ING5 Inhibits Migration and Invasion of Esophageal Cancer Cells by Downregulating the IL-6/CXCL12 Signaling Pathway

Yali Wang¹, Jiao Tan¹, Jing Li¹, Huihui Chen¹, and Wei Wang¹

Abstract
Esophageal squamous cell carcinoma (ESCC) is a common cancer in East Asia and in other parts of the world and exhibits a poor prognosis. Growth inhibitor 5 (ING5) is a new member of the growth inhibitor (ING) protein family and is involved in many important cellular functions, such as the cell cycle, apoptosis, and chromatin remodeling. As a newly discovered tumor suppressor, ING5 has been shown to inhibit lung cancer proliferation and distant metastasis through the AKT pathway. In lung cancer tumors, ING5 can attenuate the ability of cancer cells to invade normal tumor-adjacent tissues. However, ING5 has rarely been studied in ESCC. Here, we found that in ESCC EC-109 cancer cells, ING5 overexpression inhibited cell proliferation and tumor invasion, whereas, in ESCC TE-1 cancer cells, ING5 knockdown promoted cell invasion. In a nude mouse xenograft model, ING5 overexpression inhibited tumor growth and the invasion ability of ESCC cells. Further studies revealed that ING5 overexpression inhibited IL-6/CXCL12 expression at both the mRNA and protein levels as well as morphological changes. We found for the first time that ING5 inhibits ESCC cell migration and invasion by downregulating the IL-6/CXCL12 signaling pathway.

Keywords
esophageal cancer, growth Inhibitor 5, IL-6, signaling pathway

Abbreviations
ESCC, esophageal squamous cell carcinoma; ING5, growth inhibitor 5; MEF, myeloid Elf-1 like factor

Introduction
Esophageal cancer is one of the most aggressive malignancies and the sixth leading cause of cancer-related death worldwide. Esophageal squamous cell carcinoma (ESCC) or adenocarcinoma is the most common primary esophageal cancer.¹² Research has shown that 90% of esophageal cancer cases are ESCC in the highest-risk areas in southern Africa and some Asian regions.³ Due to the continuous advancement and development of medical technology, many new treatment options have been used to treat ESCC, such as early screening or surgery combined with chemotherapy and radiotherapy.⁴⁵ As the fourth most common cancer in China, esophageal cancer has a poor prognosis.³ Esophageal cancer is a multiple recurrent cancer with high mortality. Therefore, there is an urgent need to elucidate the molecular mechanisms underlying esophageal cancer proliferation and to develop effective molecular therapeutic targets.

During ESCC tumor metastasis, local tumor infiltration only affects surrounding tissues, especially interstitial cells. Tumor cells enter the circulatory system by infiltration, and tumor cells entering the circulatory system must survive in a flowing environment.⁶ Tumor cells in the circulatory system are removed from the circulatory system by extravasation, they enter distant tissues, they form tiny metastatic clones, and then they form visible metastases via proliferation.⁷⁻⁹ The IL-6/CXCL12 signaling pathway is closely related to the proliferation, metastasis, and invasion of ESCC tumors.

Preliminary studies on ING5 have shown that ING5 can interact with P300 and P53, causing apoptosis in colorectal cancer cells.¹⁰ ING5 is a new member of the ING family, and
multiple functions of ING5 have been reported since it was first discovered through a homologous search.\textsuperscript{11} ING5 overexpression inhibits the growth of myeloid Elf-1 like factor (MEF), induces cell arrest in the S phase, and increases fast-induced apoptosis.\textsuperscript{12} These features of ING5 depend on the role of INCA1. Studies have shown that the interaction of ING5 with INCA1 can inhibit the activity of cyclin A1/CDK2.\textsuperscript{13} The results of this study all pointed out that ING5 plays an important role in the processes of cell proliferation, apoptosis, and differentiation, indicating that ING5 is closely related to the occurrence and development of tumors. However, in ESCC, the exact role and potential mechanism by which ING5 acts as a tumor suppressor gene are not clear.

The IL-6/CXCL12 signaling pathway was originally discovered as a participant in embryonic development.\textsuperscript{14} During tumor cell development, this pathway causes tumor cells to lose some of their epithelial cell characteristics and to acquire mesenchymal cell characteristics; at the same time, this pathway gives tumor cells strong invasion and motility abilities.\textsuperscript{15} Studies have shown that multiple molecular signaling pathways in tumor tissues can activate the IL-6/CXCL12 signaling pathway.\textsuperscript{16} For example, nuclear translocation of the HOP/HSP90 complex-mediated transcription factor Snail promotes the IL-6/CXCL12 signaling pathway in gastric cancer; AIBP through HIF1 disrupts the IL-6/CXCL12 signaling pathway and inhibits HCC invasion and metastasis, and the ING4/miR-940/Snail1 signaling pathway regulates the IL-6/CXCL12 signaling pathway in colorectal cancer.\textsuperscript{15,16}

However, no relevant research has revealed the relationship between ING5 and IL-6/CXCL12 or the role of ING5 in tumors, especially ESCC.

In this study, we used EC-109 and TE-1 cell lines to establish ING5 overexpression and knockdown ESCC cell lines, respectively. Existing literature indicates that both EC-109 and TE-1 cells were derived from human esophageal squamous cell carcinoma organization and have the characteristics of proliferation and migration of cancer cells. It has the ability to form distal metastasis in vivo. We found that ING5 can inhibit ESCC cell proliferation, metastasis, and invasion in vitro and in vivo compared with the effects in normal cells. For animal experiments, we used a nude mouse model because the autoimmune systems of mice are incomplete, which is conducive to the successful construction of the model. The tumor-suppressive mechanism of ING5 may include downregulation of the IL-6/CXCL12 pathway. The above results indicate that ING5 acts as a tumor suppressor gene and plays an important role in ESCC proliferation and metastasis; thus, ING5 can be used as a potential therapeutic target.

**Materials and Methods**

**Cell Culture**

EC-109, TE-1, and human bronchial epithelial (HBE) cell lines were purchased from the Chinese Academy of Sciences Type Culture Collection Center, Shanghai, China. The cells were grown in DMEM (Gibco) supplemented with 10% fetal bovine serum (US HyClone), 10 mg/mL antibiotics (penicillin and streptomycin), and L-glutamine at 37°C in 5% carbon dioxide and saturated humidity.

**Cell Proliferation Assay**

The logarithmic growth phase cells were seeded into a 96-well plate at a cell density of 5 × 10^4 cells/mL. After successful transfection for 48 h, the cells were incubated for 1 to 3 days, followed by the addition of 20 μL of CCK-8 reagent per well. The Synergy H4 Hybrid microplate reader (BioTek) detects readings at 450 nm.

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**

The total RNA was isolated using TRizol reagent (Invitrogen). Total RNA extraction, reverse transcription, and polymerase chain reaction (PCR) were performed in strict accordance with the procedures of the product manual, and 1 μg of RNA was reverse-transcribed into cDNA. Upstream and downstream microRNA-21 and β-actin primers were designed. The following PCR conditions were used: predenaturation at 95°C for 30 s, followed by 95°C for 5 s and 60°C for 30 s for a total of 40 cycles. RNA (1 μg) was used with random primers and reverse transcribed using MMLV reverse transcriptase (Promega) according to the manufacturer’s protocol. Quantitative RT-PCR was performed with 1 μL cDNA, sequence-specific primers, and the SYBR green real-time PCR mixture (Applied Biosystems). The following thermal conditions were used: 95°C for 10 min and 35 cycles of 95°C for 15 min and 65°C for 1 min.

**Western Blotting**

The cells were seeded in a 6-well plate at a cell count of 1 × 10^6 cells/mL, and 3 replicate wells were set. After the cells adhered to the surface, the cells were treated as needed. After the cells were reoxygenated, the 6-well plates were removed, and the supernatant was discarded. After washing for 1 min with phosphate-buffered saline (PBS), lysis buffer containing a protease inhibitor was added, and the cell pellet was lysed and placed on ice. After lysis for 30 min, the cells were scraped off with a cell scraper, transferred to a 1.5-mL test tube, and centrifuged at high speed. The supernatant was collected to obtain the extracted protein. After protein quantification, sample buffer was added, and the samples were boiled for 5 min and then frozen at −80°C for storage. After SDS-PAGE, the protein was transferred to a membrane, which was blocked with skim milk, washed with phosphate-buffered saline with Tween20, cut into strips, and incubated with anti-IL-6 (ENZO, PSC-XP-5704-M-C500), anti-CXCL12 (Promocell, PK-AB718-7591P), and anti-β-actin (Proteintech, 20536-1-AP) antibodies (diluted 1:1000) at room temperature for 1 h and then overnight at 4°C. The strips were washed repeatedly the next day and incubated with secondary antibodies at room temperature for 90 min. The strips were washed with PBS.
We selected 2 common ESCC cell lines, EC-109 and TE-1. ING5 mRNA and protein levels were detected in EC-109 and TE-1 cells as well as in normal HBE cells. ING5 expression was higher in TE-1 cells and lower in EC-109 cells (Figure 1A and B) than in HBE cells. Therefore, we established a stable ING5 overexpressing (OE-ING5) cell line and an ING5 knockdown (KD-ING5) cell line (Figure 1C).

**Xenograft Studies**
Six-week-old male thymus nude mice were purchased from Shanghai Kai Li. They were randomly divided into a control group and an experimental group, with 5 animals in each group. Every 5 mice are kept in a $30 \times 30 \times 30$ mm$^3$ sterile cage. All animal procedures were performed in accordance with procedures approved by the Animal Care and Use Committee. The mice were injected subcutaneously with $6 \times 10^6$ EC-109 control cells or EC-109 ING5 cells. Tumor size was monitored twice weekly using calipers, and tumor volume was estimated. The model mice were injected intravenously with $6 \times 10^6$ EC-109 control cells or EC-109 ING5 cells. The mice were sacrificed 45 days after injection to examine the formation of lung tumors.

**Statistical Analysis**
For the in vitro studies, all experiments were performed in triplicate. The data were analyzed using the GraphPad Prism 6 software. Data from 2 groups were statistically compared using an unpaired Student’s t-test. $P$-values <.05 were considered significant for all analyses.

**Results**

**Successful Establishment of Stable ING5 Overexpression and Knockdown Cell Lines**

We selected 2 common ESCC cell lines, EC-109 and TE-1. ING5 mRNA and protein levels were detected in EC-109 and TE-1 cells as well as in normal HBE cells. ING5 expression was higher in TE-1 cells and lower in EC-109 cells (Figure 1A and B) than in HBE cells. Therefore, we established a stable ING5 overexpressing (OE-ING5) cell line and an ING5 knockdown (KD-ING5) cell line (Figure 1C).

**ING5 Inhibits the Proliferation, Migration, and Invasion of ESCC Cells**

Based on the ING5 overexpression and knockout cell lines, we examined the effects of ING5 on cell proliferation, migration, and invasion. The results showed that ING5 overexpression significantly inhibited the proliferation and colony formation ability of EC-109 cells, whereas ING5 knockdown in TE-1 cells promoted these effects (Figure 2A and B). Our results also indicate that ING5 overexpression inhibited the migration of EC-109 cells in a transwell migration assay, and these cells were also subjected to a healing test (Figure 2C to F). Overexpression of ING5 significantly prevented EC-109 cells from invading the transwell chamber through the matrix-coated polycarbonate filter (Figure 2D). In contrast, ING5 knockdown promoted the migration and invasion of TE-1 cells (Figure 2C to F). These results indicate that ING5 has an anti-tumor effect and inhibits cell invasion and proliferation.
ING5 Inhibits Tumor Growth and Invasion in Mouse Xenograft Models

To confirm the role of ING5 in tumor growth capacity, we established a mouse xenograft model with EC-109 ING5 and EC-109 control cells via subcutaneous injection. ING5 overexpression significantly inhibited tumor growth and formation and significantly reduced tumor volume (Figure 3A and B). The most common site of metastasis of ESCC cells in the body is the lung. We injected EC-109 ING5 and EC-109 control cells into the tail vein of mice. At 50 days after injection, 5 mice injected with EC-109 control cells produced tumors in the lung, while 5 mice injected with EC-109 ING5 cells had far fewer tumors than control group mice (Figure 3C).

ING5 Inhibits the IL-6/CXCL12 Signaling Pathway

To investigate whether ING5 reduces cancer invasion by inhibiting IL-6/CXCL12, we analyzed mRNA levels in ING5 overexpression and control EC-109 cells and in TE-1 cells. The qRT-PCR results showed that IL-6 and CXCL12 mRNA levels were decreased in ING5-overexpressing EC-109 cells, while IL-6 and CXCL12 mRNA levels were elevated in TE-1 knockdown cells (Figure 4A). We further examined changes in the protein levels of IL-6 and CXCL12 in ING5-overexpressing EC-109 cells and TE-1 knockdown cells via Western blotting and found decreased IL-6 and CXCL12 expression in ING5-overexpressing EC-109 cells elevated expression in TE-1 knockdown cells (Figure 4B).
ING5 belongs to the ING candidate tumor suppressor family. It has been reported that the overexpression of ING5 in colorectal cancer cells results in reduced colony formation efficiency through interactions with P53 and P300. In line with these previous results, the present data also showed that ING5 overexpression inhibited cell proliferation and colony formation in

**Discussion**

ING5 belongs to the ING candidate tumor suppressor family. It has been reported that the overexpression of ING5 in colorectal cancer cells results in reduced colony formation efficiency through interactions with P53 and P300. In line with these previous results, the present data also showed that ING5 overexpression inhibited cell proliferation and colony formation in

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NSCLC A549 cells, while the loss of ING5 promoted these processes in the NSCLC cell line H1299. Furthermore, we found for the first time that ING5 could inhibit ESCC migration and invasion in both in vitro and in vivo studies, suggesting a role of ING5 in preventing ESCC metastasis.

Figure 3. Overexpression of ING5 inhibits tumor growth and invasion of ESCC cells in vivo. (A) Mice were injected subcutaneously with $6 \times 10^6$ EC-109 control cells or EC-109 ING5 cells, $**P < .05$. (B) Isolation of tumors from mice, $**P < .05$. (C) Effect of ING5 overexpression on the lung colonization of tumors in xenograft model mice, $**P < .05$.

Abbreviations: ESCC, esophageal squamous cell carcinoma; ING5, growth inhibitor 5.

Figure 4. ING5 inhibits the IL-6/CXCL12 signaling pathway. (A) mRNA expression level of IL-6/CXCN12 in EC-109 cell lines and TE-1 cell lines determined by qRT-PCR, $**P < .05$. (B) Analysis of IL-6/CXCL12 protein levels in EC-109 and TE-1 cell lines, $**P < .05$.

Abbreviations: ESCC, esophageal squamous cell carcinoma; ING5, growth inhibitor 5; qRT-PCR, quantitative real-time polymerase chain reaction.
ING5 has been reported to be related to cell cycle progression and apoptosis. It has been reported that ING5 functions as a cofactor of Tip60 for the acetylation of p53 at K120, leading to the activation of p53,11,21 the expression of its target genes and subsequent apoptosis in response to DNA damage.19,22 Despite this, further studies are required to reveal the mechanisms underlying the anti-proliferation effect of ING5.

The invasive ability of ESCC cells is regulated by different signaling pathways, among which the IL-6/CXCL12 pathway is frequently activated and can result in tumorigenesis and progression.14 IL-6/CXCL12 pathway participates in the EMT process by regulating many molecules. For instance, the EMT-related transcription factor Snail has been widely investigated as a downstream molecule in the IL-6/CXCL12 pathway.23 We hypothesized that the tumor-suppressive role of ING5 was associated with IL-6/CXCL12 signaling in ESCC. The activation of the IL-6/CXCL12 signaling pathway can promote EMT and increase the distant metastasis and invasion of tumor cells, playing a key role in the metastasis of tumor cells in vivo.23,24 CXCL12 is upregulated in many solid tumors,25,26 including gastric cancer. IL-6 binds to CXCL12 in tumor tissues and induces the synthesis of a variety of inflammatory mediators, including TNF-α, IL-8, and multiple chemokines.27,28 Reports on the relationship between CXCR12 and ESCC are relatively rare, and the exact mechanism underlying how CXCR12 promotes the occurrence and development of ESCC requires further studies.

In conclusion, our study demonstrated that ING5 can inhibit the migration and invasion of esophageal cancer cells by down-regulating the IL-6/CXCL12 signaling pathway.

Animal Study Protocol
Male athymic nude mice (4 weeks old) were bought from Shanghai Kai Li. Mice were injected subcutaneously with 5 × 10⁶ EC-109 ING5 and EC-109 control cells. Tumor size was monitored with calipers twice a week. Tumor volume was estimated as (D² × d)/2, where D is the largendiameter and d is the small diameter of the tumor. For the intravenous mouse model, mice were injected with 5 × 10⁶ EC-109 ING5 and EC-109 through tail vein, and mice were sacrificed at day 45 after injection, and lungs were inspected for tumor formation.

Availability of Data and Materials
The datasets and supporting materials generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ Contributions
Yali Wang conducted most of the experiments and drafted the manuscript. J Tan, Jing L, Hh Chen collected and analyzed the data. W Wang designed the study, revised the manuscript. All authors read and approved the final manuscript.

Declaration of Conflicting Interests
The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics Approval and Consent to Participate
The study protocol was approved by the Ethics Committee of Henan Provincial Chest Hospital (CH18627). All animal research was carried out following the Declaration of Helsinki.

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