Original Article
Cyr61 overexpression induced by interleukin 8 via NF-κB signaling pathway and its role in tumorigenesis of gastric carcinoma in vitro

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Abstract: Cyr61 (CCN1) is a multifunctional matricellular protein in bridging inflammation and cancer, involved in many biological functions such as tumorigenesis and carcinogenesis. The role of Cyr61 in gastric cancer (GC) has not been fully understood and needs to be investigated and clarified. We examined Cyr61 expression in 6 GC cell lines and stable transfection of recombinants in to BGC823 specifically down regulated the Cyr61 mRNA and protein expression shown by the analysis with western blot, RT-PCR, western blot and immunofluorescence assay. The cells treated with siRNA shown markedly reduced activity in growth, migration and invasion compared with parental BGC823 cells as well as mock transfectants. The Cyr61 deficient cells demonstrated significantly inhibited colony formation in soft agar and reduced tumorigenicity was showed in nude mice, NF-κB pathway evidently inactivated respectively. However, under the stimulation of IL-8, the siRNA-treated cells can restore the capacity of proliferation and invasion. IL-8 can induce the high expression of Cyr61 and MMP11 through NF-κB signal pathway. Silencing of Cyr61 can inhibit or minimize the proliferation and invasiveness of gastric cancer cell. The results imply that Cyr61 enhance the proliferation and invasion of gastric cancer cells and this process is partially modulated by the IL-8 up-regulation. Cyr61 may mediate the proliferation and development of gastric carcinoma.

Keywords: Cyr61/CCN1, gastric cancer, MMP11, NF-κB, proliferation, invasion

Introduction

Gastric carcinoma is the fifth most common malignant tumors with high mortality worldwide [1]. Recently, the role of matricellular proteins which bridge inflammation and GC has been reported. In fact, uncontrolled and sustained inflammation may actively contribute to many chronic pathologies, including cancer [2-5]. At present, it has been reported that Cyr61 expression is remarkably overexpressed in GC cells and contributed to crucial processes involved in inflammatory response [3].

CCN growth factor family comprises six secreted proteins: Cyr61, CCN1, nephroblastoma overexpressed (NephC3), connective tissue growth factor (CTGF/CCN2), Wisp-3 (CCN6), Wisp-2/rCop1 (CCN5), and Wisp-1/elm1 (CCN4). Matricellular protein CCN regulates wide ranges of biological events, such as cell proliferation, cell adhesion, cell migration, cell differentiation, apoptosis, growth inhibition and extracellular matrix status [6, 7]. Cyr61, the first cloned member of CCN family, is consist of four domains with similar sequence to von Willebrand factor type C repeats, thrombospondin type 1 repeats, insulin-like growth factor-binding proteins, and carboxyterminal region containing cysteine knot domains. It plays important role in cardiovascular morphogenesis, inflammation, and wound healing. Cyr61 aberrant expression is involved chronic inflammatory diseases as well as a wide variety of cancers [8, 9].

Up regulation of Cry61 was initially seen in advanced mammary adenocarcinoma, pancreatic carcinoma, and gliomas, which imply the function of Cyr61 in tumorigenesis [10, 11]. In contrast, Cry61 is down-regulated in prostate and non-small-cell lung cancer. It reveals a neg-
ative correlation with the malignancy and Cyr61 is function as a tumor suppressor gene in non-small-cell lung carcinoma. Apparently, Cyr61 plays the contrasting roles in a variety of tumors, depending on different cellular milieu [12]. Nevertheless, the underlying mechanisms of Cyr61 during the progression of malignant neoplasm remain largely unclear.

In our study, the preliminary data indicated that Cyr61 was highly expressed in GC cell line BGC823. By using RNA interference (RNAi), we knock down Cyr61 at RNA level in BGC823 and studied the function of Cyr61 on the proliferation, migration, and invasion of GC cells. In addition, the crucial effect of IL-8 in Cyr61-promoted GC invasion and mobility has also been investigated. The study also reveals that NF-κB signaling transduction is crucial for Cyr61-mediated IL-8 activation and subsequent cell mobility and invasion.

Materials and methods

Cell culture and transfection

Human gastric cell line BGC823 was grown in Dulbecco’s Modified Eagle’s Medium supplemented with 5% fetal bovine serum (FBS) (DMEM; Gibco BRL, Gaithersburg, MD, United States). The cell was kept in a humidified 5% CO₂ atmosphere at 37°C. Cells were grown in 35-mm plates transfected were done when the cell density reached to 60-70% by using Lipofectamine 2000 (Invitrogen). The monoplasminids and empty vector were transfected into GC cells, while shRNA plasmids of Cyr61 were co-transfected into BGC823 cells. To screen for stable clones, cells were seeded after 48 hours of transfection for 21 d in selected medium containing 400 μg/mL G418. Immunofluorescence, Western blot and RT-PCR were used to determine the transfection efficacy.

Plasmid construction

For RNA interference, three 21-nucleotide siRNA duplexes targeting different encoding regions of Cyr61 were designed by the Ambion siRNA system and they showed no homology with other known human genes. The chemically synthetic, annealed duplexes were inserted between the BamH1 and Hind111 sites on the Psilencer™3. 1-H1-Neo (Ambion) to form a hairpin construct to generate interfering RNA in cultured cells. Human scrambled siRNA sequence (Ambion) possessing limited homology to human genes served as a negative control. The nucleotide sequences were depicted as follows: oligo1: 5’-GATCCGAGTGTCAAATCAATCTTCAAGAGATTCGATCTCTCATTGGAAA-3’; oligo2: 5’-AGCTTTTCAAAAAAAGAGGTGTAGAATCAGACTCTCTTCTTGAGATTCTGATTCTGATCGACTC-G-3’.

Western blot

Cells were scraped and collected following being washed with PBS 2 times. Proteins were extracted, and sodium dodecyl sulfate polyacrylamide gel electrophoresis were done to separate proteins (50 μg) and transferred to polyvinyl difluoride membranes (Bio-Rad, Hercules, CA, United States). The membrane treated with monoclonal antibody specifically against Cyr61 (1:2500, dilution, Abcam United States), at 4°C overnight in blocking buffer. The immunoreactivity was revealed by extended duration substrate from Super Signal West Dura (Thermo Scientific, Rockford, IL, United States).

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide assay (MTT)

Constant transfected cells (1 × 10³) in 200 μL DMEM were seeded in 96-well cultured plat in duplicate were treated with 10 μL (5 mg/ml) 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT, Gen-View, Jacksonville, FL, United States) at culture interval of 0, 24, 48, 72 and 96 hours. After incubation in the wells at 37°C in 5% CO₂ for 4 hours, 100 μL dimethylsulfoxide (Amresco, Solon, OH, United States) was added and incubation for additional 30 min. Absorbance was then analyzed by iMark Microplate Reader (Bio-Rad, Hercules, CA, United States) at 570 nm. Growth rate (%) is determined by the ratio of A570 of transfec-tants/parental BGC823 control · 100%.

Wound healing assay

Cells were incubated in 60-mm dishes for 24 h with the concentration of 5 × 10⁵ cells/ml at 80%-90% confluence. A straight line was created by scratching with a pipette tip, and the cells were incubated for an additional 24 h following being washed with phosphate buffered solution (PBS) for three times. The scratched gap was examined at the interval of 0, 2, 4, 6,
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Transwell assay

The cells were suspended in serum free DMEM at the concentration of 1 × 10^5 and transferred to matrix membranes for invasion assay by using a BD Matrigel Invasion Chamber. A chemotactrant were made with 10% FBS in DMEM. The cells were then fixed in methanol and stained 20 minutes in crystal violet solution after 48 hours of incubation and the number of membrane penetrated cells were counted.

Immunofluorescence analysis

The immunofluorescence was performed on the fixed cells grown on the glass slides. The cells were incubated with primary antibody against Cyr61 overnight at 4°C, followed by rhodamine-conjugated anti-rabbit secondary antibodies incubation for 1 hour, and 4',6-diamidino-2-phenylindole as nuclear stain. The cells were then examined under confocal fluorescence imaging microscope (TCSSSP5; Leica, Mannheim, Germany).

Statistical analysis

Student’s t test (version 16.0) was used. The analysis of variance was used for data measurement. Mean ± SD for quantitative values. The statistically significant is referred to the differences with P < 0.05.

Results

Cyr61 high expressed in gastric cancer cell line BGC823

The expression level of Cyr61 in BGC823, MGC803, SGC7901, AGS, N87, MKN45 were examined by using RT-PCR and Real-Time PCR. The expression of Cyr61 protein in these 6 cell lines showed that Cyr61 mRNA and protein were expressed differently in the above different gastric cancer cell lines, and Cyr61 relatively high expressed at the mRNA and protein level in BGC823. Immunofluorescence results showed that Cyr61 protein was distributed in cytoplasm (Figure 1).
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Stable silencing of Cyr61 by constructed the recombinant plasmids targeting Cyr61 in GC cells

Our previous study has shown that Cyr61 has transcriptional different expression in the GC and NATs (unpublished data). High expression of Cyr61 was found both in Intestinal Metaplasia and dysplasia. To investigate the biological function and mechanism of Cyr61 in GC cells, the constructed and parental plasmids was designated as psiRNA-Cyr61 and psiRNA-vect, respectively (Figure 2). G418-resistant clones were isolated, which were stably transfected cells. By using semi-quantitative RT-PCR, Western blot and immunofluorescence assays, we analyzed Cyr61 expressions respectively (Figure 3).

Cyr61 increase the proliferation of gastric cancer cell BGC823

The roles of Cyr61 on the BGC823 cell proliferation were studied by MTT and soft agar colony formation (A, B, C). The results showed that the number of cell growth and clone formation decreased significantly in the interference of Cyr61 group and the inhibition rate was 74%. (*P* < 0.01) (Figure 4) Each test has been repeated three times.

Cyr61 increase BGC823 cell invasion and migration

For examining the role of Cyr61 on cell invasion and migration, scratch healing test and Transwell assay were conducted. The results of
The scratch test showed that the speed of psiRNA-2 migration to scratch in the interference group was slower than control group Vector and the blank control group, and the Transwell invasion experiment showed that the invasion ability of psiRNA-2 cells interfered with Cyr61 was obviously weakened, and the number of cells attacked by the matrix glue to the membrane was obviously less, and the quantity of cells under the membrane was decreased. The comparative inhibition rate in the interference group was 79%. (P < 0.01) (Figure 5) Each test has been repeated three times.

Cyr61 increase the tumorigenesis of BGC823 in nude mice

To study the role of Cyr61 on the tumorigenesis of gastric cancer cell line BGC823, we conducted a nude mouse experiment, subcutaneous injection of the same number of psiRNA-2 cells that interfered with Cyr61 and Vector cells in the empty control group. After 3 weeks, the tumor volume decreased in the interference group of nude mice (Figure 6).

IL-8 induces the expression of Cyr61 and enhances the invasiveness, proliferation, and migration of gastric cancer cells

Gastric cancer has been proposed to be caused by uncontrolled or sustained inflammation. Our previous data indicated that silencing of Cyr61 can suppress the proliferation and metastasis of BGC823, indicating that Cyr61 may contribute in the invasion process of GC. To emphasize the hypothesis of Cyr61 in GC cells could be affected by inflammation factor, BGC823 cells were treated with interleukin-8 (IL-8). Cyr61 expression was elevated by IL-8 stimulation as analyzed by RT-PCR and western blot. We next explored if the inflammation factor affects the...
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MMP11 as a downstream effector of Cyr61 and Nuclear factor-κB activation is significant for transcriptional up-regulation of MMP11

Cyr61 was highly expressed in human GC cell lines and enhanced their migrating and invasiveness in our previous studies (unpublished data). Recently, it is well known that MMP11 promotes tumor progression of GC. Nevertheless, the regulation role of Cyr61 in MMP11 expression in GC cells remains unclear. The protein level of MMP11 decreased in psiRNA-Cyr61 cells. However, the MMP11 level was increased in psiRNA-Cyr61 cells after stimulated by IL-8. The result suggest that Cyr61 induces MMP11 up-regulation and lead to the migration of GC cells stimulated by IL-8. Research data proved that activated NF-κB is vital for increasing expression of MMPs and Cyr61 in MCF-7 cells. In the same fashion, our data reveals that Cyr61 overexpression activated the NF-κB signal transduction cascades and resulted in overexpression of MMP-11 in BGC823 cells (Figure 8).

Discussion

In malignant tumor cells, studies have found that Cyr61 is up-regulated, overexpressed, and it might promote early tumorigenesis and tumor progression of human cancer [6-8, 12-15]. Over expressions of Cyr61 in cell lines and primary tumors of GC were frequently found in our studies. The recombinant plasmids have been
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A

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B

Graph showing expression levels at different time points (0h, 24h, 48h, 72h, 96h) for each group.

C

Images showing cell morphology at 0h for each group.
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constructed and the permanent cell line was established to investigate the potential role of Cyr61 in the matrix metalloproteinase tumorigenesis of GC by applying a DNA vector-based

Figure 7. A. (top). The expression of Cyr61 by RT-PCR, and the expression in psiRNAcyr61 group was significantly lower, after stimulated by IL-8 for 24 h, the Cyr61 expressions were obviously enhanced in interference group psiRNAcyr61, no-load control group BGC823-vector and control group (bottom). After stimulated by IL-8 for 24 h, the Cyr61 expressions were obviously enhanced in interference group psiRNAcyr61, no-load control group BGC823-vector and control group. Beta-Actin as an internal loading control. B. MTT assay of the proliferation ability in three groups. Interference group psiRNA cells was significantly reduced (P < 0.01). And cell growth of all three-group stimulated by IL-8 is obviously enhanced. C. Wound healing assay of migration ability in three groups. The migration ability in Interference group psiRNA cells was significantly reduced compared with the Vector and BGC823 cells. However, after stimulated by IL-8 their migration ability is obviously enhanced (P<0.01). D. Transwell invasion experiments showed that the number of cells through the Matrigel decreased significantly in the Interference group psiRNA cells. However, their invasion ability is obviously enhanced after stimulated by IL-8 (P < 0.01).
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Our results from experiments using MTT indicated that Cyr61 depletion resulted in the suppression of growth of BGC823 cells. These findings have provided evidences of the role Cyr61 in the progression of GC and could be used as potential GC therapeutic target.

The current research reports that the destruction of gastric mucosal barrier contributes in the occurrence of GC, rather than a controllable inflammation or the resulting metabolic inflammation [13, 18]. Some evidence suggests that not only is Cyr61 growth factor associated with tumorigenesis, but there is an interaction between chemokines and matrix metalloproteinases [19]. Furthermore, Cyr61 is also involved in cancer related to inflammation, angiogenesis and tissue reconstruction process [14-16, 20]. Recent studies have shown that Cyr61 can modify the adhesion molecules, cell matrix and regulating the expression of gens pertaining to inflammation of macrophages related to Th1 responses via NF-kB pathway [20, 21].

To further clarify Cyr61 gene effects on gastric cancer biological behavior and the correlation with inflammatory factors, we built the plasmid of RNA interference of Cyr61 in BGC823 cell line. Through a series of biological function experiments, the results show that interference of Cyr61 in gastric cancer BGC823 cell, the proliferation and metastasis of GC cells decreased significantly. However, after the stimulation with IL-8, the biological function of gastric cancer BGC823 cell increased significantly. It suggests that Cyr61 may under the regulation of IL-8, enhances proliferation and metastatic capacity of malignant tumor cells.

Invasion and metastasis are one of the most important biological behaviors of malignant tumors, it is also the main reasons that lead to increased mortality. Invasion and metastasis of tumor is the interaction between tumor cells and tumor microenvironment. As a result, it is a very complex pathological process, which involves multiple molecular mechanism and signal transduction pathways, such as the NF-kB, MAPK/ERK and PI3K/Akt [16, 22]. Some studies show that Cyr61 can contribute to the malignant progression of gastric cancer by promoting tumor cell mobility and invasion through up regulation of functional COX-2 via an integrin alpha(v) beta (3) and NF-kB dependent path-

Figure 8. (A) MMP11 protein expression in interference expression in psiRNA-Cyr61 group, no-load control group BGC823-vector and control group by Western blot. MMP11 protein expression decreased obviously in psiRNA-Cyr61 group. (B) NF-kB, I-kB and PI-KB was detected by using Western blot, NF-kB signaling pathway was activated in psiRNA-Cyr61 group. (C) Cyr61 gene expression in three groups increased after stimulated by IL-8 for 24 h, (D) activation of NF-kB signaling pathway was increased after IL-8 stimulation.
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way. Thus, our current data strengthen the previous study showing that interaction of Cyr61 in gastric cancer cells constitutively block the NF-kB activity. On the other hand, MMP11 was initially described to be up regulated in invasive breast cancer and later confirmed to be overexpressed in other cancers, suggesting that MMP11 play a role in tumorigenesis. To address the interaction between Cyr61 and MMP11, Cyr61 was stable silenced in gastric cancer cells by using RNAi technology and our data indicated that interaction of Cyr61 could down regulate MMP11 and inhibit BGC823 cell proliferation in vitro.

In conclusion, we have demonstrated that IL-8 induces functional Cyr61 that plays an important role in human gastric cancer cells. In this mechanistic study, the results of our investigation strongly suggest that Cyr61-mediated IL-8 signaling is a requisite for MMP-11 expression. Our current findings advise that Cyr61 enhance the proliferation and invasion of gastric cancer cells and this process is, at least in part, mediated by the up-regulation of IL-8. Cyr61 acts as both an enhancer and a predictive biomarker for gastric cancer patients at early stages of their malady.

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Disclosure of conflict of interest

None.

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