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IL-37 suppresses migration and invasion of gallbladder cancer cells through inhibition of HIF-1α induced epithelial-mesenchymal transition

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Abstract. – OBJECTIVE: Gallbladder carcinoma (GBC) is the seventh most common cancer across the globe and the most common malignancy of the biliary tract. Epithelial-mesenchymal transition (EMT) is an important pre-requisite for tumor metastasis; however, its mechanism in GBC has not yet been defined. In the present study, we investigated the effects of interleukin-37 (IL-37) on the epithelial-mesenchymal transition (EMT) of gallbladder cancer cells.

MATERIALS AND METHODS: RT-qPCR and Western blotting were used to determine the expression of IL-37 in GBC cancer cells and non-tumorigenic human intra-hepatic biliary epithelial cell line. Western blotting was also used for detecting the expression of vimentin, Snail, and E-cadherin.

RESULTS: Expression level of IL-37 in GBC cells was decreased in GBC cancer cells compared with the non-tumorigenic human intra-hepatic biliary epithelial cell line. Decreased expression of vimentin and Snail and increased expression of E-cadherin were found in the groups which overexpress IL-37 when compared with the control. Mechanism study showed that IL-37 suppressed the expression of HIF1α in cells. However, HIF1α stabilization by $CoCl_2$ could attenuate the function of IL-37.

CONCLUSIONS: Our results indicate that IL-37 plays an antitumor role during the progression of gallbladder carcinoma. IL-37 could inhibit HIF1α induced EMT. Our data provide a new strategy for the treatment of gallbladder cancer.

Key Words:
Interleukin 37, HIF1alpha, Gallbladder cancer, EMT.

Introduction

Gallbladder carcinoma (GBC) has very high incidence and mortality and represents the seventh most common cancer across the world and is also the most common malignancy of the biliary tract[1,2]. Most deaths of patients occur due to metastasis to distant organs[3]. GBC is characterized by high rates of recurrence, early lymph node invasion and metastasis to distant organs including the liver. Treatments as chemotherapy and radiotherapy are largely palliative and do not help patients survive.

IL-37 (Interleukin-37) belongs to the IL-1 family and has shown anti-inflammatory and immune suppression effects[4]. Recent studies have shown that IL-37 suppresses the progression of a number of different tumors, including fibrosarcoma[5,6], cervical cancer[6], HCC[7], NSCLC[8], RCC[9], OSCC[10], and breast cancer[11]. The underlying mechanisms include recruitment of certain immune cells[4], IL-6/STAT3 signaling suppression[6], angiogenesis[8], and epithelial-mesenchymal transition inhibition[12]. However, whether IL-37 also shows anti-tumor effects in gallbladder cancer remains unknown.

Epithelial cells acquire physiological transition to motile mesenchymal cells to metastasis, which is termed as epithelial-mesenchymal transition (EMT)[13]. Very little is known about the mechanism that regulates EMT in GBC. To better

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understand and target GBC metastasis, it is important to understand what regulates EMT in the GBC cells. Several studies[2,14] have investigated the expression of genes such as vimentin, ALDH1 and topoisomerase II, and showed that they are upregulated during GBC metastasis.

HIF1α has been shown to regulate EMT in different forms of solid malignancies; in fact, HIF1α has been shown to be sufficient to cause EMT in *in vitro* cultured breast epithelial cells and human lung cancer cell line[15]. Hence our work was to evaluate whether HIF1α was a critical effector of EMT in the gallbladder cancer cells.

In this study, we demonstrated that the expression of IL-37 decreased in gallbladder cancer cell lines as revealed by qPCR and Western blot. We showed that the activation of IL-37 by overexpression could suppress cell migration and invasion *in vitro*. Further results indicated that IL-37 suppressed the expression of EMT markers in cancer cells but could not suppress cell migration and invasion in cells with HIF1α stabilization. Our results suggest that IL-37 suppresses migration and invasion of gallbladder cancer cells through inhibition of HIF-1a induced epithelial-mesenchymal transition.

Materials and Methods

Cell Culture

Human gallbladder cancer cell lines GBC-SD, NOZ, and non-tumorigenic human intra-hepatic biliary epithelial cell line H69 were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% Fetal Bovine Serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA). All the cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C. Trypsin (0.25%, Thermo Fisher Scientific, Waltham, MA, USA) was used to detach the cells from the culture flask.

Plasmid Construct

Human IL-37 (NM 014439) cDNA open reading frame was inserted into a pcDNA3 vector, and the C-terminus of IL-37b was ligated to FLAG sequence and were sequenced to exclude mutations.

Real Time-PCR

Total RNA was extracted from H69, GBC-SD, and NOZ cells. Then, cDNA samples were prepared using the PrimeScript RT reagent Kit (TaKaRa, Beijing, China). The cDNA was quantified with the 7500 fast Real Time-PCR system (Applied Biosystems, Foster City, CA USA) using SYBR Green (TaKaRa, Beijing China). Primer pairs are: IL-37-F: 5'-GATCACAAGTACTGGTCCTGG-3', IL-37-R: 5'-TCCTTTATCCTTGTCACAGTAG-3'[16]; GAPDH-F: 5'-AAGGTGAAGGTCGGAGTCA-3', GAPDH-R: 5'-GGAAGATGGTGATGGGATTT-3'[9,10]. The expression of GAPDH was used for normalization. Relative mRNA expressions of target genes were calculated by the $2^{-\Delta\Delta Ct}$ method.

Western Blotting

Total protein was extracted from cells using 1 × SDS sample buffer. The protein extracts were denatured by boiling at 95°C for 5 min and equal amounts of proteins were loaded on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). Blots were blocked with 5% non-fat dry milk and then incubated with the primary antibodies at 4°C overnight. The primary antibodies included anti-IL-37 (1:100, Abcam, Cambridge, MA, USA), anti-E-cadherin (1:200, Santa Cruz Biotechnology, St. Louis, MO, USA), anti-Vimentin (1:200, Cell Signaling Technology, Danvers, MA, USA), anti-HIF1α (1:200, BD Biosciences, Franklin Lakes, NJ USA), anti-Snail (1:200, Cell Signaling Technology, Danvers, MA USA), and anti-β-actin (1:100, Cell Signaling Technology, Danvers, MA USA) antibodies. β-actin was used as the loading control. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse (1:2000) and anti-rabbit (1:5000) secondary antibodies at room temperature for 1 h. The blots were developed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

In Vitro Migration Assay

GBC cells transfected with empty vector and GBC cells overexpressing IL-37 were treated with or without 50 μM $CoCl_2$ for 72 h. Post-treatment, treated and untreated cells were deprived of serum overnight, trypsinized and seeded into the upper chamber (1×10^5/well) of the transwell (8 μm pore size; BD Bioscience, Franklin Lakes, NJ USA). The chemoattractant in the lower chamber was medium supplemented with 10% FBS. After overnight incubation, the migratory cells in the lower chambers were fixed with 4% PFA and

stained with crystal violet. Crystal violet-stained cells were counted in five randomly different fields with an inverted microscope. The experiments were performed in triplicates.

In Vitro Invasion Assay

A modified *in vitro* Boyden chamber invasion assay with Matrigel-coated transwell chambers (8 μm pore size) was performed. GBC cells transfected with empty vector and GBC cells overexpressing IL-37 were treated with or without 50 μM $CoCl_2$ for 72 h. Post-treatment, treated and untreated cells were deprived of serum overnight, trypsinized and seeded into the upper chamber (1×10^5/well) of the transwell (8 μm pore size; BD Bioscience, Franklin Lakes, NJ USA). The chemoattractant in the lower chamber was medium supplemented with 10% FBS. After overnight incubation, the migratory cells in the lower chambers were fixed with 4% PFA and stained with crystal violet. Crystal violet-stained cells were counted in five randomly different fields with an inverted microscope. The experiments were performed in triplicates.

Statistical Analysis

All results were analyzed by SPSS Statistical Package version 16 (SPSS for Windows. SPSS Inc., Chicago, IL, USA) and all data were expressed as mean ± standard error of mean (SEM). Two independent group's analyses were made using the Student's *t*-test. $p < 0.05$ was considered statistically significant.

Results

Decreased IL-37 Expression in GBC Cells

To assess the expression of IL-37 in the progression of gallbladder cancer, we isolated total RNA from non-tumorigenic human intra-hepatic biliary epithelial cell line H69 and two human gallbladder cancer cell lines GBC-SD and NOZ, and prepared cDNA. QPCR results showed that the expression of IL-37 at mRNA level decreased in both GBC-SD and NOZ gallbladder cancer cell lines (Figure 1A). We then examined the expression of IL-37 at the protein level. The Western blotting assay result showed that IL-37 decreased significantly in both GBC-SD and NOZ cells, compared with non-tumorigenic H69 cell line (Figure 1B). These results demonstrated that the expression of IL-37 at both mRNA and protein level decreased in gallbladder cancer cells.

IL-37 Suppresses GBC Migration, Invasion in a Dose-Dependent Manner

To explore the mechanism of IL-37 in gallbladder cancer progression, we overexpressed IL-37 in GBC-SD and NOZ cells (Figure 2E), and

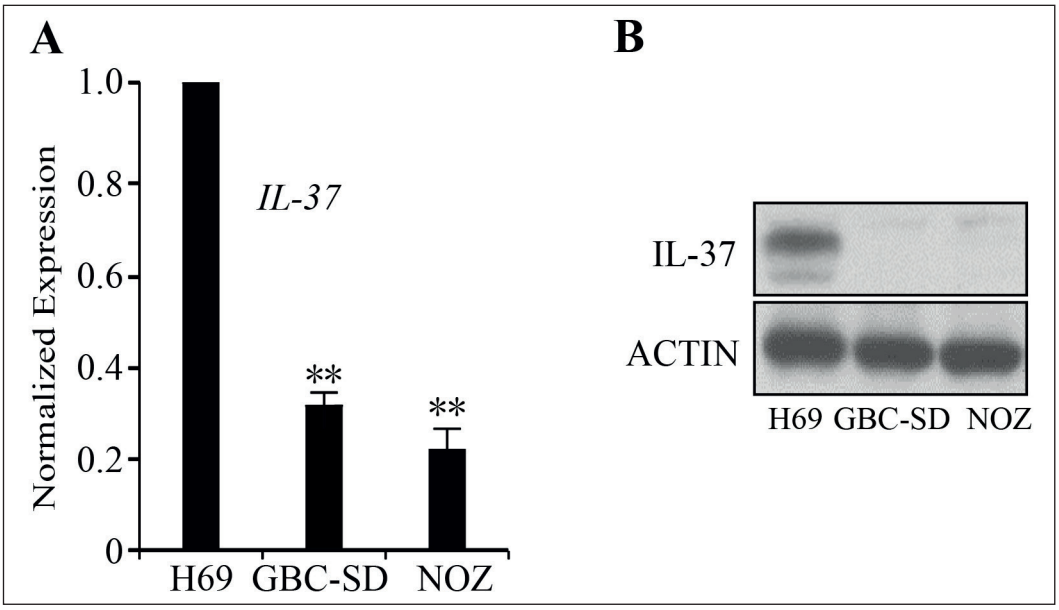

Figure 1. Decreased IL-37 expression in GBC cells. **A,** Total RNA was extracted from cells, then RT-QPCR assay were performed. IL-37 expression was decreased in GBC-SD and NOZ cells compared with H69 cells. $^{**}p < 0.01$. **B,** Western blotting showed that IL-37 expression was significantly decreased in GBC-SD and NOZ cells compared with H69 cells.

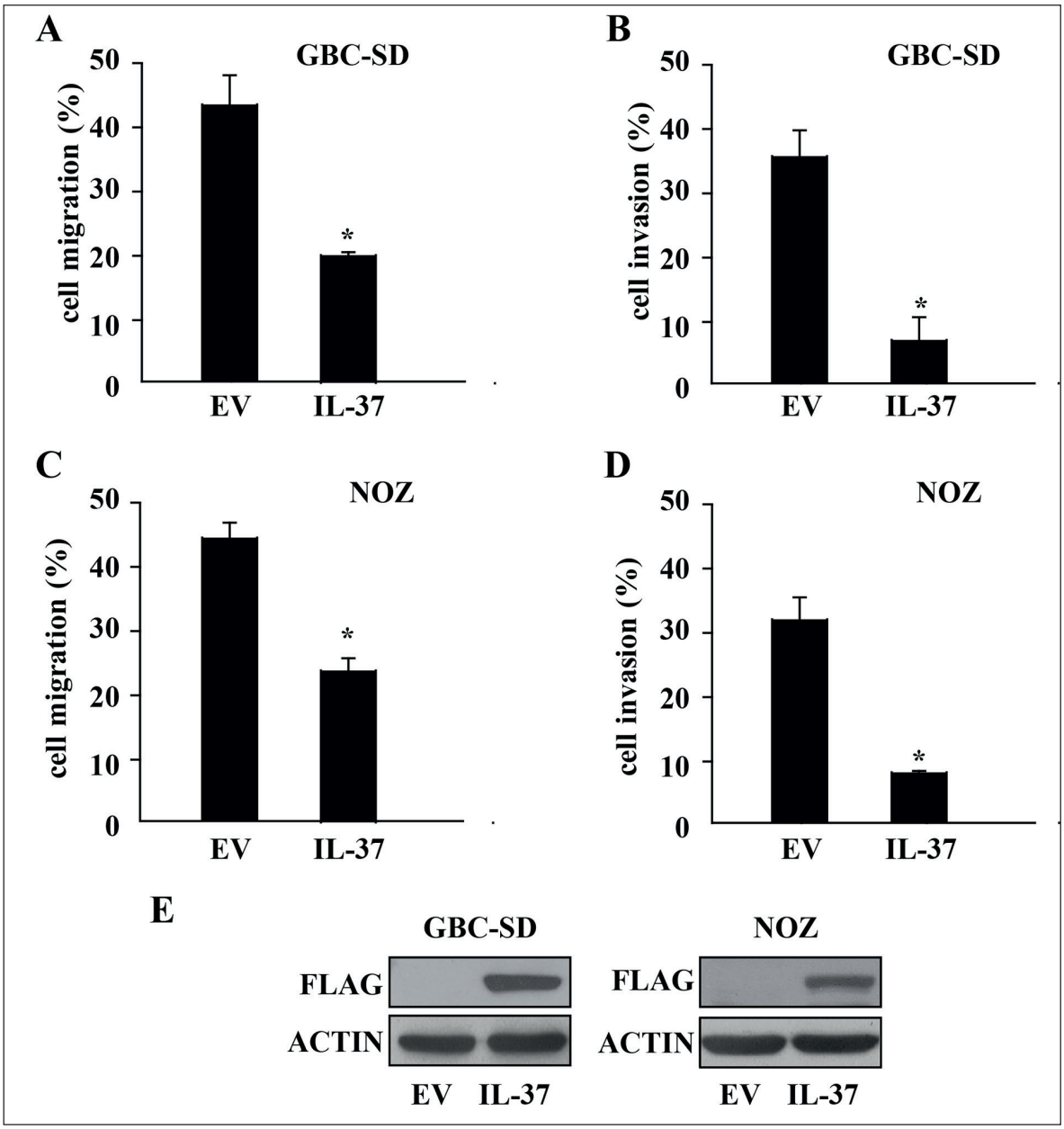

Figure 2. IL-37 suppresses GBC migration, invasion in a dose-dependent manner. Transwell assay showed that exogenous IL-37 overexpression decreased the migration of GBC-SD **(A)** and NOZ **(C)** cells, respectively. *$p < 0.05$. Invasion assay showed that IL-37 overexpression decreased the invasive capability of GBC-SD **(B)** and NOZ **(D)** cells, respectively. *$p < 0.05$.

then, cell migration and invasion were analyzed. Cells were transfected transiently with a pcDNA3-IL-37 plasmid for 48h. The transwell assay results showed that, compared with empty vector control, the IL-37 expression suppressed the migration of GBC-SD and NOZ cells, respectively (Figure 2A, C). We then tried the invasion assay and it showed that IL-37 suppressed the invasive capability of GBC cancer cells (Figure 2B, D).

IL-37 Alters the Expression of EMT Markers

Because of the crucial role of EMT in cancer progression, the expression of EMT markers in GBC-SD and NOZ cells were analyzed. Western blotting results showed that the protein levels of E-cadherin (epithelial cell marker) were increased by IL-37 overexpression, whereas Snail (EMT transcription factor) and Vimentin (mesenchymal

cell marker) were suppressed (Figure 3A, B). To further confirm the correlation between IL-37 and EMT signaling pathway, we examined the expression of HIF1α which could induce EMT. Compared with the control group, IL-37 suppressed the expression of HIF1α in GBC-SD and NOZ cells (Figure 3C). These data demonstrated that IL-37 expression might inhibit gallbladder cancer cells via HIF1α induced EMT pathway.

HIF1α Induced EMT Pathway Contributed GBC Migration, Invasion

To further study whether HIF1α is involved in the progression of gallbladder cancer, we transfected GBC-SD and NOZ cells with pcDNA3-IL37 for 48 h, and then, we treated them with $CoCl_2$ for 24 h. The transwell results showed that $CoCl_2$ significantly attenuated the function of IL-37, GBC-SD and NOZ cells treated with $CoCl_2$ maintained high migration capability compared with cells without $CoCl_2$ treatment (Figure 4A, C). Invasion assay also showed that stabilization of HIF1α attenuated the function of IL-37 which suppressed the invasive capability of GBC cancer cells (Figure 4B, D).

Discussion

Invasion and metastasis are related to most deaths of cancer patient[17,18]. EMT is a biological process by which epithelial cells lose their polarity and adhesive capabilities and gain the migratory and invasive properties of mesenchymal cells. It was shown to be an important prerequisite for tumor invasion and metastasis during tumor progression[13]. Hence, uncovering the underlying mechanism is imperative to develop better prognostic and therapeutic strategies for late-stage cancers. Our major aim was to uncover the underlying mechanism of EMT in GBC cells.

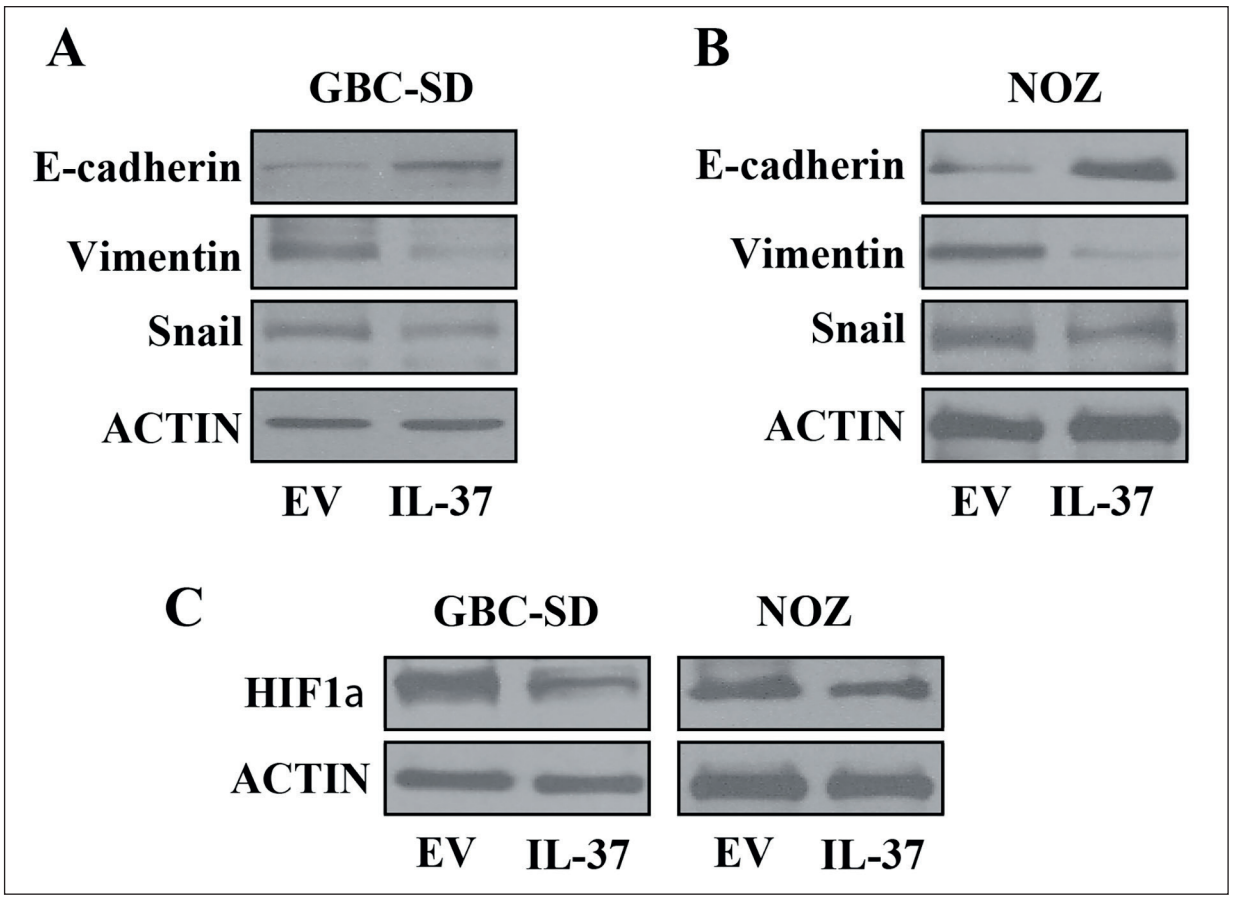

Figure 3. IL-37 alters the expression of EMT markers. Western blotting showed that IL-37 overexpression significantly increased the expression of E-cadherin whereas decreased the expression of Snail and Vimentin in GBC-SD **(A)** and NOZ **(B)** cells. **C,** Western blotting showed that IL-37 overexpression significantly decreased the expression of HIF1α.

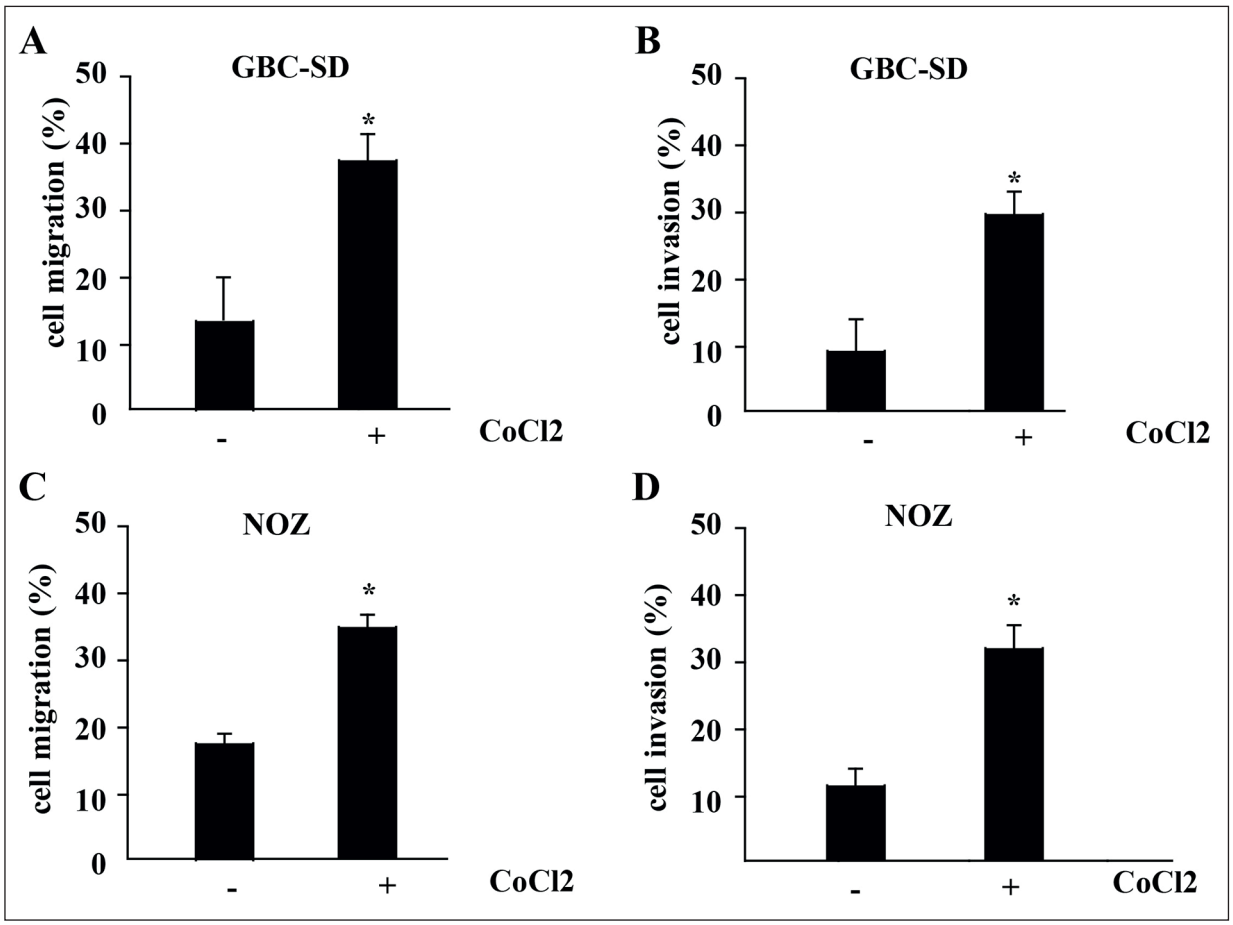

Figure 4. HIF1α induced EMT pathway contributed GBC migration, invasion. Transwell assay showed that the treatment of $CoCl_2$ attenuated the inhibitive effect of IL-37 which decreased the migration of GBC-SD **(A)** and NOZ **(C)** cells, respectively. $*p < 0.05$. Invasion assay showed that the treatment of $CoCl_2$ attenuated the inhibitive effect of IL-37 which IL-37 decreased the invasive capability of GBC-SD **(B)** and NOZ **(D)** cells, respectively. $*p < 0.05$.

Chen et al[12] showed that IL-37 could inhibit proliferation and EMT process of NSCLC cell line A549. Our results showed for the first time that the expression of IL-37 decreased in GBC-SD and NOZ cells. Of note, we observed that exogenous IL-37 overexpression decreased migration and invasive potential of GBC-SD and NOZ cells, which agrees with the studies in other cancer types such as HCC[16] and colon cancer[19]. We then found that the expression of EMT markers after IL-37 overexpression is altered in these GBC cancer cells. Western blotting results showed that the expression of E-cadherin increased whereas Snail and vimentin decreased in IL-37 overexpressing cells. HIF1α has been shown to be sufficient to cause EMT. Notably, we detected that IL-37 overexpression also downregulated the expression of HIF1α in both cell lines. Consistent with the Western blotting results, our *in vitro* experiments unequivocally established the role of HIF1α in mediating migration and invasion of GBC cells.

Further investigation on the role of IL-37 in blocking *in vivo* tumor metastasis of GBC cells needs to be addressed urgently. First, IL-37 expression levels need to be validated in GBC patient samples. It must be also determined whether non-HIF1α-mediated EMT in GBC cells are regulated through IL-37.

Conclusions

The current study shows that IL-37 is a central player in HIF1α-induced EMT in GBC cancer cells. Given the effect of IL-37 on *in vitro* invasion and migration, it might serve as a useful biomarker for predicting prognosis of gallbladder cancer metastasis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Authors' Contribution

Jing Luo and Chao Luo conceived and designed the project. Tuanjie Wu and Bin Xu acquired the data. Guihua Zhao analyzed and interpreted the data. Chao Luo and Tuanjie Wu wrote the paper.

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