Differential expression of CCN family members CYR611, CTGF and NOV in gastric cancer and their association with disease progression

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Abstract. CCN is an acronym for cysteine-rich protein 61 (CYR61), connective tissue growth factor (CTGF) and nephroblastoma overexpressed (NOV). Aberrations of certain CCN members including CYR61, CTGF, Wnt1-inducible signalling pathway protein (WISP)-1 and -3 have been reported in gastric cancer. The present study aimed to examine the clinical relevance of NOV along with CYR61 and CTGF in gastric cancer by analysing their transcript levels. *CYR61*, *CTGF* and *NOV* transcript expression in 324 gastric cancer samples with paired adjacent normal gastric tissues were determined using real-time quantitative PCR and the results were statistically analysed against patient clinicopathological data using SPSS software. NOV mRNA levels in gastric cancer tissues were significantly elevated when compared with levels in their paired adjacent non-cancerous tissues. Local advanced tumours with invasive expansion (T3 and T4) expressed higher levels of NOV (p=0.013) compared with the less invasive tumours (T1 and T2). CYR61 transcript levels were also significantly increased in gastric cancers compared with levels in the adjacent non-cancerous tissues. Kaplan-Meier survival curves revealed that patients with CYR61-low transcript levels had longer overall survival (OS) (p=0.018) and disease-free survival (DFS) (p=0.015). NOV overexpression promoted the *in vitro* proliferation of AGS cells while the knockdown resulted in a reduced proliferation of HGC27 cells. A similar effect was observed for the invasion of these two gastric cancer cell lines. NOV expression was increased in gastric cancer which was associated with local invasion and distant metastases. Taken together, the expression of NOV and CYR61 was increased in gastric cancer. The elevated expression of CYR61 was associated with poorer survival. NOV promoted proliferation and invasion of gastric cancer cells. Further investigations may highlight their predictive and therapeutic potential in gastric cancer.

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Introduction

Gastric cancer (GC) is the fourth most commonly diagnosed cancer in males and the fifth in females worldwide, with over 751,600 new cases and 723,100 deaths estimated to have occurred in 2012 (1). The highest incidence rates are found in Eastern Asia (particularly in Korea, Mongolia, Japan and China), Central and Eastern Europe, and South America, and the lowest incidences are noted in Northern America and most parts of Africa. The incidence is twice higher in males than in females (1).

The tumor microenvironment plays a pivotal role in tumourigenesis and subsequent dissemination of cancer cells by coordinating morphological transformation of cancer cells and their proliferation, survival and invasion (2,3). Recent reviews have highlighted a profound role played by a group of proteins in the tumour microenvironment which belong to the CCN family. CCN is an acronym for cysteine-rich protein 61 (CYR61), connective tissue growth factor (CTGF) and nephroblastoma overexpressed (NOV) which comprises CYR61, CTGF, NOV and another three members i.e. Wnt1-inducible signalling pathway protein (WISP)-1, -2 and -3 (4-6).

CCNs are matricellular proteins and can be secreted into the extracellular matrix. CCN3 can be detected in the culture medium, extracellular matrix (ECM) and at the cell membrane (7-9). The CCN family proteins have been preserved during evolution which can be found in vertebrates including zebra fish, chickens, mice, rats and humans. CCN proteins generally have an N-terminal secretory signal peptide and four functional domains: an insulin-like growth factor binding protein domain (IGFBP); a Von Mayebrand factor type C (VWC) domain; a thrombospondin type-1 repeat module (TSP-1) and a cysteine knot domain (CT). The two N-terminal domains are separated from the two C-domains by a linker with a variable sequence of amino acids. However, CCN5 does not have the CT domain (10).

An elevated expression of CCN1 has been demonstrated in a variety of malignancies including ovarian, prostate, breast and colorectal cancers (11-14). Similarly, an overexpression of CTGF has been noted in breast and colorectal cancer, oesophageal squamous cell carcinoma and pancreatic cancer (15-18), while increased expression of NOV has been shown in prostate and cervical cancers (19,20). In contrast to these findings, reduced expression of these three CCNs has also been observed in certain solid tumours. For example, CYR61 expression is reduced in endometrial cancer and lung cancer (21,22). A reduced expression of CYR61 has also been seen in advanced GC (23). CTGF can act as an inhibitor in lung cancer by suppressing proliferation of non-small cell lung cancer cells (24). A similar inhibitory effect on the proliferation of cancer cells has also been evident for the NOV protein in gliobastoma and malignant adrenocortical tumour cells (25,26). This suggests that the expression and function of CCNs in malignancies can be organ- or tissue-specific.

Early studies have demonstrated a profound role played by CCNs in fibrosis which has been reviewed most recently by Riser *et al* (6). Their involvement in fibrotic disorder stimulated research interest concerning their implication in a specific histologic type of gastric carcinoma, i.e. scirrhous carcinoma which is well known for a vast fibrous stroma, rapid and invasive growth and poor prognosis. Tanaka *et al* identified a novel variant of WISP1 that was highly expressed in scirrhous carcinomas. This variant lacks the von Mayebrand type C module and is named WISP1v. WISP1v can induce transformation and promote proliferation and invasion of GC cells through both autocrine and paracrine pathways (27). Tanaka *et al* also reported a loss of function mutation of WISP3 with a frequency of 10-20% in microsatellite unstable gastric carcinoma. This mutation resulted in a truncated variant of WISP3 that was lacking the TSP-1 and CT domains and was unable to suppress the invasiveness of GC cells (28). Although reduced expression of CYR61 was reported in advanced GC which was inversely correlated with the expression of MMP-7 (23), most studies demonstrated a positive role played by CYR61 in GC by promoting invasion, metastasis and also tumour-associated angiogenesis (29,30). CYR61 promotes GC cell invasion through hypoxia-inducing factor-1α (HIF-1α)-dependent upregulation of plasminogen activator inhibitor-1 (PAI-1) (31).

Elevated expression of CYR61 and CTGF has been observed in GC which is associated with lymph node metastasis, however the expression of NOV in GC is yet to be revealed. The present study aimed to determine the expression of these three CCNs in a cohort of GC tumours, in particular, by dissecting the role played by NOV in GC.

Materials and methods

Cell lines and culture conditions. Human GC cell lines AGS and HGC27 were purchased from the European Collection of Cell Cultures (ECACC; Salisbury, UK) and incubated at 37˚C, with 5% CO_2 and 95% humidity. The wild-type cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (PAA Laboratories Ltd., Somerset, UK), penicillin, streptomycin and amphotericin B.

Human gastric tissues. Gastric adenocarcinoma and Siewert type III gastrooesophageal junction adenocarcinoma tissues (n=245) with matched adjacent background tissues (n=158) were immediately collected after surgical resection at the Beijing Cancer Hospital with informed consent from the patients. All patients underwent surgery without any prior treatment. The tissue samples were stored at -80˚C at the Tissue Bank of Peking University Oncology School with a record of the relevant clinical and histopathological data. All protocols were reviewed and approved by the Beijing Cancer Hospital Research Ethics Committee (MTA10062009).

RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qPCR). RNA was extracted from confluent cells in a 25 cm^2 flask using total RNA isolation (TRI) reagent (Sigma-Aldrich, Dorset, UK). Fresh frozen tissues were also first homogenised in the TRI reagent. First strand of cDNA was synthesised from 1 μg RNA using a first-strand DNA synthesis kit (Bio-Rad, Hemel Hempstead, UK). Quantitative analysis of NOV mRNA expression in GC tissues was performed using Amplifluor™-based real-time PCR, in which a 6-carboxy-fluorescine-tagged Uniprimer™ (Biosearch Technologies, Inc.) was used as a probe along with a pair of specific primers with an addition of a Z-sequence (actgaacctgaccgtaca) to the 5'-end of the reverse primer. The quality of cDNA samples was verified using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. All the primer sequences are listed in Table I.

Quantitative analyses of transcript expression of CYR61, CTGF and NOV in human GC. Following the real-time PCR quantification of each gene transcripts, the number of samples with valid data for each individual genes were: 322 samples for *NOV*, 252 samples for *CYR61* and 320 samples for *CTGF*. These cohorts were composed of 230 men (71.4%) and 92 women (28.6%) in the *NOV* cohort, 180 men (71.4%) and 72 women (28.6%) in the *CYR61* cohort, and 228 men (71.3%) and 92 women (28.7%) in the *CTGF* cohort. Data are shown in Tables II-IV.

Construction of the ribozyme transgene targeting human NOV and the establishment of corresponding stable transfectants. Anti-human NOV hammerhead ribozymes were designed using the Zuker RNA mFold program (Zuker 2003). The ribo-

Table I. Primers used for PCR and qPCR.

Primer	Forward primer	Reverse primer
NOV (PCR)	CTCCAAGAAAAGTTGAGGTG	CTGGCTTCTTGACTATTTGC
NOV (qPCR)	CTGTGAACAAGAGCCAGAG	ACTGAACCTGACCGTACACTTGAACTGCAGGTGGAT
GAPDH (PCR)	GGCTGCTTTTAACTCTGGTA	GACTGTGGTCATGAGTCCTT
GAPDH (qPCR)	CTGAGTACGTCGTGGAGTC	ACTGAACCTGACCGTACACAGAGATGACCCTTTTG
CYR61 (qPCR)	GGGCTGGAATGCAACTTC	ACTGAACCTGACCGTACACGTTTTGGTAGATTCTGGAG
CTGF (qPCR)	GAGTGGGTGTGTGACGAG	ACTGAACCTGACCGTACAGGCAGTTGGCTCTAATCATA

NOV, nephroblastoma overexpressed; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CYR61, cysteine-rich protein 61; CTGF, connective tissue growth factor.

zymes and full-length coding sequence of human NOV were cloned into a pEF6/V5-His TOPO vector (Invitrogen, Paisley, UK). The verified ribozyme transgenes, NOV expression vectors and empty plasmids were transfected into AGS and HGC27 cells, respectively, using an Easyjet Plus electroporator (EquiBio, Kent, UK). After one week of selection with 5 μg/ml blasticidin, the selected cells were maintained in DMEM with 0.5 μg/ml blasticidin.

Western blot analysis. The protein concentrations in the cell lysates were determined using the DC protein assay kit (Bio-Rad) and the ELx800 spectrophotometer (Bio-Tek, Winooski, VT, USA). Proteins were probed with the anti-NOV antibody (1:2,000; Abcam, Ltd., Cambridge, UK) and anti-GAPDH antibody (1:2,000; Santa Cruz Biotechnology, Inc.) as housekeeping gene control, followed by a peroxidase-conjugated secondary antibody (1:2,000; Sigma). Protein bands were visualised using a chemiluminescence detection kit (Luminata, Millipore Ltd., Watford, UK) and photographed using Syngene imager (Syngene International Ltd., Bangalore, India).

In vitro cell growth assay. Cells (3,000) were seeded into 96-well plates in normal culture medium. The cells were incubated over a period of up to 4 days, and were then fixed with 4% formaldehyde followed by staining with 0.5% crystal violet. The crystal violet was dissolved in 10% acetic acid prior to a colorimetric detection of cell density at a wavelength of 540 nm using the ELx800 spectrophotometer.

In vitro invasion assay. The *in vitro* invasion assay was previously described (32). All the culture plate inserts containing 8-μm pores were pre-coated with 50 μg of Matrigel (BD Bioscience, Oxford, UK) and air-dried. Cells (30,000) were seeded to each well after a 40-min rehydration of the Matrigel. After an incubation of 72 h, cells that had migrated through the matrix to the other side of the insert were fixed and stained. The cell number was determined.

Cell-matrix adhesion assay. The cell-matrix adhesion assay was conducted as previously described (32). The 96-well culture plate was pre-coated with 5 μg of Matrigel and air-dried. Following the rehydration, 30,000 cells were seeded to each well. After an incubation of 40 min, non-adherent cells were washed-off using phosphate-buffered saline (PBS). The adherent cells were counted after a fixation and staining using crystal violet.

Wound healing assay. The assay was performed following a previously described procedure (32). In brief, the monolayer of cells was scraped with a 10 μl pipette tip. The migration of cells was photographed using a time-lapse image system (EVOS, Life Technologies Ltd., Paisley, UK).

Statistical analysis. Statistical analyses were performed using SPSS (version 11; SPSS, Inc., Chicago, IL, USA). Mann-Whitney U test and t-test were used for non-parametric and normally distributed data, respectively, including research data from the clinical cohort and cell-based experiments. Kaplan-Meier survival analysis was also performed using SPSS statistical software. Differences were considered to be statistically significant at $p<0.05$.

Results

Increased expression of NOV in human GC. Transcript levels of *CYR61*, *CTGF* and *NOV* were determined in the GC cohort using real-time PCR, respectively. The results showed that *NOV* expression was significantly upregulated in gastric tumours compared to normal tissue (p=0.009) (Table II). An increased expression of *NOV* in GC was associated with local invasion. The transcript levels of NOV were higher in the tumours with more advanced local invasion. According to the tumor-node-metastasis (TNM) staging, T4 tumours which invaded the serosa or adjacent structures expressed higher levels of NOV transcripts, p=0.0026 vs. T1. Tumours classified as T3 and T4 which invaded beyond subserosal connective tissues expressed higher levels of NOV transcripts in comparison with tumours (T1 and T2) with less local invasion (p=0.0013). According to the overall TNM staging, stage I GCs exhibited lower expression levels of NOV, p=0.016 vs. stage II, p=0.0017 vs. stage III, and p=0.0007 vs. tumours of stage II-IV. Notably, we found that high-moderately differentiated tumours exhibited significantly lower levels of NOV expression in comparison with moderately and/or poorly differentiated tumours. However, no association was observed for lymph node and distant metastases.

Table II. Expression of *NOV* in gastric cancer.

Category	No.	Mean ± SEM (copies)	P-value
Tissue			
Tumour	322	8,893±1,303	
Normal	183	3,262±1,058	0.0009
Gender			
Male	230	9,583±1,645	
Female	92	7,170±1,971	0.35
Location			
Cardia	66	8,384±2,795	
Fundus	21	8,679±4,797	0.86
Corpus	61	7,678±2,795	0.59
Pylorus	131	8,326±1,786	0.84
Differentiation			
Diff-H	1	33,009	
Diff-HM	6	172.2±56.6	
Diff-M	62	8,677±2,779	0.0033
Diff-ML	82	9,130±2,435	0.0004
Diff-L	136	8,610±1,848	<0.001
T stage			
T1	16	2,754±2,450	
T2	26	4,456±2,325	0.62
T3	41	9,478±5,181	0.25
T4	231	9,503±1,500	0.026
T1+T2	42	3,808±1,701	
T3+T4	272	9,499±1,490	0.013
N stage			
N0	70	8,735±2,479	
N1	48	9,123±3,036	0.92
N2	65	5,845±3,214	0.48
N3	133	10,311±2,106	0.63
N1+N2+N3	246	8,899±1,538	0.96
M stage			
M0	280	8,644±1,281	
M1	41	10,813±5,350	0.7
TNM stage			
I	25	1,847±1,569	
II	60	9,689±2,758	0.016
III	219	9,261±1,672	0.0017
IV	9	9,756±9,560	0.44
II+III+IV	228	9,366±1,421	0.0007
Vascular invasion			
No invasion	151	6,865±1,526	
Invasion	156	9,257±1,803	0.63
Clinical outcome			
Disease-free	119	7,464±1,832	
Metastases	15	12,921±6,376	0.42
Death	185	9,626±1,869	0.41

NOV, nephroblastoma overexpressed; SEM, standard error of the mean; TNM, tumor-node-metastasis.

Table III. Expression of *CYR61* in gastric cancer.

Category	No.	Mean ± SEM (copies)	P-value
Tissue			
Tumour	252	2,225±668	
Normal	175	120.1±50.5	0.0019
Gender			
Male	180	2,737±913	
Female	72	947±475	0.083
Location			
Cardia	50	3,558±2,288	
Fundus	12	322±172	0.86
Corpus	52	2,824±1,622	0.59
Pylorus	102	1,717±780	0.84
Differentiation			
Diff-H	1	4400.7	
Diff-HM	5	1,898±1,897	
Diff-M	52	1,890±1,426	1
Diff-ML	64	2,537±1,747	0.81
Diff-L	106	1,255±558	0.76
T stage			
T1	13	8,723±6,131	
T2	21	8.96±8.53	0.18
T3	27	2,821±1,860	0.37
T4	185	1,756±715	0.28
T1+T2	34	3,341±2,402	
T3+T4	212	1,892±666	0.56
N stage			
N0	59	3,178±1,572	
N1	42	1,050±1,035	0.26
N2	50	1,612±1,206	0.43
N3	96	2,108±1,166	0.59
N1+N2+N3	188	1,740±712	0.41
M stage			
M0	220	2,439±758	
M1	32	755±663	0.097
TNM stage			
I	21	5,400±3,855	
II	50	1,452±923	0.33
III	169	1,928±791	0.39
IV	6	450±445	0.22
II+III+IV	225	1,783±628	0.36
Vascular invasion			
No invasion	124	1,739±678	
Invasion	117	2,523±1193	0.57
Clinical outcome			
Disease-free	98	2,744±1,131	
Metastases	10	418±399	0.055
Death	142	2,026±893	0.62

CYR61, cysteine-rich protein 61; SEM, standard error of the mean; TNM, tumor-node-metastasis.

Table IV. Expression of *CTGF* in gastric cancer.

Category	No.	Mean ± SEM (copies)	P-value
Tissue			
Tumour	320	224.3±27.9	
Normal	183	247.7±38.7	0.62
Gender			
Male	228	209.5±22.92	
Female	92	261.4±79.1	0.53
Location			
Cardia	65	251.4±54.2	
Fundus	21	234.6±66.3	0.85
Corpus	61	158.2±44.0	0.18
Pylorus	130	251±52.8	1
Differentiation			
Diff-H	1	16.77	
Diff-HM	6	142.2±61.4	
Diff-M	62	259.6±53.0	0.17
Diff-ML	81	266.6±60.9	0.17
Diff-L	135	197.6±48.1	0.49
T stage			
T1	16	49±12.2	
T2	25	218.8±93.55	0.084
T3	41	252.3±90.0	0.031
T4	230	231.6±33.6	0
T1+T2	41	154.1±59.08	
T3+T4	271	234.8±31.5	0.23
N stage			
N0	70	250.7±78.5	
N1	48	120±35.1	0.13
N2	64	292.2±57.6	0.67
N3	132	218.7±43.3	0.72
N1+N2+N3	244	218.9±28.9	0.7
M stage			
M0	278	199.4±26.6	
M1	41	400±119	0.11
TNM stage			
I	25	225.8±97.01	
II	59	197.2±83.2	0.82
III	218	224.5±29	0.99
IV	9	407±349	0.63
II+III+IV	286	224.5±29.9	0.99
Vascular invasion			
No invasion	150	246.6±45.5	
Invasion	155	206.7±36.3	0.49
Clinical outcome			
Disease-free	119	237.3±48.9	
Metastases	15	119.2±47.6	0.09
Death	183	227.1±36.7	0.87

CTGF, connective tissue growth factor; SEM, standard error of the mean; TNM, tumor-node-metastasis.

CYR61 is upregulated in GCs. *CYR61* mRNA levels were significantly elevated in the GC tissues compared to levels in the non-cancerous tissues (p=0.0019), particularly in paired tissues (p=0.0013) (Table III). However, the expression of *CYR61* transcripts appeared to be lower in more advanced tumours (stage III and IV) according to the TNM staging, although it did not reach a statistically significant level. According to clinical outcomes, tumours with distant metastases had lower expression levels of *CYR61* (p=0.055) compared with that of patients who remained disease-free. No association was observed between *CYR61* expression and differentiation and local invasion.

Higher expression of CTGF in GC and the involvement in local invasion. Although no statistically significant differences were noted for *CTGF* mRNA levels in GCs compared with adjacent normal gastric tissues, a higher transcript level of *CTGF* in GCs was positively associated with local invasion (T4 vs. T1, p<0.001; T3 vs. T1, p=0.031; Table IV). However, there were no other significant correlations between *CTGF* mRNA levels and other clinical parameters.

Expression of CYR61, CTGF and NOV and survival of patients with GC. Kaplan-Meier survival curves revealed that GC patients with a low *CYR61* transcript level had longer overall survival (OS) (p=0.018) and disease-free survival (DFS) (p=0.015) than those with a higher *CYR61* transcript level (Fig. 1). Transcript levels of *CTGF* and *NOV* exhibited no correlation with either OS or DFS when individually analysed; however, analysis of the combination of *CYR61* and *CTGF* showed that patients with lower transcript levels of these two genes had longer OS (p=0.033) and DFS (p=0.025). Similarly, analysis of combined *CYR61*, *CTGF* and *NOV* showed that patients with higher transcript levels of all three genes had a poorer OS (p=0.027) and DFS (p=0.021) compared to patients with lower expression of all these genes.

Knockdown and overexpression of NOV in GC cells. The expression profile of NOV in AGS and HGC27 cell lines was assessed using RT-PCR (Fig. 2A). For assessing the effect of NOV on cellular functions, overexpression of NOV was performed in the AGS cells which had an almost undetectable level of NOV as determined using PCR, while knockdown of NOV was carried out in the HGC27 cells which highly expressed NOV. The overexpression and knockdown of NOV in AGS and HGC27 transfectants were confirmed using RT-PCR (Fig. 2B) and western blotting (Fig. 2C).

Effect of NOV knockdown and overexpression on cell growth in vitro. Overexpression of NOV increased the growth of AGS cells over the periods of 3 (p<0.01) and 4 days (p<0.001) compared with the control cells. An opposite effect was observed in the HGC27 NOV-knockdown cells over the periods of 3 (p<0.01) and 4 days (p<0.01) (Fig. 3).

Effect of NOV knockdown and overexpression on the invasion, adhesion and migration of GC cells. Overexpression of NOV resulted in increased invasion in the AGS cells, while NOV knockdown exhibited reduced invasiveness in the HGC27 cells (p<0.001) (Fig. 4). Knockdown and overexpression of

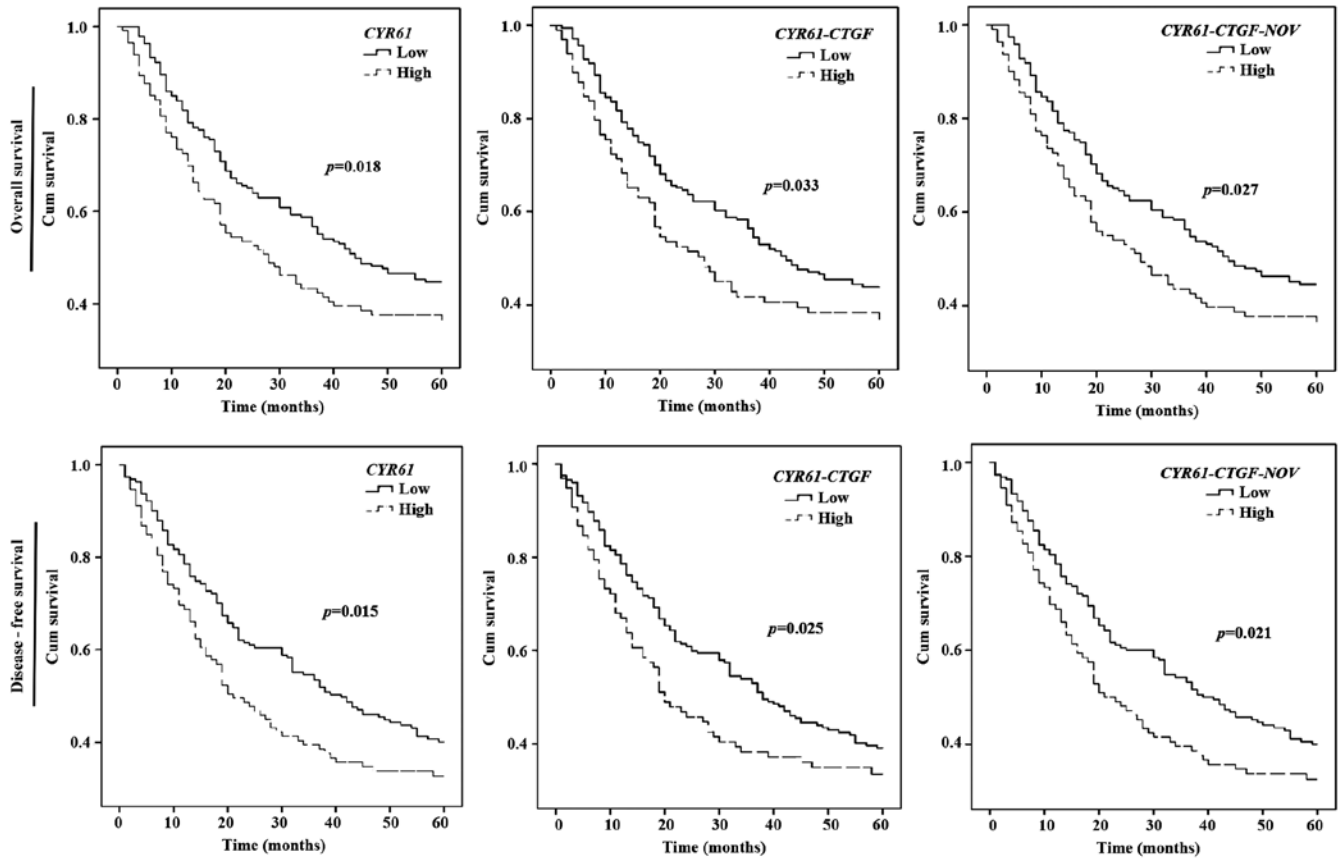

Figure 1. Association of CYR61, CTGF and NOV expression with the survival of patients. The average expression levels for CYR61, CTGF and NOV transcripts were used as thresholds. The survival of patients with higher or lower expression levels of each was analysed individually or in combination as two or three using the Kaplan-Meier survival analysis.

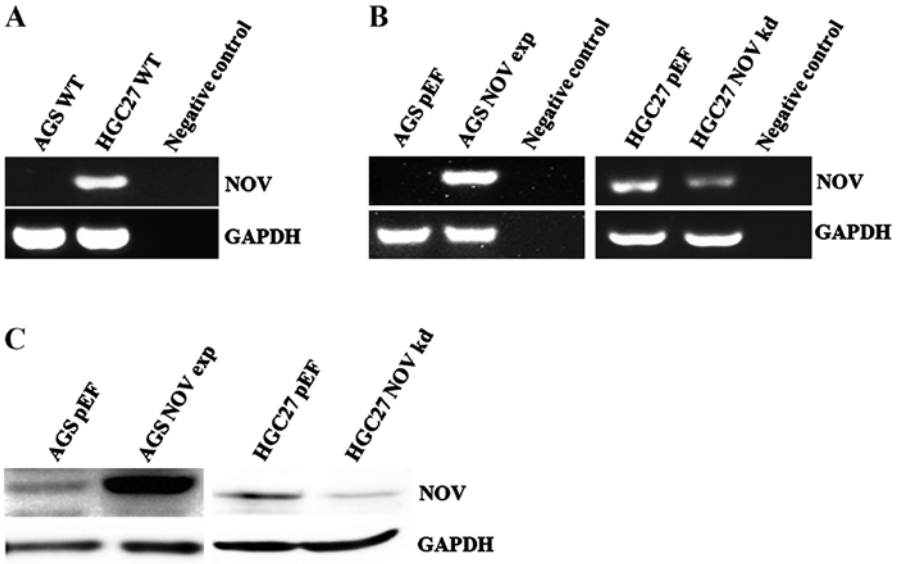

Figure 2. Knockdown and overexpression of NOV in GC cell lines. (A) The expression of NOV in the AGS and HGC27 cell lines using RT-PCR. (B) Overexpression of NOV in the AGS cell line and knockdown of NOV in the HGC27 cell line were verified using RT-PCR. (C) Verification of the knockdown and overexpression was further confirmed using western blotting.

NOV did not enhance or reduce the adhesion of the GC cells to Matrigel in comparison with the control cells. A wound healing assay was employed to determine the influence of NOV on cell migration. There was no obvious effect observed in our experiments (data not shown).

Discussion

This is the first study to assess the role played by NOV (CCN3) in gastric cancer (GC). In the present study, we determined the expression of NOV, along with CYR61 and CTGF tran-

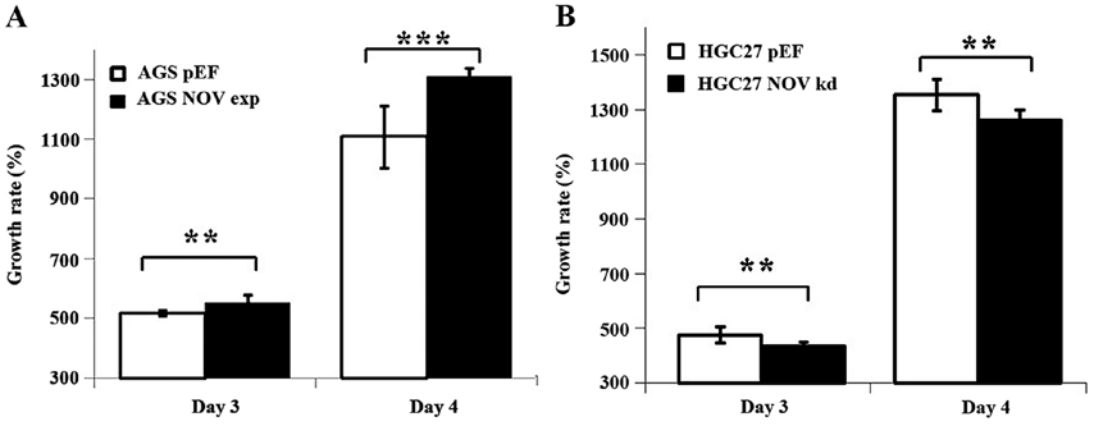

Figure 3. Influence of altered NOV expression on *in vitro* proliferation of the GC cell lines. The proliferation of (A) AGS and (B) HGC27 cells with knockdown or overexpression of NOV was determined using an *in vitro* growth assay over a culture period of up to 4 days. Three independent experiments were performed. Shown are representative results from these experiments and the error bars show standard deviations; $^{**}p<0.01$ and $^{***}p<0.001$.

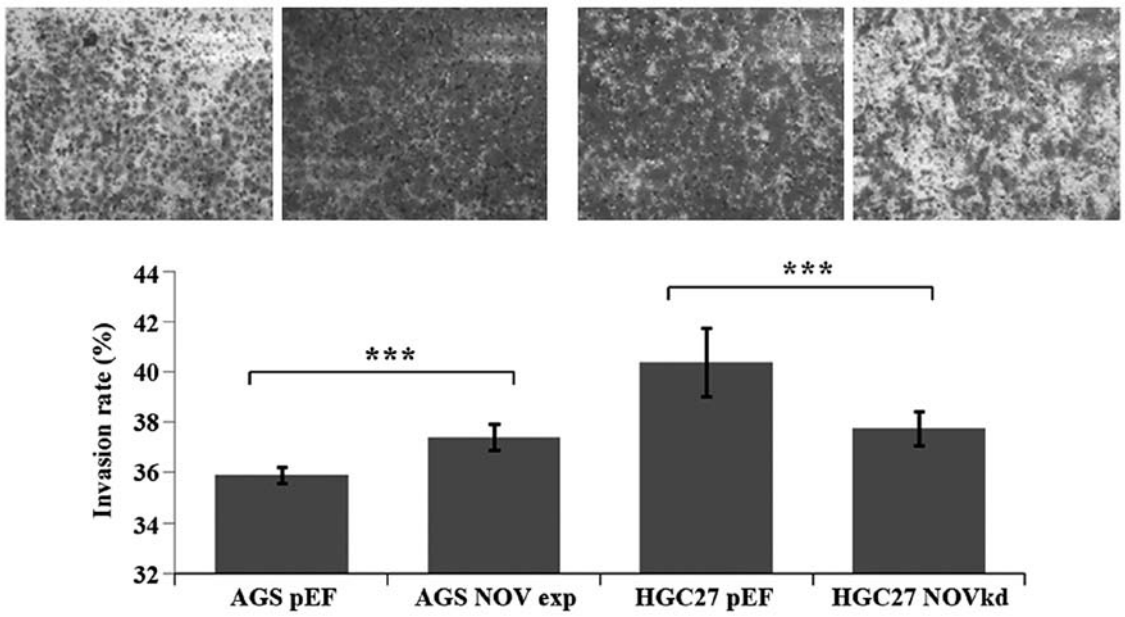

Figure 4. Effect of the altered expression of NOV on the invasiveness of GC cells.

scripts in a cohort of human GCs and paired adjacent normal gastric tissues. Increased expression of NOV was observed in the GC tissues compared with the expression noted in the adjacent normal gastric tissues. Increased expression was found to be associated with local invasion and poorer differentiation. Elevated expression of NOV in GC is consistent with observations in other cancers. For example, overexpression of NOV is evident in prostate cancer and cervical cancer and is involved in tumourigenesis and disease progression (19,20). Overexpression of NOV in cervical cancers was found to be associated with lymph node metastases and poorer prognosis of cervical cancer patients (20). This suggests that NOV is positively involved in tumourigenesis and disease progression by regulating morphological transformation and the invasiveness of GC cells. Our previous study showed reduced expression of NOV in breast cancer which was associated with poor prognosis and mortality of the disease (15). Differential expression patterns and roles can be played by NOV in different malignancies which can be organ- or tissue-specific.

In the present study, we also determined the transcript levels of CYR61 and CTGF in GC samples. Similarly, overexpression of CYR61 transcripts was noted in GC although no difference was observed for the expression of CTGF. No association was clearly evident for elevated expression of CYR61 and disease progression in the present cohort of GC cases. Overexpression of CYR61 has been observed in many different cancers, including prostate, breast, ovarian, endometrial and colorectal carcinomas (11,12,33-35). In contrast to the elevated expression of CYR61 in these malignancies, reduced expression has also been noted in various types of cancers, in particular GC which is associated with local invasion (23). In the present study, a trend of reduced expression of CYR61 was also noted in the more advanced GCs appearing to be in line with observations made by Maeta *et al* (23). A controversy may

still exist for its role in GC as experimental evidence using GC cell lines indicates that CYR61 can promote invasion, metastasis and angiogenesis in GC (29-31). The present study also showed a link between higher CYR61 expression and poorer survival outcomes of patients with GC. This was also reflected in the survival analysis of the combined expression of CYR61 with CTGF, or CTGF and NOV (Fig. 1). CTGF and NOV alone or combined did not exhibit any correlation with survival (data not shown). This suggests that a more profound role is played by CYR61 in GC which is also supported by studies of this molecule in GC (30,36). In a comparison with NOV expression and corresponding association between elevated NOV expression and local invasion and poorer differentiation of GCs, the increased expression CYR61 exhibited little implication with these clinicopathological features of the disease apart from its correlation with poor prognosis. A larger cohort of GC tissue samples may help to clarify this. Better understanding of the molecular mechanisms of GC may shed light on this issue in the near future.

Although the expression of CTGF transcripts in the present cohort of GCs was not different from its expression in the paired adjacent normal gastric tissues, its expression was increased in more invasive tumours. This tends to concur with observations from other studies focusing on this molecule and its role in GC. Higher expression of CTGF in GC exhibits involvement in local lymph node metastasis and also peritoneal metastasis (37,38). Suppression of CTGF inhibits the growth and invasion of GC cells, and also their peritoneal dissemination (39). In addition to its role in invasion and metastases, CTGF also promotes angiogenesis to facilitate tumour growth (40).

In addition to the evaluation of NOV, CYR61 and CTGF transcripts in the GC samples, we further examined the impact of NOV on cellular functions in GC cell lines. The two GC cell lines examined in the present study exhibited differential expression of NOV, where NOV was highly expressed in the HGC27 cells and almost absent in the AGS cells. This allowed us to establish contrasting models, i.e. overexpression of NOV in AGS cells and knockdown of NOV expression in HGC27 cells, for examining the consequent effect on the cellular functions. NOV overexpression promoted the *in vitro* proliferation of AGS cells while the knockdown resulted in reduced proliferation of the HGC27 cells. A similar effect was observed in regards to the invasion of these two GC cell lines. These results indicate a positive role played by NOV in promoting proliferation and invasion of GC which is consistent with its increased expression in the GC tumour samples. Increased expression of NOV has also been observed in other malignancies, such as cervical and prostate cancer (19,20). Certainly, an inhibitory effect on cellular functions has also been noted for NOV in a variety of cancer cells. For example, NOV had an antiproliferative effect on glioblastoma cells by interfering with S/G2 transition of the cell cycle leading to an accumulation at the S phase (25). Gap junction protein connexin 43 was also found to be involved in its inhibitory effect on the proliferation of glioma cells (41). NOV has also been reported to decrease the transcription and activation of matrix metalloproteinases and suppress the invasion of melanoma cells (42). However, our *in vitro* experimental data indicate that NOV promotes both proliferation and invasion of GC cells. Further investigation may shed light on the underlying molecular mechanisms for such a differential impact.

In summary, the expression of NOV and CYR61 was increased in GC. The elevated expression of CYR61 was associated with poorer survival, and NOV promoted the proliferation and invasion of GC cells.

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