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Cyr61 Induces Gastric Cancer Cell Motility/Invasion via Activation of the Integrin/Nuclear Factor- κ B/Cyclooxygenase-2 Signaling Pathway

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Abstract Purpose: Cysteine-rich 61 (Cyr61/CCN1) is involved in many different types of tumor development and progression. Nonetheless, the role of Cyr61 in human gastric cancer has not yet been fully characterized.

Experimental design: We addressed the issue by immunohistochemical staining of 81 gastric adenocarcinoma specimens. Liposome-mediated transfection was used to introduce a Cyr61 expression vector into gastric cancer AGS cell lines. Transfectants were tested in invasion assay by a Boyden chamber. Furthermore, a cyclooxygenase-2 (COX-2) reporter assay and gel mobility shift assay were done to investigate the potential signal pathway of Cyr61.

Results: Patients with gastric adenocarcinoma whose tumor displayed high expression of Cyr61 correlated well with aggressive lymph node metastasis, more advanced tumor stage, histologic diffuse type, and early recurrence. Stable transfection of Cyr61 into the AGS cell line strongly enhanced its invasive activity. The overexpression of Cyr61 into AGS cells significantly increased the expression of COX-2 mRNA, protein, and enzymatic activity. Gel mobility shift assays further showed that the nuclear factor- κ B (NF- κ B) pathway was evidently activated in Cyr61-expressing AGS cells. Function-neutralizing antibody to α v β 3 but not α v β 5 effectively suppressed Cyr61-mediated NF- κ B activation, COX-2 gene expression, and cell invasiveness.

Conclusions: Cyr61 may contribute to the malignant progression of gastric cancer by promoting tumor cell motility/invasion through up-regulation of the functional COX-2 via an integrin α v β 3/NF- κ B-dependent pathway.

Gastric cancer is one of the most frequent and lethal malignancies worldwide. In the United States, gastric cancer is the 13th most common cause of cancer mortality, with an estimated 12,100 deaths in 2003 (1). Of malignant gastric tumors, 95% are adenocarcinoma and the remaining includes lymphoma, stromal tumors, and other rare tumors (2). The 5-year survival rate for patients with gastric adenocarcinoma is only about 20%. Death from gastric cancer is mainly due to recurrent disease, and the most common form is loco-regional

recurrence (3). The prolonged use of aspirin and other nonsteroidal anti-inflammatory drugs is associated with reduced incidence of gastrointestinal cancers (4, 5). Cyclooxygenase (COX) is the most well known molecular target of nonsteroidal anti-inflammatory drugs. Elevated COX-2 expression, an induced form of COX, in gastric adenocarcinoma is frequently observed (6, 7). Several clinical studies show that overexpression of COX-2 protein is associated significantly with depth of invasion (8, 9), lymph node metastasis, advanced stage (8, 10, 11), and tumor angiogenesis (12, 13).

Cysteine-rich 61 (Cyr61) is the first cloned member of the CCN family (14), which comprises Cyr61/CCN1, connective tissue growth factor (CTGF/CCN2), nephroblastoma overexpressed (Nov/CCN3), Wisp-1/elm1 (CCN4), Wisp-2/rCop1 (CCN5), and Wisp-3 (CCN6). Most members of the CCN family share a uniform modular structure and exhibit diverse cellular functions such as regulation of cell division, chemotaxis, apoptosis, adhesion, motility, and ion transport (15–18). Cyr61 has been reported to mediate cell adhesion, stimulate chemotaxis, augment growth factor-induced DNA synthesis, foster cell survival, and enhance angiogenesis (14, 19, 20). Elevated Cyr61 expression is associated with advanced breast adenocarcinoma pathogenesis, pancreatic cancer, and gliomas (21–25). Interestingly, down-regulated Cyr61 expression is also noted in prostate cancer, uterine leiomyoma, rhabdomyosarcoma, and non-small cell lung carcinoma (26–29). The

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contrasting expression of Cyr61 in different types of cancer suggests that Cyr61 may exert a sophisticated function depending on cellular context.

In this study, we found that Cyr61 was highly expressed in more advanced gastric adenocarcinoma specimens and over-expressed Cyr61 in human gastric cancer cell lines significantly increased their invasion abilities. We also present evidence that COX-2 plays a crucial role in Cyr61-promoted gastric cancer invasion and motility. The mechanistic study further showed that the integrin $\alpha\beta3$ /nuclear factor- κ B (NF- κ B) signaling cascade is essential for Cyr61-mediated COX-2 up-regulation and subsequent cell motility and invasion.

Materials and Methods

Human samples and clinicopathologic analysis. In this retrospective analysis, the study population consisted of patients with gastric adenocarcinoma who underwent surgery from 1996 to 1998. None of the patients had received preoperative or adjuvant chemotherapy. Additional inclusion criteria were radical gastrectomy with a minimum of D1 lymphadenectomy, availability of paraffin-embedded specimens of the primary tumor, and complete follow-up information. The follow-up program included interim history, physical examination, hematologic studies, carcinoembryonic antigen levels, and diagnostic imaging (chest X-ray and abdominal sonography) every 3 months in the first year and every 6 months in the second through fifth years. Patients underwent upper endoscopy 6 months after surgery and every 12 months thereafter. Abdominal and pelvic computed tomography was done for any evidence of relapse. The recurrence of gastric carcinoma had to be proven by cytologic biopsy or surgery. The 1997 revision of American Joint Committee on Cancer manual was used for the classification of each case. The study was done in a blind fashion so that patient outcome was unknown to the pathologist examining the immunohistochemical stains.

Cell culture. Human gastric carcinoma cells (AGS, N87, TSGH, and SNU-16) were obtained from the American Type Culture Collection (Manassas, VA and Rockville, MD). All gastric carcinoma cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mmol/L l-glutamine, 100 μ g/mL streptomycin, and 100 units/mL penicillin (all from Life Technologies, Rockville, MD and Manassas, VA). Cell cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. Adherent cells were detached from the culture dishes with trypsin/EDTA (Sigma, Deisenhofen, Germany).

Antibodies and reagents. Anti- $\alpha\beta3$ integrin functional blocking antibodies (LM609) and anti- $\alpha\beta5$ integrin functional blocking antibodies (B1F6) were obtained from Chemicon International, Inc. (Temecula, CA). Anti-Cyr61, anti-COX-2, anti- α -tubulin, anti-glyceraldehyde-3-phosphate dehydrogenase, anti-p65, and anti-p50 polyclonal antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Prostaglandin E₂ (PGE₂), COX inhibitors, celecoxib (NS398), and valeryl salicylate were all obtained from Cayman Chemical (Ann Arbor, MI). 3-4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (Sigma), specific RGD-blocking peptides (Gly-Arg-Gly-Asp-Ser, GRGDS), and RGD-control peptides (Ser-Asp-Gly-Arg-Gly, SDGRG) were all obtained from Cayman Chemical.

Boyden chamber assay. Invasion assays were done using modified Boyden chambers with filter inserts for 24-well dishes containing 8- μ m pores (Nucleopore Corp., Pleasanton, CA). Matrigel (40 μ g, Collaborative Biomedical, Becton Dickinson Labware, San Jose, CA)-coated filters were used for invasion assays. Cells (2.5×10^4) were plated into 100 μ L of complete RPMI in the upper chamber, and the lower chamber was filled with 1 mL of RPMI. After 48 hours in culture, cells were fixed in methanol for 15 minutes and stained with 0.05% crystal violet in PBS for 15 minutes. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed in PBS. Cells on the underside of the filters were viewed and counted under a

microscope (type 090-135.001, Leica Microsystems, Wetzlar, Germany). Each clone was plated in triplicate in each experiment and each experiment was repeated at least thrice.

Western blot analysis. Proteins in the total cell lysate (75 μ g) or after trichloroacetic acid precipitation of conditioned medium were collected after 48 hours, once they became confluent, and were separated by SDS-PAGE in 10% gels and electrotransferred to polyvinylidene difluoride membrane (Immobilon-P membranes; Millipore, Bedford, MA). After the blot was blocked in a solution of 5% skim milk, 0.1% Tween 20, and PBS, the membrane-bound proteins were probed with primary antibodies against COX-2, Cyr61, glyceraldehyde-3-phosphate dehydrogenase, or α -tubulin (Santa Cruz Biotechnology). The membrane was washed and then incubated with horseradish peroxidase-conjugated secondary antibodies for 60 minutes. Antibody-bound protein bands were detected with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and photographed with Kodak X-Omat Blue autoradiography film (Perkin-Elmer Life Sciences, Boston, MA).

Transient and stable transfection. The expression vector Cyr61 was constructed by placing the human Cyr61 cDNA into the pcDNA3.1 eukaryotic expression vector containing the *neomycin* gene. The dominant-negative 32/36A mutated form of I κ B (DN-I κ B), decoy NF- κ B, 1.6-kb wild-type COX-2 promoter (pR-COX-2-Luc) and its mutated NF- κ B site (*dNF- κ B mut*, *pNF- κ Bmut*, and *dpNF- κ Bmut*) of COX-2 promoter-luciferase constructs were gifts from Dr. Shuang-En Chuang. Expression plasmid DNA (Cyr61, DN-I κ B, Decoy NF- κ B, pR-COX-2-Luc, *dNF- κ B mut*, *pNF- κ Bmut*, and *dpNF- κ Bmut*) or pcDNA3) and the transfection reagent TransFast (Promega, Madison, WI) were mixed and the transfection protocol was done according to the manufacturer's instructions (Promega). The transfection efficiency varied between 40% and 60%. After 48 hours of transfection, stable-transfected cells were trypsinized and replated in RPMI 1640 with 10% FCS and 0.8 mg/mL gentamicin (G418; Life Technologies, Carlsbad, CA). G418-resistant clones were selected and expanded.

Cell proliferation assay. The growth rates of the AGS/Cyr61 single clones were determined by using 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide as a substrate. The 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide assay is based on the activity of mitochondrial dehydrogenases, which reduce the water-soluble tetrazolium salt to a purple insoluble formazan product. The amount of 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide formazan product was analyzed spectrophotometrically at a wavelength of 570 nm. Each individual experiment was repeated thrice.

RNA isolation and reverse transcriptase-PCR. The method had been described previously (21). Briefly, total RNA was isolated from cells in a final reaction volume of 20 μ L containing 5 μ g of total RNA in Moloney murine leukemia virus reverse transcriptase buffer (Promega). The reaction mixture was incubated at 37°C for 2 hours and was terminated by heating at 70°C for 10 minutes. One microliter of the reaction mixture was then amplified by PCR with the following pairs of primers: Cyr61 primers, 5'-CGAGGTGGAGTTGACGAGAAAC-3' (forward) and 5'-AGGACTGGATCATCATGACGTTCT-3' (reverse) to produce a 550-bp fragment of the *Cyr61* gene; COX-2 primers, 5'-TTCAAATGAGATTGTGGGAAAT-3' (forward) and 5'-AGATCATCTCTGCCCTGAGTATCTT-3' (reverse) to produce a 305-bp fragment of the *COX-2* gene; and β -actin primers, 5'-GATGATGATATCGCCGCGCT-3' (forward) and 5'-TGGGT-CATCTTCTCGCGGTT-3' (reverse) to produce a 320-bp fragment of the β -actin gene, which was used as the internal control. PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis.

Prostaglandin E₂ assay. The PGE₂ level was assessed in the conditioned medium of Cyr61-expressed clones, using a commercially available ELISA kit (Cayman Chemical). The conditioned medium of Cyr61-expressed clones was applied to 96-well plates precoated with goat anti-mouse IgG and incubated with PGE₂ monoclonal antibody for 18 hours. After incubation, plates were rinsed five times with washing buffer and developed using Ellman's reagent. The PGE₂

concentration was determined spectrophotometrically and calculated by plotting (percentage of sample or standard bound/maximum bound) the protein standard versus PGE₂ concentration in pg/mL.

Cell fractionation. Cells in ice-cold PBS, recovered by centrifugation at $500 \times g$ for 3 minutes and resuspended in 200 μ L of CER I buffer (NE-PER Nuclear and Cytoplasmic Extraction Reagents, Pierce, Rockford, IL) and then vigorously vortexed for 15 seconds. After incubation

on ice for 10 minutes, CER II buffer was added to the tube. The tube was then vortexed for 5 seconds and the cytoplasmic fraction was pelleted at 4°C by centrifugation for 5 minutes at $16,000 \times g$. The insoluble fraction, which contains nuclei, was resuspended in 100 μ L of ice-cold NER. After vortexing for 15 seconds, the sample was returned to ice. Vortexing for 15 seconds was continued at 10-minute intervals for a total 40 minutes. Finally, the tube was centrifuged at maximum speed

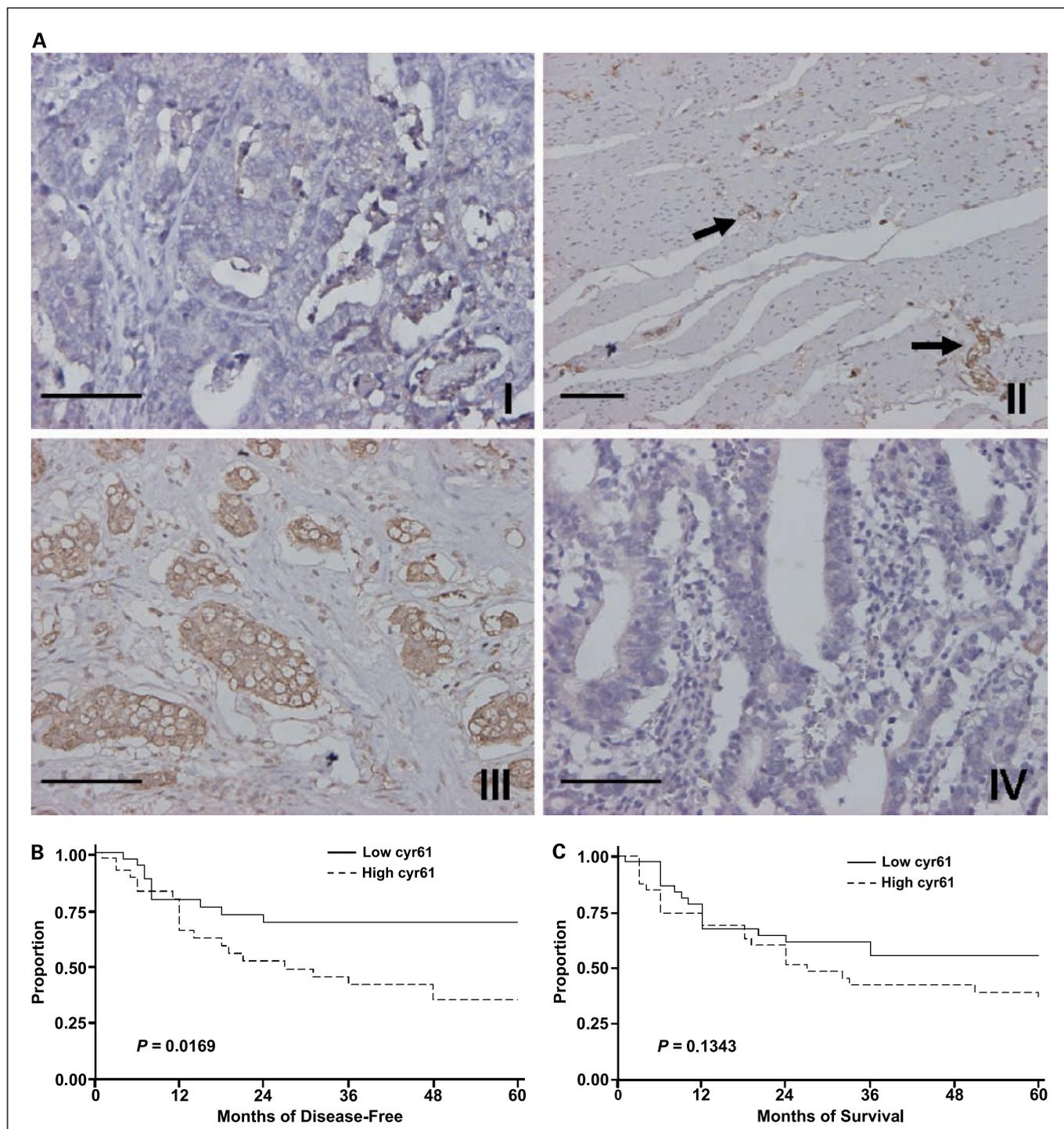


Fig. 1. Representative Cyr61 immunohistochemical staining of human gastric carcinomas. A, I, carcinomatous areas staining negative for IgG control are shown surrounded (magnification, $200\times$). II, moderate Cyr61 expression is found in vascular endothelial cells (black arrow, $100\times$). III, high Cyr61 expression is shown in invasive and advanced gastric tumors ($200\times$). IV, low Cyr61 protein expression is noted in a noninvasive gastric tumor specimen ($200\times$). Bar, 100 μ m. B and C, Kaplan-Meier plots for patients with gastric carcinoma, grouped according to overall survival and recurrence-free survival.

Table 1. Clinical and pathologic characteristics for high and low Cyr61 expression in gastric cancer

Characteristics	Low Cyr61 (n = 40)	High Cyr61 (n = 41)	P
Age, y (mean ± SD), median (range)	62.9 ± 10.8, 63.5 (40-86)	60.5 ± 11.3, 60 (39-86)	0.294
Sex			
Male	28	20	0.071
Female	12	21	
Stage			
I	10	2	0.011*
II	7	10	
III	9	20	
IV	14	9	
Lymph node			
Negative	16	4	0.002*
Positive	24	37	
Lymph node stage			
0	16	3	0.004*
1	11	16	
2	8	17	
3	5	5	
Tumor status			
T1	3	0	0.074
T2	12	15	
T3	14	21	
T4	11	5	
Tumor site			
Distal	16	23	0.184
Proximal	24	18	
Histologic type [†]			
Intestinal	27	11	<0.001*
Diffuse	13	30	

*Statistical significance ($P < 0.05$).
†Histologic classification by Lauren's system.

for 10 minutes and the nuclear fraction was immediately transferred to a prechilled tube and stored.

Immunofluorescence staining. Cells grown on degreased glass coverslips to 80% confluence in culture medium were fixed in methanol/acetic acid (3:1, v/v) for 15 minutes at 4°C and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes. These cells were then rinsed and blocked for 1 hour in 5% fetal bovine serum at room temperature. The cells were then incubated with anti-p65 monoclonal antibody

(Santa Cruz Biotechnology) and diluted 1:100 in PBS at 4°C overnight. After washing in PBS, the cells were incubated with a secondary FITC-conjugated antibody (1:200, Sigma) for 1 hour at room temperature. After washing, the coverslips were inverted onto glass slides using Mowiol (Sigma) as a mounting medium. The slides were observed using fluorescence microscopy.

Electrophoretic mobility shift assay. Nuclear extracts were prepared using a nonionic detergent method as described previously. In brief, nuclear extracts were prepared from AGS/Cyr61 clones in extraction buffer [10 mmol/L KCl, 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl₂, and 0.5 mmol/L DTT] plus protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride). After centrifugation at 14,000 rpm in a microcentrifuge for 1 minute, the cytosol proteins were removed, and the nuclei were placed into extraction buffer [420 mmol/L NaCl, 20 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, 25% glycerol, 0.5 mmol/L DTT, and 0.5 mmol/L phenylmethylsulfonyl fluoride]. After centrifugation at 14,000 rpm for 5 minutes, the supernatant fraction was harvested as the nuclear protein extract and stored at -70°C. The electrophoretic mobility shift assay for NF-κB DNA binding in AGS/Cyr61 clones cells was done using the annealed and [³²P]dCTP end-labeled κB consensus probe (5'-AGCTTCAGAGGGGACTTTCCGAGAGG-3'/3'-TCGACCTCTC-GGAAAGTCCCCTCTGA-3') in a 20-μL reaction mixture [containing 10-15 μg of protein of nuclear extract and 2 μg of poly(deoxyinosinic-deoxycytidylic acid) for 20 minutes at room temperature]. In competition experiments, 10- to 100-fold excess of unlabeled oligonucleotide was added to the binding reactions. The reaction products were analyzed by 5% nondenaturing PAGE using 12.5 mmol/L Tris, 12.5 mmol/L boric acid, and 0.25 mmol/L EDTA (pH 8.3), for 4 to 5 hours at 280 to 300 V and 10 to 12 mA. The gels were dried and exposed to Amersham film (Amersham Pharmacia Biotech) at -70°C using an intensifying screen.

Promoter activity and luciferase assay. After various drug treatments, the transfected cells were harvested with passive lysis buffer (Promega) at room temperature for 10 minutes. The lysed cells were centrifuged at 14,500 × g for 30 minutes to remove cell debris, and the supernatant was collected and used in the reporter assay. Luciferase activity was determined by using a Dual-Luciferase Reporter Assay System (Promega) and measured with a luminometer.

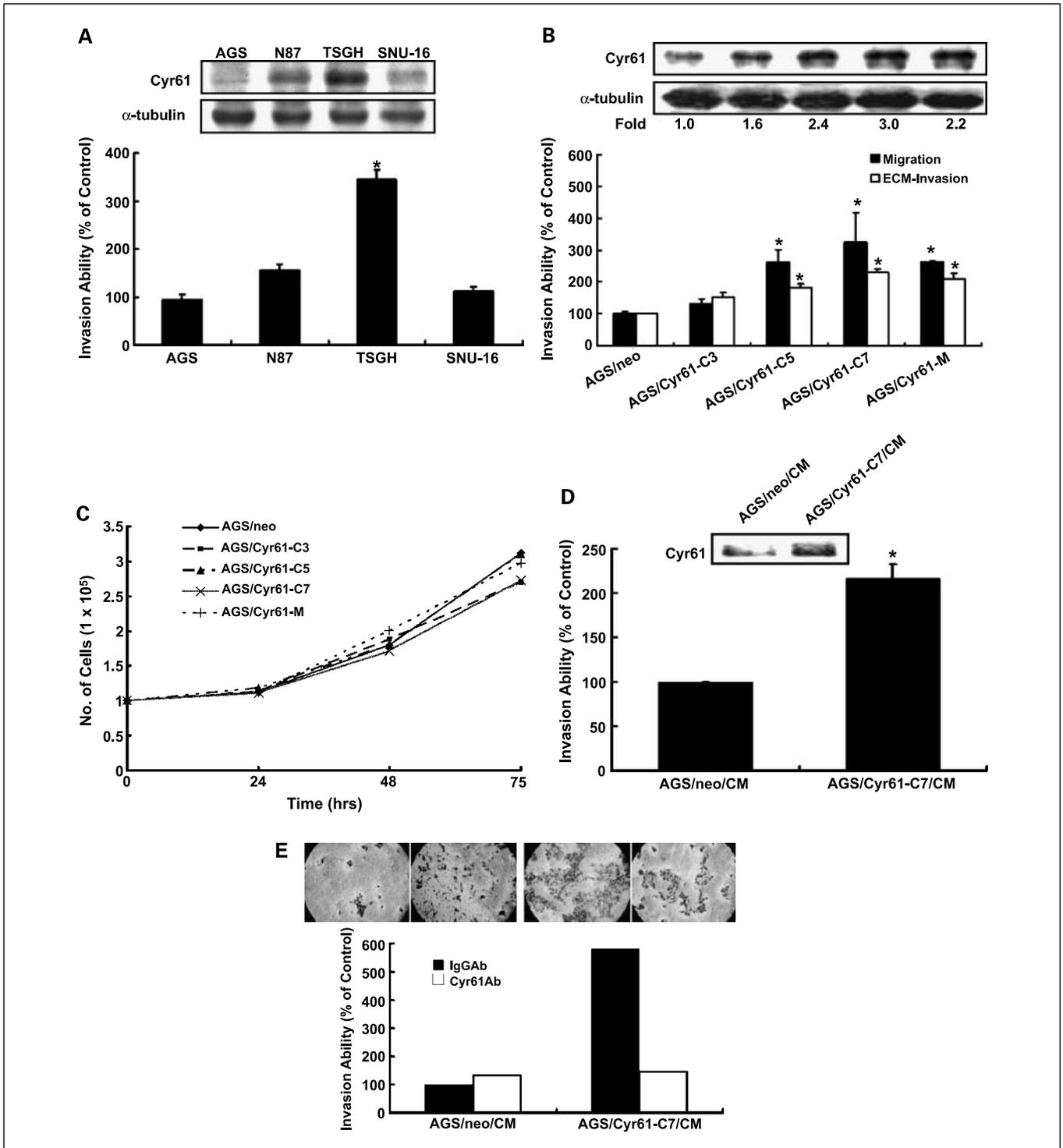
Chromatin immunoprecipitation analysis. Cells were cultured in confluence overnight and added formaldehyde directly to culture medium to cross-link histone proteins to DNA. After centrifugation, cell pellet was lysed in two consecutive lysis buffers: LB1 [50 mmol/L HEPES-KOH (pH 7.5), 140 mmol/L NaCl, 1 mmol/L EDTA, 10% glycerol, 0.5% NP40, 0.25% Triton X-100, protease inhibitor cocktail] and LB2 [10 mmol/L Tris-HCl (pH 8.0), 200 mmol/L NaCl, 1 mmol/L EDTA, 0.5 mmol/L EGTA]. After the second lysis, the pellet was suspended and sonicated. For immunoprecipitation, 1 μg antibodies, prebound protein A, were added to 400 μL of the purified chromatin sample and incubated overnight at 4°C. After wash with TE, the immunocomplexes were recovered by adding elution buffer [1% SDS, 1%, 10 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.0)] for 10 minutes

Fig. 2. Cyr61 expression and *in vitro* invasion ability in human gastric cancer cell lines and Cyr61-overexpressing clones. *A*, top, Western blot analysis of the protein level expressions of Cyr61. Total proteins were extracted and probed with Cyr61-specific polyclonal antibody; 75 μg of total protein was analyzed per sample. β-Actin was used as an internal loading control. *Bottom*, invasion activity of each cell was measured *in vitro* with the Boyden chamber after 48 hours. *Columns*, mean number of cell harvested for three experiments done in triplicate; *bars*, ±SE. *B*, top, representative immunoblot demonstrating levels of Cyr61 protein expression in mock-transfected and Cyr61-transfected AGS clones. To facilitate comparison of the relative Cyr61 protein expression between mock-transfected clone (AGS/neo), mix population of Cyr61-transfected clones (AGS/Cyr61-M) and single clones of Cyr61-transfected clones (AGS/Cyr61-C3, C5, C7), fold values were normalized to neo-control clone values as indicated below the panel. *Bottom*, expression of Cyr61 increased the *in vitro* invasion and migration activities of AGS cells. *In vitro* invasion was measured by determining the percentage of cells that migrated through Matrigel-coated transwell chambers. Cyr61-transfected clones had statistically significantly increased invasive and migration activities ($P < 0.05$) compared with the AGS/neo vector control clone. Each clone was assayed in three experiments carried out in triplicate. *C*, effect of Cyr61 overexpression on the growth properties of AGS cells. Monolayer *in vitro* growth in medium containing 10% FCS of the vector- and Cyr61-transfected cell lines. *Columns*, mean; *bars*, ±SE. All experiments were carried out in triplicate on separate occasions with similar results. *D*, top, Western blot analysis of Cyr61 expression in conditioned medium (CM) of mock-transfected AGS cells (AGS/neo) and AGS/Cyr61-C7 clones. *Bottom*, conditioned medium of AGS/Cyr61-C7 cells increased the invasion activity of AGS cells. To compare the relative invasiveness of AGS cells between treatment of condition medium of AGS/neo and AGS/Cyr61-C7 clones, values were normalized to the relative invasion activity of AGS clone. *Columns*, means of three independent experiments; *bars*, ±SE. *E*, top, crystal violet staining of the invasive cells in Boyden chamber. *Bottom*, pretreatment of Cyr61 blocking antibody for 1 hour inhibited the *in vitro* invasion activity of AGS cell incubated with condition medium of AGS/Cyr61-C7 clone. *Columns*, means.

at 65°C, then centrifugation and recovered by phenol/chloroform extraction and resuspended in TE. PCR primer sets were designed to overlap and span the proximal NF-κB site of the COX-2 promoter, primer set forward 5'-CAAGGCGATCAGTCCAGAAC-3' and reverse 5'-GGTAGGCTTTGCTGTCTGAG-3'; activator protein site, forward 5'-CGCTTGATGATCAGCCGGAA-3' and reverse 5'-TTCCGGCTGACTCATCAAGCG-3'. Conventional PCR was then done Amplification was carried out for 35 cycles (28 cycles for unprecipitated chromatin input lanes) with denaturation at 94°C for 1 minute, annealing at 55°C for

45 seconds, and extension at 72°C for 1 minute. PCR products were run in 2% agarose gel.

Statistical analysis. A comparison of the background data was carried out between the low-Cyr61 and the high-Cyr61 groups. This was done for scale variables (expressed as mean ± SD) by a Mann-Whitney test and for nominal variables by a Fisher's exact test. Analysis of the survival data was by the Kaplan-Meier method. Kaplan-Meier curves were compared by a log-rank test. *P* values were two-sided and the significant level was 0.05.



Results

Overexpression of Cyr61 in human gastric cancer. The study population included 81 patients with gastric adenocarcinoma treated at National Taiwan University Hospital from 1996 to 1998. There were 48 men and 33 women patients. Their average age was 61.7 ± 11.01 years (median, 63; range, 39-86). The median follow-up time was 15 months for recurrence and 24 months for death. Twelve patients had stage I, 17 had stage II, 29 had stage III, and 23 had stage IV disease. The 5-year survival rates were 91.7%, 75.6%, 26.7%, and 10.5% for stages I to IV, respectively.

The expression level of Cyr61 in gastric adenocarcinoma was determined by immunohistochemistry using a Cyr61-specific antibody. The results of immunohistologic staining were classified as intensity level 0 (negative or <5% of tumor cells stained), intensity level 1 (5-50% of tumor cells stained), intensity level 2 (50-80% of tumor cells stained), and intensity level 3 (>80% of tumor cells stained). The high level of immunoreactivity for Cyr61 (intensity level 3) was detected in invasive and advanced gastric tumors (Fig. 1A, III). In contrast, very weak immunoreactivity of Cyr61 was observed in noninvasive gastric tumors (Fig. 1A, IV). The Cyr61 protein seemed predominantly localized in the cytoplasm or the membrane of tumor epithelial cells. Consistent with previous observations (30), Cyr61 was also found to be positively expressed on vascular endothelial cells (Fig. 1A, II) of nontumor tissues. Negative staining for gastric tumors was shown when immunostaining using an IgG control (Fig. 1A, I). Of the 81 gastric adenocarcinoma analyzed, low expression levels (intensity levels 0 and 1) and high expression levels (intensity levels 2 and 3) of Cyr61 in gastric adenocarcinoma specimens were 49% (40 of 81) and 51% (41 of 81), respectively. The incidence of high Cyr61 expression among the four tumor-node-metastasis stages was 17% (2 of 12) in stage I, 59% (10 of 17) in stage II, 69% (20 of 29) in stage III, and 39% (9 of 23) in stage IV disease. The relationships between the high and low levels of Cyr61 expression and the baseline clinicopathologic characteristics are summarized in Table 1. No significant association was found for age, sex, tumor site or tumor status, but significant relationships with Cyr61 expression were found for tumor stage ($P = 0.011$), lymph node status ($P = 0.002$), and histologic type ($P < 0.001$). In patients with high- and low-expression Cyr61, the 5-year recurrence-free survival rate was 68% and 33%, respectively; the 5-year overall survival rate was 40% and 58%, respectively. As shown in Fig. 1B, the difference between the groups for recurrence-free Kaplan-Meier curves was significant ($P = 0.0169$), whereas the overall survival Kaplan-Meier curve between high and low expression of Cyr61 expression was not statistically different (Fig. 1C).

Forced expression of Cyr61 enhanced the invasiveness of gastric cancer cells. Our study indicated a strong association between the level of Cyr61 protein and lymph node metastasis, suggesting that Cyr61 may have a role in the invasiveness of gastric adenocarcinoma. To address this, four monolayer gastric cancer cell lines, including AGS, N87, SNU-16, and TSGH cells, were examined for their basal expression levels of Cyr61 protein and *in vitro* invasion capacity. Figure 2A (top) shows that the level of Cyr61 protein was highly elevated in TSGH cells but was moderately expressed in SNU-16 and N87

cells. AGS cells displayed an extremely low level of Cyr61 protein compared with other gastric cancer cells. In a modified Boyden chamber assay, TSGH cells exhibited the strongest invasive ability among these gastric cancer cell lines. In contrast, AGS cells, which expressed trace amounts of Cyr61, had very weak invasive potency (Fig. 2A, bottom). These results led us to hypothesize that Cyr61 could be directly involved in the malignant progression of human gastric cancer cells.

To address this hypothesis, AGS cells stably expressing Cyr61 were established by transfection with pcDNA-3 comprising human Cyr61 cDNA, pcDNA-3-Cyr61. After G418 selection, we isolated three single clones (AGS/Cyr61-C3, AGS/Cyr61-C5, and AGS/Cyr61-C7), a pool mixture (AGS/Cyr61-M), and vector control clone (AGS/neo), and then we assessed the levels of Cyr61 expression in each. Western blot analysis revealed that these stable single clones and the pooled clones expressed a 1.6- to 3.0-fold increase of Cyr61 protein compared with the vector control cells (Fig. 2B, top). Examination of migratory activity using a Transwell migration assay without a Matrigel coating showed that all Cyr61 transfectants, except Cyr61-C3, showed significantly increased migratory ability compared with vector control cells (Fig. 2B, bottom). These Cyr61 protein expressions in transfected cells paralleled their migratory activity and exhibited more invasive activity compared with vector control cells in the invasion assay using Matrigel-coated filters (Fig. 2B, bottom). However, the proliferation of these Cyr61 transfectants were similar to that of vector transfectants (Fig. 2C), suggesting that the changed invasiveness of Cyr61 transfectants was not attributable to alteration of their growth properties.

Because Cyr61 is a secreted protein, we further questioned whether the invasive activity of parental AGS cells would be affected by the conditioned medium from Cyr61 transfectants (AGS/Cyr61-C7). Western blot analysis showed a substantial level of secreted Cyr61 protein was detected in 48-hour cultured medium of AGS/Cyr61-C7 cells (Fig. 2D, top). AGS cells treated with the conditioned medium from AGS/Cyr61-C7 cells showed significantly increased invasiveness compared with conditioned medium from AGS/neo cells (Fig. 2D, bottom). To exclude the possibility that expression of Cyr61 induces the synthesis of another secreted factor, which in turn mediated invasion, we pretreated the Cyr61 blocking antibody to neutralize the secreted Cyr61 in condition medium for 1 hour. The result showed that treated antibody against Cyr61 could significantly inhibit the increased invasion abilities of the cells (Fig. 2E). These results show that Cyr61 is directly involved in invasion in human gastric cancer cell lines.

Cyclooxygenase-2 acts as a downstream effector of Cyr61. It is well recognized that COX-2 plays a critical role in malignant progression of gastric cancer by increasing metalloproteinase activity as well as enhancing angiogenesis (31, 32). We questioned whether Cyr61 regulated COX-2 gene expression in gastric cancer cells. To address this, we examined the expression level of COX-2 protein and determined its enzymatic activity in the Cyr61-transfected cells using immunoblotting and ELISA, respectively. As shown in Fig. 3A (top), the level of COX-2 protein significantly increased in Cyr61-overexpressing AGS cells compared with the neo control cells. However, the COX-1 protein level was unchanged among these Cyr61-transfectants and neo control cells. As expected, elevated

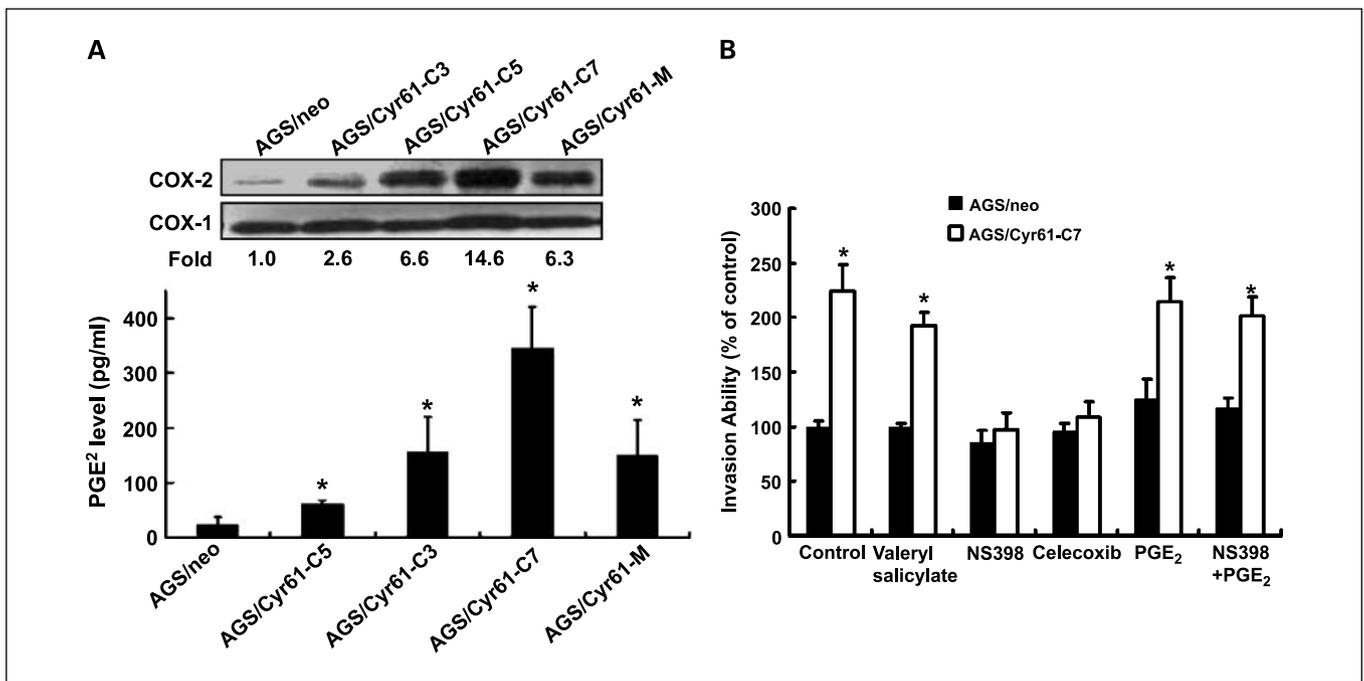


Fig. 3. Cyr61 induced COX-2 up-regulation in human gastric cancer cells. *A*, top, COX-2 protein expression in AGS cells stably transfected with Cyr61-expressing vector and control vector. COX-1 was used as an internal loading control. Fold values were normalized to neo-control clone. Bottom, production of PGE₂ in Cyr61-overexpressed AGS cells. The post-cultured medium was collected and assayed for PGE₂ by enzyme immunoassay. PGE₂ production levels were from three independent experiments. Columns, means; bars, \pm SD. *, $P < 0.05$ versus AGS/neo clone. *B*, numbers of invaded cells of AGS/Cyr61-C7 was strongly decreased by treatment with COX-2 inhibitors, NS398 and celecoxib, but not with valeryl salicylate. *, $P < 0.05$ versus AGS/neo control group. PGE₂ (2 μ g/mL) reversed the COX-2 inhibitors-provoked invasion inhibition in AGS/Cyr61-C7 cells. The independent experiments were done at least four times.

levels of PGE₂, assessed by ELISA (Fig. 3A, bottom), paralleled the increased expression of COX-2 protein in these Cyr61-transfected cells. This implies that the COX-2, induced by Cyr61, displayed authentic enzymatic activity in AGS cells.

To further exploit the role of COX-2 in Cyr61-mediated gastric cancer cell invasiveness, we treated Cyr61 transfectants (AGS/Cyr61-C7) with inhibitors that specifically targeted COX-1 or COX-2 and subsequently examined their change in invasive ability. Interestingly, the invasive capacity of AGS/Cyr61-C7 cells was strongly attenuated by treatment with COX-2 inhibitors, NS398 (20 μ mol/L) and celecoxib (5 μ mol/L) but not with COX-1 inhibitor, valeryl salicylate (25 μ mol/L; Fig. 3B). Addition of 2 μ g/mL of PGE₂ significantly reversed the COX-2 inhibitor-provoked invasion inhibition in AGS/Cyr61-C7 cells (Fig. 3B). Taken together, these results suggest that Cyr61 stimulates the invasiveness of gastric cancer cells by up-regulating functionally active COX-2.

Nuclear factor- κ B activation is important for transcriptional up-regulation of cyclooxygenase-2. Previous studies have shown that activation of transcriptional factor NF- κ B is critical for COX-2 gene up-regulation (33–38). In an earlier study, we also showed that overexpression of Cyr61 in breast cancer MCF-7 cells activated the NF- κ B signaling pathway (21). Accordingly, we examined whether Cyr61 overexpression would activate the NF- κ B pathway and, in turn, up-regulate downstream COX-2 in AGS cells. As shown in Fig. 4A (left) Western blotting revealed significant levels of NF- κ B p65 subunits detected in nuclear fractions of AGS/Cyr61-C7 cells but not in neo control cells. However, only a slight increase in nuclear p50 subunits in AGS/Cyr61-C7 cells occurred. The level of constitutive proliferating

cell nuclear antigen, as an internal control, remained the same in both cell lines. Consistently, immunofluorescent analysis showed an evident nuclear staining for p65 (Fig. 4A, right) and p50 NF- κ B (data not shown) in Cyr61-overexpressing cells but not in neo control cells.

The electrophoretic mobility shift assay was done to examine the DNA-binding activity of NF- κ B in Cyr61-overexpressing AGS cells. Figure 4B shows that DNA binding activity of NF- κ B increased notably in nuclear extracts of Cyr61-expressing cells (lanes 1–4), and the increment in NF- κ B DNA binding activity in AGS/Cyr61-C7 cells was strongly abrogated when it was specifically competed against by a nonradiolabeled probe.

In addition, the DNA binding activity of NF- κ B in Cyr61-expressing cells was also reduced after transfection with DN- κ B, which effectively inhibits NF- κ B activity, or treatment with NF- κ B decoy oligonucleotide, which acts as a transcription factor decoy and inhibits NF- κ B binding to any native DNA sites (Fig. 4B, lanes 5–9). To quantify the NF- κ B transcriptional activity, we transiently transfected an NF- κ B luciferase reporter into Cyr61-overexpressed and neo control cells. The data for the luciferase activity (Fig. 4C) were correlated with the DNA binding activity of NF- κ B in a gel shift assay and showed that Cyr61 strongly activated NF- κ B transactivation activity in AGS cells and that the Cyr61-enhanced NF- κ B reporter activity was completely blocked by DN- κ B or NF- κ B decoy. The above data suggest that overexpression of Cyr61 activated the NF- κ B signaling pathway constitutively in AGS cells.

The exact role of NF- κ B in Cyr61-mediated COX-2 up-regulation was further examined. As shown in Fig. 5A, the level of COX-2 mRNA in AGS/Cyr61-C7 cells was strongly reduced

when treated with decoy NF- κ B or transfected with DN-I κ B, suggesting that NF- κ B activation is important for COX-2 gene up-regulation by Cyr61. To identify whether the NF- κ B site is actually involved in transcriptional regulation of COX-2 by Cyr61, the 5'-flanking COX-2 promoter region between -1647 to +6 was cloned into the pGL3-basic luciferase vector. Figure 5B shows that Cyr61 expression vector activated 3.5-fold induction of COX-2 promoter activity, which could be abolished by cotransfection with DN-I κ B or decoy NF- κ B. Two putative NF- κ B sites, one proximal and one distal, were located at -223/-214 and -422/-412 on the COX-2 promoter region, respectively, and were further mutated separately or simultaneously by site-directed mutagenesis. As shown in Fig. 5C, when the proximal or distal NF- κ B sites mutated COX-2 promoters (e.g., *dNF- κ B mut*, *pNF- κ Bmut*, and *dpNF- κ Bmut*) were transfected into AGS cells, their luciferase activities were decreased by about 60%, 50%, and 95% induced by Cyr61 when compared with the pGL3-1.6-kb wild-type COX-2 promoter. These experiments show that both NF- κ B sites are equally important for Cyr61-mediated COX-2 gene up-regulation. To further confirm the data, we examined whether NF- κ B p65 subunit would directly bind to the NF- κ B site within the COX-2 gene promoter by chromatin immuno-

precipitation assay (Fig. 5D). Overexpression of Cyr61, followed by chromatin immunoprecipitation assay, showed that p65 of NF- κ B were constitutively bound to this region of the COX-2 promoter.

Integrin/nuclear factor- κ B signaling is involved in Cyr61-induced cell invasion. Previous studies showed that Cyr61 exhibited, in part, its biological functions through binding to cell surface integrin receptors (20, 39-43). Thus, we tested whether integrin was involved in Cyr61-mediated NF- κ B activation by using a luciferase reporter assay. We first transiently transfected AGS/Cyr61-C7 and neo control cells with the NF- κ B reporter and then treated these cells with RGD peptides or function-blocking monoclonal antibodies to the $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins. RGD peptide treatment but not control DGR peptide significantly inhibited NF- κ B reporter activity in Cyr61-expressed cells (Fig. 6A). Function-blocking monoclonal antibodies to integrin $\alpha_v\beta_3$ (LM609) but not $\alpha_v\beta_5$ (PIF6) reduced greatly NF- κ B activity (Fig. 6A). Consistently, the increased level of COX-2 mRNA in Cyr61-overexpressing cells was also significantly attenuated by treatment with RGD peptide or anti- $\alpha_v\beta_3$ integrin antibody (Fig. 6B). To further link the effect of integrin and NF- κ B to Cyr61-mediated cell invasiveness, we examined whether the inhibition of $\alpha_v\beta_3$

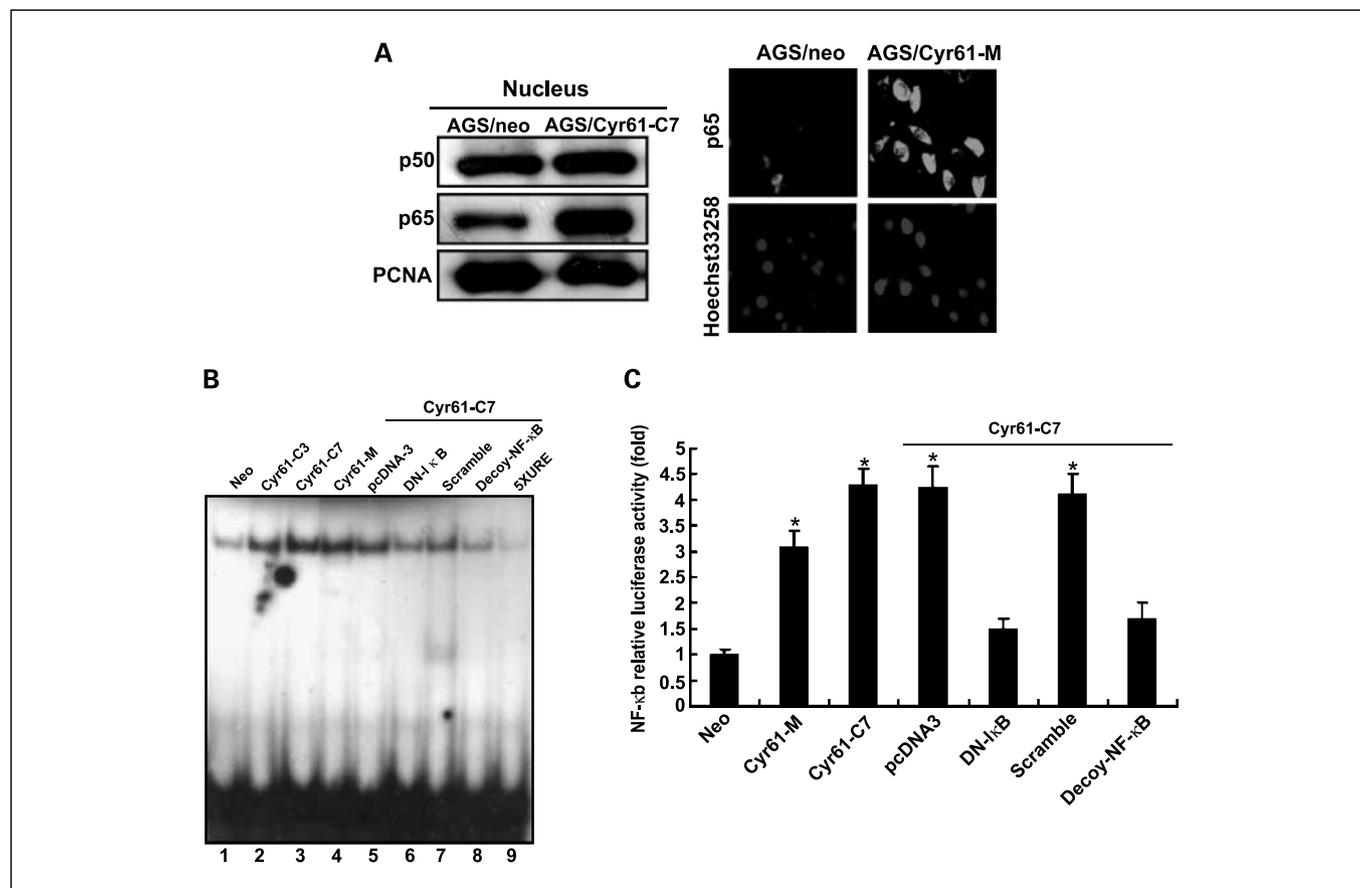


Fig. 4. Cyr61 overexpression activated NF- κ B pathway. **A**, left, Western blots show the nuclear protein expression of p50, p65, and proliferating cell nuclear antigen (*PCNA*) in stable-transfected-Cyr61 AGS cells. Proliferating cell nuclear antigen acts as a nuclear protein positive control. Right, immunofluorescence staining of NF- κ B p65 subunit in AGS/neo and AGS/Cyr61-M clones. The cell nuclei were visualized by Hoechst 33258 staining. **B**, Cyr61-overexpressing (Cyr61-M and Cyr61-C3, Cyr61-C7) and neo cells were transiently transfected with 1 μ g of pcDNA3, DN-I κ B, 1 μ mol/L scramble, or Decoy-NF- κ B, and then nuclear extracts were isolated and subjected to electrophoretic mobility shift assay analysis. To test for specificity of binding, samples were incubated with 5 \times excess unlabeled wild-type (*URE*). **C**, NF- κ B transcriptional activity was measured by a luciferase assay using an optimal NF- κ B-binding site in tandem following by luciferase. The luciferase activity value was normalized with internal control (transfection with β -galactosidase). Average of three independent experiments.

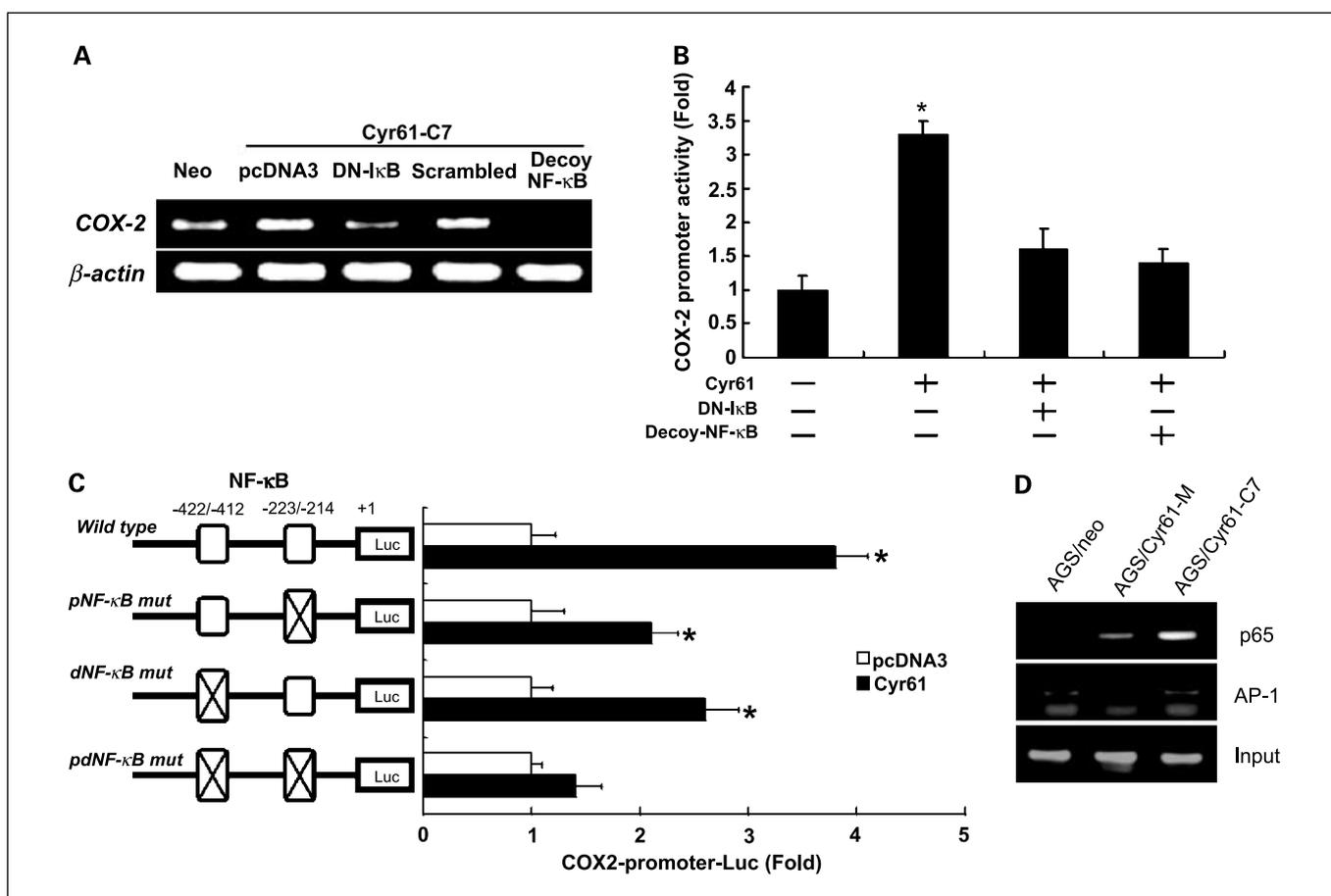


Fig. 5. NF- κ B played a role in Cyr61-induced COX-2 gene up-regulation. *A*, AGS/Cyr61-C7 cells were transiently transfected with 1 μ g of pcDNA3, DN-I κ B, 1 μ mol/L scramble, or decoy-NF- κ B expressing vector. After 24 hours, cells were harvested and processed to detect the mRNA level of COX-2. β -Actin was used as internal control. *B*, AGS cells were transiently cotransfected with the Cyr61 vector and COX-2 promoter luciferase reporter plasmids. After 24 hours, cells were harvested and processed to determine the luciferase gene-reporter activity. *C*, proximal or distal NF- κ B sites mutated COX-2 promoters and Cyr61 or pcDNA3 were transiently cotransfected into AGS cells. After 24 hours, cells were harvested to verify the luciferase gene-reporter activity. The luciferase activity value was normalized with internal control (transfection with β -galactosidase). *Points*, means of triplicate experiments. *D*, chromatin immunoprecipitation analysis was done in Cyr61-stable-transfected-AGS cells using specific for the indicated protein. Following immunoprecipitation (*IP*) of cross-linked lysates, conventional PCR analysis of eluted DNA was done. All PCR were normalized to the input control. Data were shown as binding relative to wild-type COX-2 for each individual antibody used.

integrin or NF- κ B would impair the invasive activity of Cyr61-overexpressing cells. As expected, treatment of AGS/Cyr61-C7 cells with decoy NF- κ B or anti- $\alpha_v\beta_3$ antibody significantly reduced the invasive ability of the cells (Fig. 6C). Taken together, the above data show that the signaling pathway integrin $\alpha_v\beta_3$ /NF- κ B is critical for Cyr61-mediated COX-2 up-regulation and invasiveness.

Discussion

Members of the CCN family are multifunctional growth factors, and the nature of their effects seemingly depends on the cellular context. Recent clinical evidence shows that Cyr61, one of CCN family members, is substantially involved in the development and progression of human malignancies such as, breast, lung, central nervous system, and prostate (20, 21, 23–25, 29, 45–48). The significant importance of Cyr61 in human malignant alterations can, at least in part, be explained by its active regulation of multifaceted biological activities including cell survival, cell motility, and cell proliferation (20, 23, 29, 46, 48), although the opposing function of Cyr61 has been reported in different types of cancer.

In the present study, we showed for the first time, that high expression levels of Cyr61 are frequently observed in invasive gastric adenocarcinoma but are rarely detected in normal gastric mucosa or noninvasive gastric adenocarcinoma. In addition, the level of Cyr61 positively correlates with the status of lymph node metastasis of gastric cancer patients and its level also predicts disease recurrence well. The *in vitro* experiments supported our clinical data revealing that more invasive gastric cancer cell lines exhibited higher levels of Cyr61. Enforced expression of Cyr61 in AGS cells, which expressed trace level of Cyr61, directly promoted cell motility and invasiveness. Collectively, our data provide evidence that Cyr61 plays a novel role in regulating gastric cancer cell motility/invasiveness and that its expression level also seems associated with recurrence of the disease.

How does Cyr61 affect gastric cancer cell invasiveness? Under examination of possible downstream genes induced by Cyr61, we found that the COX-2 gene was functionally linked to Cyr61. This connection was evidenced by either stable or transient transfection of gastric cancer cells with the Cyr61 expression vector, which substantially increased COX-2 mRNA and protein levels. Furthermore, inhibition of COX-2 activity

by COX-2 inhibitors strongly reduced the Cyr61-mediated invasive ability in gastric cancer cells. This experiment was the first to show that functional COX-2 can be up-regulated by Cyr61 overexpression in gastric cancer cells. The importance of COX-2 in gastric cancer is well established. For example, elevated COX-2 expression is correlated with depth of invasion (38, 49, 50), lymph node metastasis (51, 52), and advanced

stage (51, 52) of gastric adenocarcinoma, although it correlated only weakly with prognosis among gastric cancer patients (53). Inhibition of COX-2 by nonsteroidal anti-inflammatory drugs or its specific inhibitors suppressed proliferation and migration in gastric cancer cells (54, 55). COX-2-specific inhibitors also effectively abolished gastric carcinogenesis in the rat model (56) and reduced tumor growth in immunodeficient mice (57). Many of COX-2 effects are mediated by its product, PGE₂, including inhibition of apoptosis, promotion of invasion and metastasis, stimulation of angiogenesis, and induction of immunosuppression (34, 43, 58). Indeed, our current data showed that Cyr61-expressing gastric cancer cells significantly elevated the amount of PGE₂, which was greatly reduced by treatment with COX-2 inhibitors. Of interest, the COX-2 inhibitor-mediated invasion inhibition of Cyr61-expressing cells was reversed by addition of PGE₂. Thus, we suggest that the invasiveness of Cyr61-expressing cells is partially attributable to the function of PGE₂. In dissecting the role of Cyr61 in human malignancies, only a limited number of downstream genes have been identified to date (21, 23, 36, 38, 52, 55). Our present findings on COX-2 provide further insights into the action mechanism of Cyr61 in the human malignant process.

Activation of transcriptional factor NF- κ B is significantly involved in many pathologic processes including drug resistance (59, 60), inflammation (61, 62), angiogenesis (63, 64), and tumor invasion (65). Supportively, constitutive activation of NF- κ B was observed in various types of cancer (60, 63, 64, 66); however, little information is available concerning NF- κ B activation in gastric cancer. Until recently, a study by Sasaki et al. showing that NF- κ B is constitutively activated in human gastric cancer and this activation is also correlated with the tumor aggression and patients' survival (67). Of interest, our current data strengthen the previous study showing that Cyr61-overexpressing gastric cancer cells constitutively activated NF- κ B as showed by enhancing p65 subunit nuclear translocation, increasing DNA-binding activity and inducing NF- κ B reporter activity. Blockage of NF- κ B activity by transfection with DN-I κ B vector or NF- κ B decoy significantly reduced Cyr61-mediated increase in COX-2 mRNA. The human COX-2 promoter has two NF- κ B consensus sites, one located within -422 to -412 bases, and the other located within -223 to -214 bases from the transcriptional start site (68). Using the site-directed mutagenesis assay, we determined that both sites are equally required for Cyr61-induced COX-2 transcription. In addition, our results showed that overexpression of DN-I κ B or transfection with NF- κ B decoy substantially inhibited the COX-2 promoter activity in response to Cyr61. Taken together, our results suggest that the NF- κ B pathway is indispensable for the Cyr61-mediated transcriptional activation of COX-2 in gastric cancer cells. Our earlier study (21) showed that Cyr61 induced antiapoptotic protein XIAP and chemoresistance in breast cancer cells was mediated through activation of NF- κ B. However, we did not detect the induction of XIAP in Cyr61-expressing gastric cancer cells. This implies that Cyr61-induced NF- κ B activation in different types of cell may lead to activating distinct downstream genes.

Integrins are recognized as innate receptors for Cyr61 protein, and integrin activation may engage a variety of cellular effects. Cyr61 binds to integrins α v β 3, α v β 5, α 6 β 2, and α 6 β 1 (39–42). Interaction of Cyr61 with integrins triggers cell adhesion, migration, and survival (21, 23, 43). In the present

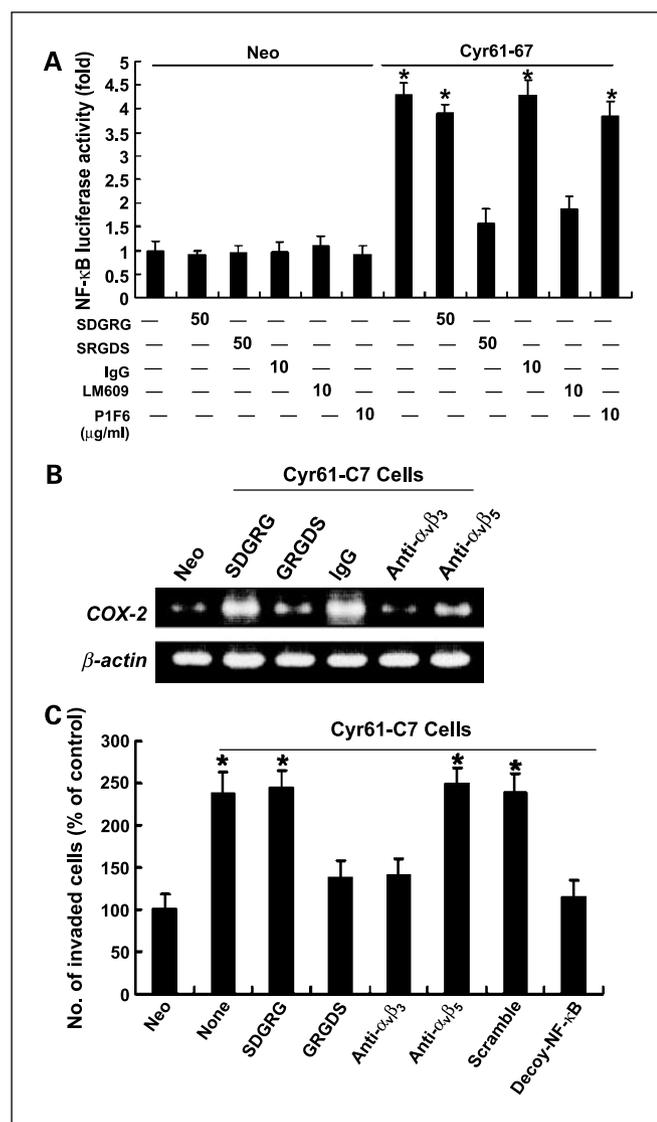


Fig. 6. Integrin is involved in Cyr61-mediated NF- κ B activation, COX-2 up-regulation, and cell invasion. *A*, effects of RGD-functional blocking peptide (SRGDS) and anti-integrin antibodies (LM609 and P1F6) on the NF- κ B transcriptional activity in Cyr61-overexpressing cells. Cyr61-C7 and Neo cells were transfected with NF- κ B-Luc reporter (1 μ g), and 24 hours after transfection, cells were treated with 50 μ g/mL SRGDS (RGD functional blocking peptide) or SDGRG (control peptide) or treated with 10 μ g/mL integrin functional blocking antibody (LM609, anti- α v β 3; P1F6, anti- α v β 5) or IgG for 12 hours, and then the luciferase activities of transfectants were measured. Average of three independent experiments. *Bar*, SE. *B*, Cyr61-C7 cells were treated with 50 μ g/mL SRGDS, SDGRG, 10 μ g/mL IgG, LM609, or P1F6. After 24 hours, the transfected cells were harvested and subjected to reverse transcription-PCR analysis. *C*, decreased invasion abilities of decoy NF- κ B or anti- α v β 3 antibody treatment in AGS/Cyr61-C7 cells. Cyr61-C7 cells were treated with SDGRG, GRGDS, anti- α v β 3, α v β 5 blocking antibodies, decoy-NF- κ B, and scramble. After indicated time, the invaded cells were fixed and counted. Each experiment was done in triplicate. *Columns*, mean of three experiments; *bars*, \pm SD. Statistical significance was determined with Student's *t* test ($P < 0.05$).

study, we showed that Cyr61-mediated NF- κ B activation and COX-2 up-regulation are effectively abolished by anti-integrin α v β 3 but not by anti-integrin α v β 5 antibody. Subsequent experiment further showed the invasiveness of Cyr61-over-expressing cells was inhibited by anti-integrin α v β 3 antibody. These findings provide a novel mechanism by which Cyr61-induced NF- κ B activation, COX-2 up-regulation, and the subsequent cell invasion seem dependent on the integrin α v β 3. Consistent with this finding, our previous study also showed that integrin α v β 3 is required for Cyr61-induced NF- κ B activation and *XIAP* gene expression in breast cancer MCF-7 cells (21). A similar finding made by Xie et al., revealing that over-expression of Cyr61 in glioma cells increased expression of integrin α v β 3, which is also requisite for activation of integrin-linked kinase and subsequent β -catenin/TCF signaling induced by Cyr61 (23). The detailed signaling cascade from integrin α v β 3 to NF- κ B in gastric cancer AGS cells is currently under investigation.

In conclusion, the expression and function of Cyr61 in gastric cancer was analyzed at both the cellular and pathologic levels. The results suggest that Cyr61 is highly expressed in a majority of advanced stage and more invasive gastric adenocarcinomas (lymph node metastasis) and its levels also correlated with the recurrence of the disease. In the mechanistic study, Cyr61 promoted gastric cancer cell migration and invasion through elevating functional COX-2 by an integrin α v β 3/NF- κ B-dependent pathway. Pharmacologic or genetic inhibition of the signaling pathway impairs Cyr61-induced invasiveness in gastric cancer cells. These findings significantly advance our understanding not only of Cyr61 but also on the progression of gastric cancer.

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