The interleukin-mediated Janus kinase (JAK)/STAT pathway plays a crucial role in carcinogenesis. Recently, increased STAT3 activity was found in hepatocellular carcinoma and multiple myeloma in which there was silencing of SOCS-1 (suppressor of cytokine signalling-1) by gene promoter hypermethylation. We investigated the expression level of interleukin-6 (IL-6) and SOCS-1 in gastric cancer cell lines. Expression of SOCS-1 correlated with IL-6 level in most of the cell lines, except for AGS cells in which SOCS-1 was absent despite a high level of IL-6 production. Methylation analysis by methylation-specific polymerase chain reaction and bisulphite sequencing revealed that CpG island of SOCS-1 was densely methylated in AGS cells. Demethylation treatment by 5’aza-deoxycytidine restored SOCS-1 expression and also suppressed constitutive STAT3 phosphorylation in AGS cells. Moreover, methylation of SOCS-1 was detected in 27.5% (11 of 40) of primary gastric tumour samples, 10% (one of 10) of adjacent noncancer tissues but not in any (zero of nine) normal gastric mucosa. Methylation of SOCS-1 also correlated with the loss of mRNA expression in some primary gastric cancers. In conclusion, this is the first report to demonstrate that hypermethylation of SOCS-1 led to gene silencing in gastric cancer cell line and primary tumour samples. Downregulation of SOCS-1 cooperates with IL-6 in the activation of JAK/STAT pathway in gastric cancer.

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Gastric cancer is the second most common cause of cancer death worldwide (Pisani et al., 1993). One of the well recognised environmental risk factors for gastric cancer is Helicobacter pylori infection (Graham, 1997). Although this bacterium has been classified as a type I carcinogen by the World Health Organisation (Kuipers and Meuwissen, 1996), the mechanisms by which H. pylori causes gastric cancer is not fully understood. It is recognised that there is a strong inflammatory response in H. pylori-infected gastric cancer tissues. In particular, upregulation of interleukin-6 (IL-6) levels is observed in gastric cancer tissue (Yamaoka et al., 1996, 2001). Furthermore, serum IL-6 levels is shown to correlate with prognosis of gastric cancer patients (Wu et al., 1996). These data suggest that the activation of IL-6 signalling pathway may be important in the development of gastric cancer.

Interleukin-6 and other interleukin family proteins are thought to be involved in host defense mechanism as well as cancer development (Kabir and Daar, 1995; Wu et al., 1996; Schneider et al., 2000; Giri et al., 2001). The activation of IL-6 signal transduction involves binding to its transmembrane receptor and subsequent activation of the Janus kinase (JAK), which is followed by phosphorylation of STAT (STAT1/3) (O’Shea et al., 2002). Phosphorylated STAT protein then translocates into the nucleus with subsequent activation of target genes. One of the STAT-activated genes is SOCS-1 (suppressor of cytokine signalling-1). Suppressor of cytokine signalling-1 and its family are proteins containing the SH2 domains that interact with JAK and prevent activation of STAT, as well as downregulate the JAK/STAT signalling pathways (Endo et al., 1997; Starr et al., 1997). Specifically, SOCS-1 can be rapidly upregulated by IL-6 and is involved in the downregulation of the IL-6-induced activation of STAT3 (Starr et al., 1997; Nicholson et al., 1999).

Recent findings suggest that the inactivation of SOCS-1 was one of the targets in cancer development. Suppressor of cytokine signalling-1 is downregulated by methylation of the CpG island in human hepatocellular carcinoma (HCC), multiple myeloma and pancreatic ductal neoplasm (Yoshikawa et al., 2001; Nagai et al., 2002; Fukushima et al., 2003; Galm et al., 2003; Okochi et al., 2003). On the other hand, restoration of SOCS-1 suppressed growth in HCC cell lines and oncogene-activated haematopoietic cells (Yoshikawa et al., 2001; Rottapel et al., 2002). Taken together, these data suggest that SOCS-1 functions as a tumour suppressor in the JAK/STAT pathway.

In the present study, we found that there was downregulation of SOCS-1 gene in gastric cancer cell line AGS due to gene promoter
Methylation of SOCS-1 in gastric cancer

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Introduction

Hypermethylation. Furthermore, demethylation treatment by 5′aza-deoxycytidine (5′azaDC) not only restored SOCS-1 expression in AGS cell but also suppressed constitutive STAT3 phosphorylation. Methylation of SOCS-1 was detected in 27.5% (11 of 40) of primary gastric tumours samples. We further showed that downregulation of SOCS-1 correlated with the methylation status in primary gastric cancer. This study provides evidence that the activation of JAK/STAT pathway by aberrant SOCS-1 methylation in gastric cancer.

Materials and methods

Gastric cancer cell lines and tissues

Gastric cancer cell lines AGS (CRL-1739), SNU-16 (CRL-5974), KATO III (HTB-103), and NCI-N87 (CRL-5822) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), while MKN28 (RCB1000) and MKN45 (RCB1001) were obtained from Riken Cell Bank (Tsukuba, Japan). All cell lines except Kato III were grown in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL). Kato III was grown in RPMI 1640 medium supplemented with 20% FBS. All cell lines were kept at 37°C in a humidified incubator with 5% CO₂ in air.

In all, 40 primary gastric tumour samples and 10 adjacent noncancerous tissues were obtained in Prince of Wales Hospital, Shatin, Hong Kong. Nine normal gastric mucosa from individuals without gastric cancer were also obtained as control. All patients gave informed consent for obtaining the specimens. The median age of gastric cancer patients at the time of diagnosis was 70 years old (range from 34 to 83). The male to female ratio was 1.5:1. The H&E-stained sections were reviewed by a pathologist to confirm the diagnosis. In all, 19 cases were intestinal type, 17 cases were diffuse type, and four cases were mixed-type gastric adenocarcinoma.

DNA extraction

DNA from formalin-fixed paraffin-embedded sections were extracted using High Pure PCR Template Preparation Kit (Boehringer Mannheim, Indianapolis, IN, USA). For extraction of cell lines DNA, phenol/chloroform method was used. H&E-stained sections from each tumour sample were examined by an experienced pathologist to confirm their histological diagnosis and assess the tumour content. If tumour content was less than 80%, tumour content was enriched by microdissection using a fine needle under a dissection microscope as described previously (Chen et al, 2000).

Table 1  

| Primer          | Forward primer (5′ → 3′)         | Reverse primer (5′ → 3′)         | Annealing temperature (°C) | Product size (bp) |
|-----------------|----------------------------------|----------------------------------|-----------------------------|-------------------|
| SOCS-1          | M: TCCGGGTTATTTTTAGTGCTTGC      | M: CGACACAACCTCCCCAGAC           | 60                          | 93                |
| MSP             | U: TTATGAGTTTGTGTTTATTTTAGTGGTT | U: CACTAACAAACAACCTCCCCAACCAACCA | 60                          | 105               |
| Bisulphite      | TGAGGATGATTATAATTTAGGGTT         | TAATACCCAAACACTCTAAAAAACCTC     | 60                          | 471               |
| sequencing      |                                  |                                  |                             |                   |
| RT – PCR        | CGCCTCGGATTCCTACTG              | AGCAGGGCGAGAGGAGGAGG             | 60                          | 227               |
| IL-6            | CACACAGACACACCTCACATC           | CTCGAGGCTGAGGTGCCAGAA           | 65                          | 495               |
| β-actin         | GGGTGGGTTTAGGAGGGGGG            | GGGTGGGTTTAGGAGGGGGG            | 65                          | 53                |

M = methylated primer; U = unmethylated primer; SOCS-1 = suppressor of cytokine signalling-1; MSP = methylation-specific PCR; RT – PCR = reverse transcription–polymerase chain reaction; IL-6 = interleukin-6. *Touch-down programme starting from 65°C.

Methylation-specific polymerase chain reaction (PCR) (MSP) for SOCS-1

Extracted DNA was bisulphite modified by CpGenome DNA Modification kit (Intergen, Purchase, NY, USA). The modified DNA was subjected to MSP using specific primers for SOCS-1 as described previously (Yoshikawa et al, 2001). Primer sequences, annealing temperatures and the expected product size were listed in Table 1. A measure of 2μl of bisulphite-modified DNA were amplified in a total volume of 25μl containing 1× PCR buffer II (Applied Biosystems, Foster City, CA, USA), 2 mM MgCl₂, 0.25 mM dNTP, 1μM of each primer and 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) at 95°C for 10 min, 38 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by a final extension of 72°C for 10 min. In vitro methylated DNA (IVD) (Intergen, Purchase, NY, USA) was used as a positive control for methylation and water was used as a negative control. A measure of 10μl of PCR products were loaded onto nondenaturing 10% polyacrylamide gels. The gels were then stained with ethidium bromide and visualised under UV illumination.

Bisulphite sequencing for SOCS-1

Bisulphite-treated DNA was amplified using specific primers for exon 1 region of SOCS-1 gene as reported by Yoshikawa et al (Table 1). The PCR products were cloned into Topo TA cloning kit (Invitrogen, Carlsbad, CA, USA). Five randomly picked clones were sequenced using the dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). The sequencing products were separated on an Applied Biosystems 377 automated sequencer (Applied Biosystems, Foster City, CA, USA) and analysed using Applied Biosystems sequencing analysis software.

RNA isolation and reverse transcription – PCR (RT – PCR) for SOCS-1 and IL-6

Total RNA was extracted from frozen human gastric tissues and cell lines by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (2μg) was reverse transcribed into cDNA by MMLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The expression of SOCS-1 and IL-6 was examined by PCR using specific primers as listed in Table 1. For amplification of IL-6, a touch-down PCR cycle as described by Lin et al (2000a) was used. As an internal control, amplification of β-actin was performed. The PCR products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide and visualised under ultraviolet illumination.

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IL-6 protein measurement

*In vitro* IL-6 production from AGS gastric cancer cell line was performed as described previously (Hwang et al., 2003). AGS (5 x 10^5 cells ml\(^{-1}\)) were plated into 24-well plate and cultured for 2 days in triplicate. Interleukin-6 in the supernatant was measured by Quantikine HS human IL-6 immunoassay (R&D systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. The ELISA sensitivity of IL-6 is 0.15 pg ml\(^{-1}\).

Demethylation treatment of gastric cancer cells

To determine if SOCS-1 expression can be restored by demethylating agent, gastric cancer cell lines were subjected to 5’azaDC treatment. Cells were plated and incubated for 4 days with 5 \(\mu\)M of 5’azaDC (Sigma Chemical Co, St Louis, MO, USA).

IL-6 and anti-IL-6 antibody treatment of gastric cancer cells

After 24 h of serum starvation, 10ng ml\(^{-1}\) of recombinant IL-6 (R&D systems, Minneapolis, MN, USA) was then added into AGS cell for 15 min and proteins were extracted for further analysis. For antibody treatment, 10 \(\mu\)g ml\(^{-1}\) of anti-IL-6 antibody (R&D systems) was added into the medium for 24 h. Cells were then harvested for protein extraction and determination of the phosphorylation status of STAT3.

Western blotting analysis for phospho-STAT3 and total STAT3

Cells are washed with PBS and lysed on ice in buffer containing 1% NP-40, 50 mM Tris at pH 8, 150 mM NaCl, 5 mM EDTA, 10 \(\mu\)g ml\(^{-1}\) of aprotinin and pepstatin, 100 \(\mu\)g ml\(^{-1}\) of PMSF, and 100 mM NaVO\(_4\). Protein concentration was determined using the Bio-Rad protein assay. Protein (50 \(\mu\)g) are separated on 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane at 100 V for 2 h at 4°C using Bio-Rad transfer unit. The transfer buffers used are 25 mM Tris, 192 mM glycine, and 20% methanol. After transfer, the blots are blocked in 10% milk (fatty acid free) with TBS-T (0.1% Tween-20, 20 mM Tris, 137 mM NaCl, and 1 mM HCl). The blots are then hybridised with anti-phospho-STAT3 or anti-STAT3 polyclonal antibody (Cell Signaling, Beverly, MA, USA) in 1 : 1000 dilution overnight at 4°C followed by washes in TBS-T, and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1 : 2000 dilution for 1 h at room temperature. After several washes, blots are developed using ECL Western blotting detection kit (Amersham Biosciences, Uppsala, Sweden).

**RESULTS**

Expression of IL-6 in gastric cancer cell lines

Expression of IL-6 in six gastric cancer cell lines was assessed by RT – PCR. Our result showed that AGS, N87, and MKN45 expressed IL-6 (Figure 1, Table 2), while MKN28 had barely detectable IL-6 expression. IL-6 expression was absent in KATOIII and SNU16.

Expression of SOCS-1 in gastric cancer cell lines

By RT – PCR analysis, SOCS-1 was expressed in MKN45, N87, MKN28, and KATOIII (Figure 1, Table 2). Since IL-6 and other cytokines are known to upregulate the level of SOCS-1 gene (Starr et al, 1997; Song and Shuai, 1998; Schuringa et al, 2000), we also examine IL-6 level in these cell lines. Expression of IL-6 were noticed in MKN45, N87, and MKN28, suggesting that the SOCS-1 expression may be related to a negative feedback mechanism of IL-6 activation in these cell lines. In SNU16, both SOCS-1 and IL-6 was not detected. Although KATO III does not express IL-6, other cytokines may also upregulate SOCS-1 in this cell line. Notably, despite a high level of IL-6 expression, SOCS-1 expression was not found in AGS cells (Figure 1).

IL-6 protein production in AGS cell

To further confirm that IL-6 protein is produced in AGS cells, we examined IL-6 level secreted by AGS cells by high-sensitivity ELISA assay. Our result showed that IL-6 production in AGS cells was 1004 ± 130 ng ml\(^{-1}\).

Methylation of SOCS-1 in gastric cancer cell lines

By MSP among the six cancer cell lines, methylation of SOCS-1 could only be detected in AGS cell (Figure 2). Dense methylation pattern in the CpG islands of the exon 1 region of AGS cells was...
confirmed by bisulphite DNA sequencing, while other cell lines were essentially free of methylation (Figure 3).

Demethylation study of SOCS-1 in the AGS gastric cancer cell line

Demethylation study was carried out in the AGS cell. After treatment with 5’azaDC, expression of SOCS-1 was restored as demonstrated by RT–PCR (Figure 4, lower panel). Furthermore, MSP detected both methylated and unmethylated allele of SOCS-1, indicating that demethylation of the gene occurred (Figure 4, upper panel). These results confirmed that loss of expression of SOCS-1 in the AGS cell was related to gene methylation.

STAT3 activation in AGS gastric cancer cell line

Constitutive activation of STAT3 was found in the AGS cells in which IL-6 was highly expressed (Figure 5, lane 1). We further restored SOCS-1 expression in AGS cell by treating the cell with 5’azaDC. In addition to the re-expression of SOCS-1, it was accompanied by reduction in phosphorylation of STAT3 (Figure 5, lane 2). Pretreatment with anti-IL-6 antibody for 24 h also resulted in inhibition of phosphorylation of STAT3 but not as marked as 5’azaDC treatment (Figure 5, lane 5). Addition of recombinant IL-6 in the medium for 15 min resulted in the reactivation of STAT3 phosphorylation in 5’azaDC-treated AGS cells (Figure 5, lane 4), suggesting that IL-6 was responsible for STAT3 activation. Taken together, these findings suggested that SOCS-1 play an important role in the inhibition of IL-6-mediated STAT3 activation in AGS gastric cancer cell line.
Methylation status and expression of SOCS-1 in primary gastric cancer

Methylation of SOCS-1 was found in 27.5% (11 of 40) of gastric cancer samples and 10% (one of 10) of adjacent normal mucosa (Figure 6). Among these 40 gastric cancer samples, no correlation between methylation status of SOCS-1 with H. pylori status, histological type or staging was observed (Table 3). To further investigate the expression status of SOCS-1 in these gastric cancer tissues, total RNA from 14 gastric cancer samples and the corresponding normal tissues were extracted for RT-PCR analysis. Downregulation of SOCS-1 was observed in two (14%) gastric tumour tissues, which also showed SOCS-1 methylation (Figure 7, Table 4). These result suggested that methylation of SOCS-1 gene was responsible for the downregulation of the gene in primary gastric cancer.

DISCUSSION

Activation of interleukin-mediated JAK/STAT pathway has been recently described to play a crucial role in human cancer development. Constitutive activation of STAT3 has been observed in breast cancer, prostate cancer and leukaemia (Schuringa et al., 2000; Lin et al., 2000b; Campbell et al., 2001; Li and Shaw, 2002). Recent studies also found that blockade of STAT3 activity by the expression of the dominant-negative STAT3 can inhibit growth of AGS gastric cancer cell line, thus further suggesting that JAK/STAT may play an important role in development of gastric cancer (Kanai et al., 2003). On the other hand, inactivation of the negative regulator, SOCS-1, also leads to the activation of the JAK/STAT pathway. Downregulation of SOCS-1 by gene promoter hypermethylation has been recently reported in 65% of HCC cell line, 62.9% of multiple myeloma patients samples and 31.6% of pancreatic cancer cell lines with resultant activation of STAT3 (Yoshikawa et al., 2001; Fukushima et al., 2003; Galin et al., 2003). Moreover, restoration of SOCS-1 suppresses tumour growth in

Table 3 Association between SOCS-1 methylation and clinicopathological parameters of gastric cancer

| Total no. | SOCS-1 methylation (%) | P-value |
|-----------|-------------------------|---------|
| Sex       |                         | 0.347   |
| Male      | 24                      | 6 (25.0) |
| Female    | 16                      | 5 (31.3) |
| Age (years) |                       | 0.404   |
| ≥60       | 26                      | 8 (30.7) |
| <60       | 14                      | 3 (21.4) |
| H. pylori status |               | 0.305   |
| Positive  | 19                      | 4 (21.1) |
| Negative  | 21                      | 7 (33.3) |
| Site      |                         | 0.510   |
| Antrum    | 21                      | 6 (28.5) |
| Body      | 11                      | 4 (36.3) |
| Cardiac   | 8                       | 1 (12.5) |
| Staging<sup>a</sup> |           | 0.596   |
| I         | 7                       | 2 (28.5) |
| II        | 6                       | 3 (50)   |
| III       | 19                      | 4 (21.1) |
| IV        | 7                       | 2 (28.5) |
| Lauren classification |         | 0.875   |
| Intestinal type |             |         |
| Mixed type |             |         |

SOCS-1 = suppressor of cytokine signalling-1. Number of methylated cases, numbers within parentheses are percentages.<sup>a</sup>Comparison were made by 2-test or Fisher’s exact test (SPSS 10.0).<sup>b</sup>Staging according to American Joint Committee on Cancer. Data (staging were not available in one case).
In summary, loss of expression of SOCS-1 in AGS cell line was related to gene promoter hypermethylation. This phenomenon together with endogenous IL-6 expression leads to the activation of STAT3 protein. The increase of STAT3 activity together with overexpression of cytokine in this gastric cancer cell line suggested that alteration of JAK/STAT was important in a subset of gastric cancer.

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