoted proliferation and migration of breast cancer cells.**

(A) Expression of mRNA of IL-19 and its receptors, IL20R1/R2, in human (MCF-7 and Hs578T) and mouse (67NR and 4T1) breast cancer cell lines were determined using RT-PCR. (B) Immunocytochemical staining showed that IL-19 and its receptors, IL-20R1 and IL-20R2, were expressed in 4T1 cells (magnification, ×400). Rat IgG was the negative control. (C) 4T1 cells (2 × 10^5) were treated with mIL-19 (200 ng/mL) for the indicated times. Cell lysate was then collected and phosphorylation of STAT3, JNK, Erk, AKT, and NFκB was determined using Western blotting. (D) MCF-7 and Hs578T cells (2 × 10^5) were cultured for 24 h in 2% FBS with or without hIL-19 protein (200 or 400 ng/mL). 1BB1 (8 μg/mL) was...
used to inhibit IL-19. Cell proliferation was determined using a BrdU ELISA colorimetric assay. (E) IL-19 increased the G2/M stage of the cell cycle of MCF-7 cells determined using propidium iodide staining with flow cytometry. (F) Hs578T cell migration was measured after the cells had been treated with hIL-19 (400 ng/mL) for 6 h. 1BB1 (8 μg/mL) was used to inhibit IL-19. Cell migration was evaluated using a modified Boyden chamber assay.

**Figure 3. The effect of IL-19 on fibronectin expression and assembly in breast cancer cells.** MCF-7 cells were treated with hIL-19 (200 or 400 ng/mL) for 24 h, and then fibronectin was determined using (A) immunostaining (magnification, ×100) or (B) Western blotting. 1BB1 (8 μg/mL) was used to inhibit hIL-19. Data are means ± SEM of triplicate experiments. *P < 0.05 vs. PBS group; #P < 0.05 vs. hIL-19 group. (C) 4T1 and Hs578T cells were treated with mIL-19 (300 ng/mL) and hIL-19 (300 ng/mL) respectively for 24 h, and then fibronectin assembly was analyzed using immunofluorescence staining (magnification, ×400). IL-19 increased fibronectin assembly (arrows) on the cells. (D) IL-19 protein expression in control-shRNA- and IL-19-shRNA-4T1 (4T1 IL-19 knockdown) cells was analyzed using immunoblotting. (E) There was less fibronectin assembly on IL-19-shRNA-4T1 cells than on control-shRNA-4T1 cells (arrows) (magnification, ×400). (F) Control-shRNA- or IL-19-shRNA-4T1 cells were treated with PBS or mIL-19, and cellular migratory activity was evaluated using a modified Boyden chamber assay. Data are means ± SEM of triplicate experiments. *P < 0.05 vs. control shRNA group, #P < 0.05 vs. PBS treatment. (G) Proliferation of control-shRNA- and IL-19-shRNA-4T1 cells. Data are means ± SEM of the number of cells counted in triplicate experiments. *P < 0.05 vs. IL-19-shRNA group.

**Figure 4. IL-19 promoted hypoxia-induced CXCR4 and upregulated MMP2, MMP9, TGF-β, IL-1β, and IL-6 in breast cancer cells.** (A) 4T1 cells were treated with mIL-19
IL-19 upregulated in breast tumors (400 ng/mL) for the indicated times, and then CXCR4 mRNA and protein were analyzed using (a) RT-qPCR and (b) Western blotting. *P < 0.05 vs. untreated group (UT). (B) (a) MCF-7 cells were treated with CoCl₂ (300 ng/mL) for 6 h, and then the mRNA levels of IL-19 and CXCR4 were analyzed using RT-PCR. (b) MCF-7 cells were treated with CoCl₂ (300 ng/mL) or CoCl₂ plus 1BB1 for 24 h, and then HIF-1α and CXCR4 protein levels were analyzed using Western blotting. The expression of HIF-1α protein was a control to monitor the mimetic of hypoxia. (C) 4T1 cells were treated with mIL-19 (400 ng/mL) for the indicated times, and the expression levels of IL-1β, IL-6, TGF-β, MMP2, and MMP9 was analyzed using RT-qPCR. *P < 0.05 vs. untreated group. All data are means ± SEM of triplicate experiments.

Figure 5. IL-19 overexpression promoted breast cancer cell proliferation and migration in vitro and induced tumor growth and metastasis in vivo. We constructed the IL-19-overexpressing stable clones of MCF-7 (MCF-7/IL-19) and 67NR (67NR/IL-19) cells. (A) Proliferation of MCF-7/IL-19 and 67NR/IL-19 cells was significantly higher than that of control cells. Data are means ± SEM of triplicate experiments. *P < 0.05 vs. control-vector cells. (B) Cell migration, determined using a wound healing assay, was greater in MCF-7/IL-19 and 67NR/IL-19 cells than in control-vector cells. Quantitative results are shown as a wound healing index. Data are means ± SEM. *P < 0.05 vs. control-vector cells. (C) 67NR cells (2 × 10⁶) were injected into the left mammary fat pads of BALB/c mice and growth of tumor was determined (a). Fifteen days later, the tumors were removed and tumor size was measured (b). IL-19 mRNA expression levels in the tumors were analyzed using RT-PCR (c). Expression of mIL-19 and fibronectin in tumors were determined using immunohistochemical staining (d). (D) Thirty days after surgical removal of tumor, the mice were killed and histological analysis showed that lung metastasis (arrows) was significantly higher in 67NR/IL-19 cells than in control cells (magnification, ×100). Data are means ±
SEM (n = 6 mice in each group). *P < 0.05 vs. control mice.
IL-19 upregulated in breast tumors
IL-19 upregulated in breast tumors

References


17. Hsing CH, Hsieh MY, Chen WY, Cheung So E, Cheng BC, Chang MS. Induction of


Table 1. Associations between IL-19 protein and mRNA expressions in 60 breast invasive duct carcinoma tumors with other important clinicopathologic variables.

<table>
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<th>Parameters</th>
<th>Category</th>
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<th>IL-19-H (^2) (n = 38)</th>
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</table>

IL, interleukin; HER2, human epidermal growth factor receptor 2;

\(^1\)IL-19-L: Low grade immunostaining, H score < 200;

\(^2\)IL-19-H: High grade immunostaining, H score ≥ 200;

\(^3\)Data were normalized to β-actin and relative to healthy breast tissue;

\(^4\)HER scoring was done using standard HercepTest guidelines;

\(^#\), Expressed as mean ± SD; *, Statistically significant.
Figure 1. Hsing et al

A

B

C

D

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Figure 2. D.-F. Hsing et al

D

MCF-7

Hs578T

E

Events

Propidium Iodide

PBS

IL-19

IL-19+5B1

F

PBS

IL-19

IL-19+5B1

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Figure 4. Hsing et al

A

\[\text{CXCR4 mRNA (fold of increase)}\]

![Graph showing CXCR4 mRNA levels](image)

\[\text{CXCR4} \quad \text{mL-19} \]

PBS 12 24 h

CXCR4

\[\beta\text{-Actin}\]

B

\[\text{IL-19} \quad \text{CoCl}_2 \]

![Graph showing IL-19 levels](image)

\[\text{CXCR4} \quad \text{CoCl}_2 +1 \text{BB1} \quad \text{CoCl}_2 +1 \text{gG}\]

HIF-1 \(\alpha\)

\[\beta\text{-Actin}\]

C

\[\text{IL-1\beta} \quad \text{IL-6} \quad \text{TGF-beta} \quad \text{MMP2} \quad \text{MMP9}\]

![Bar charts showing mRNA levels](image)
Figure 5. Hsing et al

A

B

C
Figure 5 Ccd&D. Hsing et al

C

C

D

D
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Upregulated IL-19 in breast cancer promotes tumor progression and affects clinical outcome


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