Interleukin-22 regulates gastric cancer cell proliferation through regulation of the JNK signaling pathway

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Abstract. Inflammation is considered as one of the major hallmarks of cancer and is associated with gastric cancer. Interleukin-22 (IL-22), a member of the IL-10 family, serves an important role in inflammatory diseases and tumors. The aim of the present study was to examine the effects of IL-22 on the proliferation of gastric cancer cells (AGS cells) *in vitro* and explore the associated molecular mechanism. The results of a Cell Counting kit-8 assay using AGS cells transfected with an IL-22-plasmid indicated that IL-22 could promote AGS cell viability. However, when IL-22 was knocked down by IL-22-short hairpin (sh)RNA, the viability of AGS cells was significantly impaired. Western blotting results indicated that IL-22 decreased the activation of the mitogen-activated protein kinase (MAPK) signaling pathway. Furthermore, IL-22-shRNA transfection increased the activation of MAPK, as evidenced by the upregulated phosphorylation of ERK and JNK. Taken together, the results of the present study suggest that IL-22 regulated the viability of gastric cancer cells through the JNK signaling pathway, suggesting a therapeutic approach for gastric cancer via targeting IL-22.

Introduction

Gastric cancer is one of the primary causes of cancer-associated mortality worldwide and is responsible for over 700,000 fatalities per year (1). It is the second most common type of cancer and the third leading cause of fatality amongst patients with cancer in China (2). The gastric cancer incidence in China and Japan account for more than 40% of the worldwide occurrences (3).

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A wide range of cytokines, chemokines and growth factors, as well as the extracellular matrix can affect the carcinogenesis and progression of gastric cancer (4). Recently, it has been suggested that the interaction between cancer cells and the surrounding tumor microenvironment serves a pivotal role during tumor progression (5). As one of the cytokines secreted by T helper 17 cells in the tumor microenvironment, interleukin (IL)-22 is a cytokine that structurally associated with IL-10 and produced predominantly by activated lymphocytes in chronically inflamed tissues (6). IL-22 exerts its biological actions via the IL-22 receptor (IL-22R) (7). IL-22R is a heterodimeric receptor consisting of two chains: IL-22R1 and IL-10R2. IL-10R2 is ubiquitously expressed in various organs, whereas IL-22R1 is restricted to epithelial cells in the skin, pancreas, kidney, liver and gastrointestinal tract (8). It has been reported that the expression of IL-22 is elevated in several types of gastrointestinal cancer (9,10) and that increased IL-22 expression is associated with cancer development (8).

JNK, a member of the mitogen-activated protein kinase (MAPK) family, can respond to a variety of environmental stresses, including cytokines, ultraviolet irradiation and heat shock, and has been implicated in multiple cellular events, including apoptosis and autophagy (11). There are three JNK genes, namely JNK1, JNK2 and JNK3, which encode 2-4 JNK isoforms (12). JNK1 has been revealed to be involved in apoptosis, neurodegeneration, cell differentiation and proliferation, as well as inflammatory conditions (13-16). It can also regulate several important cellular functions, including cell growth, differentiation, survival and apoptosis (17,18). It has been documented that IL-22 can trigger the nuclear factor-κB, MAPK and PI3K/Akt/mTOR signaling pathways (19). IL-22-mediated signaling enhances the expression of genes with anti-inflammatory, mitogenic, proliferative and anti-apoptotic effects, which are cellular effects that promote local tissue regeneration and host defense (20).

The aim of the present study was to analyze the role of IL-22 in gastric cancer cell progression and explore its underlying molecular mechanism. The effects of IL-22-plasmid and IL-22-short hairpin (sh)RNA on the viability of gastric cancer cells were therefore investigated.

Materials and methods

Cell culture. The gastric cancer cell line AGS and the human normal gastric epithelial cell line GES-1 were obtained from the American Type Culture Collection. GES-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences). AGS cells were maintained in Roswell Park Memorial Institute 1640 (Gibco; Thermo Fisher Scientific, Inc.). All media were supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin (Sigma-Aldrich; Merck KGaA) and 100 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA). Cell lines were cultured at 37˚C in a humidified incubator with 5% CO_2.

Cell transfection. IL-22-shRNA, control shRNA and IL-22-plasmids control plasmid were designed and constructed by Genechem Corporation. IL-22-shRNA, IL-22-plasmid and the corresponding control were transfected into AGS cells using Lipofectamine™ 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. 1 μl shRNA or 0.5 μg plasmid was diluted with 50 μl serum-free Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.) respectively, gently mixed and incubated at room temperature for 5 min. 1 μl Lipofectamine™ 2000 reagent (Invitrogen; ThermoFisher Scientific Inc.) was diluted with 50 μl serum-free Opti-MEM, lightly mixed and incubated at room temperature for 5 min. Then, the above mixtures were mixed again gently and incubated at room temperature for 20 min. The mixture of shRNA-Lipofectamine™ 2000 or plasmid-Lipofectamine™ 2000 was added into 400 μl medium and incubated for 6 h. The medium was replaced with fresh medium at 37˚C for 48 h. Untreated cells served as the control group. Following incubation for 48 h, the cells were subjected to subsequent experiments. Transfection efficiency was determined using reverse transcription-quantitative PCR (RT-qPCR).

Western blot analysis. The anti-ERK monoclonal antibody (mab; MAB1230; 1:1,000), anti-phosphorylated (p)-ERK mab (MAB18251; 1:1,000), anti-JNK mab (AF1387; 1:2,000), anti-p-JNK mab (AF1205; 1:500), anti-STAT3 mab (MAB1799; 1:1,000), anti-p-STAT3 mab (AF4607; 1:500), anti-Bcl-2 mab (AF810; 1:1,000), anti-GAPDH mab (MAB5718; 1:2,000) and mouse or rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (HAF007, HAF008; 1:1,000) were purchased from R&D Systems, Inc.

Cells were lysed in radioimmunoprecipitation assay buffer containing phenylmethane sulfonyl fluoride and protease inhibitors (Beyotime Institute of Biotechnology). The protein concentration of each sample was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology). Following this, 30-50 μg of whole-cell protein from each sample was loaded and separated using 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (EMD Millipore). The membranes were blocked with 5% non-fat milk or bovine serum albumin at room temperature for 1 h, and incubated with primary antibodies as mentioned above overnight at 4˚C, according to the manufacturer's instructions, followed by HRP-conjugated secondary antibody at room temperature for 2 h. An enhanced chemiluminescence detection system (GE Healthcare) was used for the visualization of the target proteins. GAPDH was used as the loading control. Relative quantification of protein expression was conducted using Image-Pro Plus 6.0 (Media Cybernetics, Inc.).

Cell viability assay. Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) was used to assess cell viability. Cells were seeded onto 96-well culture plates ($2x10^3$ cell/well), then transfected with IL-22-plasmid, IL-22-shRNA and corresponding negative control as described above. Cells were cultured for a further 24, 48, 72 h, allowing for transcription, translation and secretion. Subsequently, CCK-8 was added to the culture media. The optical density value of each well was measured at a wavelength of 450 nm using a plate reader (Infinite 200 PRO NanoQuant; Tecan Austria GmbH).

RT-qPCR. Total RNA was extracted from the cells using the TRIzol Plus RNA Purification kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. A reaction mixture (20 μl) containing total RNA (1 μg) was transcribed to cDNA at 37˚C for 15 min and 85˚C for 5 sec using PrimeScript RT-polymerase (GE Healthcare Life Sciences).

qPCR was performed using the SYBR Green qPCR kit (Takara Bio, Inc.), and the reaction system was established according to the manufacturer's instructions. qPCR was performed at 95˚C for an initial 10 min followed by 40 cycles of denaturation for 30 sec at 95˚C, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec. GAPDH was used as the internal control. All reactions were performed on the CFX96 Touch™ System (Bio-Rad Laboratories, Inc.). Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (21). The following primers were used: GAPDH forward, 5'-ATTGAT GGATGCTAFGAGTATT-3' and reverse, 5'-AGTCTTCTG GGTGGCAGTGAT-3'; and IL-22 forward 5'-GCTAAGGAG GCTAGCTTG-3' and reverse, 5'-CAGCAAATCCAGTTC TCC-3'.

Statistical analysis. Statistical analysis was performed using SPSS 13.0 software (SPSS, Inc.). Experimental values are expressed as the mean ± SEM of at least three independent experiments performed in triplicate. Data were analyzed using one-way analysis of variance followed by Dunnett's post hoc test for multiple comparisons or a Student's t-test for comparisons between two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

IL-22 is overexpressed in the AGS cell line. In order to explore the role of IL-22 in gastric cancer, the expression level of IL-22 in GES-1 and AGS cells was compared. As depicted in Fig. 1, the expression of IL-22 was significantly higher in AGS cells compared with GES-1 cells at the protein and mRNA levels. Therefore, AGS was chosen as the target cell line in the present study and subjected to further analysis.

IL-22 promotes AGS cell viability. To investigate the effect of IL-22 on cell viability, AGS cells were transfected with control plasmid, IL-22 plasmid, control shRNA or IL-22 shRNA. The

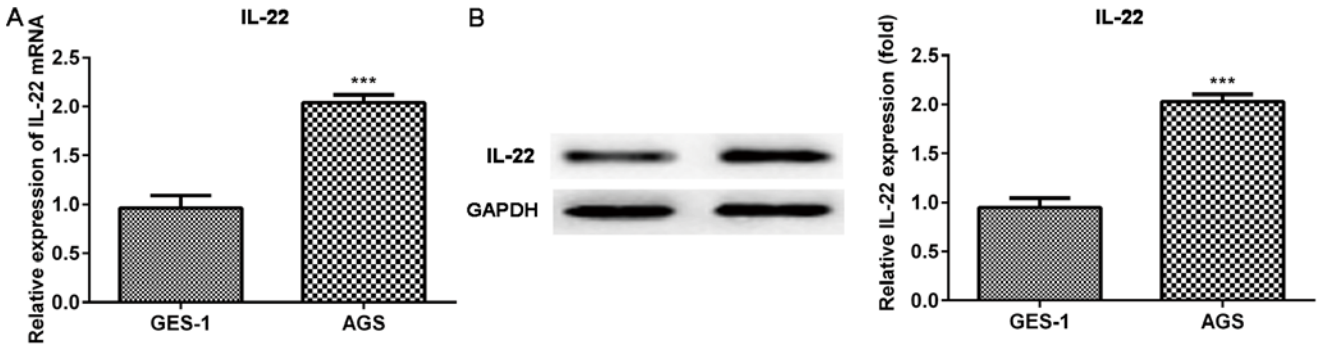

Figure 1. IL-22 is overexpressed in AGS cells. (A) Reverse transcription-quantitative PCR revealed that the mRNA expression level of IL-22 was significantly higher in AGS cells than in GES-1 cells. (B) Western blot analysis demonstrated that the protein expression of IL-22 was significantly enhanced in AGS cells compared with GES-1 cells. ***P<0.001 vs. GES-1 group. IL, interleukin.

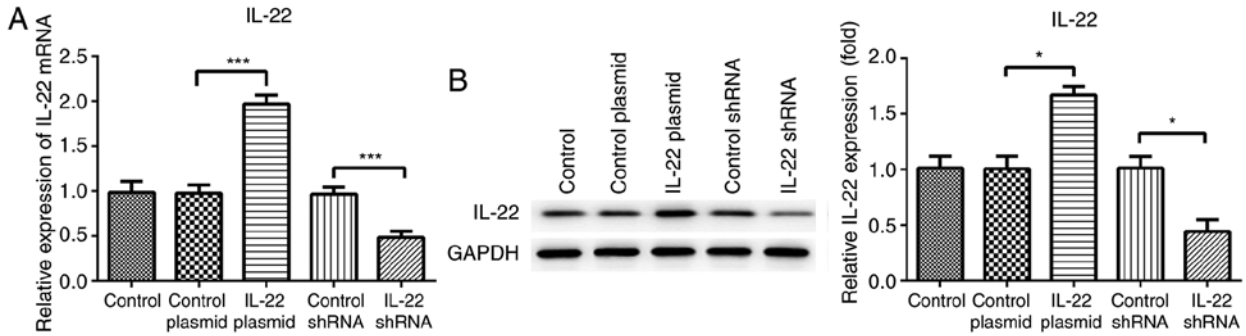

Figure 2. mRNA and protein expression levels of IL-22 in different treatment groups following transfection. (A) Reverse transcription-quantitative PCR revealed that IL-22 expression was higher in the IL-22 plasmid group and downregulated in the IL-22 shRNA group compared with the respective control group. ***P<0.001 as indicated. (B) Western blot analysis demonstrated that protein expression of IL-22 was increased in the IL-22 plasmid group and decreased in the IL-22 shRNA group compared with the respective control group. *P<0.05 as indicated. IL, interleukin; shRNA, short hairpin RNA.

expression level of IL-22 in each treatment group was detected using western blot analysis and RT-qPCR. As indicated in Fig. 2, significantly higher IL-22 expression and an enhanced transcriptional level of IL-22 mRNA was observed in the IL-22 plasmid treatment group compared with the control plasmid treatment group. Furthermore, shRNA IL-22 reduced the translation of IL-22 in AGS cells, as the mRNA and protein expression levels of IL-22 were knocked down significantly compared with those in the control shRNA group.

The viability of AGS cells at different time points following transfection for 48 h was examined using the CCK-8 assay. As indicated in Fig. 3, the overexpression of IL-22 significantly increased AGS cell viability compared with that in the control plasmid group. In addition, IL-22 knockdown significantly decreased the viability of AGS cells when compared with the control shRNA group.

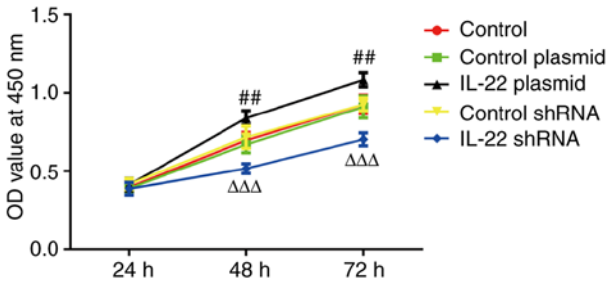

Figure 3. IL-22 increases the viability of AGS cells. The viability of AGS cells was assessed at 24, 48 and 72 h following transfection. The viability of AGS cells was increased in the IL-22 plasmid group and decreased in the IL-22 shRNA group compared with the respective control group. ##P<0.01 vs. control plasmid group; ΔΔΔP<0.001 vs. control shRNA group. IL, interleukin; shRNA, short hairpin RNA; OD, optical density.

MAPK signaling pathway is involved in the effects of IL-22 on AGS cells. To investigate the molecular mechanism underlying the effects of IL-22, activation of the MAPK signaling pathway in AGS cells was investigated following transfection with IL-22 plasmid and IL-22 shRNA (Fig. 4). Western blot analysis revealed that the IL-22 plasmid decreased the phosphorylation of ERK and JNK, increased the phosphorylation of p-STAT3 and increased the expression of Bcl-2, when compared with the control plasmid. By contrast, transfection with IL-22 shRNA increased the phosphorylation of ERK and JNK, decreased the phosphorylation of STAT3 and decreased the expression of Bcl-2 when compared with the control shRNA.

Discussion

The incidence of gastric cancer is gradually declining in developed countries; however, it remains one of the most common types of cancer worldwide (22). Although the overall survival of patients with gastric cancer has been prolonged in the last decades, due to an increase in early detection and a wider use

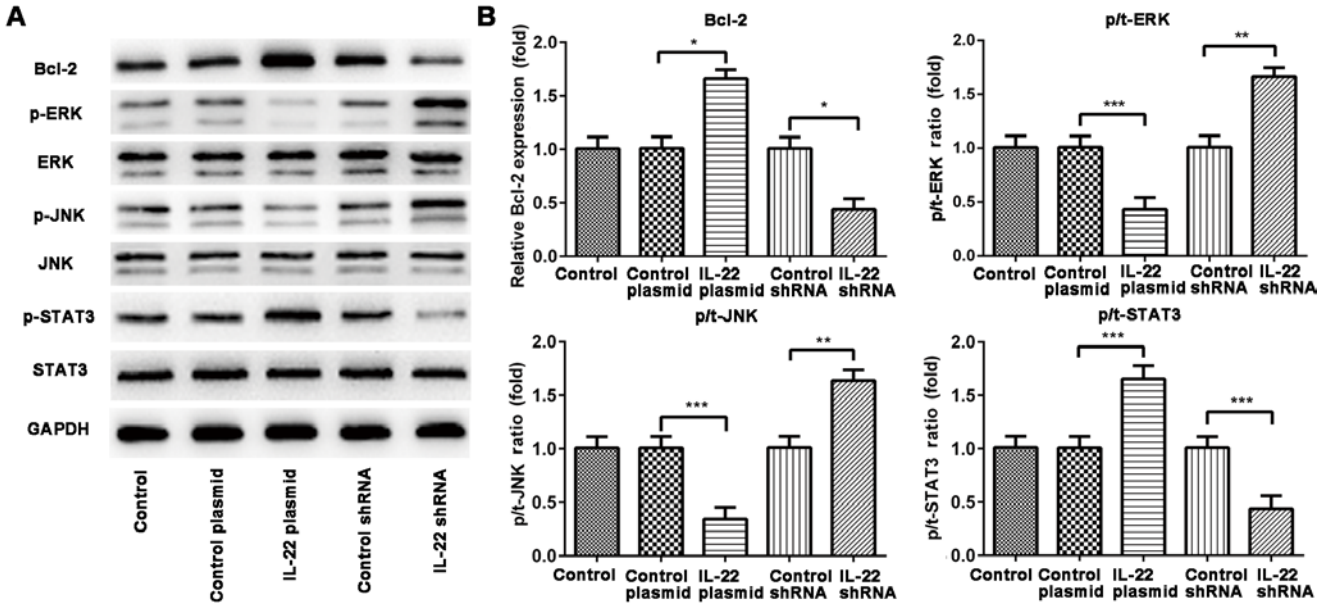

Figure 4. Activation of the MAPK signaling pathway in AGS cells following transfection with IL-22 plasmid and IL-22 shRNA. (A) Representative western blots and (B) quantification results demonstrated that IL-22 plasmid decreased the phosphorylation of ERK and JNK, increased the phosphorylation of STAT3 and increased the expression of Bcl-2, whereas IL-22 shRNA increased the phosphorylation ERK and p-JNK, decreased the phosphorylation of STAT3 and decreased Bcl-2 expression. *P<0.05, **P<0.01 and ***P<0.001 as indicated. MAPK, mitogen-activated protein kinase; IL, interleukin; shRNA, short hairpin RNA; p-, phosphorylated; t-, total.

of radical surgery (23), the prognosis of this advanced type of cancer remains poor as safe and effective treatment options are limited (24). It is urgently necessary to explore the molecular mechanism of gastric cancer in order to aid the progress of clinical practice and drug development.

While it is clear that various cytokines are associated with the proliferation of human cancer cells, the mechanisms of cytokine action remain largely unknown. IL-22 and other members of the IL-20 subfamily (IL-20, IL-24 and IL-26) belong to the larger IL-10 family of cytokines that have important roles in tissue remodeling and wound healing (25). Previous studies have demonstrated that IL-22 is released by infiltrating immune cells and by the tumor microenvironment, thereby promoting the progression of tumor cells. As these functions are also central to several types of cancer, it is not surprising that IL-22 has been implicated in cancer development and progression (26-28). Increased numbers of blood-circulating and intratumoral IL-22-secreting cells have been reported in lung, gastric, colorectal, pancreatic and hepatocellular carcinomas (20). Although IL-22 has been well documented in the aforementioned cancer types, the underlying mechanism of IL-22 regulation of gastric cancer cell is not clear. In the present study, the pro-survival effect of IL-22 and its underlying molecular mechanism of action in AGS cells were revealed for the first time. It was identified that IL-22 promoted AGS cell proliferation.

The signaling pathway underlying the effect of IL-22 on AGS cells was also investigated in the present study. IL-22 has various functions in multiple cell types by activating a wide range of signaling cascades, such as the Janus kinase (JAK)/STAT, ERK, JNK, p38 and PI3K/Akt/mTOR pathways (29-32). Several important signaling pathways can be regulated downstream of IL-22R1 activation. For instance, IL-22 can stimulate the PI3K/AKT pathway in epidermal keratinocytes (31). It can also activate the JAK/STAT signaling pathway in human hepatocellular carcinoma cells (33) and ERK1/2, p38 and JNK in H4IIE cells (29). Furthermore, IL-22 induces matrix metalloproteinase-9 production via activation of IL-22R1 and AKT, further confirming the involvement of IL-22R1/AKT signaling in the IL-22-mediated migration and invasion of gastric cancer cells (34). JNKs have a critical role in death receptor-initiated extrinsic and mitochondrial intrinsic apoptotic pathways, and can activate apoptotic signaling through the upregulation of pro-apoptotic genes via the transactivation of specific transcription factors upon stimulation (18). Therefore, it may be hypothesized that IL-22 could protect AGS cells from apoptosis through the JNK signaling pathway. In the present study, western blot analysis and RT-qPCR demonstrated that IL-22 downregulated the phosphorylation of ERK and JNK simultaneously, which indicated multiple inhibition of MAPK activity in AGS cells. The activated MAPK pathway has been detected in many types of cancer, including breast, prostate, lung and gastric cancer (35). In addition, the present findings showed that IL-22 induced a robust activation of STAT3. The expression of Bcl-2, an anti-apoptotic protein, was also increased in AGS cells transfected with IL-22-plasmid, which may indicate a potential anti-apoptotic effect of IL-22, in accordance with a previous study (36). However, further experiments such as flow cytometry and TUNEL staining are necessary to verify the anti-apoptotic effect of IL-22, and whether alternation of JNK activity affects the role of IL-22 in cell apoptosis is worth exploring.

In conclusion, the present study demonstrated that IL-22 acts as an endogenous resistance factor in human gastric cancer cell lines. By modulating the IL-22 expression levels with IL-22 plasmid or IL-22 shRNA, it was demonstrated that IL-22 enhanced the viability of AGS cells, which may involve the JNK signaling pathway.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HD designed the research. HD and FZ performed the experiments. SJ and JT analyzed the data. FZ drafted the manuscript and analyzed data. SJ and JT collected and interpreted data and revised the final manuscript. HD wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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