Elevated Expression of Cyr61 Enhances Peritoneal Dissemination of Gastric Cancer Cells through Integrin $\alpha_2\beta_1$ *

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Cysteine-rich 61 (Cyr61/CCN1) is involved in human gastric cancer development and progression. Nonetheless, the role of Cyr61 as regards peritoneal dissemination of such cancers has not yet been completely characterized. We used liposome-mediated transfection to establish Cyr61, or antisense Cyr61, expression vectors into gastric cancer AGS or MKN45 cell lines. Transfectants were tested by means of a cancer-cell adhesion assay in vitro and ex vivo. Furthermore, a functional integrin fluorescence-activated cell sorting assay, reverse transcription-PCR, and an AP-1 reporter assay were performed to investigate the potential signaling pathway of Cyr61. It was shown that stable transfection of Cyr61 into the AGS cell line strongly enhanced its adhesion ability. The overexpression of Cyr61 within AGS cells significantly increased the functional expression of integrin $\alpha_2\beta_1$. Function-neutralizing antibody to integrin $\alpha_2\beta_1$ effectively suppressed the Cyr61-mediated enhanced adhesion of AGS cells to peritoneal tissue. Promoter assays of integrin $\alpha_2$ gene further revealed that the AP-1 pathway was evidently activated within Cyr61-expressing AGS cells. Animal studies have revealed that mice injected with Cyr61-overexpressed AGS cells featured a greater number of peritoneal seeding nodules and a lower survival rate than the Neo control cell lines, and when such cells were treated with functional blocking antibody to integrin $\alpha_2\beta_1$, they were able to elicit a decline in the peritoneal dissemination. The data suggest that Cyr61 may contribute to the peritoneal dissemination of gastric cancer by promoting tumor-cell adhesion ability through the up-regulation of the functional integrin $\alpha_2\beta_1$ via an AP-1-dependent pathway.

Gastric cancer is reputed to be one of the most frequent and lethal malignancies worldwide (1). Although the range of therapeutic strategies available for treatment of this malady has improved over the past decades, the median survival time for advanced gastric cancer patients still appears to remain at around seven months (2, 3). Peritoneal dissemination of tumor cells is the most common form of recurrence for cases of advanced gastric cancer, and for most such situations, severe treatment is not recommended (3). At the mechanism level, peritoneal dissemination typically involves several steps, including tumor cell attachment, invasion, and growth within the peritoneum. Many cytokines, growth factors, matrix metalloproteinases, and angiogenic factors have been shown to play an essential role in the peritoneal dissemination processes (4–8); however, the one or more critical factors influencing the first steps of cancer cell attachment to the peritoneum remain to be established.

To the best of our knowledge, Cyr61 is the first cloned member of the CCN family (9), a family that comprises cysteine-rich 61 (Cyr61/CCN1), connective-tissue growth factor (CTGF/CCN2), nephroblastoma-overexpressed (Nov/CCN3), Wisp-1/elm1 (CCN4), Wisp-2/rcp1 (CCN5), and Wisp-3 (CCN6). Most members of the CCN family share a uniform modular structure and exhibit diverse cellular physiological and pathological functions (10, 11). Cyr61 has been reported to mediate various cellular processes, including cell adhesion, stimulation of chemostasis, enhancement of growth factor-induced DNA synthesis, cell survival, and angiogenesis (12–14). Elevated Cyr61 expression has been reported to be highly correlated with advanced breast adenocarcinoma, pancreatic cancer, and glioma (15–17). Our previous study demonstrated that overexpressing Cyr61 in human gastric cancer cell lines significantly increased the invasion ability of such cancer cell lines (18). The biological significance of Cyr61 as regards human malignant alterations can be explained by Cyr61’s active regulation of multifaceted biological activities, including cell adhesion, cell motility, cell survival, and cell proliferation (12–14), such activity arising through direct binding to distinct integrin receptors, including $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_4\beta_1$, $\alpha_6\beta_4$, $\alpha_6\beta_7$, $\alpha_6\beta_{13}$, and $\alpha_6\beta_5$ (19–22). The adhesion of gastric cancer cells to the peritoneum is, reportedly, a key step in the initial process of peritoneal metastasis (23). Adhesion molecules such as CD44 and $\beta_1$ integrin receptor have been previously reported to be functionally involved in the peritoneal adhesion of gastric cancer cells (24).

In the present study, we present further evidence to suggest that Cyr61 promotes gastric cancer cell adhesion to the peritoneum during the metastasis process. Functional dissection has revealed that the integrin $\alpha_2\beta_1$/AP-1 signaling cascade is essential for Cyr61-mediated cell adhesion, this having been reported to be the initial step of peritoneal dissemination for cases of human gastric cancer. A reduction of cellular adhesion to the

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peritoneum is expected to inhibit the metastasis or dissemination of gastric cancer cells. Our findings provide potential support for the development of a new therapeutic regimen to be directed against cancer progression in gastric cancer.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Antibodies were obtained from the following sources: anti-α-tubulin antibody (Sigma), anti-p-Akt1/2/3 (Ser-473)-R antibody, anti-Akt-1 antibody, anti-p-ERK1/2 antibody, anti-ERK1/2 antibody, anti-p-c-Jun antibody, anti-c-Jun antibody, anti-Cyr61 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal antibod BHA2.1 (anti-α₂β₁), and anti-α₂β₁, anti-integrin subunits α₂, α₃, and β₁ (Chemicon International, Temecula, CA). Thiol-reactive fluorescent probes and 5-chloromethylfluorescein diacetate (Cell Tracker Green) were products of Molecular Probes (Eugene, OR).

**Cell Culture**—The human gastric-carcinoma cell lines AGS and N87 were purchased from American Type Culture Collection (Manassas, VA), and MKN45 and TSGH were purchased from the Health Science Research Resources Bank (Osaka, Japan). All of these gastric cancer cell lines were cultured in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum (Bioserum, Victoria, Australia), penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and sodium pyruvate (1 mM), all such reagents deriving from Invitrogen. The medium was refreshed every 48 h subsequent to seeding.

**Transfection and Established Stable Clone Cells**—The cloning process of cyr61 expression plasmid was described previously (14). Briefly, total RNA was extracted and Cyr61 complementary DNA was cloned and amplified by RT-PCR with the primer with cloning sites 5'-TATAGGATCCGATGGAGATGTG-3' (forward) and 5'-AGGACTGGATCAGTGCCTGTT-3' (reverse) to produce a 550-bp fragment of the Cyr61 gene; human integrin α₂ primers, 5'-CTTCTCTGGTGGAATGCTGG-3' (forward) and 5'-CTATGTGCTGGTGTGGTGA-3' (reverse) so as to produce a fragment of the human integrin α₂ gene; human integrin β₁ primers, 5'-GGCGGGGCCCCTCCAT-3' (forward) and 5'-ATGGAGGCGGGGCCCCTCCAT-3' (reverse) to produce a fragment of the human integrin β₁ gene, and glyceraldehyde-3-phosphate dehydrogenase primers, 5'-GATGATGATATCGCCGCT-3' (forward) and 5'-TGGGTCATCTTCTCCGCGT-3' (reverse) to facilitate the production of a 448-bp fragment of the glyceraldehyde-3-phosphate dehydrogenase gene, which was used as the internal control. The PCR amplification was performed in a reaction buffer containing Tris-HCl (20 mM, pH 8.4), KCl (50 mM), MgCl₂ (1.5 mM), all four dNTPs (each at 167 μM), Taq polymerase, and appropriate primers (0.1 μM). The reactions were performed in a Biometra Thermoblock (Biometra, Hamburg, Germany) featuring the following program: denaturing for 1 min at 95 °C, annealing for 1 min at 52 °C, and elongating for 1 min at 72 °C for a total of 30 cycles; the final extension being conducted at 72 °C for a period of 10 min. PCR products were visualized by ethidium bromide staining following agarose-gel electrophoresis.

**Western Blot Analysis**—Gastric cancer cells were incubated in serum-free RPMI for a period of 16 h and collected in radio-immune precipitation assay buffer (Tris-HCl (50 mM, pH 7.5), NaCl (120 mM), Nonidet P-40 (0.5%), NaF (100 mM), Na₃VO₄ (200 mM), phenylmethylsulfonyl fluoride (1 mM), leupeptin (10 μg/ml), and aprotinin (10 μg/ml)) for a period of 15 min on ice. The total cell lysates were centrifuged in a microtube at 4 °C for 20 min. Following this, equal amounts of protein from the cell lysates were resolved by SDS-PAGE in 10% gels and electrophoresis to a polyvinylidene difluoride membrane (Immobilon-P membranes, Millipore, Bedford, MA). Subsequently, the blot was blocked in a 3% solution of bovine serum albumin, 0.1% Tween 20, and phosphate-buffered saline, the blot then being incubated with primary antibodies as indicated for 4 °C overnight, and then washed in phosphate-buffered saline incorporating 0.1% Tween 20 Renaissance® (NEN™ Life Science Products). The membrane was then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h, and then washed with phosphate-buffered saline with 0.1% Tween 20. The antibody-bound protein bands were detected with appropriate enhanced chemiluminescent reagents (Amersham Biosciences) and photographed using Kodak X-Omat Blue autoradiography film (PerkinElmer Life Sciences).
Flow Cytometry—Integrin expression was measured by means of the use of a flow cytometer (FACSCalibur, BD Biosciences), using monoclonal antibodies against human integrin α3β1 subunits and fluorescein isothiocyanate-labeled secondary antibodies.

Cell Adhesion Assay—The excised partial rat peritoneum (≈1.6 cm²), which had been put in a coverslip, was placed in a 6-well tissue-culture plate and filled with 1.0 ml of RPMI 1640 medium containing 1% bovine serum albumin. Gastric cancer cells (1.5 × 10⁶ cells) were fluorescently labeled with 5-chloromethylfluorescein diacetate (1.77 mm) at 37 °C for 30 min and then washed twice with RPMI 1640 medium containing 1% bovine serum albumin. Then the cells were overlaid onto pieces of rat peritoneum contained within a 6-well plate and were incubated at 37 °C for 40 min. Following gentle washing of the pieces, adherent to the peritoneum with phosphate-buffered saline, the cells were fixed using 4% paraformaldehyde, or their fluorescence intensity was determined using a fluorescence spectrophotometer (Ex = 490 nm, Em = 520 nm). For the inhibition experiments, the cells were pre-treated with neutralizing antibody to different integrin subunits at 37 °C for 30 min prior to their being incubated with the section of peritoneum. This procedures was described before by Asao et al. (25).

Animal Study of Peritoneal Implantation—8-week-old female SCID mice (supplied by the animal center of the National Defense Medical Center, Taiwan) were acclimatized over a period of 1–2 weeks while being caged in groups of five. The animals were housed under pathogen-free conditions and fed a diet of animal chow and water throughout the conduct of this series of experiments. AG5/Neo, AG5/Cyr61, MKN-45/Neo, and MKN-45/Cyrl-AS cells (5 × 10⁶ cells, 0.5 ml) were suspended in RPMI 1640 and incubated with antibodies directed against α3β1 integrin at 37 °C for a period of 20 min and then inoculated into the abdominal cavity of mice. The mice were sacrificed 4 weeks later, and any disseminated nodules present on the mesentery and diaphragm were evaluated. All mouse studies were performed using protocols approved by the Institutional Animal Care and Use Committee of the College of Medicine, National Taiwan University.

Integrin α32Luciferase Plasmid Construction—The integrin α32 promoter fragment was screened using full-length human integrin α32 cDNA as a probe. An Nhel-HindIII genomic fragment (−944 to +92 in relation to the transcription start site at +1) was prepared upstream to the Luc structural sequences in pGL3-Basic (Promega), which lacks natural Luc regulatory elements. The promoter and enhancer activity of the 5' flanking region was analyzed by inserting the nested deletion mutants of the 1.0-kb construct (pc2944-Luc) mutants being generated by means of restriction-enzyme digestion of pc2944-Luc at the relevant restriction enzyme sites, by using the QuikChange site-directed mutagenesis kit (Stratagene). Co-transfection with β-galactosidase served as a control for determining transfection efficiency for all assays. All transfection experiments were performed on at least three occasions.

Chromatin Immunoprecipitation Analysis—Cells were cultured in 10-cm dish in confluence overnight, and treated with formaldehyde and added directly to culture medium (to a final concentration of 1%) at room temperature for 10 min to cross-link histone proteins to DNA, then glycine (to a final concentration of 0.125 M) was added to plates to quench formaldehyde. Soluble chromatin was made as follows: Cells were washed and detached from the dish by scraping after addition of ice-cold phosphate-buffered saline, then pelleted by centrifugation for 4 min at 700 × g. The resultant cell pellet was then lysed, pelleted, and lysed in two consecutive lysis buffers: LB1 (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 0.25% Triton X-100, protease inhibitor mixture) and LB2 (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA). After the second lysis, the pellet was suspended in LB3 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate) and sonicated. Samples were then centrifuged at 13,000 rpm for 10 min, and supernatant was collected. For immunoprecipitation, 1 μg of antibodies and a prebound protein A were added to 400 μl of the purified chromatin sample, and the mixture was incubated overnight at 4 °C. Immunocomplexes with the beads were washed with immunoprecipitation assay buﬀer followed by a wash with TE (10 mM Tris, 1 mM EDTA, pH 7.4), then the immunocomplexes were recovered by adding elution buﬀer (1% SDS, 1%, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) for 10 min at 65 °C, then by centrifugation at 14 K rpm for 10 min. Antibody-immunocomplexed DNA was then recovered by phenol/chloroform extraction and ethanol precipitation and resuspended in TE. PCR primer sets were designed to overlap and span the AP-1 site of the integrin α32 promoter: primer set, forward (5'- AAAAAGGCGCCCTAC- CAGTTTCG-3') and reverse (5'-GCCCTCATTAGCTACCCATCCA-3'). The primers were first evaluated using the integrin α32 construct as DNA template. Conventional PCR was then performed with eluted immunocomplexed DNA, Titanium TaqPCR kit (Clontech Laboratories, Palo Alto, CA). PCR was performed on unprecipitated chromatin as a positive control and to correct for input volume. Amplification was carried out for 35 cycles (28 cycles for unprecipitated chromatin input lanes) with denaturation at 94 °C for 1 min, annealing at 58 °C (64 °C for FGF8 primers) for 30 s, and extension at 72 °C for 1 min. PCR products were run in 2% agarose gel.

Surgical Specimens and Immunohistochemistry—Serosal-invasive (T3 and T4) gastric cancer tissues were collected from 125 consecutive patients who underwent surgery at the National Taiwan University Hospital during the period 1995–1998. All patients underwent complete surgical resection, and their clinical and pathological data were available. The expression level of Cyr61 in gastric adenocarcinoma was determined by immunohistochemistry using a Cyr61-specific antibody that was described before (18). If >50% of the tumor cells were positively stained, the specimen was grouped as “positive.” All other staining results were regarded as negative. The pathologist assessing immunostaining intensity was blinded to patients’ information.

Statistics—For statistical analysis, p values were based upon the two-tailed, parametric Student’s t test using Excel (Microsoft Corp., Redmond, WA). A resultant value for p of <0.05 (on the basis of at least three independent sets of experiments) was considered to represent a statistically significant difference between test data sets. The relative dimension of
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RESULTS

Because Cyr61 belongs to the family of extracellular matrix-associated proteins, we hypothesize that Cyr61 may play some sort of role in influencing the relative adhesion ability of gastric cancer cells to the peritoneum of gastric cancer-afflicted individuals. To address this issue, initially we examined the intracellular level of Cyr61 for four different human gastric cancer cell lines (AGS, N87, MKN-45, and TSGH) using RT-PCR and Western blotting assays. Of the cell lines analyzed, AGS and N87 cells featured lower RNA- and protein-expression levels for Cyr61 than did the remainder, whereas, MKN-45 and TSGH cells revealed a stronger expression level of Cyr61 than was the case for AGS and N87 (Fig. 1A). We next tested the adhesion ability of these cell lines using a specific ex vivo peritoneum assay, and it had been shown that the gastric cancer cell line MKN-45 had the highest ratio of cells adherent to the peritoneum among the colon and gastric cancer cell lines (25). Our results showed MKN-45 and TSGH cells displayed strong intracellular adhesion ability, whereas, by contrast, AGS and N87 cells exhibited relatively weak adhesion ability (Fig. 1B). Under such experimental condition, MKN-45 and TSGH cells exhibited a 3-fold more pronounced level of adhesion to the peritoneum derived from test rats. Cells were fluorescently labeled with 5-chloromethylfluorescein diacetate cell-tracker dye before seeding, 40 min following which, the pictures were photographed by means of fluorescence microscopy following fixation. C. in vitro adhesion was measured by determining the proportion (percentage) of cells adherent to the section of peritoneal membrane as compared with AGS wild-type cells. Each cell was assayed in three separate experiments, each carried out in triplicate. Bar graphs represent average number of cells harvested ± S.E.

FIGURE 1. Cyr61 expression and adhesion ability of human gastric cancer cell lines. In A, upper panel, RT-PCR analysis for Cyr61 mRNA expression. The 550-base-coding regions of Cyr61 cDNA were used as probes. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used as an internal control for RNA quantity. Lower, Western blot analysis of Cyr61 protein in these human gastric cancer cell lines as described under “Experimental Procedures.” Total proteins were extracted from gastric cancer cell lines and probed with a Cyr61-specific polyclonal antibody. 40 µg of total protein was analyzed per sample. α-Tubulin was used as an internal loading control. B, the adhesion activity of each cell was measured in vitro using an excised partial peritoneum derived from test rats. Each bar represents the mean (± S.E.) number of cells harvested for three experiments performed in triplicate. C, in vitro adhesion was measured by determining the proportion (percentage) of cells adherent to the excised rat peritoneal membrane. Each bar represents the mean (± S.E.) number of cells harvested for three experiments performed in triplicate. α-Tubulin was used as an internal loading control. In B, upper, representative immunoblot demonstrating levels of Cyr61 protein expression in mock transfected and Cyr61 antisense (AS)-transfected MKN45 clones. Lower, expression of Cyr61-AS decreased the in vitro adhesion activity of MKN45 cells. In vitro adhesion was measured by determining the proportion (percentage) of cells that adhered to excised rat peritoneal membrane. Cyr61-AS-transfected clones revealed statistically significantly diminished adhesion activity (p < 0.05) compared with the MKN45/Neo vector control clone. Each clone was assayed in three separate experiments carried out in triplicate.

human gastric cancer cell lines. To explore whether Cyr61 is involved in the regulation of peritoneal adhesion of human gastric cancer cells, a population of AGS cells stably expressing Cyr61 was established by transfection of AGS cells with Cyr61-overexpressing plasmid. Following G418 selection, we obtained the pooled transfectants (AGS/Cyr61), and the vector control clone (AGS/Neo), and then we assessed the levels of Cyr61 expression within these stably transfected cells. Western blot analysis revealed that the AGS/Cyr61 cells expressed a 3-fold greater level of Cyr61 protein than was the case for the vector control cells (Fig. 2A, right panel). From the peritoneal adhesion assay, the AGS/Cyr61 transfectants revealed a 2.5-fold greater adhesion ability as compared with vector control cells (Fig. 2A, left panel). On the other hand, we also established the presence of MKN45 cells stably expressing antisense Cyr61 following transfection of such cells with pcDNA3 vector carrying an antisense orientation of the Cyr61 gene. Western blotting analysis indicated an 80% lower level of Cyr61 protein in the antisense Cyr61-transfected MKN-45 cells (MKN-45/Cyr61-AS) as compared with vector control cells (MKN-45/Neo) (Fig. 2B, upper panel). Similarly, the peritoneal adhesion ability of MKN45/
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Cyr61-AS cells was significantly lower than that of MKN45/Neo cells (Fig. 2B, lower panel). These data clearly show that the increased Cyr61-expression level within gastric cancer cells results in an enhancement of the ability of such cells to adhere to the peritoneum.

It has been often reported that certain integrins, including \( \alpha_3 \beta_1 \) and \( \alpha_2 \beta_1 \), have revealed the ability to act as critical molecules as regards determining the ability of gastric cancer cells to adhere to the peritoneum (24). We thus examined whether antibodies directed against the \( \alpha_2 \), \( \alpha_3 \), and \( \beta_1 \) subunit, and their combinations, affected the peritoneal adhesion ability of AGS/Cyr61 cells or MKN-45 cells, both of which exhibit a substantial level of Cyr61. The peritoneal adhesion ability of AGS/Cyr61 cells appeared to be effectively inhibited by the action of certain antibodies directed against integrin \( \alpha_2 \beta_1 \) and \( \beta_1 \), and to a lesser extent, by antibodies directed against integrin \( \alpha_3 \) whereas antibodies directed against integrin \( \alpha_3 \) and \( \alpha_2 \beta_1 \) failed to affect the adhesion ability of AGS/Cyr61 cells (Fig. 3A). For MKN-45 cells, similar inhibitory effects upon the peritoneal adhesion ability were also noted as a consequence of the actions of antibodies directed against integrins \( \alpha_2 \beta_1 \), \( \beta_1 \), and \( \alpha_2 \) (Fig. 3B).

To identify whether Cyr61 is able to directly regulate the \( \alpha_2 \) integrin gene, the 5' flanking region of the Cyr61 promoter region
between −944 and +92 was cloned into the pGL3-basic luciferase vector. In addition, a series of 5’T deletion mutants, such as pα2/944-Luc, pα2/240-Luc (−240 to +92), pα2/90-Luc (−90 to +92), and pα2/31-Luc (−31 to +92) were constructed and used for experimentation, as shown in Fig. 4A. Luciferase plasmids containing pα2/944-Luc or deleted α2 integrin upstream DNA fragments were transiently cotransfected with β-galactosidase expression plasmid into AGS/Cyr61 and AGS/neo cells, following which cellular luciferase activity was measured within the cell lysate. Fig. 4B revealed that the longest promoter fragment, pα2/944-Luc, featured a 3-fold induction of promoter activity within AGS/Cyr61 cells as compared with the corresponding level for AGS/Neo cells, however, this increased promoter activity within AGS/Cyr61 cells was not observed following transfection with other shorter length promoter fragments, including pα2/240-Luc (−240 to +92), pα2/90-Luc (−90 to +92), and pα2/31-Luc (−31 to +92). Such results suggest that the cis-regulatory element is located somewhere between bp −240 and −944. Based upon our data, two potentially responsive elements, estrogen receptor (−802/−798) and AP-1 (−765/−760), are located within this region. To evaluate the role of these two sites in promoter activity, mutations in the AP-1 site, the estrogen receptor site, or both sites was used by means of PCR-directed mutagenesis. Fig. 4B illustrates the site-
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directed mutation that arose at the AP-1 site, but not the estrogen receptor site, led to the induction of a dramatic impairment of α2,944-Luc promoter activity within Cyr61-overexpressing cells, this thus demonstrating that the AP-1 binding site is critical for Cyr61-induced α2 integrin gene expression. To further corroborate these data, using a chromatin immunoprecipitation assay, we examined whether the AP-1 transcription factor would directly bind to the AP-1 site within the α2 integrin gene promoter (Fig. 4C). Overexpression of Cyr61, followed by a chromatin immunoprecipitation assay, indicated that the AP-1 protein was constitutively bound to this region of the α2 integrin promoter, as evidenced by the immunoprecipitation by anti-Jun antibody. The increased level of binding of AP-1 to α2 integrin promoter was effectively abolished by transfection with the AP-1 decoy oligonucleotide (Fig. 4C). We confirmed again that the elevation of α2 integrin protein within Cyr61-expressing cells was significantly reduced by transfection of such cells with the AP-1 decoy (Fig. 4D). Subsequently, we wanted to know the possible signaling pathways involved in the Cyr61-mediated activation of AP-1. To address this, AGS cells were exposed to extracellular Cyr61 recombinant protein in combination with Mek or phosphatidylinositol 3-kinase/Akt inhibitor and, in turn, we checked the expression of the related target molecules. As shown in Fig. 4E, we found MAPK/ERK signaling pathway is involved in the Cyr61-induced c-Jun phosphorylation (AP-1 activation). Mek inhibitor PD98059, but not wortmannin, effectively blocked AP-1 activation after recombinant Cyr61 treatment. Collectively, the above data suggest that the Cyr61-mediated elevation of the α2 integrin gene proceeds through an AP-1-dependent pathway.

To explore the effects of Cyr61 and its downstream effector α2β1 integrin on the peritoneal dissemination of gastric cancer cells, we inoculated different transfecants (1 × 10^7 cells) into SCID mice and/or administered treatment either with or without anti-α2β1 integrin antibodies once a week. As a consequence of such treatment, the apparent development of peritoneal carcinomatosis in mice injected with AGS/Cyr61 cells as compared with those injected with AGS/neo cells was noted (Fig. 5A). Quantitatively, ~98 ± 30 disseminated nodules were noted for test mice inoculated with AGS/Cyr61 cells, and by contrast, no disseminated nodules were able to be observed for mice injected with AGS/neo cells (Fig. 5B). Injection of test mice with MKN-45/neo cells effectively produced about 151 ± 32 nodules, however, we did observe that the number of peritoneal dissemination incidents so produced was substantially lower (82 ± 17 nodules) when test mice were injected with MKN-45/Cyr61-AS cells (Fig. 5B). These data indicate that Cyr61 expression typically influences the peritoneal dissemination of gastric cancer cells for test mice. In a parallel experiment, we determined whether α2β1 integrin antibody was highly applicable for the therapeutic treatment of disseminated gastric cancer cells, i.e. AGS/Cyr61 and MKN-45. The results presented in Table 1 reveal that treatment with anti-α2β1 integrin antibodies reduced the development of peritoneally disseminated tumors for AGS/Cyr61 and MKN-45 cells ~65 and 50%, respectively. In addition, the survival time of AGS/Cyr61- and MKN-45-cell-injected mice was significantly prolonged by

![FIGURE 5. Cyr61 induced peritoneal dissemination by enhanced α2β1 expression in vivo. A, effects of AGS/Neo (panels I and III) and AGS/Cyr61 (panels II and IV) clones on peritoneal dissemination for SCID mice. Various transfected cells were injected intraperitoneally as described under “Experimental Procedures.” 4 weeks later, the mice were sacrificed, photographed, and dissected, and any disseminated nodules present on the mesentery and diaphragm were counted. B, the disseminated nodules were evaluated for AGS/Neo versus AGS/Cyr61-stable-overexpressing clones (left), and for MKN45/Neo- versus MKN45/Cyr61-AS-overexpressing clones (right). Each bar represents the mean ± S.E. C, Kaplan-Meier survival plots for mice intraperitoneally injected with human gastric cancer cell lines treated with IgG control or anti-α2β1 functional blocking antibody (dotted with square line, AGS/Cyr61 plus anti-α2β1 function-blocking antibody; solid with square line, AGS/Cyr61 plus IgG control antibody; solid with circle line, MKN45 plus IgG control antibody; and dotted with circle line, MKN45 plus anti-α2β1 function-blocking antibody). p value was determined by two-sided log-rank test.]
treatment with anti-αβ1 integrin antibodies compared with the IgG control (Fig. 5C).

Our previous study (18) demonstrated that the expression level of Cyr61 is positively correlated with the extent of lymph-node metastasis, a more-advanced tumor stage and early recurrence of metastasis in patients suffering from gastric adenocarcinoma. Here we further analyze the possible association between the level of Cyr61 in primary tumors and the occurrence of peritoneal carcinomatosis. Immunohistochemical staining of 125 primary tumors of T3 and T4 stage gastric carcinoma was performed herein. Representative results are presented in Fig. 6A. Expression of Cyr61 protein was negative for T3 stage patients not featuring peritoneal dissemination (Fig. 6A, panel I). The expression of Cyr61 was significantly increased in peritoneal dissemination T3 patients with diffuse-type gastric cancer (Fig. 6A, panel II). For these tumors, the protein was predominantly localized within the cytoplasm. As summarized in Table 2, the expression level of Cyr61 protein is significantly correlated with the development of peritoneal dissemination of gastric cancer (p = 0.001). Among these 125 gastric tumors, 70 tumors were scored as positives for Cyr61 expression (60%) and 55 tumors were revealed as being negative for Cyr61 expression (40%). Furthermore, 25 of 65 Cyr61-expressing primary tumors developed peritoneal carcinomatosis (35.5%), whereas only three of 55 Cyr61-negative tumors were associated with the development of peritoneal metastases (5.4%). Calculation of the survival duration of the 125 involved patients by the Kaplan-Meier method revealed that the patients who featured Cyr61-positive tumors demonstrated a shorter survival when compared with those patients who suffered from Cyr61-negative tumors (Fig. 6B, p = 0.0065). Finally, to further investigate the potential interaction of Cyr61 and integrin αβ1 in vivo, we examined the relationship in primary cancer tissues by immunohistochemical staining. As shown in Fig. 6C (panels I and II), positive high staining of integrin αβ1 expression was noted in gastric tumors with high Cyr61 expression. The arrow indicates the membranous distribution of integrin αβ1. In contrast, negative low staining of integrin αβ1 expression was found in gastric tumors with low Cyr61 expression in Fig. 6C. There is a positive correlation between Cyr61 and integrin αβ1 expression (R² value = 0.585, p = 0.003) in Table 3. These data suggest that Cyr61 not only promotes the development of peritoneal metastasis but also acts as an effective prognostic factor for advanced-stage gastric cancer patients.

**DISCUSSION**

Peritoneal metastasis is not only the most frequent pattern of gastric cancer recurrence, but it is also a major cause of death among advanced gastric cancer patients (3). Although the presence of peritoneal metastasis reveals a strong impact for patient prognosis, the molecular mechanisms by which gastric cancer cells actually acquire the ability to undergo peritoneal dissemination remains to be clarified. In the present study, we established, we believe, for the first time, that Cyr61, an extracellular matrix-associated protein, is critically involved in the peritoneal adhesion/metastasis of gastric cancer cells. These findings are supported by compelling evidence that the enforced expression of Cyr61 within human AGS cells greatly enhanced these cells’ adhesion ability to excised sections of peritoneum. On the one hand, reduced levels of endogenous Cyr61 within MKN-45 cells strongly impaired these cells’ adhesion to sections of peritoneum. Consistent with in vitro data, we found that Cyr61-overexpressing AGS cells had acquired the ability to form tumor nodules in the peritoneal cavity for SCID mice; by contrast to the significantly diminished peritoneal metastatic ability of MKN-45 cells subsequent to these cells having been stably transfected.

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**TABLE 1**

Effect of αβ1 integrin antibody treatment on peritoneal carcinomatosis

| Cell line | Treatment                  | No. of disseminated nodule |
|-----------|---------------------------|----------------------------|
| AGS/Cyr61 | IgG                       | 82 ± 28                    |
| MKN45     | Anti-αβ1 integrin antibody | 33 ± 15*                   |
|           | IgG                       | 135 ± 36                   |
|           | Anti-αβ1 integrin antibody | 68 ± 21*                   |

* p values of <0.05 were considered statistically significant.

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**FIGURE 6.** Immunohistochemistry of gastric cancer patients for Cyr61 expression. A, representative section of specimen from gastric cancer patient undergoing primary surgical excision (stage III). Carcinomatous areas featuring no peritoneal dissemination reveal a level-1 stain intensity for Cyr61 (left, magnification, 200×), and level-3 intensity Cyr61 staining for the specific tumor component that does feature peritoneal dissemination (right, magnification, 200×). B, Kaplan-Meier survival plots for 125 patients afflicted with T3 and T4 gastric cancer, grouped according to Cyr61 protein expression. p was determined by the log-rank test. C, positive high staining of integrin α2β1 expression in gastric tumors with high Cyr61 expression (panel I, 100×; panel II, 200×). The arrow indicates the membranous distribution of integrin α2β1. Panel III, negative low staining of integrin αβ1 expression in gastric tumors with low Cyr61 expression (100×).
Correlation of Cyr61 and integrin $\alpha_2\beta_1$ in gastric cancer primary tissues ($R^2$ value = 0.585, $p$ = 0.003)

|                | Cyr61, low | Cyr61, high | Total |
|----------------|------------|------------|-------|
| Integron $\alpha_2\beta_1$, low | 3 (13.0%) | 4 (17.4%) | 7 (30.4%) |
| Integron $\alpha_2\beta_1$, high | 0 (0%) | 16 (69.6%) | 16 (69.6%) |
| Total          | 3 (13.0%) | 20 (87.0%) | 23 (100%) |

TABLE 2
Correlation between Cyr61 expression level and peritoneal dissemination from patients with gastric cancer

| Peritoneal dissemination | Cyr61 (-), $n = 55$ | Cyr61 (+), $n = 70$ | $p$ |
|--------------------------|---------------------|---------------------|-----|
| Negative                 | 52                  | 45                  | 0.001 |
| Positive                 | 3                   | 25                  |     |

With antisense Cyr61. Further evidence from clinical analysis revealed that the levels of Cyr61 correlated significantly with the extent of peritoneal metastasis among 125 cases of advanced gastric adenocarcinoma. Collectively, the interpretation of our data would appear to encourage us to conclude that Cyr61 plays a novel role in regulating gastric cancer cell peritoneal adhesion in a clinical/experimental setting, and it would also appear to be feasible as a biological marker to predict the relative likelihood/extent of peritoneal metastasis following gastric carcinoma.

In respect to the mechanism by which Cyr61 regulates the peritoneal adhesion ability of gastric cancer cells, our results suggested an intriguing mechanism by which the expression of Cyr61 within cells could transcriptionally up-regulate the $\alpha_2\beta_1$ integrin subunit and, in turn, promote the formation of the $\alpha_2\beta_1$ integrin receptor. The predominant expression of $\alpha_2\beta_1$ integrin associated with peritoneal metastasis of gastric carcinomas has already been reported on elsewhere (26), although, to the best of our knowledge, the specific function would appear to have not yet been accurately defined. Using function-neutralizing antibodies to the $\alpha_2$, $\alpha_3$, and $\beta_1$ subunits, or their combinatorial dimer, we observed that only antibodies directed against $\alpha_2\beta_1$ integrin, but not antibodies directed against $\alpha_3\beta_1$, could block the peritoneal adhesion/metastasis of Cyr61-expressing cells (i.e. AGS/Cyr61 and MKN-45), either in vitro or in vivo. In addition, the survival time of test mice transplanted with AGS/Cyr61 or MKN-45 cells was significantly prolonged when mice were pre-treated with anti-$\alpha_2\beta_1$ integrin antibodies. Our results suggest that certain therapeutic modalities incorporating the administration of antibodies to $\alpha_2\beta_1$ integrin in gastric cancer patients may, potentially, prevent peritoneal metastasis for a certain subset of such patients subsequent to the surgical treatment of primary tumors. Integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ are the most important integrins in peritoneal dissemination, implantation, or adhesion of gastric cancers (23, 24, 26). Although integrin $\alpha_3\beta_3$ signaling has been found to be involved in the invasive promoting pathway of gastric cancer in our previous study (18), we did not find any literature about integrin $\alpha_2\beta_3$ in gastric cancer peritoneal dissemination. We consider that integrin $\alpha_2\beta_3$ is involved in the invasive potential, but not in the peritoneal adhesion/dissemination, of gastric cancer.

The $\alpha_2\beta_1$ integrin receptor is commonly expressed on certain types of cells, such as platelets (27), leukocytes (28), endothelium (29), and, particularly, within many types of cancer cells (30). $\alpha_2\beta_1$ integrin is a collagen-binding integrin, which engages in various biological functions, including collagen-induced platelet activation and aggregation (31), acting as a mediator for vascular endothelial growth factor-induced angiogenesis (32), and facilitating the invasion and adhesion of squamous cell carcinoma cells (33). The authors of several earlier studies have suggested that the increased level of $\alpha_2\beta_1$ integrin is mediated by the up-regulation of $\alpha_2$ integrin mRNA abundance (34–36). For example, hepatocyte growth factor-induced $\alpha_2\beta_1$ integrin-dependent tubulogenesis is reported to result from an increase of $\alpha_2$ integrin mRNA (34). The $\alpha_2\beta_1$ integrin functional involvement in a three-dimensional collagen lattice-activated fibroblast contraction is also due, reportedly, to the up-regulation of the $\alpha_2$ integrin subunit (35). The compound 12-O-tetradecanoylphorbol-1,3-acetate, a protein kinase C activator, can modulate the $\alpha_2\beta_1$ integrin-dependent adhesion of MCF-7 cells by altering the gene expression of integrin $\alpha_2$ (36). Consistent with the results of the above-mentioned studies, we have also determined that Cyr61 promotes the $\alpha_2\beta_1$ integrin-dependent peritoneal metastasis of gastric cancer cells, a process that is mediated by the activation of $\alpha_2\beta_1$ integrin gene expression. In addition, using a promoter reporter gene assay, we have determined an essential role of the AP-1 site (−765/−760) during the Cyr61-induced up-regulation of the $\alpha_2$ integrin gene. It has been previously reported that several binding sites (including AP-1, AP-2, SP-1, ER, GATA, and NF-1) for various transcription factors are present within the integrin $\alpha_2$ promoter (37). These elements are involved in the transcriptional activation of integrin $\alpha_2$ gene expression by numerous factors, including hepatocyte growth factor and certain proteoglycans (38–40). It has been reported quite recently that AP-1 binding site activity is induced during megakaryocytic differentiation within human leukemia cells so as to increase integrin $\alpha_2\beta_1$ expression within these cells (41). The promoter construct in this study is not the full-length $\alpha_2$ integrin promoter (37, 42, 43). Previous studies have shown that most important regulatory binding sites, including transcription factors AP-1, AP-2, SP-1, ER, GATA, and NF-1, are present within the 1-kb promoter construct (42, 43). The 5′-flanking region of the $\alpha_2$ integrin gene had been characterized to have three different regulatory regions, including a core promoter (−30 to −92), a silencer (−92 to −351), and megakaryocyte enhancers in the distal 5′ flank (−1426 to −2592) (42). Although a megakaryocytic enhancer in the distal 5′-end of the promoter region −1426 to −2592 had been found to play an important role in $\alpha_2$ integrin gene activity in cells undergoing megakaryocyte differentiation, this distal regulatory element is not involved in $\alpha_2$ integrin gene activity of epithelial cells (43). However, we could not exclude the possibility that there are more cis-regulating elements further upstream of our promoter construct. Interpreted collectively, our findings reveal that Cyr61 plays a major role in regulating integrin $\alpha_2$ gene expression through AP-1 and that such a function contributes importantly to the peritoneal dissemination of gastric cancer cells.

It has been often reported that Cyr61 is an extracellular matrix-signaling molecule that promotes cell proliferation, migration, invasion, and adhesion (10, 11). We propose that...
Cyr61 is a signaling cell-adhesion molecule being able to augment carcinoma-cell adhesion and metastatic activities, and it may dictate specificity in the biological and pathological roles. To verify our hypothesis, the clinical implications of Cyr61, as regards peritoneal dissemination, were investigated for 125 consecutive T3 and T4 gastric cancer patients who underwent surgical resection of serosa-invasive gastric cancer. Inspection of T3 stage tumors revealed penetration of the serosa of gastric wall and T4 stage revealed the invasion of adjacent structures. Herein, we have found that Cyr61, the first cloned member of the CCN family, is significantly associated with the phenotype of peritoneal seeding for patients with gastric cancer and may be used to act as an appropriate prognostic factor for serosa-invasive advanced-stage gastric cancer.

As far as we are currently aware, the local production of high concentrations of cytokines at the (gastric cancer) tumor site may directly alter tumor-adhesion properties associated with invasive and metastatic abilities of various cancers. For example, it has been previously reported that interleukin-2 and interferon α may enhance the expression of certain cell-adhesion receptors, such as CD44 and EGFR (44). From our current investigation, we concluded that Cyr61 is a pivotal adhesion-regulating cytokine involved in the development of peritoneal dissemination for human gastric cancer, and that incremental changes to intracellular levels of cell-adhesion integrin αβ1 play a key role in this step. The extracellular matrix molecule implicated in the processes of cancer invasion and tumor progression and metastasis may provide the focus for an effective strategy for the treatment of patients afflicted with advanced gastric cancer.

It has recently been reported that CYR61 is down-regulated in a proteomics analysis study in human gastric carcinomas using two-dimensional gel studies, independent of antibody (45). In that study, the authors used a proteomic approach to search for genes that may be involved in gastric carcinogenesis and that might serve as diagnostic markers. They collected 14 pairs of gastric carcinoma–corresponding noncancerous gastric mucosa tissue specimens. All of the carcinoma specimens were histologically identified as advanced carcinoma. They found CYR61 protein was down-regulated in gastric carcinoma tissues as compared with normal gastric mucosa (45). The discrepancy between that study and the current one might be due to different study design. In the current study, only T3 and T4 gastric cancers, but not gastric carcinoma tissues and normal gastric mucosa, were compared. In our previous study, we also found that Cyr61 did not alter the growth properties in AGS cells (18). All these findings suggest that Cyr61 may suppress the growth of normal gastric epithelial cells and/or prevent cancer development, but when it was secreted by gastric cancer cells, it may not influence the growth properties of gastric cancer cells (which may have more other factors to promote their growth or anti-apoptosis) but may contribute to invasiveness and/or adhesion and peritoneal dissemination in advanced gastric cancer. Another possibility is that the down-regulation of Cyr61 in gastric cancer tissues compared with normal gastric mucosa might be a phenomenon secondary to some primary events during carcinogenesis. Further studies on the roles of Cyr61 in normal gastric mucosal cells are needed to elucidate its role on carcinogenesis.

In conclusion, we have demonstrated that Cyr61 induces functional integrin αβ that plays an important role in the peritoneal dissemination of human gastric cancer cells. In this mechanistic study, the results of our investigation strongly suggest that Cyr61-mediated AP1 signaling is a requisite for integrin αβ2 expression. Our current findings provide a substantial body of evidence that suggests that Cyr61 acts to enhance gastric cancer-cell peritoneal dissemination, and this process is, at least in part, mediated by the up-regulation of integrin αβ2. Cyr61 acts as both an enhancer and also a predictive biomarker for the peritoneal dissemination of cancer cells for gastric cancer patients at advanced stages of their malady. Collectively, our data also reveal that Cyr61 and its downstream effector integrin αβ2 could constitute a potential target for future treatment of peritoneal dissemination of cancer cells.

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