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ORIGINAL ARTICLE

Down-Regulation of CXCL12 by DNA Hypermethylation and Its Involvement in Gastric Cancer Metastatic Progression

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Abstract
Background Chemokine receptors are now known to play an important role in cancer growth and metastasis. However, there is little information regarding chemokine expression in gastric cancer. In this study, we examined CXCL12 expression in gastric cancer and also evaluated whether the down-regulation of CXCL12 is due to aberrant methylation of the gene.
Methods CXCL12 expression was examined using real-time reverse-transcription polymerase chain reaction (RT-PCR), immunofluorescence, flow cytometry, and immunohistochemistry, and the methylation status of the gene was evaluated by methylation-specific PCR (MSP) in normal gastric and gastric cancer cell lines and 35 primary gastric carcinomas and corresponding nonmalignant gastric tissues.
Results The down-regulation of CXCL12 was observed in gastric cancer cell lines and primary gastric carcinomas, while decreased expression of CXCL12 protein was significantly associated with lymph node metastasis and histological grade. And this down-regulation was found to be in accordance with aberrant methylation of the gene. Hypermethylation of the gene was observed in 65.7% (23 of 35) of the primary gastric carcinomas, while it was found in only 11.4% (4/35) of the corresponding nonmalignant tissues. Furthermore, CXCL12 expression was restored in gastric cancer cell lines after treatment with the demethylating agent, 5-aza-2′-deoxycytidine (5-Aza-dC), and demethylation of the highly metastatic cells SGC-7901 induced invasion suppression of the cells. For two CXCL12 receptors, CXCR4 and CXCR7, the mRNA levels remained almost unchanged with the 5-Aza-dC treatment.
Conclusions Collectively, our results suggest that the aberrant methylation of CXCL12 frequently occurs in the down-regulation of CXCL12 in gastric cancers and that it may play a role in the metastasis of gastric cancer.

Keywords CXCL12 · Methylation · Gastric cancer · Chemokine · Chemokine receptor

Abbreviations

5-Aza-dC	5-Aza-2′-deoxycytidine
RT-PCR	Reverse-transcription polymerase chain reaction
MSP	Methylation-specific PCR

Introduction

Chemokines have multiple roles in many kinds of physiologic processes, such as hematopoiesis, lymphocyte development, and wound healing [1]. Recent findings demonstrated that there is a close relationship between tumor cells and chemokines [2]. Cancer cell invasion and metastasis shares many similarities with the process by

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which leukocytes enter inflamed tissues [3]. Numerous studies indicate that chemokine receptors are expressed by tumor cells, while chemokines are expressed at organs that turn into metastatic targets. To date, the CXCL12–CXCR4 pair was found to be involved in almost all malignancies that were studied, including many solid cancers and tumors of a hematopoietic origin [4–7]. In most cases, the CXCL12–CXCR4 pair was found to be associated and/or involved with increased malignancy and metastasis, acting at many different levels [4–6, 8–15], and another CXCL12 receptor, CXCR7, was shown to promote the survival of tumor cells by preventing apoptosis, increased adhesion properties and dissemination, but did not mediate chemotaxis towards CXCL12 [16].

Recent studies have also reported that the epigenetic down-regulation of CXCL12 modulates the metastatic potential of breast, colon carcinoma, and non-small cell lung cancer [17–21]. Also, a previous study showed that many gastric cancer cell lines do not express CXCL12 [22]; however, CXCL12 protein levels were present in gastric cancer tissues by immunohistochemistry [23]. It is meaningful to understand the role of CXCL12 in gastric carcinogenesis. We investigated the expression of CXCL12 in gastric cancer and we also explored whether an epigenetic mechanism was involved in CXCL12 inactivation.

Materials and Methods

Cell Lines and Tissue Samples

Three human gastric cancer cell lines, MGC-803, BGC-823, and SGC-7901, and an immortalized normal gastric cell line, GES1, were cultured in RPMI 1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 IU/ml), and streptomycin (100 μg/ml), and incubated in a humidified incubator containing 5% CO_2 at 37°C. MGC-803, BGC-823, and SGC-7901 cells were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The GES1 cells were obtained from the Oncology Institute of China Medical University.

Tumor and corresponding nonmalignant gastric tissue specimens were obtained from 73 patients who underwent curative resection at the First Affiliated Hospital of China Medical University (Shenyang, China) between July 2009 and June 2010. None of these patients had undergone chemotherapy or radiotherapy before surgery. The local institutional review board approved our protocol for the use of patient samples; all patients provided written informed consent prior to participation in the study. Hematoxylin and eosin (H&E)-stained sections were examined histologically for the presence or absence of tumor cells. The nonmalignant gastric tissue specimens were obtained from either the opposite end of resected surgical samples or as far as possible away from the tumor. All of the macroscopically normal samples were confirmed to be normal by H&E staining.

RNA Extraction and Real-Time RT-PCR

Total RNA was isolated from cultured cells using Trizol (Invitrogen, Carlsbad, CA, USA) according to the protocol supplied by the manufacturer. Two micrograms of DNase I-treated (Fermentas, Vilnius, Lithuania) total RNA were converted to cDNA using a Reverse Transcription System kit (Fermentas). RNA was excluded in cDNA synthesis reactions as a negative control. The polymerase chain reaction (PCR) was performed in a volume of 25 μl using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) with an initial denaturing at 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 30 s, annealing for 30 s at 56°C, extension for 30 s at 72°C, and then a final extension for 10 min at 72°C. The following primers were used (5′–3′): GTC AAG CAT CTC AAA ATT CTC AAC AC (sense) and CAC TTT AGC TTC GGG TCA ATG C (antisense) for CXCL12 [24], CAG TTT CAG CAC ATC ATG GTT GG (sense) and GTG ACA GCT TGG AGA TGA TAA TGC (antisense) for CXCR4 [25], CTG CGT CCA ACA ATG AGA CCT (sense) and CCG ATC AGC CAC TCC TTG A (antisense) for CXCR7 [26], and CAT GAG AAG TAT GAC AAC AGC CT (sense) and AGT CCT TCC ACG ATA CCA AAG T (antisense) for GAPDH [27]. Relative gene expression to an internal GAPDH control and, hence, fold changes were calculated using the equation $2^{-\Delta\Delta Ct}$ method [28].

Methylation-Specific PCR

The genomic DNA was prepared from cell lines and tissues by the phenol/chloroform protocol and was modified by bisulfite treatment as described previously [29]. Then, DNA (2 μg) was purified using a Wizard DNA Clean-Up System (Promega Corporation, Madison, WI, USA), precipitated with ethanol, and resuspended in 30 μl of Tris-EDTA buffer. PCR amplification was performed using 2.0 μl bisulfite-modified DNA in a volume of 50 μl containing 10× DreamTaq buffer (Fermentas), 2 mM dNTP Mix, 0.4 μM of each primer, and 1.25 U of DreamTaq (Fermentas). The primers of CXC12 used for methylation-specific PCR (MSP) are located in the promoter region and the CpG map was based on a previous study [18]. The primers for the methylated CXCL12 CpG island were 5′-GGA GTT TGA GAA GGT TAA AGG TC-3′ (sense) and 5′-TTA ACG AAA AAT AAA AAT AGA CGA T-3′ (antisense). The primers for the unmethylated CXCL12 CpG islands were 5′-GAG TTT GAG AAG GTT AAA

GGT TGG-3′ (sense) and 5′-TAA CAA AAA ATA AAA ATA CAA CAA T-3′ (antisense). The PCR conditions were as follows: 95°C for 10 min, followed by 39 cycles of denaturing at 95°C for 30 s, annealing for 30 s at 60°C, extension for 30 s at 72°C, and then a final extension for 10 min at 72°C. CpGenome Universal Methylated and Unmethylated DNA (Chemicon, Temecula, CA, USA) was used as a positive control for the methylated and unmethylated genes, respectively. The amplification products were separated on 2.5% agarose gels.

Immunofluorescence

Indirect immunofluorescences were done on CXCL12 cells grown in 24-well plates (Costar Corp, Corning, NY, USA). Cells were fixed in 4% paraformaldehyde at 25°C for 15 min and permeabilized with 0.2% Triton X-100, and then washed three times with PBS. Cells were incubated with 25 μg/ml murine anti-human CXCL12 monoclonal antibody (MAB350 R&D Systems, Minneapolis, MN, USA) or an isotype control (MAB002 R&D Systems) overnight, followed by incubation with a 1:100 dilution of goat anti-murine TRICT-conjugated IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h in the dark. Nuclei were visualized with Hochest33258 counterstain and examined using a fluorescence microscope (Olympus BX-40).

Flow Cytometry

The cells were fixed in 4% paraformaldehyde for 15 min on ice and permeabilized with 0.1% saponin. Then, they were washed and incubated with 25 μg/ml murine anti-human CXCL12 monoclonal antibody for 1 h on ice. After being washed three times, bound primary antibody were detected by incubation with FITC-labeled anti-murine IgG (Santa Cruz Biotechnology) for 1 h on ice in the dark. Cells were washed extensively, resuspended in PBS, and analyzed by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA, USA). Murine IgG served as an isotype-negative control. The data were expressed as a ratio between the mean fluorescence intensity (MFI) of specific antibody and the MFI of the relative isotype control. The results were obtained from three independent experiments for each cell line.

Immunohistochemistry

For the immunohistochemical examination, we followed the normal protocol in preparing the tissues and the epitope retrieval was performed in citrate buffer (pH 6) using a microwave for 2 min at 100°C. After blocking endogenous peroxidase with 3% H_2O_2, we followed all of the protocols according to the Ultra Sensitive S-P secondary antibody kit (KIT-9701, Maixin Bio, Fuzhou, China). The primary antibody or an isotype control was incubated overnight at 4°C at 1.25 μg/ml. Finally, detection was carried out with the DAB Kit (DAB-003, Maixin Bio, Fuzhou, China) as described by the manufacturer.

Following immunostaining, the slides were first analyzed manually. Any cytoplasmic staining for CXCL12 was considered to be positive. The immunostaining results were evaluated by defining a threshold of positive staining for all sections before automated processing. The immunohistochemical parameter assessed in the area detected was the mean density and the intensity was averaged from five fields of view with a magnification of 400×. This was performed using software Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA).

Treatment of Cells with 5-Aza-dC

Three tumor cell lines were incubated in culture media with 5 μmol/l of the demethylating agent 5-aza-2′-deoxycytidine (5-Aza-dC) (Sigma Chemical Co., St. Louis, MO, USA) for 3 days using daily media changes. Cells were harvested and RNA and DNA were extracted on day 3.

Transwell Chamber Invasion Assay

The transwell chamber (Corning Life Sciences, Corning, NY, USA) containing an 8-μm pore size polycarbonate membrane filter was coated with a Matrigel (BD Biosciences, San Jose, CA, USA) and inserted in a 24-well culture plate. SGC-7901 cells and SGC-7901 cells incubated with 5-Aza-dC for 3 days were separately detached from the tissue culture plates, washed, and plated at the density of 5×10^4 per upper well in 200 μl of culture medium (RPMI 1640, 1% FBS), and the lower chamber was filled with 500 μl of medium (RPMI 1640, 20% FBS). After being recultured with 5% CO_2 at 37°C for 24 h, the non-invading cells with Matrigel matrix were removed from the upper surface of the membrane by scrubbing with a cotton-tipped swab. Cells on the lower surface of the filter were fixed for 30 min in methanol and glacial acetic acid mixture (3:1), air-dried briefly, and stained with Giemsa. The number of invaded cells was counted from three preselected microscopic fields at 200× magnification; all experiments were performed in triplicate.

Statistical Analysis

Statistical analysis was performed using the statistical software package SPSS version 12.0 (LEAD Technologies Inc., Chicago, IL, USA). Each data point shows a representative result from a series of experiments done on at

least three independent occasions. A P-value < 0.05 was considered to be statistically significant. Significance between controls and treated cell lines were calculated by Student's t-test. Significance between controls in different cell lines was calculated by one-way analysis of variance (ANOVA). The correlations of the CXCL12 protein level immunostained in gastric cancers and clinicopathological characteristics were assessed by the Kruskal–Wallis test and the Mann–Whitney test. The correlations of the methylation alterations of the *CXCL12* gene with clinicopathological parameters were analyzed by the Chi-square test and Fisher's exact test.

Results

Down-Regulation of CXCL12 in Gastric Cancer Cell Lines

We first sought to define the CXCL12 expression pattern in three gastric cancer cell lines and GES1. As an initial step, we performed real-time RT-PCR to assess the mRNA expression of CXCL12. Quantitative RT-PCR revealed a considerable down-regulation of CXCL12 in the tumor cell lines MGC-803, BGC-823, and SGC-7901 compared with the normal cell line GES1 ($P = 0.00$) (Fig. 1a).

We next analyzed CXCL12 protein expression using two parallel approaches, flow cytometry and immunofluorescence, to determine if dysregulated protein expression was paralleled in those cell lines. Perinuclear cytoplasmic CXCL12 immunofluorescence was observed in GES1, but not in other cancer cell lines. No specific immunofluorescences were observed using murine isotype control antibodies for CXCL12 (Fig. 1b). To detect intracellular CXCL12 protein using flow cytometry, permeabilized GES1 cells were immunostained with monoclonal antibodies and were shown to express CXCL12. However, lower CXCL12 expression levels were detected in MGC-803, BGC-823, and SGC-7901 ($P = 0.00$) (Fig. 1c).

Correlation Between the Down-Regulation of CXCL12 and Clinical Factors in Primary Gastric Carcinomas

CXCL12 protein was detected in the cytoplasm of normal gastric epithelial cells and well-differentiated gastric cancer cells, especially in the most differentiated surface

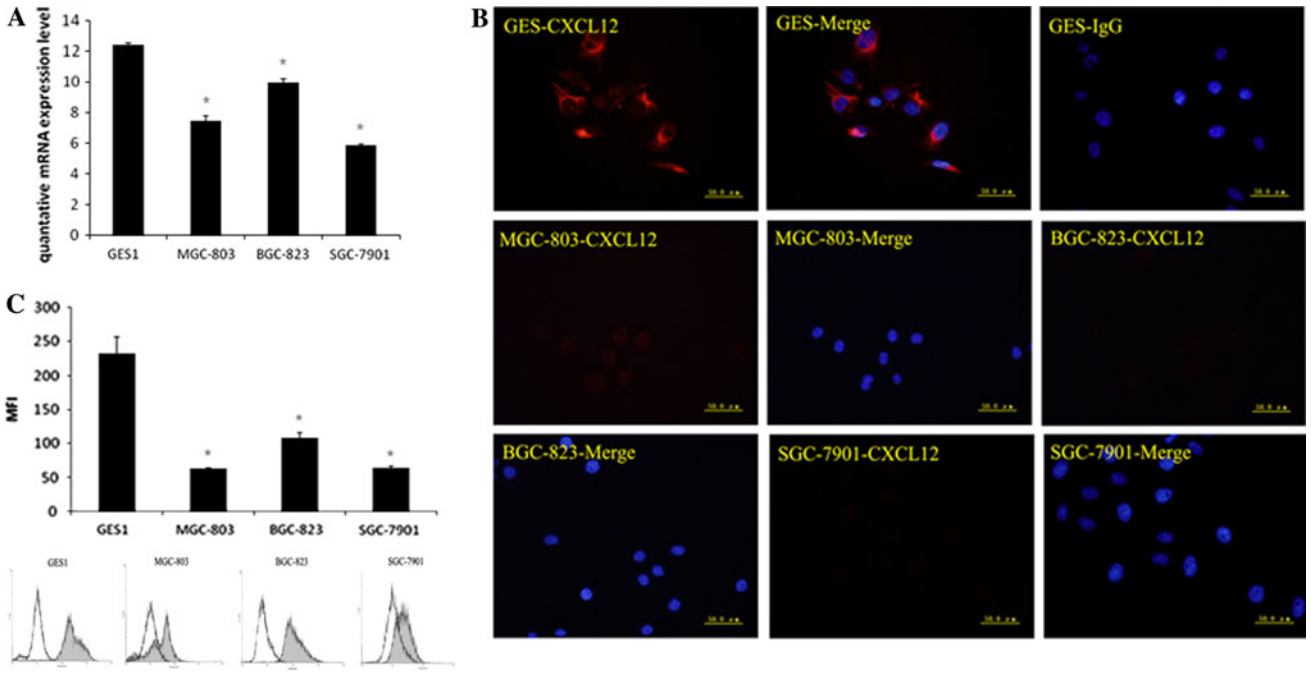

Fig. 1 Decreased expression of CXCL12 in gastric cancer cell lines. **a** Quantitative reverse-transcription polymerase chain reaction (RT-PCR) analyses on the expression of CXCL12 in GES1 and three gastric cancer cell lines (MGC-803, BGC-823, and SGC-7901). The columns indicate the mean CXCL12 expression levels normalized to GAPDH expression. *$P < 0.05$ compared with GES1. **b** The GES1 cells permeabilized and immunostained with antibody to CXCL12 (*red*) were characterized by perinuclear cytoplasmic immunofluorescence. Cell nuclei were visualized with Hochest33258 (*blue*). CXCL12 signals were not detected on MGC-803, BGC-823, and SGC-7901. Original magnification 400×. **c** Intracellular protein expression of CXCL12 was detected in permeabilized GES1 cells using flow cytometry (*solid histograms*). The *clear histograms* correspond with immunostaining with the appropriate isotype control IgG, whereas lower CXCL12 expression levels were detected in MGC-803, BGC-823, and SGC-7901. The columns indicate the mean fluorescence intensity (MFI) of CXCL12 protein expression levels. *$P < 0.05$ compared with GES1

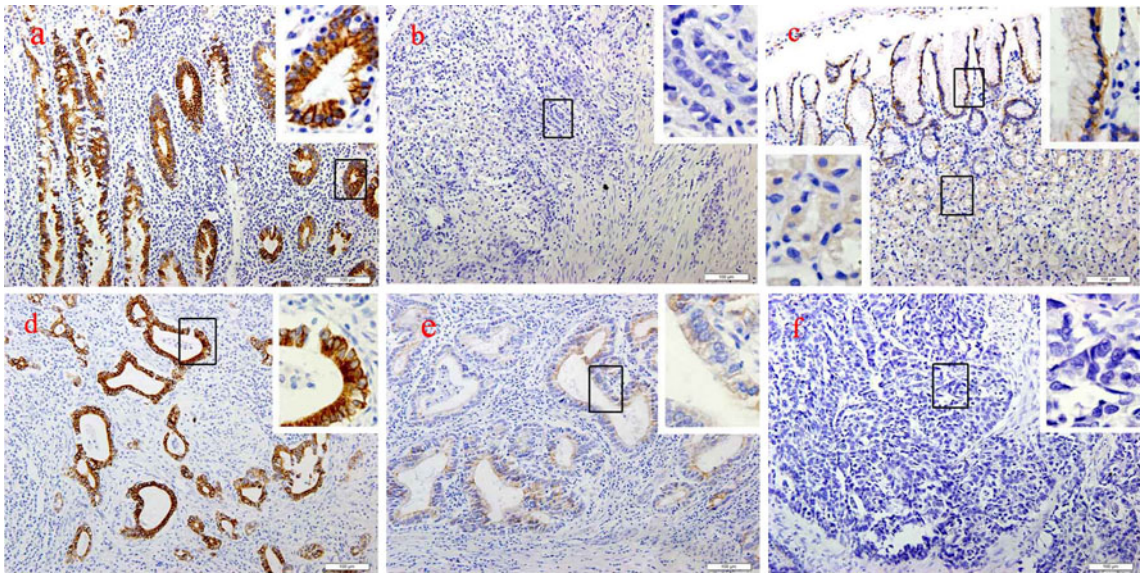

Fig. 2 Immunohistochemical analysis of CXCL12 protein in primary gastric carcinomas and corresponding nonmalignant tissues. Representative tissue samples stained with an anti-CXCL12 antibody demonstrate weak staining in cancerous tissues (**d**, **e**, **f**), but strong staining in normal tissues (**a**, **c**). CXCL12 was strongly expressed in the cytoplasm of the most differentiated surface gastric epithelial cells, but moderate in the less differentiated epithelial cells far from the mucosal side (**c**). CXCL12 expression by primary gastric carcinoma cells was variable and reduced, CXCL12-specific staining was strong in well-differentiated gastric cancer cells (**d**), while there was weak or no staining in less-differentiated gastric cancer cells (**e**, **f**). Immunoglobulin G (IgG) control images for CXCL12 (**b**) were shown negatively. The images are shown at 100× magnification, with the *boxed areas* indicating the 400× images

gastric epithelial cells, whereas lower CXCL12 expressions were detected in the less differentiated epithelial cells distant from the mucosal side and the less differentiated gastric cancer cells (Fig. 2).

Quantitative analysis of the CXCL12 protein by the mean density of staining in 73 pairs of primary gastric carcinomas and corresponding nonmalignant gastric tissue revealed significantly ($P = 0.00$) lower levels in gastric carcinoma tissues (median = 0.006150, range = 0.000090–0.147000) than in normal gastric tissues (median = 0.0258000, range = 0.000050–0.227000). Furthermore, lower protein expression levels of CXCL12 in tumors were related to lymph node metastasis ($P = 0.04$) and histological grade ($P = 0.00$) (Table 1).

CXCL12 Silencing by DNA Methylation

The MGC-803 and SGC-7901 gastric cancer cell lines only showed methylated bands (hypermethylated, both alleles methylated), which were in agreement with the observed low levels of CXCL12 expression. In contrast, the GES1 cell line expressing high CXCL12 levels showed unmethylated bands (unmethylated, neither allele is methylated) and the BGC-823 cell line showed both methylated and unmethylated bands (partially methylated, only one allele methylated) (Fig. 3a).

We then examined the methylation status of the *CXCL12* promoter in 35 primary gastric carcinomas as well as nonmalignant gastric tissues from the same individuals by MSP (Fig. 3b). Hypermethylation of the *CXCL12* gene was detected in 23 (65.7%) of the 35 primary gastric carcinomas, while hypermethylation in nonmalignant gastric tissue was only found in 4 cases (11.4%). And *CXCL12* was partially methylated in 8 cases (22.9%) and non-methylated in 4 cases (11.4%) in primary gastric tumors and 4 cases (11.4%) of partially methylation and 27 cases (77.1%) of non-methylation in noncancerous tissues, respectively.

We also examined the relationship between gastric tumor *CXCL12* methylation status and the clinicopathologic features of the patients, including age, gender, depth of invasion, lymph node status, histological grade, and pathologic staging of the tumor, but no correlation with any of the clinicopathologic features of the patients was found (Table 1).

Reactivation of CXCL12 Expression After Treatment with 5-Aza-dC

To confirm that aberrant methylation was responsible for silencing CXCL12 expression, we treated the MGC-803, BGC-823, and SGC-7901 gastric cancer cell lines with the demethylating agent 5-Aza-dC. The methylation status of CXCL12 of gastric cancer cells was modified from methylated to unmethylated by the 5-Aza-dC treatment (Fig. 4d). The expression of CXCL12 mRNA (Fig. 4a) and protein (Fig. 4b, c) were restored in MGC-803 ($P = 0.00$;

Table 1 Clinicopathological features and CXCL12 protein expression and *CXCL12* methylation in primary gastric carcinomas

	Number, $N = 73$	CXCL12 protein expression			Number, $N = 35$	*CXCL12* methylation			
		Median	Range	*P*-value		$M^{\dagger}$	$U^{\ddagger}$	$M + U^{\S}$	*P*-value***
Age (years)				0.303					0.389
<50	14	0.005390	0.000508–0.030100		5	4 (80.0%)	1 (20.0%)	0 (0.0%)	
≥50	59	0.007950	0.000097–0.147000		30	19 (63.3%)	3 (10.0%)	8 (26.7%)	
Gender				0.333					0.977
Male	52	0.008020	0.000090–0.147000		27	18 (66.7%)	3 (11.1%)	6 (22.2%)	
Female	21	0.004740	0.000500–0.064600		8	5 (62.5%)	1 (12.5%)	2 (25.0%)	
Depth of invasion				0.800					0.421
T1–2	4	0.004795	0.000940–0.011400		1	1 (100.0%)	0 (0.0%)	0 (0.0%)	
T3	15	0.005730	0.000310–0.053100		6	3 (50.0%)	0 (0.0%)	3 (50.0%)	
T4	54	0.007275	0.000090–0.147000		28	19 (67.9%)	4 (14.3%)	5 (17.9%)	
Lymph node status				0.043*					0.320
0	14	0.021200	0.000140–0.147000		8	4 (50.0%)	2 (25.0%)	2 (25.0%)	
1–2	13	0.010800	0.001300–0.132000		6	3 (50.0%)	1 (16.7%)	2 (33.3%)	
3–6	20	0.006275	0.000090–0.051300		12	8 (66.7%)	0 (0.0%)	4 (33.3%)	
≥7	26	0.004895	0.000130–0.030100		9	8 (88.9%)	1 (11.1%)	0 (0.0%)	
TNM stage									0.485
I–II	10	0.004180	0.000940–0.053100	0.665	5	3 (60.0%)	0 (0.0%)	2 (40.0%)	
IIII–IV	63	0.007520	0.000090–0.147000		30	20 (66.7%)	4 (13.3%)	6 (20.0%)	
Histological grade				0.005**					0.145
1–2	40	0.011750	0.000140–0.147000		19	14 (73.7%)	3 (15.8%)	2 (10.5%)	
3–4	33	0.003500	0.000090–0.132000		16	9 (56.3%)	1 (6.3%)	6 (37.5%)	
Tumor location				0.844					0.687
Upper	6	0.011475	0.000130–0.092700		2	1 (50.0%)	0 (0.0%)	1 (50.0%)	
Middle	24	0.009245	0.000170–0.147000		13	7 (53.8%)	3 (23.1%)	3 (23.1%)	
Lower	35	0.005030	0.000140–0.132000		13	9 (69.2%)	1 (7.7%)	3 (23.1%)	
Whole	8	0.008085	0.000090–0.026300		6	5 (83.3%)	0 (0.0%)	1 (16.7%)	

* *P*-value obtained from the Kruskal–Wallis test; ** *P*-value obtained from the Mann–Whitney test; *** *P*-value obtained from the χ^2 test; † "*M*" indicates methylated cases; ‡ "*U*" indicates unmethylated cases; § "$M + U$" indicates partially methylated cases

$P = 0.00$) and SGC-7901 ($P = 0.00$; $P = 0.00$) cells, but not in BGC-823 ($P = 0.89$; $P = 0.12$) cells. The mRNA levels of the two receptors CXCR4 and CXCR7 in MGC-803 ($P = 0.12$; $P = 0.30$) and SGC-7901 ($P = 0.45$; $P = 0.44$) cells and the CXCR7 mRNA levels in BGC-823 ($P = 0.40$) cells remained almost unchanged with the 5-Aza-dC treatment, except for CXCR4 mRNA in BGC-823 cells ($P = 0.01$) (Fig. 4a).

Reactivation of CXCL12 Expression with 5-Aza-dC Inhibits the Invasion Ability of Highly Metastatic SGC-7901 Cells

To further examine whether the reactivation of CXCL12 expression can regulate gastric cancer invasion, we analyzed the invasion capability of the highly metastatic SGC-7901 cells using the methods described above. The number of SGC-7901 cells in the untreated group that migrated through the membrane was 140.00 ± 12.12. The number of invading cells was significantly decreased when SGC-7901 cells were treated with 5-Aza-dC (63.67 ± 6.03). A significant reduction in the number of invasive cells was observed for 24 h when the cells were treated with 72 h of 5-Aza-dC exposure compared to the control ($P = 0.001$) (Fig. 5).

Discussion

Various types of cancer cells express chemokine receptors and the chemokines may play a role in cancer progression and/or organ-selective metastasis. It is assumed that disseminated tumor cells expressing chemokine receptors invade the circulation and are then attracted and arrested by the corresponding ligand. The specific metastatic sites to which tumor cells preferred to metastasize expressed more chemokines, and these chemokines are then able to induce the migration of tumor cells [10–12, 30–32]. The ability of a specific chemokine to act on chemokine receptor-

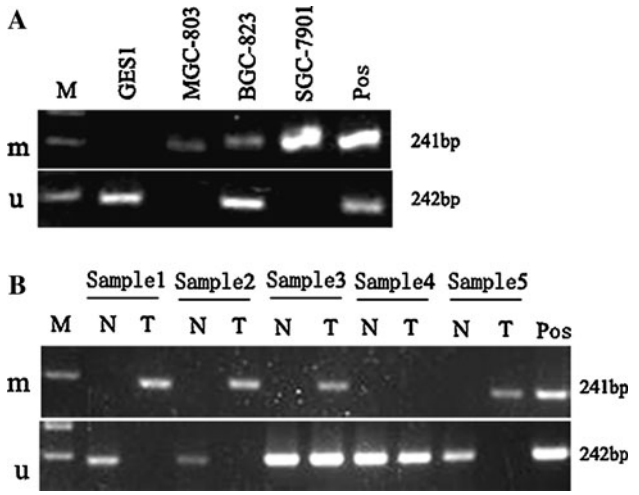

Fig. 3 *CXCL12* methylation analyses in cell lines and tumors and their corresponding nonmalignant gastric tissues by methylation-specific PCR (MSP). **a** Methylated bands were detected in MGC-803 and SGC-7901. Unmethylated bands were detected in GES1, and both methylated and unmethylated bands were detected in BGC-823. **b** Representative examples of the methylation analysis of *CXCL12* in tumors and their corresponding nonmalignant gastric tissues. *M* size marker, *N* nonmalignant gastric tissue, *T* tumor, *u* amplified product with primers recognizing an unmethylated sequence, *m* amplified product with primers recognizing a methylated sequence, *Pos* positive control

expressing tumor cells and to support their directionality requires that chemokine-induced cellular changes occurring in the tumor cells would culminate into motility in response to chemokine gradients. Solid evidence to such a mechanism was provided by the study of Müller et al., demonstrating that a highly potent functional axis exists between CXCL12 and its CXCR4 receptor in breast cancer metastasis [30]. In addition to this role, CXCR4 signaling is also a key regulator of organogenesis as well as lymphopoiesis and myelopoiesis [33, 34]. Previous studies have defined the co-expression of both CXCR4 and CXCL12 by the cells of the human intestinal epithelium [35], and, also, many studies reported the different expression levels of CXCL12 in the cancer cells and tissues [36, 37].

In this study, we demonstrated that lower expression levels of CXCL12 mRNA and protein occur in gastric cancer cell lines, which is similar to the results found by Yasumoto et al. [22], who showed the role of CXCR4-expressing gastric cancer cells in peritoneal carcinomatosis. Also, these investigators found no mRNA and protein expression of CXCL12 in any gastric cancer cell lines, which definitely showed no autocrine of CXCL12 in the gastric cancer cells. Moreover, we also found that the expression of CXCL12 was frequently reduced in gastric primary carcinomas when compared to corresponding normal gastric tissues. Other researchers found the same phenomenon in colon cancer, mammary carcinoma, and non-small lung cancer, and the CpG islands of the *CXCL12* gene was hypermethylated [17–20]. One of our objectives was to verify whether the aberrant methylation findings were in gastric carcinomas. We evaluated the CpG islands of the *CXCL12* gene that had already been analyzed by another group that found DNA methylation in colon cancer and mammary carcinoma [17, 18, 20]. In our research, the CpG islands of the *CXCL12* gene was hypermethylated in 2 of 3 gastric cancer cells, but not in GES1 cells, and as high as 65.7% in the 35 primary gastric tumors, whereas only 11.4% hypermethylation of *CXCL12* was observed in corresponding normal gastric tissues. To further explore the DNA methylation, we treated cancer cells with 5-Aza-dC, a DNA methyltransferase inhibitor, which was sufficient to cause demethylation of the promoter region and reactivate the expression of the hypermethylated silenced gene [38]. After 5-Aza-dC treatment, we observed a complete reversal of CXCL12 mRNA and protein expression in MGC-803 and SGC-7901 cells. As shown in this study, the action of 5-Aza-dC on the gastric cancer cell lines MGC-803 and SGC-7901 resulted in the demethylation of the *CXCL12* gene, accompanied by the up-regulation of mRNA and protein. This confirmed that 5-Aza-dC regulated the transcription of the *CXCL12* gene. In addition, the effect of 5-Aza-dC on the invasion ability of the highly metastatic gastric cancer cells SGC-7901 was demonstrated in vitro using the transwell assay. Our data suggest that the invasion ability suppression effect of 5-Aza-dC may result from the demethylation and reactivation of CXCL12. The down-regulation of CXCL12 was correlated with its promoter methylation in both gastric cancer cell lines and gastric tumor tissues. These results suggest that the aberrant methylation of *CXCL12* might be involved in the metastasis of gastric cancer.

In this study, we found that the *CXCL12* was frequently down-regulated by the methylation of its promoter in gastric cancer. Loss of CXCL12 with maintained expression of CXCR4 imparts to metastatic cancer cells a phenotype similar to that of circulating highly migratory leukocytes and lymphocytes [39]. Our data have also shown that lower CXCL12 protein levels in gastric tumors were related to lymph nodes metastasis, suggesting that carcinoma cells with no autocrine of CXCL12 may have a selective advantage to receive endocrine CXCL12 signals, promoting their exit and driving more active metastasis to ectopic sources of the CXCR4 ligand [17, 18]. In our study, we also found that the CXCL12 expression was restored by 5-Aza-dC treatment with no changes of the two cognate receptors CXCR4 and CXCR7, which is a new CXCL12 receptor that has been recently identified [16, 40]. This finding indicates that the CXCL12 changes in the gastric cancer cells could not affect its receptors, which reinforces the notion that the endogenous and exogenous CXCL12

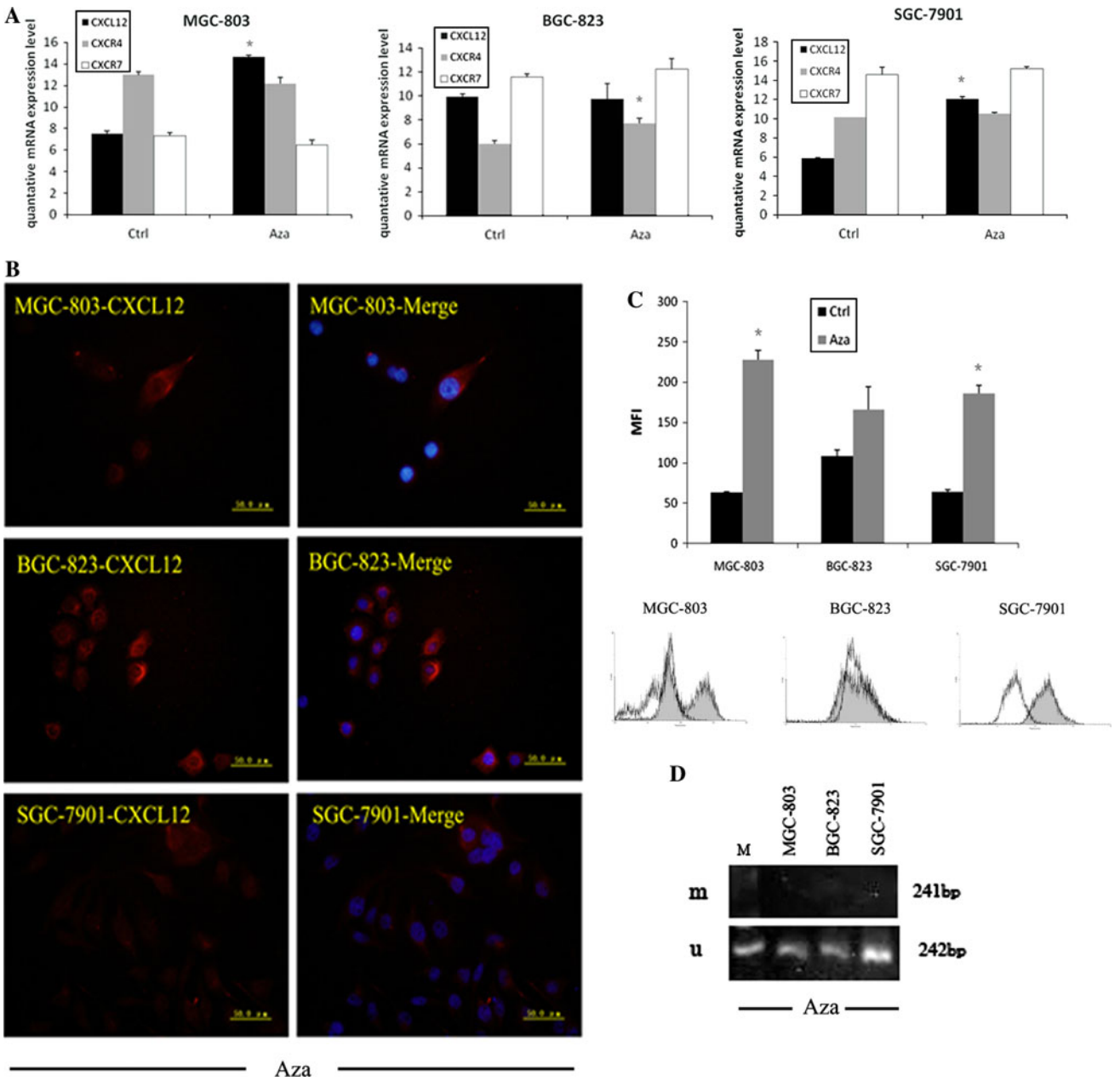

Fig. 4 Demethylation analysis using gastric cancer cell lines. **a** CXCL12 and its two receptors CXCR4 and CXCR7 mRNA expression evaluation after the 5-aza-2′-deoxycytidine (5-Aza-dC) treatment in MGC-803, BGC-823, and SGC-7901 cell lines using real-time RT-PCR. $*P < 0.05$ compared with cells before the 5-Aza-dC treatment. **b** CXCL12 fluorescent immunocytochemical signals were re-visualized in MGC-803, BGC-823, and SGC-7901 cells after 5-Aza-dC exposure. 400× original magnification. **c** Effect of the 5-Aza-dC treatment on CXCL12 protein expression analyzed by flow cytometry in MGC-803, BGC-823, and SGC-7901 cell lines. The *clear histograms* represent cell lines before the 5-Aza-dC treatment, whereas the *solid histograms* indicate corresponding cells after the treatment. The *columns* indicate the MFI of CXCL12 protein expression levels. $*P < 0.05$ compared with cells before the 5-Aza-dC treatment. **d** CXCL12 MSP indicating the presence of unmethylated bands of CXCL12 in MGC-803, BGC-823, and SGC-7901 cell lines treated with 5 μmol/l 5-aza for 3 days. *M* size marker, *u* amplified product with primers recognizing an unmethylated sequence, *m* amplified product with primers recognizing a methylated sequence

play different roles. Endogenous CXCL12, in marked contrast to exogenous ligand, inhibits tumor metastasis through increased anoikis [39]. Drury et al. found that CXCL12 expression induced apoptosis specifically in nonadherent colorectal carcinoma cells by increasing anoikis via the activation of a Bim-mediated intrinsic apoptotic pathway [41].

In our research, the CpG islands of the *CXCL12* gene were partially methylated in BGC-823 and were not hypermethylated in 13 of the 35 primary gastric carcinomas,

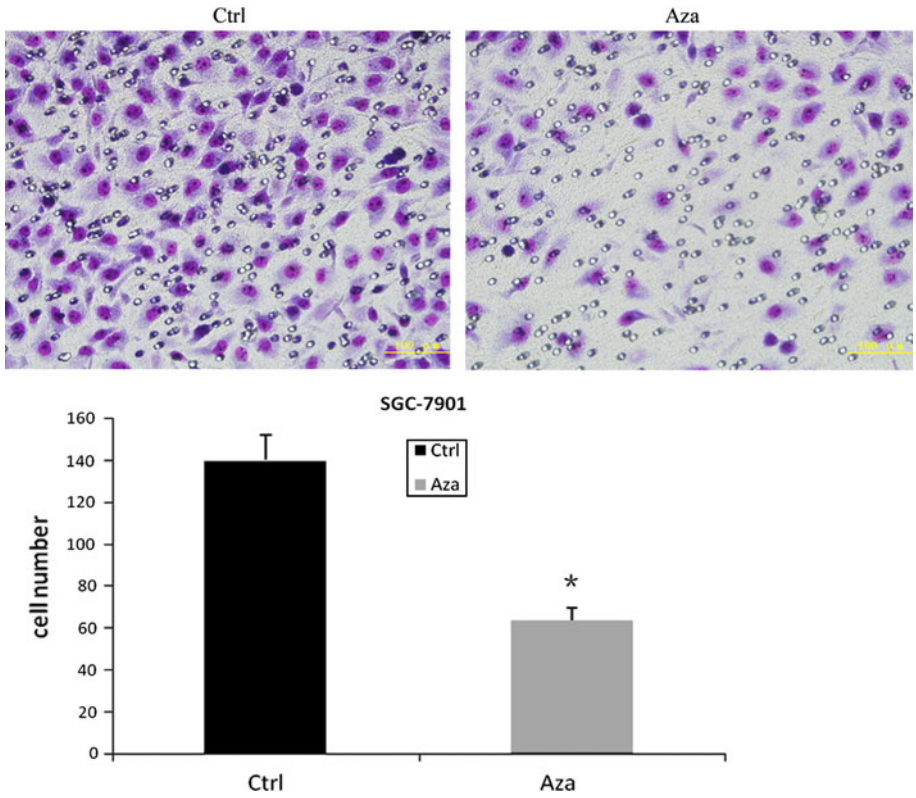

Fig. 5 Effect of 5-Aza-dC on SGC-7901 cells invasion. The transwell assay was performed to assess the effect on cell invasion. Cells were treated or untreated with 5 M 5-Aza-dC. Representative photographs of treated and untreated cells are presented (200× magnification). The *columns* indicate the number of cells invaded at the 24-h time point. $*P < 0.05$ compared with untreated SGC-7901 cells. The values represent the mean values ± standard deviation (SD)

which suggested to us that the aberrant methylation of the gene might not be the only reason to down-regulate the expression of CXCL12. An understanding of the precise mechanism of the down-regulation of endogenous CXCL12 in these gastric cancer cells needs to be explored.

Conclusions

In conclusion, we examined endogenous CXCL12 expression in gastric cancer cells and tissues. We also explored whether the decreased CXCL12 expression occurred as a result of aberrant methylation of the gene. We found that the *CXCL12* was frequently down-regulated by the methylation of its promoter in gastric cancers, which suggests that endogenous CXCL12, in marked contrast to exogenous ligand, inhibits tumor metastasis in gastric cancer, which was the first research to explore the mechanism of endogenous CXCL12 down-regulation in gastric cancer. Our results, together with recent findings, emphasize the importance of the CXCL12-CXCR4 signaling axis in an organ-specific pattern of metastasis. Targeting CXCL12 signal may also be a novel and efficient strategy for treating human gastric cancers.

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Conflict of interest We declare that we have no conflict of interest.

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