IL-22 produced by cancer-associated fibroblasts promotes gastric cancer cell invasion via STAT3 and ERK signaling

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Background: Interleukin-22 (IL-22) has been recently highlighted owing to its biological significance in the modulation of tissue responses during inflammation. However, the role of IL-22 in carcinogenesis has remained unclear. Here, we investigated the pathophysiological significance of IL-22 expression in gastric cancer tissues and examined the mechanism by which IL-22 promotes gastric cancer cell invasion.

Methods: Human gastric cancer specimens were analysed by immunohistochemistry for expression of IL-22 and IL-22 receptor 1 (IL-22R1). The effects of IL-22-induced STAT3 and ERK signalling on invasive ability of gastric cancer cells were examined using a small-interfering RNA system and specific inhibitors. AGS cells were co-cultured with cancer-associated fibroblasts (CAFs) from human gastric cancer tissues and assessed by invasion assay.

Results: Interleukin-22 and its receptor were expressed in α-smooth muscle actin-positive stromal cells and tumour cells at the invasive front of gastric cancer tissues, respectively. The expression of IL-22 and IL-22R1 was significantly related to lymphatic invasion. Interleukin-22 treatment promoted the invasive ability of gastric cancer cells through STAT3 and ERK activation. The invasive ability of gastric cancer cells was significantly enhanced by co-culture with IL-22-expressing CAFs.

Conclusions: Interleukin-22 produced by CAFs promotes gastric cancer cell invasion via STAT3 and ERK signalling.

Gastric cancer, the second leading cause of cancer-related death worldwide (Kamangar et al., 2006), is known to have a poor prognosis due to its marked propensity for invasion and metastasis. Recently, accumulating evidence has suggested that cross-talk between cancer cells and their surrounding stromal cells has a pivotal role in invasion and metastasis during tumour progression (Bhowmick et al., 2004; Xing et al., 2010). Indeed, gastric cancer tissues consist of not only cancerous cells but also stromal cells including fibroblasts, immune cells and endothelial cells, and those cells are likely to communicate via cytokines, chemokines, growth factors or extracellular matrix (Girri and Chiarugi, 2012).

Interleukin-22 (IL-22) is a recently identified IL-10 superfamily cytokine produced mainly by activated lymphocytes in chronically inflamed tissues (Dumoutier et al., 2000a; Liang et al., 2006; Zheng et al., 2007), and its receptor consists of two chains: IL-22 receptor 1 (IL-22R1) and IL-10 receptor 2 (IL-10R2) (Xie et al., 2000; Dumoutier et al., 2000b). Interestingly, IL-10R2 is expressed ubiquitously in various organs, whereas expression of IL-22R1 is...
restricted to epithelial cells, and not immune cells, in the skin, pancreas, kidney, liver, and gastrointestinal tract (Aggarwal et al., 2001; Wolk et al., 2004). In this regard, IL-22 may have pivotal roles as a biological mediator in signalling from the immune system to epithelial cells in the gastric mucosa. Detailed studies of the biological roles of IL-22 have suggested that IL-22 is likely to exert multiple functions under inflammatory conditions because its signalling modulates the expression of many genes involved in chemotaxis, proliferation, innate immunity and inflammation (Zenzewicz and Flavell, 2011; Rutz et al., 2013). On the other hand, the role of IL-22 in carcinogenesis remains somewhat unclear, even though chronic inflammation is a process involved in the development of various cancers. At the beginning of this study, we examined the expression of IL-22 in gastric cancer tissues and found that IL-22-positive stromal cells were increased at the invasive tumour front. Therefore, in the present study, we investigated whether IL-22 has a role in gastric cancer cell invasion and examined in vitro the process of intracellular signalling by which IL-22 promotes gastric cancer cell invasion. Moreover, we examined whether gastric cancer-associated fibroblasts (CAFs) are a possible source of IL-22 and whether they are associated with the promotion of gastric cancer cell invasion.

**MATERIALS AND METHODS**

**Reagents and cell culture.** Human recombinant IL-22 protein, anti-human IL-10R2 antibody and anti-human IL-22 neutralising antibody were purchased from R&D Systems (Minneapolis, MN, USA). MEK inhibitor PD98059 and PI3K–Akt inhibitor wortmannin were from Sigma (St Louis, MO, USA). Anti-STAT3, anti-phospho-specific STAT3 (p-STAT3; Tyr705), anti-Akt, anti-phospho-specific Akt (p-Akt; Ser473), anti-ERK, and anti-phospho-specific ERK (p-ERK) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-β-actin antibody was purchased from Sigma.

The gastric cancer cell lines MKN1, MKN28, MKN45, MKN74, and GCIY were maintained in RPMI 1640 medium (Invitrogen, Grand Island, NY, USA) with 10% fetal bovine serum (Invitrogen, Sigma).

**Tissue specimens and histological examination.** A total of 36 gastric cancer tissues were obtained from specimens resected surgically at Dokkyo University School of Medicine. The tissue specimens were fixed in 10% formalin solution and embedded in paraffin. This study was approved by the Dokkyo University Surgical Pathology Committee, and informed consent was obtained from all patients.

Multiple hematoxylin–eosin-stained sections of all 36 lesions were examined (Supplementary Table 1). The following factors were determined for all patients and lesions; age, sex, tumour size, tumour location, Lauren's histological classification, tumour invasion, lymph node metastases, and tumour stage according to the American Joint Committee on Cancer system.

**Immunohistochemistry.** Immunohistochemical staining for IL-22, IL-22R1, and phosphorylated STAT3 (p-STAT3) was performed with an Envision kit (DAKO, Kyoto, Japan) or an a brief, the sections were incubated with goat anti-IL-22 antibody (1: 50) or mouse anti-z smooth muscle actin (zSMA) for 60 min at room temperature. Then, the sections were incubated with fluorescein isothiocyanate-conjugated anti-goat immunoglobulin (1: 100; Dako) and Alexa Fluor 566 (1 : 100; Invitrogen) for 30 min at room temperature. After washing in phosphate-buffered saline (PBS), the sections were observed by confocal laser microscopy (LSM510; Carl Zeiss Japan, Tokyo, Japan).

**RNA extraction and RT-PCR.** Total RNA was extracted from each cell line using Trizol reagent (Invitrogen). Five micrograms of total RNA was reverse-transcribed using oligo dT primer (Applied Biosystems, Branchburg, NJ, USA) and 200 U of Superscript II reverse transcriptase (Invitrogen) in a total volume of 20 μl. For the following PCR, pairs of oligonucleotide primers for human IL-22 (Ziesche et al., 2009), human IL-22R1 (Naher et al., 2012), human IL-10R2 (Naher et al., 2012), and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Ogata et al., 2010) were prepared. Human IL-22: 5'-GCTAAGGAGGCTAGCTTG-3' (sense) and 5'-CACCAAAATCTCGTTTCC-3' (antisense); human IL-22R1: 5'-CTCACAGGGCGATAGCTC-3' (sense) and 5'-ACATGCACTCTGCTCAGTCG-3' (antisense); human IL-10R2: 5'-GGCTGAATTTCGAGATGAGCA-3' (sense) and 5'-GAAGACCCAGGCATGAG-3' (antisense); human GAPDH: 5'-GGCTGCTTTAACTCTGGTA-3' (sense) and 5'-ATGACGTCCTCCCAG-3' (antisense). One microtitre of RT product (cDNA) was amplified by PCR in a 50-μl reaction volume containing 20 pmol of the above sets of primers, 1.25 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA), and the final PCR buffer: 20 μmol Tris-HCl (pH 8.4), 50 μmol KCl, 2.5 μmol MgCl2, 10 μmol dithiothreitol, and 1 μmol dNTP. The PCR amplification was performed as follows: at 95 °C for 5 min once; 35 cycles at 95 °C for 1 min, at 60 °C for 1 min, and at 72 °C for 1 min; then at 72 °C for 7 min.

**Real-time RT-PCR.** Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using 7900HT Fast Real-Time PCR System (Applied Biosystems) as previously described (Sekikawa et al., 2010). The following sets of primers for human matrix metalloproteinase 7 (MMP7), MMP13, IL-22, IL-22R1, and GAPDH, were prepared (Supplementary Table 2). Real-time RT-PCR assays were carried out with 200 ng RNA equivalent cDNA, SYBR Green Master Mix (Applied Biosystems), and 500 nmol1 L gene-specific primers. The PCR cycling conditions were 50 °C for 15 s and 60 °C for 60 s. The intensity of the fluorescent dye was determined, and each of mRNA expression levels was normalised to GAPDH mRNA expression levels.

**Western blot analysis.** Proteins were extracted as previously reported (Hoshino et al., 2007). In brief, following treatment with or without reagents, cells were lysed in protein extraction buffer. Proteins from membrane and cytoplasm were extracted from the supernatants and the precipitation was additionally treated with H2O2 for 3 min and then counterstained with Mayer's haematoxylin. To evaluate the immunoreactivity of IL-22, at least five different visual fields were observed at the invasive front of gastric cancer lesions. A specimen was considered positive when IL-22-positive fibroblastic nests were observed in the visual fields examined. IL-22R1 was focally expressed at the invasive front or diffusely at whole parts of gastric cancer lesion. Both of the immunostaining patterns were considered as positive, whereas no immunoreactivity was considered as negative. Immunoreactivity of p-STAT3 was evaluated as previously described (Sekikawa et al., 2008). A specimen was considered positive when >10% of the tumour cell nuclei were positively stained.

Immunohistochemical double staining was performed in gastric cancer tissues as previously described (Sekikawa et al., 2010). In brief, the sections were incubated with goat anti-IL-22 antibody (1: 50) or mouse anti-z smooth muscle actin (zSMA) for 60 min at room temperature. Then, the sections were incubated with fluorescein isothiocyanate-conjugated anti-goat immunoglobulin (1: 100; Dako) and Alexa Fluor 566 (1 : 100; Invitrogen) for 30 min at room temperature. After washing in phosphate-buffered saline (PBS), the sections were observed by confocal laser microscopy (LSM510; Carl Zeiss Japan, Tokyo, Japan).
nuclear lysis buffer. After centrifugation, nuclear protein was extracted from the treated supernatants.

Western blot analyses were performed using each primary antibody as previously described (Sekikawa et al., 2008). Protein extract (25 μg) was fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with a primary antibody and then with a peroxidase-conjugated secondary antibody. Proteins were detected using an enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK).

**RNA interference.** Small-interfering RNA for human STAT3 (STAT3 siRNA) was obtained from Qiagen (Hilden, Germany). AGS and MKN28 cells were seeded in 6-cm dish (Iwaki, Funabashi, Japan) and maintained for 24 h. Then, the cells were transfected with 25 nM STAT3 siRNA or non-silencing siRNA (as a control) using the Oligofectamine reagent (Qiagen) according to the manufacturer’s recommendation (Sekikawa et al., 2010). After incubation for 48 h, cells were washed with PBS, harvested, and subjected to invasion assay.

**Invasion assay.** Cell invasion assay was performed using BioCoat Matrigel invasion chambers (BD Biosciences, Bedford, MA, USA) in accordance with the manufacturer’s protocol. Briefly, AGS (5 × 10^4), MKN28 (5 × 10^5), or those with RNA interference treatment were seeded in the upper matrigel-coated invasion chamber (24 wells, 8-μm pore size), and the lower chamber was filled with serum-free medium containing different concentrations of IL-22 (0–100 ng ml⁻¹). To inhibit the effects of IL-22 (10 ng ml⁻¹) or its related ERK signalling, anti-IL-22 antibody (0–40 μg ml⁻¹) or MEK inhibitor PD98059 (20 μM) was added to the upper chamber. After incubation for 36 h, non-invading cells were removed using a cotton swab and the cells that had invaded into the lower surface of the membrane were fixed with 10% buffered formalin. The invading cells were then stained with hematoxylin and counted using a microscope in five different visual fields (magnification, × 200).

**Isolation of CAFs and ELISA.** Human gastric cancer specimens were obtained from patients who underwent gastrectomy at Hyogo College of Medicine Hospital in 2012. Tissue specimens were prepared from the cancerous portion (CAFs) and non-cancerous portion at least 50 mm distant from the tumour (normal gastric fibroblasts; NGFs) in the stomach. The tissue specimens were trimmed of fat and necrotic tissue, minced with scalpels and washed in PBS containing antibiotic–antimycotic reagent (Anti-Anti, GIBCO, Grand Island, NY, USA). The tissue pieces were transferred to a 12-well microplate (IWAKI, Tokyo, Japan) at one fragment per well. The cells were cultured in DMEM medium (GIBCO) with 10% heat-inactivated FCS (Biowest, Nuaille, France) at 37 °C in an atmosphere of 5% CO₂. The fibroblasts that initially grew in a monolayer were collected, transferred to another dish and used for experiments within the 10th passage.
Table 1. Relationship between clinicopathological features and IL-22 or IL-22R1 expression in patients with gastric cancer

| Tumour location       | Number of IL-22 positive/total number of patients | P-value | Number of IL-22R1 positive/total number of patients | P-value |
|-----------------------|-------------------------------------------------|---------|-----------------------------------------------------|---------|
| Lower                 | 7/9 (77.8%)                                      | 0.8975  | 9/9 (100%)                                         | 0.4784  |
| Mid                   | 11/13 (84.6%)                                    |         | 12/13 (92.3%)                                      |         |
| Upper                 | 11/14 (78.6%)                                    |         | 12/14 (85.7%)                                      |         |
| Lauren’s classification|                                                | 0.5515  |                                                    | > 0.9999|
| Intestinal type       | 9/12 (75.0%)                                     |         | 11/12 (91.7%)                                      |         |
| Diffuse type          | 20/24 (83.3%)                                    |         | 22/24 (91.7%)                                      |         |
| Stage                 |                                                | 0.0035  |                                                    | 0.3729  |
| I                     | 3/8 (37.5%)                                      |         | 7/8 (87.5%)                                        |         |
| II                    | 5/5 (100%)                                       |         | 5/5 (100%)                                        |         |
| III                   | 12/12 (100%)                                     |         | 12/12 (100%)                                       |         |
| IV                    | 9/11 (81.1%)                                     |         | 9/11 (81.1%)                                       |         |
| Lymphatic invasion    |                                                | 0.0031  |                                                    | 0.0282  |
| None                  | 0/2 (0.0%)                                       |         | 1/2 (50.0%)                                        |         |
| Present               | 29/34 (85.3%)                                    |         | 32/34 (94.1%)                                      |         |
| Venous invasion       |                                                | 0.3464  |                                                    | 0.4185  |
| None                  | 4/6 (66.7%)                                      |         | 5/6 (83.3%)                                        |         |
| Present               | 25/30 (83.3%)                                    |         | 28/30 (93.3%)                                      |         |
| Lymph node metastasis |                                                | 0.0016  |                                                    | 0.7277  |
| None                  | 4/9 (44.4%)                                      |         | 8/9 (88.9%)                                        |         |
| Present               | 25/27 (92.6%)                                    |         | 25/27 (92.6%)                                      |         |

Abbreviations: IL-22 = interleukin-22; IL-22R1 = IL-22 receptor 1. P-values < 0.05 are indicated by bold entries.
Interleukin-22 stimulation promotes the invasive ability of gastric cancer cells. We pursued a function of IL-22 invasive ability of gastric cancer cells as IL-22-positive stromal cells were increased at the invasive tumour front. We examined the invasive ability of gastric cancer cells stimulated with IL-22 using a Matrigel invasion assay. Gastric cancer cells that had invaded across the membrane were observed below the membrane (Figure 3A). When AGS cells were stimulated with IL-22, the number of invasive cells was significantly increased in a dose-dependent manner (Figure 3A). Similarly, the invasive ability of MKN28 cells was significantly enhanced dose-dependently by IL-22 stimulation (Figure 3A). Then, to inhibit the effect of IL-22, AGS cells were treated concomitantly with anti-IL-22 antibody (10–40 μg ml⁻¹). This abolished the increase in the number of invasive AGS cells stimulated with IL-22 (Figure 3B), and similar findings were also obtained for MKN28 cells (Figure 3C). Moreover, we examined whether IL-22 upregulates the expression of MMP7 and MMP13, which are likely to promote cell invasion in the downstream of IL-22 signalling (Howlett et al., 2005; Fukuda et al., 2011). As shown in Figure 3D, IL-22 stimulation enhanced the expression of MMP7 and MMP13 in MKN28 and AGS cells, respectively. In contrast, we showed that the increase of MMP7 and MMP13 expression was abolished by the addition of anti-IL-22 antibody. Regarding the effect of IL-22 on cell proliferation and survival, IL-22 treatment did not show any promoting effects for those cell lines under this experimental condition (Supplementary Figure 2).

**Interleukin-22 stimulation promotes gastric cancer cell invasion via STAT3 and ERK signalling.** Inhibition of STAT3 signalling by STAT3 siRNA significantly decreased the number of invasive AGS cells accelerated by IL-22 stimulation, showing that IL-22 promotes AGS cell invasion via STAT3 signalling. In addition, STAT3 siRNA significantly inhibited the invasive ability of AGS cells under unstimulated conditions, suggesting that STAT3 signalling is crucial for AGS cell invasion (Figure 4A).
With regard to MKN28 cells, STAT3 siRNA partly but not completely decreased the number of invasive cells accelerated by IL-22 stimulation, suggesting that not only STAT3 but also some other forms of signalling may mediate the promotion of MKN28 cell invasion by IL-22 (Figure 4B). We furthermore examined whether MAPK signalling is involved in the promotion of gastric cancer cell invasion by IL-22. The increase in the number of invasive AGS cells after the treatment with IL-22 was partly attenuated by concomitant administration of the MEK inhibitor PD98059 (Figure 4C). On the other hand, the increase in the number of MKN28 cells after treatment with IL-22 was reduced to a level equivalent to that in the absence of stimulation upon treatment with PD98059 (Figure 4D).

Throughout the invasion assay, cell viability was 48.6% by trypan blue (data not shown). These findings suggest that IL-22 may promote gastric cancer cell invasion via STAT3 and MAPK signalling, although the predominant intracellular signalling mechanism may differ according to the cell line.

**Discussion**

Although IL-22 has recently been highlighted in the pathophysiology of inflammatory diseases, its biological role in carcinogenesis is poorly understood. In the present study, we first confirmed that IL-22 promotes gastric cancer cell invasion. We examined whether CAF cells produce IL-22 protein by ELISA. The concentration of IL-22 in culture supernatant was lower than the serum concentration from UC, whereas it was higher than the serum concentration from healthy control (Figure 5A). This finding suggests that CAF cells are at least possible to produce IL-22 protein. Moreover, we demonstrated that the level of IL-22 expression was significantly greater in CAFs than in NGF cells (Figure 5B).

To establish an in vitro model for the invasive front of gastric cancer tissues, we prepared co-culture system using CAF1 and AGS cells. The number of invasive AGS cells was significantly increased when AGS cells were co-cultured with CAF1 cells (Figure 5C). Furthermore, addition of IL-22 antibody abolished the increase in number of invasive AGS cells under co-culture with CAF1 cells (Figure 5C).
IL-22 promotes gastric cancer cell invasion

To clarify whether IL-22 promotes the invasion ability of gastric cancer cells, we stimulated gastric cancer cell lines with IL-22 in an *in vitro* invasion assay. As shown in Figure 3, IL-22 stimulation significantly promoted the invasive ability of gastric cancer cells, and its effect was abolished by addition of IL-22 antibody, confirming that IL-22 is an invasion-promoting factor for gastric cancer cells. Although we did not test the effect of IL-22 in *in vivo* models, it is noteworthy that hepatocellular carcinoma co-transplanted with IL-22-expressing lymphocytes showed a high potential for invasion and metastasis (Jiang et al., 2011). Gastric cancer cells are known to have higher invasion and metastasis potential than other solid tumours. Therefore, IL-22 warrants further study as a potentially important mediator of invasion/metastasis in gastric carcinogenesis.

Previous studies have indicated that IL-22 may activate various pathways such as STAT3, MAPK, Akt and/or NF-κB signalling in different types of cells (Lejeune et al., 2002; Andoh et al., 2005; Zhang et al., 2008). In this regard, we examined the signalling pathways activated by IL-22 in human gastric cancer cells and subsequently showed that STAT3 and ERK phosphorylation was enhanced in two of the gastric cancer cell lines examined. Accumulating evidence suggests that IL-22 is possible to promote cell proliferation and anti-apoptosis via STAT3 and/or ERK signalling (Brand et al., 2006; Ziesche et al., 2007; Zhang et al., 2008; Sekikawa et al., 2010; Jiang et al., 2011). In addition, we have clarified in the present study that IL-22 significantly promoted the invasive ability of two gastric cancer cell lines via STAT3 and ERK signalling. These effects of IL-22 seem to be advantageous to tumour progression, and indeed, recent studies has reported that IL-22 is associated with tumour progression and prognosis in human malignancies (Kobold et al., 2013; Wen et al., 2014).

Interleukin-22 is normally expressed in immune cells such as IL-17-producing T (Th17), NK, dendritic, and lymphoid...
tissue-inducer cells (Zenewicz and Flavell, 2011). However, it was noteworthy that IL-22 was strongly expressed in αSMA-positive fibroblasts neighbouring gastric cancer cells at the invasive front. The fibroblasts in such a tumour microenvironment are referred to as CAF cells and can be detected by their αSMA expression (Xing et al, 2010; Cirri and Chiarugi, 2012). In this context, it is tempting to speculate that IL-22 is produced in CAF cells in gastric cancer tissues. In support of this, we confirmed that IL-22 is expressed in isolated CAF cells from gastric cancer tissues. Although we are unable to explain why IL-22 is expressed in CAF cells, recent evidence has suggested that CAF cells originate from not only resident fibroblasts but also bone marrow-derived progenitors or transformed cells from endothelial or cancer cells (Xing et al, 2010; Cirri and Chiarugi, 2012). Thus, the heterogeneity of CAF cells may explain the unexpected expression of IL-22 in CAF cells.

CAF cells have recently received attention because of their pivotal roles in tumour growth, angiogenesis, invasion, and metastasis by interacting with tumour cells (Bhowmick et al, 2004; Xing et al, 2010; Cirri and Chiarugi, 2012). Although the mechanism of the interaction between CAF and tumour cells is not fully understood, growth factors, chemokines or extracellular matrix are thought to be important mediators by which such cells communicate with their microenvironment (Bhowmick et al, 2004; Xing et al, 2010; Cirri and Chiarugi, 2012). Regarding the role of IL-22 in cancerous cells, a few studies have indicated that IL-22 can function as a cell growth and/or anti-apoptotic factor in vitro (Brand et al, 2006; Zhang et al, 2008; Jiang et al, 2011), although the source of IL-22 in human cancerous tissues has remained unclear. In this context, we have shown for the first time that IL-22 promotes the invasive ability of gastric cancer cells via STAT3 and ERK activation, and that CAF is a possible source of IL-22 at the invasive tumour front. Although these two important findings were confirmed in different series of experiments, our co-culture experiment lent further support to the possibility that IL-22-expressing CAFs actually have a role in the promotion of gastric cancer cell invasion.

In summary, we have clarified that IL-22 promotes the invasive ability of gastric cancer cells via STAT3 and ERK activation. Moreover, we have shown that IL-22 is expressed in CAFs at the invasive front of gastric cancer lesions and that IL-22-expressing CAFs isolated from human gastric cancer promote invasion of the cancer cells. Together, these results suggest that IL-22 produced by CAFs promotes gastric cancer cell invasion via STAT3 and ERK signalling.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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