

World Journal of *Gastrointestinal Oncology*

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- 1892 Go-Ichi-Ni-San 2: A potential biomarker and therapeutic target in human cancers
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- 1903 Neoadjuvant therapy in resectable pancreatic cancer: A promising curative method to improve prognosis
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- 1918 Transcriptional factor III A promotes colorectal cancer progression by upregulating cystatin A
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- 1933 VCAN, expressed highly in hepatitis B virus-induced hepatocellular carcinoma, is a potential biomarker for immune checkpoint inhibitors
Wang MQ, Li YP, Xu M, Tian Y, Wu Y, Zhang X, Shi JJ, Dang SS, Jia XL
- 1949 Overexpression of ELL-associated factor 2 suppresses invasion, migration, and angiogenesis in colorectal cancer
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- 1968 Interleukin-34 promotes the proliferation and epithelial-mesenchymal transition of gastric cancer cells
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- 1981 Cuproptosis-related long non-coding RNAs model that effectively predicts prognosis in hepatocellular carcinoma
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WJGO mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal oncology and covering a wide range of topics including liver cell adenoma, gastric neoplasms, appendiceal neoplasms, biliary tract neoplasms, hepatocellular carcinoma, pancreatic carcinoma, cecal neoplasms, colonic neoplasms, colorectal neoplasms, duodenal neoplasms, esophageal neoplasms, gallbladder neoplasms, *etc.*

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Basic Study

Interleukin-34 promotes the proliferation and epithelial-mesenchymal transition of gastric cancer cells

Chuan-Hong Li, Zhang-Ming Chen, Pei-Feng Chen, Lei Meng, Wan-Nian Sui, Song-Cheng Ying, A-Man Xu, Wen-Xiu Han

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Abstract

BACKGROUND

Interleukin (IL)-34 is a pro-inflammatory cytokine involved in tumor development. The role of IL-34 in the proliferation and epithelial-mesenchymal transition (EMT) of gastric cancer (GC) remains to be investigated.

AIM

To investigate whether and how IL-34 affects the proliferation of GC cells and EMT.

METHODS

Using immunohistochemical staining, the expression of IL-34 protein was detected in 60 paired GC and normal paracancerous tissues and the relationship between IL-34 and clinicopathological factors was analyzed. The expression of IL-34 mRNA and protein in normal gastric epithelial cell lines and GC was detected using quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting, respectively. Stable IL-34 knockdown and overexpression in AGS cell lines were established by lentiviral infection and validated by qRT-PCR and western blotting. The cholecystokinin-8 assay, clone formation assay, cell scratch assay, and transwell system were used to detect GC cell proliferation, clone formation, migration, and invasion capacity, respectively. The effects of IL-34 on the growth of GC transplant tumors were assessed using a subcutaneous transplant tumor assay in nude mice. The effects of IL-34 on the expression level of EMT-associated proteins in AGS cells were examined by western blotting.

RESULTS

Expression of IL-34 protein and mRNA was higher in GC cell lines than in GES-1 cells. Compared to matched normal paraneoplastic tissues, the expression of IL-34 protein was higher in 60 GC tissues, which was correlated with tumor size, T-stage, N-stage, tumor, node and metastasis stage, and degree of differentiation. Knockdown of IL-34 expression inhibited the proliferation, clone formation, migration, and invasion of AGS cells, while overexpression of IL-34 promoted cell proliferation, clone formation, migration, and invasion. Furthermore, the reduction of IL-34 promoted the expression of E-cadherin in AGS cells but inhibited the expression of vimentin and N-cadherin. Overexpression of IL-34 inhibited E-cadherin expression but promoted expression of vimentin and N-cadherin in AGS cells. Overexpression of IL-34 promoted the growth of subcutaneous transplanted tumors in nude mice.

CONCLUSION

IL-34 expression is increased in GC tissues and cell lines compared to normal gastric tissues or cell lines. In GC cells, IL-34 promoted proliferation, clone formation, migration, and invasion by regulating EMT-related protein expression cells. Interference with IL-34 may represent a novel strategy for diagnosis and targeted therapy of GC.

Key Words: Gastric cancer; Interleukin-34; Proliferation; Epithelial-mesenchymal transition; Metastasis

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Core Tip: Our study provides novel evidence that interleukin (IL)-34 contribute the growth and metastasis of gastric cancer (GC). IL-34 is up-regulated in GC cell lines and tissues, which is correlated with tumor size, grade of differentiation and tumor, node and metastasis stage. IL-34 enhances the ability of proliferation, clone formation, migration and invasion of GC cells and regulates the expression levels of epithelial-mesenchymal transition-associated proteins, suggesting that IL-34 may be an effective target for the therapy of GC.

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INTRODUCTION

Gastric cancer (GC) is one of the most aggressive cancers, with approximately 1 million new cases diagnosed worldwide in 2020 and an estimated 769000 deaths, ranking fifth in incidence and fourth in mortality globally[1]. Rates are two-fold higher in men than in women[1]. East Asia, including China, Japan, and Korea, is the hotspot of incidence and mortality of GC[2]. In Japan and Korea, screening programs have led to substantial reductions in GC-associated mortality[2]. However, in China, due to the lack of extensive initial screening for early GC, most patients present with advanced GC[3]. Radical surgery is the main modality for the treatment of locally progressive GC, and the combination of chemotherapy and targeted therapy can improve the prognosis. During 2007-2021, the 5-year relative survival rate of patients with GC continued to increase[4]. The overall 5-year age-standardized relative survival rates in 2007-2011, 2012-2016, and 2017-2021 were 38.3%, 40.6%, and 42.9%, respectively[4]. However, the overall relative survival of patients with GC remains very low[4]. In particular, the survival rate of patients with distant-stage GC remains very low (10%)[4]. Targeted therapy alone or in combination with chemotherapy has shown potential advantages in the treatment of advanced GC[5]. Trastuzumab is a targeted therapy that effectively improves the prognosis of HER2-positive patients with GC; however, less than 20% of patients with GC are HER2-positive, which means that the remaining 80% of patients with GC do not benefit from the drug[1]. Therefore, identifying more effective targets will provide new ideas for targeted therapy for GC.

Epithelial-mesenchymal transition (EMT) refers to the biological process in which epithelial cells are transformed into cells with mesenchymal properties through a complex series of mechanisms in response to relevant factors[6-8]. In cancer, EMT is associated with tumorigenesis, invasion, metastasis, and resistance to therapy. Different states of EMT exhibit different functional characteristics in cancer and are associated with tumor proliferation, propagation, plasticity, invasion, and metastasis[9]. Exosomes have been proposed as a therapeutic tool to control the development of EMT and influence

the progression of cancer[10]. In addition, Pinin induces EMT and malignant progression in hepatocellular carcinoma[11]. Furthermore, Wu *et al*[12] found that interleukin (IL)-6 secreted by cancer-associated fibroblasts promotes EMT and GC metastasis through the JAK2/STAT3 signaling pathway, Li *et al*[13] reported that tumor-associated neutrophils induce EMT by IL-17a to promote migration and invasion in GC cells, and Tian *et al*[14] reported that SERPINH1 regulates EMT and GC metastasis through the Wnt/ β -catenin signaling pathway. Clarifying the regulatory mechanism of EMT in the development of GC is of great practical importance in attempting to understand the occurrence and metastasis of GC.

IL-34 is a new cytokine discovered in 2008 that binds to its functional receptor and plays a role in the regulation of cell differentiation, proliferation, angiogenesis, inflammation, and the immune response [15-17]. IL-34 has been found to play a pro-cancer role in a variety of tumors, including thyroid, colorectal, and liver cancers[18-20]. In particular, Zhang *et al*[18] found that IL-34 promotes tumor proliferation and activates the ERK signaling pathway in papillary thyroid cancer cells. However, it remains unclear whether IL-34 regulates GC cell migration and invasion. Furthermore, it is also unknown whether IL-34 can regulate GC proliferation *in vitro* and *in vivo*. This study aimed to elucidate the relationship between IL-34 and the clinicopathological characteristics of patients with GC and the role of IL-34 in the proliferation and the EMT of GC cells.

MATERIALS AND METHODS

Patients and samples

A total of 60 patients diagnosed with GC (age >18 years) were collected from patients who underwent surgical resection at The First Affiliated Hospital of Anhui Medical University from November 2019 to June 2020. Patients with a history of preoperative radiotherapy, long-term drug treatment, inflammatory diseases, rheumatic immune diseases, and genetic-related diseases were excluded. Information collected included sex, age, tumor diameter, T-stage, N-stage, M-stage, tumor, node and metastasis (TNM) stage, and grade (well, moderate, and poor). All tumors were staged according to the Eighth Edition of American Joint Committee on Cancer TNM classification. All procedures performed in studies with human participants were reviewed and approved by the Ethics Committee of The First Affiliated Hospital of Anhui Medical University and were carried out in accordance with the Declaration of Helsinki of 1964 and its subsequent amendments or comparable ethical standards. Written informed consent was obtained from all individual participants included in the study. Approval for the ethical use of clinical samples was obtained from the Institutional Review Board (No: Quick-PJ2019-09-11).

Immunohistochemical staining

Tumor tissues collected from patients with GC were fixed in 4.0% paraformaldehyde, embedded in paraffin, and sliced into 4- μ m sections. The sections were incubated with 3.0% hydrogen peroxide to inactivate endogenous peroxidase and then blocked in 5.0% bovine serum albumin after antigen retrieval. After blocking, sections were incubated with anti-IL-34 monoclonal antibody (863800; ZEN-BIOSCIENCE; 1:150) overnight at 4°C, followed by secondary antibody (ab150077; Abcam; 1:500). Sections were visualized with a diaminobenzidine solution and hematoxylin counterstain. Images were acquired with a microscope (Nikon Eclipse E200). Two pathologists blinded to clinical data independently scored tissue staining. The percentage of staining was evaluated and scored as follows: 0, < 1% staining; 1, 1%-25% staining; 2, 26%-50% staining; 3, 51%-75% staining; and 4, > 75% staining. The intensity of staining was defined as follows: 0, no signal; 1, weak; 2, moderate; and 3, strong. Scoring formula = percentage of positive tumor cell score \times positive cell staining score. A total score of < 5 was considered negative and > 6 was considered positive.

RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was isolated from tissues and cells with the TRIzol reagent (Thermo Fisher). Reverse transcription was achieved using Vazyme Biotech kits. The cDNA amplification was then performed using SYBR Premix Ex Taq (Vazyme Biotech) using a Roche 480 light cycler. PCR was carried out for 40 cycles according to the following procedure: 95°C for 60 s, and 60°C for 30 s. Relative mRNA expression of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization with GAPDH expression. Primer sequences were as follows: IL-34, 5'-TTGACGCAGAATGAGGAGTG-3'(forward); 5'-CCCTCGTAAGGCACACTG AT-3'(reverse); GAPDH, 5'-CAGGAGGCATTGCTGATGAT-3'(forward); 5'-GAAGG CTGGGGCTCATTT-3'(reverse).

Western blotting

Protein was obtained by lysis of tissues or cells using RIPA buffer containing protease inhibitors (Thermo Fisher), and protein concentration was quantified using the BCA Protein Assay kit (Beyotime Biotechnology). Protein was separated by SDS-PAGE and transferred to a polyvinylidene fluoride

membrane according to standard protocols and then blocked with 5% skim milk in TBST for 1 h. The membranes were incubated overnight at 4°C with the following antibodies: anti-E-cadherin (1:5000, Abcam), anti-N-cadherin (1:5000, Abcam), anti-Vimentin (1:5000, Abcam), anti-GAPDH (1:5000, Abcam), anti-Vimentin (1:5000, Abcam), anti-GAPDH (1:5000, Abcam), anti-IL-34 (1:1000, ZEN-BIOSCIENCE) and then incubated with horseradish peroxidase conjugated secondary antibodies (1:2000, Proteintech Group) at room temperature for 1 h after washing 3 times using TBST. The relative band density was determined with the ECL Western Blotting Substrate Kit (EMD Millipore) using Tanon 5200 Multifunctional Imaging System (Shanghai, China). GAPDH was used as an internal control.

Cell culture and transfection

The human normal gastric mucosal epithelial cell line (GES-1) and human GC cell lines (AGS, MKN-45 and HGC-27) were provided by Prof. Aman Xu (The First Affiliated Hospital of Anhui Medical University). Cells were grown in RPMI 1640 medium supplemented with 10% FBS (BI), 100 U/mL penicillin (Gibco). The cells were kept in a humidified incubator at 37°C with a mixture of 95% air and 5% CO₂.

The short hairpin RNA (shRNA) and IL-34 overexpression plasmid sequences were synthesized by the Public Protein/Plasmid Library. The shRNA sequences were as follows: shRNA-IL-34, 5'-CAGAGC-CCTCATGTCAGTATG-3' and Negative control, 5'-GTTCTCCGAACGTGTCACGTT-3'. The IL-34 overexpression plasmid is a lentiviral expression vector for human IL-34 with an inserted sequence size of 729 bp and no fused CopGFP protein at its C-terminus. Target plasmids with IL-34, shRNA-IL-34, or shRNA control were transfected into AGS cells using lentivirus and selected by adding puromycin after 48 h, then viable cells were diluted to 50 cells/mL and inoculated into 96-well plates, respectively, and continued to be screened with drugs until satisfactory monoclonal cell lines were selected.

Cell proliferation and clone formation assay

For the cholecystokinin (CCK) assay, cells were seeded in 96-well culture plates at about 3000 cells per well. At 0, 24, 48, 72, and 96 h, CCK-8 agent was added to each well and cells were treated for 2 h. Absorbance values at 450 nm were measured using an enzyme labeler (USCN KIT INC, China). For the clone formation assay, cells were seeded in six-well plates at 400 cells per well and then cultured for 2 wk. Cells were fixed and stained for 30 min in a 35% methanol solution with 1% crystal violet, and then the number of stained cells was counted.

Cell migration and invasion assays

The cells were seeded in six-well plates (2×10^4 /well). A scratch was made through the cell layer along the central axis using a sterile plastic tip after complete cell attachment. After scratching, the cells were washed 3 times with PBS to remove floating cells and to make the scratch visible, and then the medium was replaced with fresh medium to continue the culture. The scratch was photographed at 0 h and 24 h. The photos were imported into Image J software and the cell closure was calculated according to the software instructions.

The cell invasion assay was performed using Transwell chambers with a pore size of 8 μm. A 200 μL cell suspension (2×10^3 /L) was added to the upper chamber with Matrigel and placed in a 24-well plate containing 700 μL medium with 10% FBS. After 24 h of incubation at 37°C and 5% CO₂, cells that invaded toward the lower surface of the membrane were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and counted.

Animal experiments

All applicable national and institutional guidelines for the care and use of animals were followed. The ethics approval for the animal protocols for this study was obtained by the Animal Research Committee of Anhui Medical University (No. SC20200513). Ten 4-wk-old female nude mice purchased from Shanghai SLAC Laboratory Animal Co., Ltd were randomly divided into two groups (experimental and control groups), five in each group. Pre-cultured AGS cells (overexpression group and control group) in good growth condition were digested separately using trypsin, centrifuged, and a cell suspension (1×10^6 cells) was injected into the inguinal region of mice. The mice in both groups were maintained in the same feeding environment and nutritional conditions. The animals were sacrificed at the end of the experiments for an observation period of 28 d. Tumor weights were recorded per mouse and tumor volumes were calculated using the modified formula of $(\text{length} \times \text{width}^2)/2$.

Statistical analyses

SPSS statistical software (version 22.0) statistical software was used for statistical analysis and GraphPad Prism (version 8.0.2) was used for graphing. Measurement data were first examined using the Kolmogorov-Smirnov test to check whether the measurement data of each group had a normal distribution. Results are expressed as the mean ± SE for measurement data with a normal distribution. Comparisons between two groups were performed using the two-tailed Student's *t* test. One-way analysis of variance was used for multiple group comparisons, and the LSD-*t* test was used for pairwise

comparisons. All results are from three independent replicate experiments. P values < 0.05 were used to indicate statistically significant differences.

RESULTS

IL-34 expression increased in GC tissues and cell lines

The results of immunohistochemical staining showed that IL-34 expression increased in GC tissues compared to paired normal gastric tissues (Figure 1A and B, $P < 0.01$). Meanwhile, as shown in Figure 1C-E, the expression of IL-34 mRNA and protein in GC cell lines (AGS, HGC-27, and MKN-45) were higher than in normal gastric mucosal cells GES-1 ($P < 0.01$). Furthermore, the relationship between IL-34 and clinicopathological characteristics was also analyzed, and the results showed that tumor size in GC patients with higher expression of IL-34 was greater than with lower expression of IL-34 ($P < 0.05$, Table 1). Furthermore, IL-34 expression correlated with the depth of invasion, the degree of differentiation, lymph node metastasis, and the TNM stage of GC patients, but not with age, sex, or distant metastasis ($P > 0.05$, Table 1). Altogether, these findings suggested that IL-34 might function as an oncogene in the development of GC.

IL-34 accelerated the formation and proliferation of clones of GC cells

AGS cell lines with stable knockdown or overexpression of IL-34 were successfully obtained by introducing plasmids containing shRNA-IL-34 or the cds fragment (coding sequence) of the IL-34 gene into AGS cells by lentiviral infection, followed by puromycin pressurization screening. As shown in Figure 1F-J. To identify the effects of IL-34 on GC cell proliferation, we evaluated the formation of CCK-8 and plate clones in AGS cells with different expression of IL-34. The results showed that the number of AGS cell clones in the stable knockdown group (shRNA-1) was less than in the control group (shNC) (Figure 2A and B, $P < 0.01$). Meanwhile, the proliferation rate of AGS cells in the shRNA-1 group was also slowed compared to the shNC group (Figure 2C, $P < 0.05$). Furthermore, as shown in Figure 2C and D, the clone formation was significantly increased following the stable overexpression of the IL-34 compared to cells transfected with Vector alone (Figure 2E, $P < 0.05$). In addition, the CCK-8 assay also showed that the proliferation ability of AGS cells in the stable IL-34 overexpression group was significantly enhanced compared to the Vector-transfected cells, and the difference was statistically significant (Figure 2F, $P < 0.05$). Therefore, IL-34 accelerated the proliferation and clone formation of AGS cells.

IL-34 improved the migration and invasion of AGS cells

To verify the effect of IL-34 on GC migration and invasion ability, we examined the migration and invasion capacity of AGS cell lines in the shNC group and in the shRNA-1 group. As shown in Figure 3A and B, the migration capacity of AGS cells in the shRNA-1 group was reduced compared to the shNC group ($P < 0.05$). Meanwhile, the transwell assay showed that the invasion of AGS cells in the shRNA-1 group was weaker than that of the shNC group (Figure 3C and D, $P < 0.05$). As shown in Figure 3E-H, the migration and invasion of AGS cell lines was enhanced following the stable overexpression of IL-34 compared to cells transfected with Vector alone ($P < 0.05$). Taken together, IL-34 overexpression promoted migration and invasiveness of AGS cells.

Overexpression of IL-34 accelerated the growth of subcutaneous transplantation tumors in nude mice

To further verify the effects of IL-34 on GC transplant tumors *in vivo*, we constructed a subcutaneous transplant tumor model in nude mice. As shown in Figure 4A and B, the tumors of the nude mice in the IL-34 group grew faster compared to the Vector implanted group ($P < 0.05$). Furthermore, tumor weight in the IL-34 group was higher than in the Vector group (Figure 4C, $P < 0.05$). Therefore, IL-34 overexpression promoted the growth of subcutaneous transplantation tumors in nude mice.

IL-34 regulated the expression of EMT-associated proteins

The EMT process is characterized by a decreased expression of the epithelial cell marker (E-cadherin) and an elevated expression of the mesenchymal cell marker (vimentin) and the tumor invasion promoter marker (N-cadherin)[24]. As shown in Figure 5A-D, knockdown increased the expression of the E-cadherin protein expression but decreased the expression of vimentin and N-cadherin in AGS cells ($P < 0.01$). In contrast, overexpression of IL-34 suppressed E-cadherin expression and increased vimentin and N-cadherin protein expression in AGS cells (Figure 5E-H, $P < 0.01$). Overall, IL-34 promoted the migration and invasion of AGS cells by regulating the expression of EMT-associated proteins.

Table 1 Correlations between expression of interleukin-34 proteins and clinicopathologic features in 60 patients with gastric cancer

Characteristics	Expression of IL-34		χ^2/t	P value
	Lower (n = 29)	Higher (n = 31)		
Age (yr)	62.14 ± 11.23	63.87 ± 10.642	0.976	0.945
Tumor size (cm)	4.69 ± 1.74	6.52 ± 3.06	0.003	0.006
Sex				
Male	16	21	1.001	0.317
Female	13	10		
T-stage				
T1 + T2	13	4	7.520	0.006
T3 + T4	16	27		
N-stage				
N0	17	8	6.638	0.010
N1 + N2 + N3	12	23		
M0 stage				
Yes	1	2	0.000	1.000
No	28	29		
TNM stages				
I + II	17	10	4.207	0.040
III + IV	12	21		
Grade				
Well + moderate	11	4	5.006	0.025
Poor	18	27		

IL: Interleukin; TNM: Tumor, node and metastasis.

DISCUSSION

In recent years, IL-34, a newly discovered cytokine, has been reported to influence tumorigenesis and progression by binding to receptors triggering multiple intracellular pathways, which mediate cellular processes such as cell proliferation, the cell cycle, and protein phosphorylation[16]. Zhou *et al*[17] found that IL-34 binds to the colony stimulating factor 1 receptor (CSF-1R) to control the survival, proliferation, and differentiation of tumor-associated macrophages, and patients with higher expression of IL-34 and higher density of tumor-associated macrophages had a poorer prognosis and shorter overall survival and recurrence-free period. Additionally, Zhang *et al*[18] found that IL-34 Levels were significantly up-regulated in serum and tissue samples from patients with thyroid cancer, which were strongly correlated with tumor size, TNM stage, and lymph node metastasis. Kobayashi *et al*[19] found that IL-34 is a prognostic factor in colorectal cancer. Irie *et al*[20] found that IL-34 promotes hepatoblastoma cells progression *via* autocrine and paracrine mechanisms. Kajihara *et al*[21] found that IL-34 expression was upregulated in triple-negative breast cancer and that overall survival was worse in patients with triple-negative breast cancer with high expression of IL-34. Consistent with the above findings, we found that IL-34 expression levels were correlated with the clinicopathological characteristics of the patients, including tumor size, T-stage, N-stage, stage TNM, and tumor grade of differentiation. More specifically, patients with GC with lower expression of IL-34 had smaller tumors, earlier tumor stages (T-stage and N-stage, and TNM stage) and well-differentiated tumors. Additionally, CCK-8 assays and plate clone formation assays showed that the capacity for proliferation and clone formation of GC cells was enhanced after IL-34 overexpression, while the knockdown of IL-34 suppressed the proliferation and clone formation of GC cells. Similarly, Franzè *et al*[22] also found that IL-34 promoted colorectal cancer cell proliferation by regulating the growth of tumor-associated macrophages. In this study, we also attempted to further validate the effects of IL-34 on GC growth *in vivo* by constructing subcutaneous tumor nude mice models. The results showed that the overexpression of IL-34 accelerated the growth rate of GC tumors compared to cell xenograft containing only Vector. Regrettably, due to the limitations of nude mouse subcutaneous tumor models, no supporting evidence was observed with

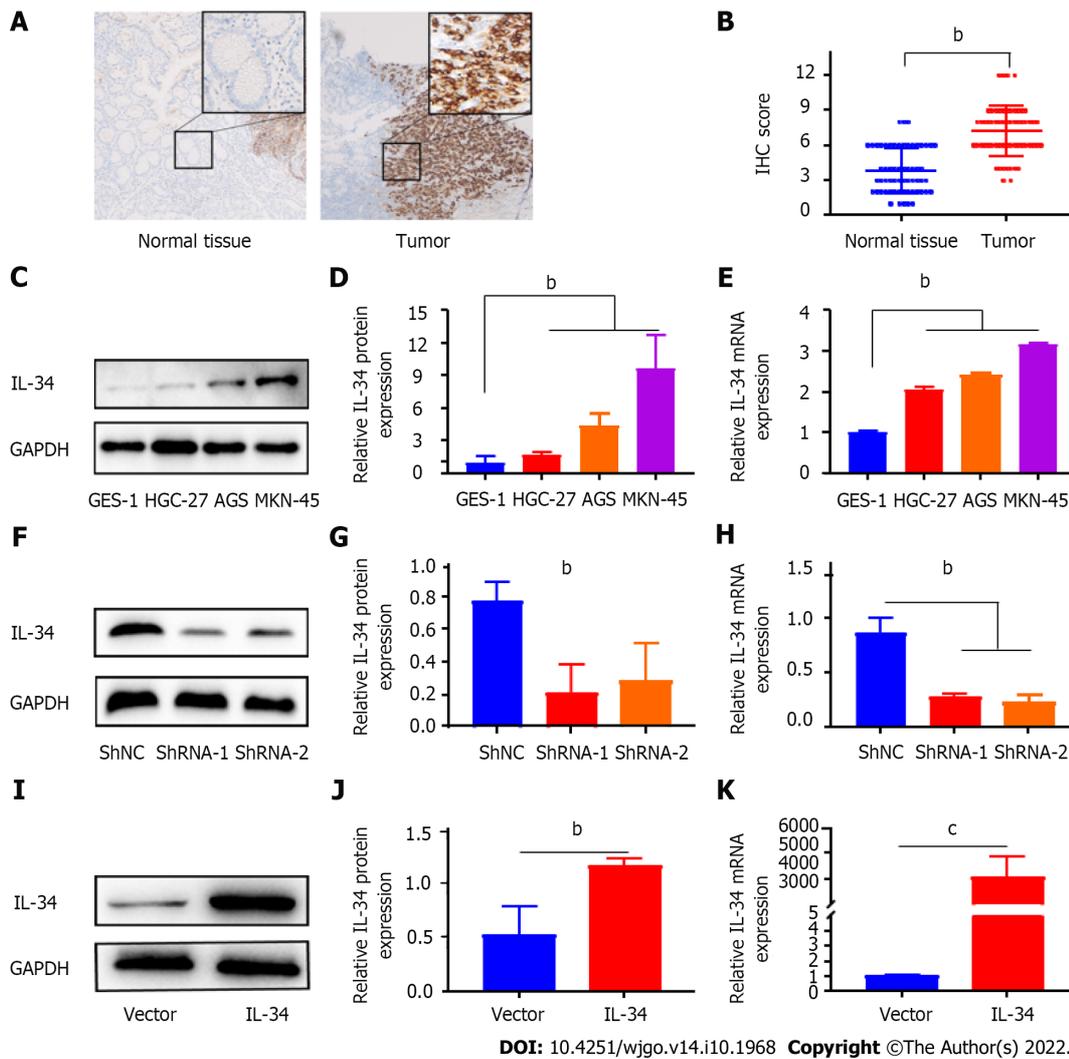
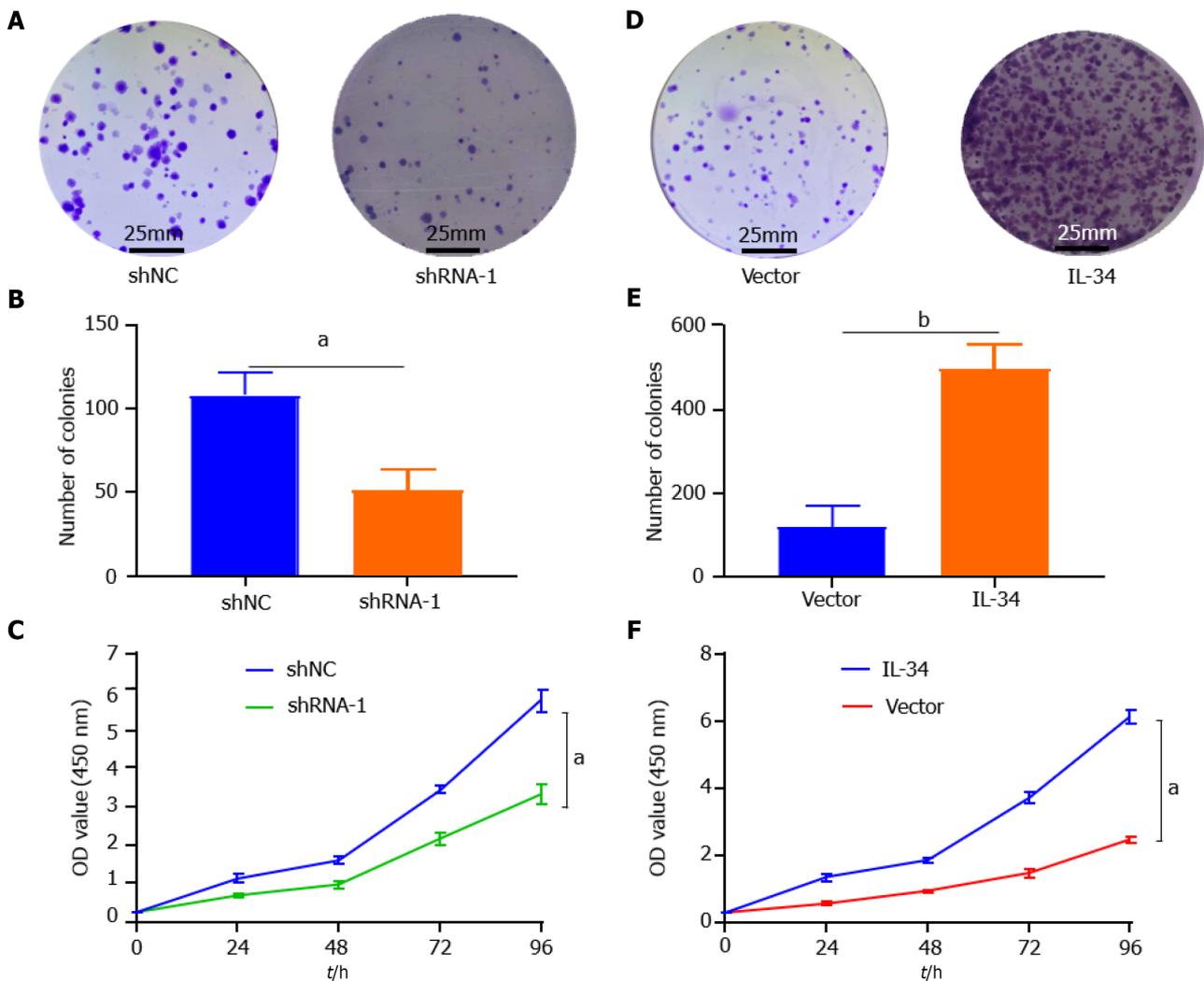


Figure 1 Expression of interleukin-34 in gastric cancer tissues and cell lines, and construction of AGS cell lines with stable knockdown or overexpression of interleukin-34. A: Representative immunohistochemistry (IHC) staining of interleukin (IL)-34 in adjacent normal tissue, gastric cancer (GC) tissues, (scale bar = 25 μ m); B: IHC staining scores were used to evaluate IL-34 expression in GC tissues and adjacent normal tissue; C: Western blotting was used to detect IL-34 protein expression in gastric normal epithelial cells (GES-1) and GC cell lines (AGS, HGC-27, and MKN-45); D: The relative densitometric analysis of protein bands was calculated; E: IL-34 mRNA expression was detected by quantitative real-time polymerase chain reaction (qRT-PCR); F and G: Western blotting was used to verify the downregulation of IL-34 in AGS cell lines; H: qRT-PCR was used to verify the downregulation of IL-34 in AGS cell lines; I and J: Western blotting was used to verify the overexpression of IL-34 in AGS cell lines; K: qRT-PCR was used to verify the overexpression of IL-34 in AGS cell lines. ^b $P < 0.01$, ^c $P < 0.001$.

regard to macrophage and lymphocyte infiltration into tumors (data was not shown), making it difficult to investigate the role of tumor-associated macrophages in IL-34 mediating GC progression.

It is well known that EMT is involved in GC metastasis, which is characterized by changes in migration, invasion, and expression of proteins associated with EMT, including E-cadherin, vimentin, and N-cadherin[6,23-25]. Previous studies have found that EMT is strongly associated with the proliferation and metastasis of GC[12-14]. For example, cancer-associated fibroblasts promote EMT and GC metastasis *via* the JAK2/STAT3 signaling pathway[12], while tumor neutrophils induce EMT to promote migration and invasion in GC cells[13]. SERPINH1 regulates the progression of EMT and GC through the Wnt/ β -catenin pathway[14]. Furthermore, ZMYM1 promotes EMT and metastasis of GC cells by recruiting the CtBP/LSD1/CoREST complex to bind to the E-cadherin promoter and mediating its repression[26]. IL-34 has been reported to promote EMT and activate the ERK signaling pathway in papillary thyroid cancer cells[15]. However, it remains unclear whether IL-34 regulates the EMT process of GC. In the present study, overexpression of IL-34 enhanced migration and invasion ability, while knockdown of IL-34 impaired metastasis in GC cells. Furthermore, we also found that the N-cadherin and vimentin proteins were up-regulated in GC cells after overexpression of IL-34, while the knockdown of IL-34 decreased the expression of N-cadherin and vimentin. In human papillary thyroid cancer, it has also been reported that IL-34 regulates the expression of E-cadherin, vimentin, and N-cadherin, which is consistent with our results.



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Figure 2 Interleukin-34 modulates clone formation and proliferation in AGS cells. A and B: Downregulation of endogenous interleukin (IL)-34 reduced the mean colony number in the colony formation assay; C: Downregulation of endogenous IL-34 reduced the mean number of AGS cells in the proliferation assay; D and E: Upregulation of endogenous COL5A2 increased the number of invasion cells in the colony formation assay; F: Upregulation of endogenous IL-34 increased the mean number of AGS cells in the proliferation assay. Data shown on graphs were obtained from three independent replicates of the experiments and expressed as mean \pm SD, ^a $P < 0.05$, ^b $P < 0.01$.

Previous studies have shown that interactions between IL-34 and its functional receptors trigger several intracellular pathways that ultimately control the growth and progression of many types of cancers. For example, multiple studies have found that IL-34 promotes tumor cell growth and invasion through the activation of the ERK signaling pathway[27-29], while inhibition of the ERK signaling pathway inhibits the tumor-promoting effects of IL-34[30]. Currently, the factors and molecular mechanisms that regulate IL-34 in cancer cells are still unknown. We speculate that IL-34 induction is associated with oncogenic mutations that trigger signal that sustain carcinogenesis, and that production of cytokines and chemokines sustains carcinogenesis[31]. Furthermore, it is possible that the altered synthesis of IL-34 depends on changes in miRNA expression, a class of small noncoding RNAs that regulate a wide range of biological processes by altering the expression and translation of their target messenger RNA genes[32]. Finally, immune and stromal cells present in the tumor microenvironment secrete many inflammatory mediators, growth factors such as TNF- α , IL-1b, and IL-6, and these small molecules stimulate tumor cells to secrete IL-34[33,34].

A limitation of the present study is that we did not further investigate the mechanism of action of IL-34 in GC. During the novel coronavirus epidemic, we were temporarily unable to conduct further mechanistic studies due to lack of time and funding constraints. However, we have identified nuclear factor-kappa B as a potential mechanism that may regulate IL-34 expression in GC by single cell sequencing. In the next step, we will continue to validate the mechanism of IL-34 regulation of proliferation, migration and EMT expression in GC.

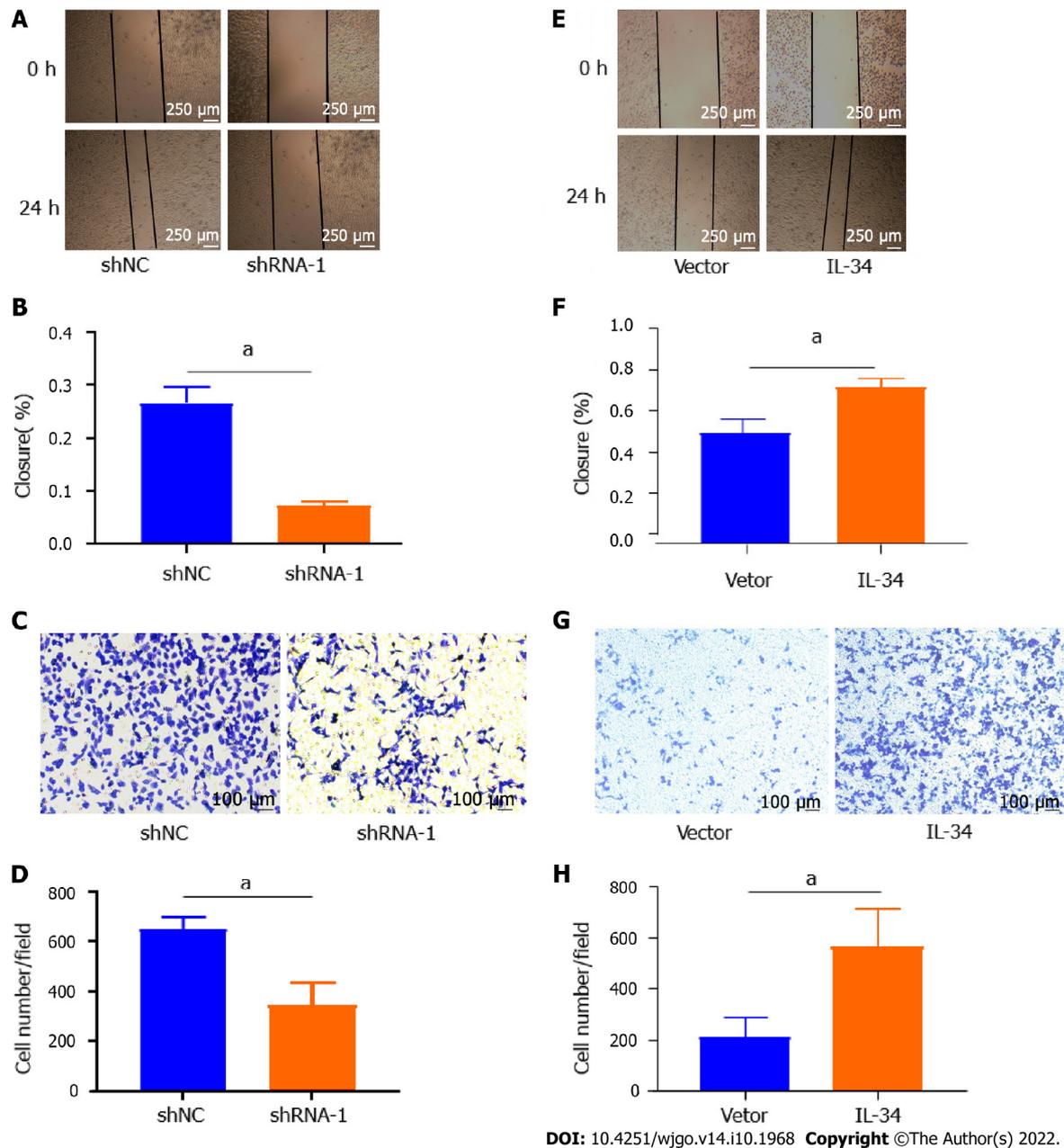


Figure 3 Interleukin-34 regulates the migration and invasiveness of AGS cells. A and B: Wound-healing assay revealed that downregulation of endogenous interleukin (IL)-34 significantly reduced the migration rate; C and D: Downregulation of endogenous IL-34 reduced the number of invaded cells in the transwell assay; E and F: Wound-healing assay revealed that upregulation of endogenous IL-34 significantly increased the migration rate; G and H: Upregulation of endogenous IL-34 increased the number of invaded cells in the transwell assay. Data derived from three independent experiments performed in triplicate and expressed as mean ± SD, and **P* < 0.05.

CONCLUSION

Our study provides novel evidence that IL-34 contributes to GC growth and metastasis. IL-34 is up-regulated in GC cell lines and tissues, and is correlated with tumor size, grade of differentiation, and TNM stage. IL-34 enhances the capacity for cancer cell proliferation, clone formation, migration, and invasion, and regulates the expression of EMT-associated proteins, suggesting that IL-34 may be an effective target for the therapy of GC.

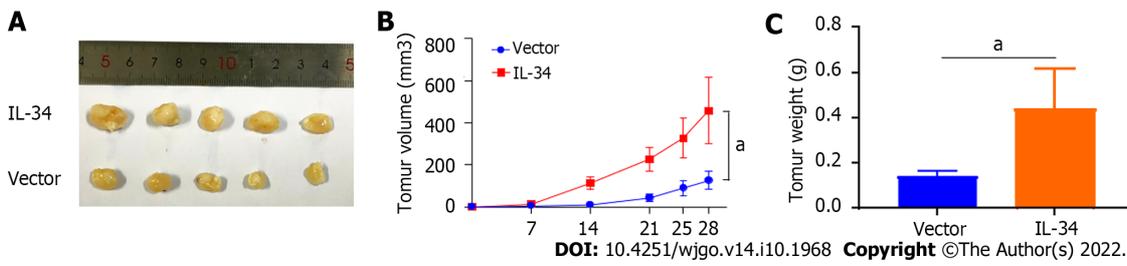


Figure 4 Interleukin-34 regulates the growth of subcutaneous transplantation tumors in nude mice. A-C: Upregulation of endogenous interleukin-34 promotes the growth of transplanted tumors in nude mice. ^a*P* < 0.05.

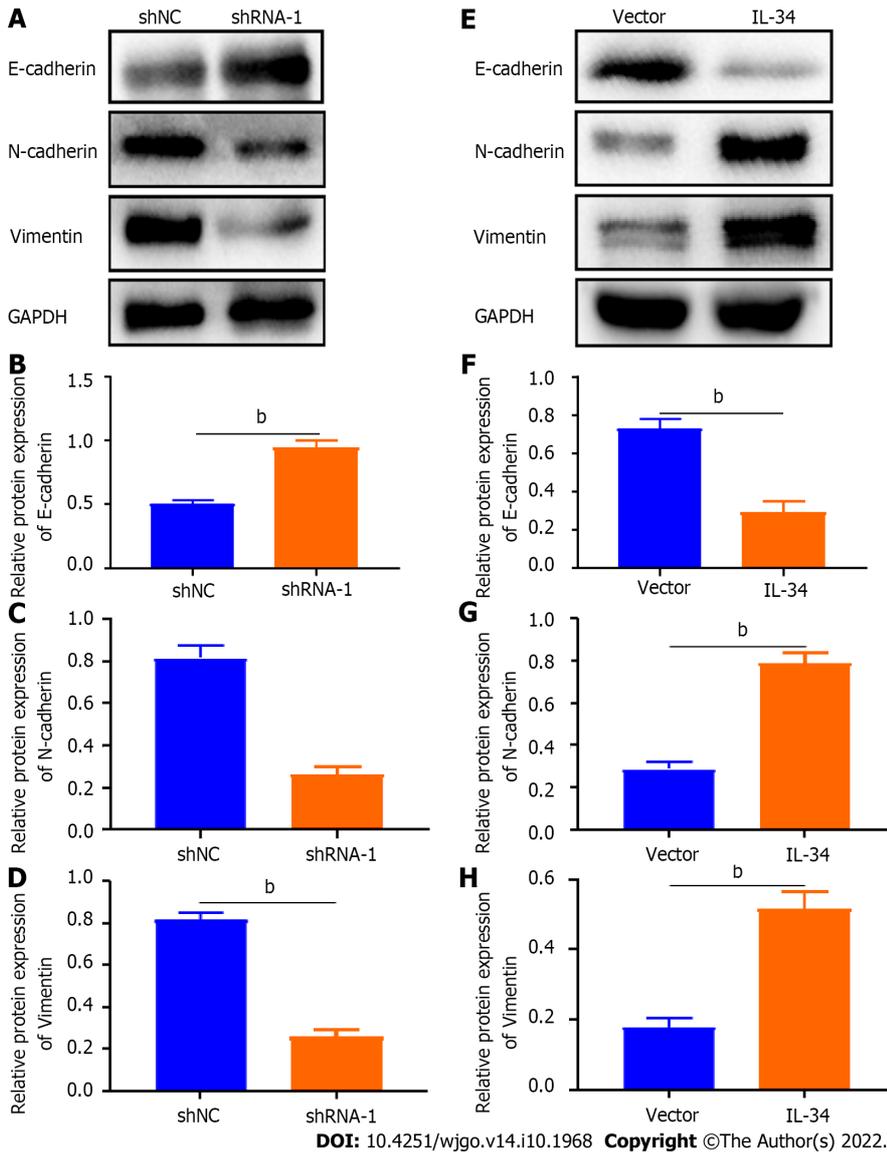


Figure 5 Interleukin-34 regulates the expression of epithelial-mesenchymal transition-related proteins in AGS cells. A-D: Downregulation of endogenous interleukin (IL)-34 increases E-cadherin expression, and reduces the expression of N-cadherin and vimentin in AGS cells; E-H: Upregulation of endogenous IL-34 reduces the E-cadherin expression, but increases the expression of N-cadherin and vimentin in AGS cells. Data was experiments performed in triplicate and expressed as mean ± standard deviation. ^b*P* < 0.01.

ARTICLE HIGHLIGHTS

Research background

Interleukin (IL)-34 is an inflammatory cytokine that is also involved in the development of several tumors. However, the role of IL-34 in the proliferation and epithelial-mesenchymal transition (EMT) of gastric cancer (GC) remains to be investigated.

Research motivation

To investigate the effect of IL-34 on the proliferation of GC cells and to find new therapeutic targets for the treatment of GC.

Research objectives

To clarify the effect of IL-34 on the prognosis of GC patients and the effect of IL-34 on the proliferation and EMT of GC cells.

Research methods

The expression of IL-34 protein in GC tissues and cells was detected using immunohistochemical staining and Western blotting. *In vitro*, stable IL-34 knockdown and overexpressed GC cell lines were cultured, and the proliferation, clone formation, migration and invasion ability of GC cells were examined using cholecystokinin-8 assay, clone formation assay, cell scratch assay and transwell assay, respectively. *In vivo*, the effect of IL-34 on GC transplantation tumor growth was assessed using a subcutaneous tumor transplantation assay in nude mice. Western blotting was used to detect the association of IL-34 protein with EMT-related protein expression levels.

Research results

IL-34 expression is elevated in GC cells and tissues, and IL-34 expression levels correlated with tumor size, T stage, N stage, tumor, node and metastasis stage, and degree of differentiation. *In vitro*, endogenous upregulation of IL-34 promoted GC cell proliferation and EMT. *In vivo*, IL-34 overexpression promoted subcutaneous graft tumor growth in nude mice.

Research conclusions

IL-34 expression is increased in GC tissues and cell lines. IL-34 promotes the proliferation and epithelial-mesenchymal transition of GC cells.

Research perspectives

IL-34 may represent a new strategy for the diagnosis and targeted treatment of GC. Further search for potential cancer-promoting mechanisms of IL-34 is needed in the future.

FOOTNOTES

Author contributions: Li CH, Chen ZM, and Chen PF collected the data and wrote the manuscript; Sui WN participated in data analysis; Ying SC participated in discussion and language editing; Xu AM provided funding support; Han WX reviewed the manuscript and provided funding support; All authors contributed to the article and approved the submitted version.

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