Expression of CCN family members correlates with the clinical features of hepatocellular carcinoma

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Abstract. Studies have reported that the CCN family of proteins plays an important role in stimulating tumorigenesis. However, the relationship between the CCN protein family members and the features of hepatocellular carcinoma (HCC) remains unclear. The objective of this study was to determine the relationship between the expression levels of CCN protein family members and the features of HCC. Expression levels of the CCN family of proteins in 80-paired primary HCC samples and 11 normal liver samples were determined by a quantitative real-time PCR assay. Enhanced expression of nephroblastoma overexpressed protein (NOV) and decreased expression of Wnt-induced secreted protein 1 (WISP1), cysteine-rich protein 61 (CYR61) and connective tissue growth factor (CTGF) were found in HCC samples when compared to levels in matched non-cancerous tissues. No significant difference in WISP2 was found between matched-pair samples; only a few samples showed WISP3 expression. Furthermore, the expression levels of NOV, WISP1 and CYR61 were closely correlated with certain clinical features, including venous invasion, cellular differentiation, pTNM stage, disease-free survival and overall survival. Our results suggest that HCC progression may be enhanced by NOV and suppressed by WISP1 and CYR61. Our statistical analysis suggests that these proteins may be valuable in determining the prognosis of this deadly disease and directs attention to modulating the levels of these proteins as a potential mode of therapy.

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Introduction

Primary hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third most frequent cause of worldwide cancer mortality (1,2). Hepatocarcinogenesis consists of multiple steps leading to the accumulation of genetic and epigenetic changes in hepatic cells, which eventually cause malignant progression (3). Recent studies revealed that the Wnt/β-catenin and p53 pathways are the two most important pathways in the malignant development of HCC (4).

The CCN family of proteins was named for its first three described members: cysteine-rich protein 61 (CYR61/CCN1), connective tissue growth factor (CTGF/CCN2) and nephroblastoma overexpressed protein (NOV/CCN3) (5). It includes six members: CYR61, CTGF, NOV, Wnt-induced secreted protein 1 (WISP1/CCN4), WISP2/CCN5 and WISP3/CCN6 (6-9). All of the CCN proteins consist of 4 specific structural domains: insulin-like growth factor binding protein, thrombospondin type 1, Von Willebrand type C and a COOH-terminal domain. These specific domains display a high homology to the conserved regions of many types of extracellular matrix proteins (5,10).

Previous studies have demonstrated that the CCN proteins play an important role in stimulating tumorigenesis in many cancers (10-12). Babic *et al* suggested that CYR61 may activate integrins and induce cell progression in endothelial cells (13). CTGF may stimulate the production of collagen and fibronectin due to its involvement in the TGF-β pathway and SMAD signaling (14,15). WISP1 is upregulated in breast cancers of Wnt-1 transgenic mice (8). Additionally, the upregulation of WISP1 may induce the transformation of kidney fibroblasts in normal rats (9). Accumulating evidence has shown that the CCN family proteins participate in many fundamental cellular processes and that their expression is altered during cancer

development (6,7). Few studies have explored the level of NOV involvement in oncogenesis.

However, correlations between CCN gene expression and clinical features of HCC remain unexplored. In the present study, we used real-time qPCR to quantify the mRNA levels of the CCN family of genes in HCC samples. Expression levels of these genes were measured in 80 primary HCC samples and matched non-cancerous tissues, as well as in 11 human normal liver samples. We then examined the correlations of CCN protein expression with clinical and pathological characteristics, as well as survival data in HCC patients using several statistical methods.

Materials and methods

Patients and samples. Eighty primary HCC tissue samples with matched non-cancerous tissues and 11 normal liver tissue samples were obtained from the tissue bank of Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University (GuangZhou, China) after obtaining informed consent for utilizing the tissue specimens derived from patients. Tissues were obtained during surgical procedures. The matched non-cancerous tissues (NC) were resected at a minimum distance of 2 cm away from the cancerous tissues. The pathology of each sample was confirmed by histological examination, and the samples were stored at -80˚C until RNA was extracted. The use of clinical specimens in the present study was approved by the Ethics Committee of Sun Yat-Sen Memorial Hospital of Sun Yat-sen University. Two of the 80 patients died during the preoperative period, 1 patient was lost during the follow-up period and all other patients participated in regular postoperative follow-up evaluations.

Cellular differentiations of HCC samples were characterized by Edmondson's classification and divided into stages I/II and III/IV. The pTNM stages of patients were divided into two groups: stages I/II and III/IV, as described in the International Union Against Cancer (UICC), 6th edition, 2002.

RNA extraction and cDNA synthesis. Total RNA was extracted from all tested samples using the TRIzol reagent (#15596-026; Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality of the RNA was determined by electrophoresis on an agarose gel stained with GoldView (Tiangen Biotech Co., Ltd., Beijing, China). A total of 2.5 μg of each RNA in a total volume of 50 μl was used to generate cDNA by reverse transcription with the PrimeScript™ RT-PCR kit (#RR014B Ax4; Takara Biotechnology, Dalian, China), following the manufacturer's instructions.

Real-time quantitative PCR. Real-time qPCR was used to determine the initial detection of the PCR product during the amplification cycle. The parameter C_t was defined as the detected cycle number at which the fluorescence reached a fixed threshold value above the baseline. The amount of CCN mRNA in test samples was quantified by measuring C_t and calculating the mRNA concentration from the matched standard curve. Additionally, the amount of β-actin mRNA was quantified as an endogenous control. Once mRNA concentrations were determined, the quantity of the target gene was divided by the endogenous reference to obtain a normalized value. Finally, the relative expression levels (termed ΔCCN) of the target genes in the HCC samples were calculated according to the following formula:

$$\Delta CCN = (CCN_{HCC}/\beta\text{-actin}_{HCC})/(CCN_{matched\ NC}/\beta\text{-actin}_{matched\ NC})$$

Table I. Oligonucleotide primer sequences used for qPCR.

Gene	Oligonucleotide sequences	Product size (bp)
CYR61		
5'-primer	5'-TCACCCTTCTCCACTTGACC-3'	153
3'-primer	5'-AGTCCTCGTTGAGCTGCTTG-3'	
CTGF		
5'-primer	5'-CAAGGGCCTCTTCTGTGACT-3'	129
3'-primer	5'-CAGCTGCTCTGGAAGGACTCT-3'	
NOV		
5'-primer	5'-AAGAGCTGTGGTATGGGGTTC-3'	175
3'-primer	5'-GGTGGATGGCTTTGAGTGAC-3'	
WISP1		
5'-primer	5'-CCATACACTCATTAAGGCAGGGAA-3'	105
3'-primer	5'-GGTTGATAGGAGCGTGTGCTG-3'	
WISP2		
5'-primer	5'-AGCCCAAGGACCCCAGTT-3'	155
3'-primer	5'-TCTCCAGTCGGCAGAAGC-3'	
WISP3		
5'-primer	5'-CTGGCCTGGCACAGTTCT-3'	175
3'-primer	5'-TCTCTCACCAGGCTCACTCC-3'	
β-actin		
5'-primer	5'-CTCCCTGGAGAAGAGCTACG-3'	115
3'-primer	5'-ACAGGACTCCATGCCCAG-3'	

CYR61, cysteine-rich 61; CTGF, connective tissue growth factor; NOV, nephroblastoma overexpressed protein; WISP, Wnt-induced secreted protein.

Primers. Primers (Table I) were designed by performing BLAST searches within the GenBank database to confirm the specificity of the nucleotide sequences and the absence of DNA polymorphisms. At least one of the two primers corresponded to a different exon or the junction between 2 exons to avoid amplification of genomic DNA contamination.

Standard curve construction. The standard templates were constructed according to the manufacturer's instructions described in the qPCR Technical Guideline (16). The standard curve was produced by 10-fold serial dilution of standard templates. The linear relationship between the C_t value and the log of the initial copy number was clearly demonstrated in all cases. The standard curves for CYR61 were constructed and displayed in Fig. 1.

PCR amplification. The PCR reaction mixture for DNA amplification included 3 μl of cDNA, 10 μl of GoTaq® qPCR Master Mix (Promega Biotech Co., Ltd., Madison, WI, USA),

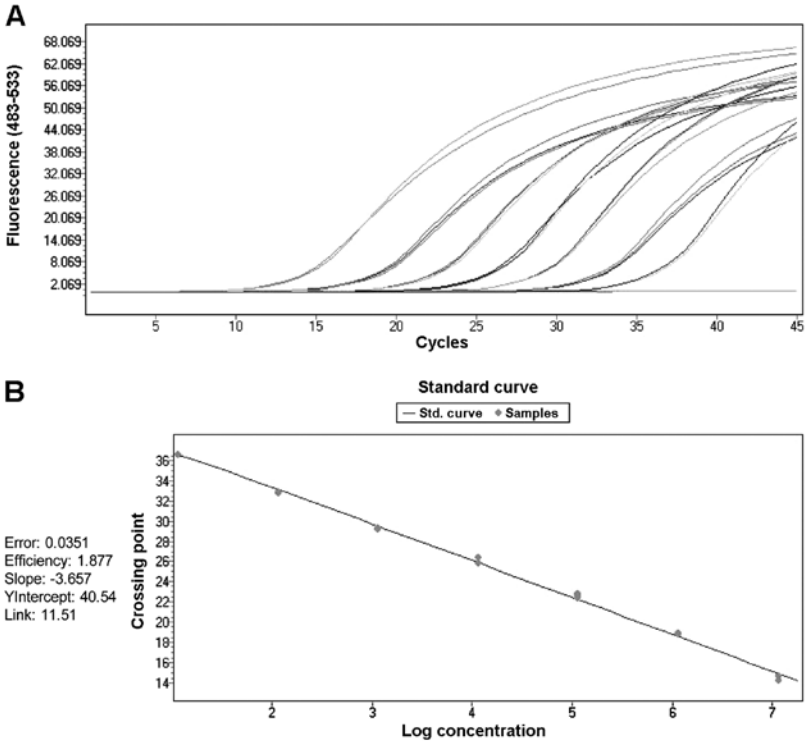

Figure 1. The standard curve of CYR-61. (A) Amplification plots for reactions with 7 points of the CYR-61 standard curve; (B) Standard curve generated after determination of C_t values plotted against the starting quantity of target DNA. CYR61, cysteine-rich 61

1 μl of each specific primer (10 nmol/μl), and 5 μl of nuclease-free water. All reactions were performed in triplicate in a LightCycler® 480 System (F. Hoffmann-La Roche Ltd., Basel, Switzerland). The cycling conditions for the reaction were: 5 min at 95˚C, followed by 45 cycles at 95˚C for 10 sec, 20 sec at the Tm of each gene and 72˚C for 30 sec.

Statistical analysis. Student's t-test, the χ^2 test, the continuity correction test, Fisher's exact test, the log-rank test and the Cox proportional hazard model were used in this study to explore the relationship between the expression level of each gene and the clinical details, including age, gender, tumor size, α-fetoprotein (AFP), HBsAg status, direct liver invasion, bile duct invasion, venous invasion, tumor microsatellite, cellular differentiation, pTNM stage, relapse and survival data. For each gene, Kaplan-Meier analysis of disease-free and overall survival curves for patients were constructed and a log-rank test was used to analyze the equality of the curves. Kappa statistical analysis was used to study the correlations between all pairs of CCN genes. Cox regression for multivariate survival analysis was used for evaluating the prognosis covariate.

Results

We used qPCR to examine the relative expression of CCN family members. A relative expression level of ΔCCN >1 was set as the criterion for enhanced expression of the CCN genes in HCC, which resulted in the sample being classified into a positive group, while an expression level of ΔCCN ≤1 was considered a decrease in expression and the sample was classified into a negative group.

Expression of NOV in HCC. Normalized mean expression levels (CCN/β-actin) of NOV relative to standard curves are shown in Fig. 2A. Statistical analysis showed that NOV was upregulated in primary HCC samples compared to that in the paired non-cancerous tissues (P=0.037). Enhanced expression of NOV (ΔNOV >1) was found in 44 of 80 (55%) HCC samples compared to the matched non-cancerous tissues (Fig. 3A).

Univariate analysis showed a significant association between the enhanced expression of NOV and several clinical features (venous invasion, cellular differentiation, pTNM stage, disease-free and overall survival) in HCC samples. No significant difference was noted between age, gender, tumor size, AFP, HBsAg status, direct liver invasion, bile duct invasion, tumor microsatellite instability and the expression level of NOV in the HCC samples (Figs. 4A and 5A; Table IIA and Table III).

Expression of WISP1 in HCC. Normalized mean expression levels of WISP1 relative to standard curves are shown in Fig. 2B. Statistical analysis showed that the expression of WISP1 was downregulated in primary HCC samples compared to the levels in the paired non-cancerous (P<0.001) and normal liver tissues (P<0.001). Decreased expression of WISP1 (ΔWISP1 ≤1) compared to matched non-cancerous tissues was found in 65 of 80 (81%) HCC samples (Fig. 3B).

Univariate analysis showed significant associations between the decreased expression of WISP1 and several clinical features (tumor size, direct liver invasion, venous invasion, tumor microsatellite instability, pTNM stage, disease-free and overall survival) in HCC samples. No significant differences were noted between the age, gender, AFP, HBsAg status, bile duct

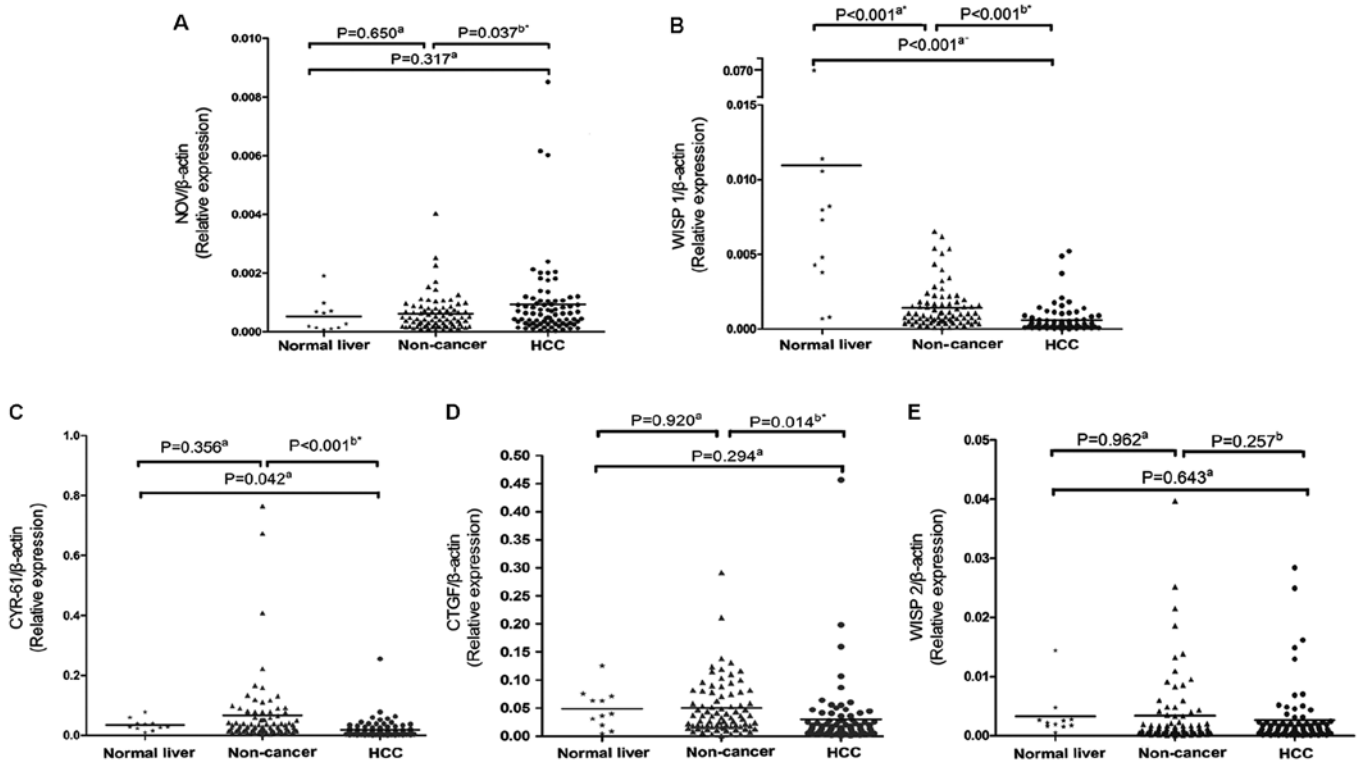

Figure 2. Normalized expression of NOV (A), WISP1 (B), CYR61 (C), CTGF (D), and WISP2 (E) in HCC patients. Normalized expression levels are displayed as a ratio of the expression of CCN genes in all samples divided by β-actin. [a]Independent-sample t-test. [b]Paired-sample t-test. HCC, hepatocellular carcinoma; CYR61, cysteine-rich 61; CTGF, connective tissue growth factor; NOV, nephroblastoma overexpressed protein; WISP, Wnt-induced protein.

invasion and cellular differentiation and the expression level of WISP2 in the HCC samples (Figs. 4B and 5B; Table IIA and Table III).

Expression of CYR61 in HCC. Normalized mean expression levels of CYR61 relative to standard curves are shown in Fig. 2C). Statistical analysis showed that CYR61 was down-regulated in the primary HCC samples compared with that in the paired non-cancerous tissues (P<0.001). Decreased expression of CYR61 (ΔCYR61 ≤1) compared to matched non-cancerous tissues was found in 71 of 80 (88%) HCC samples (Fig. 3C).

Nineteen patients with either enhanced expression of CYR61 (ΔCYR61 >1, n=9) or expression of CYR61 that was decreased <2-fold (ΔCYR >-1, n=10) were defined as the CYR61 high-expression group. Samples from these patients were compared to the samples from patients whose CYR61 level was decreased >2-fold (ΔCYR <-1, n=61), which were defined as the CYR61 low-expression group. The results of univariate analysis showed a significantly disease-free and longer overall survival in the CYR61 high-expression group. However, no significant differences were noted between the level of CYR61 expression and other clinical features (age, gender, tumor size, AFP, HBsAg status, direct liver invasion, bile duct invasion, venous invasion, tumor microsatellite, cellular differentiation and pTNM stage) (Figs. 4C and 5C; Table IIB and Table III).

Expression of CTGF in HCC. Normalized mean expression levels of CTGF relative to standard curves are shown in Fig. 2D. Statistical analysis showed that CTGF was downregulated in the primary HCC samples compared with that in the paired non-cancerous tissues (P=0.014). Enhanced expression of CTGF (ΔCTGF >1) compared to matched non-cancerous tissues was found in 20 of 80 (25%) HCC samples (Fig. 3D). Univariate analysis showed that no statistically significant correlations existed between the level of CTGF and any of the clinical features (Figs. 4D and 5D; Table IIB and Table III).

Expression of WISP2 in HCC. Normalized mean expression levels of WISP2 relative to standard curves are shown in Fig. 2E. Statistical analysis showed that the expression levels of WISP2 in primary HCC samples showed no difference from the paired non-cancerous tissues (P=0.257). Enhanced expression of WISP2 (ΔWISP2 >1) compared to matched non-cancerous tissues was found in 38 of 80 (47.5%) HCC samples (Fig. 3E). Univariate analysis demonstrated no statistical correlations between the level of WISP2 expression and any of the clinical features (Figs. 4E and 5E; Table IIB and Table III).

Expression of WISP3 in HCC. Among all of the samples examined, including HCC, NC and normal liver (NL) tissues, only a few (12/171, 7%) expressed WISP3. Therefore, we concluded that WISP3 was not expressed in the liver tissues.

Correlation among the expression levels of the CCN family of genes in HCC samples. The kappa statistical analysis, which defines a significant correlation at a κ-value >0.5, and implies no significant association at a κ-value ≤0.5, was used to examine the correlation among the expression levels of the

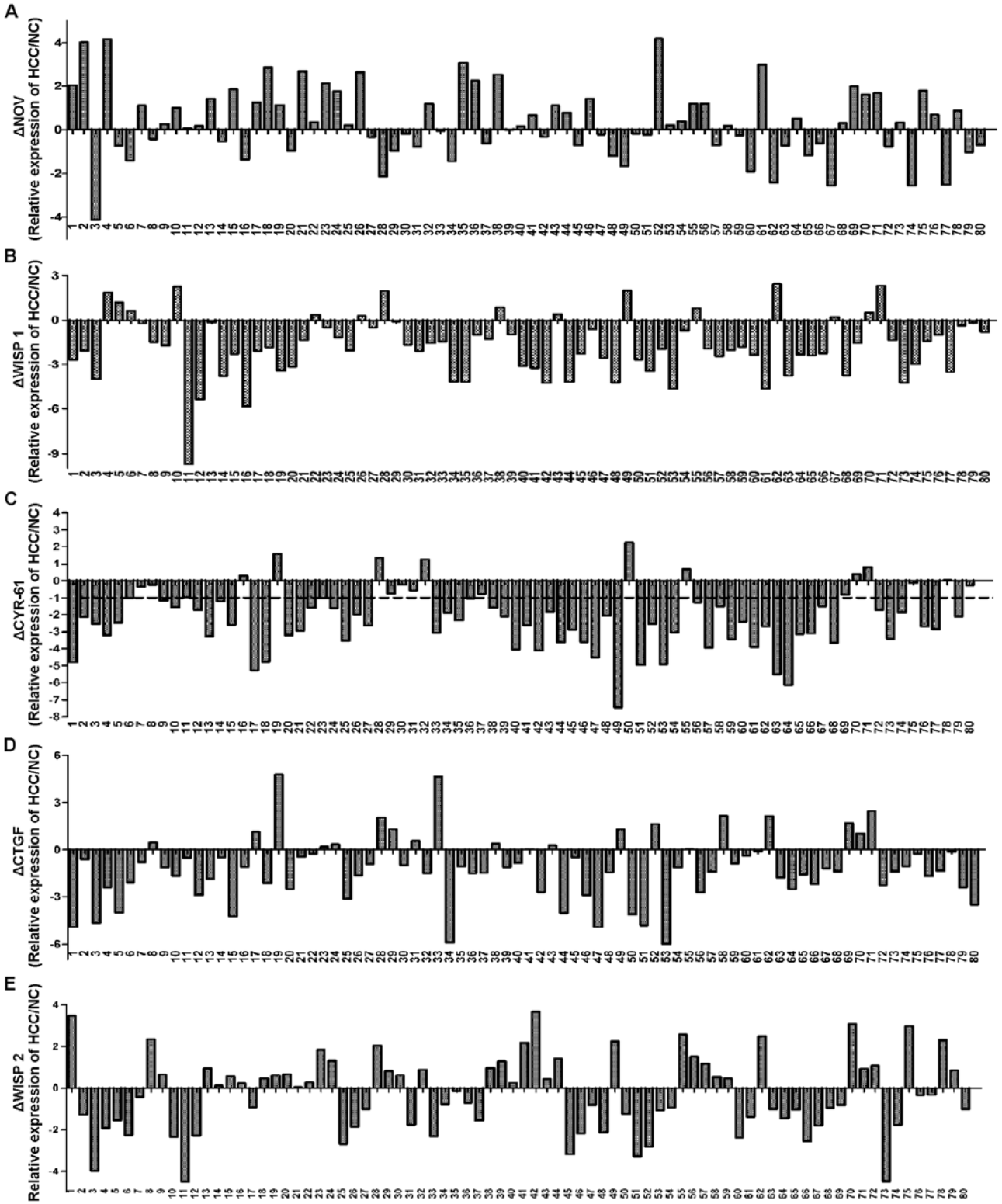

Figure 3. Relative expression of the CCN family in HCC samples compared to matched non-cancer (NC) tissues: NOV (A), WISP1 (B), CYR61 (C), CTGF (D), and WISP2 (E). HCC, hepatocellular carcinoma; CYR61, cysteine-rich 61; CTGF, connective tissue growth factor; NOV, nephroblastoma overexpressed protein; WISP, Wnt-induced protein.

NOV, WISP1, CYR61, CTGF and WISP2 genes. The results showed that no correlation existed among the expression levels of these 5 CCN genes in the HCC patients (Table IV).

Correlation between overall survival and expression of the CCN gene family in HCC samples. Univariate survival analysis with a log-rank test and the Cox proportional hazard model for continuous variables was used to study the relationship between overall survival and the expression of each CCN gene in the HCC samples (Table III). The overall length of survival was significantly correlated with the expression levels of NOV, WISP1 and CYR61, but not CTGF or WISP2.

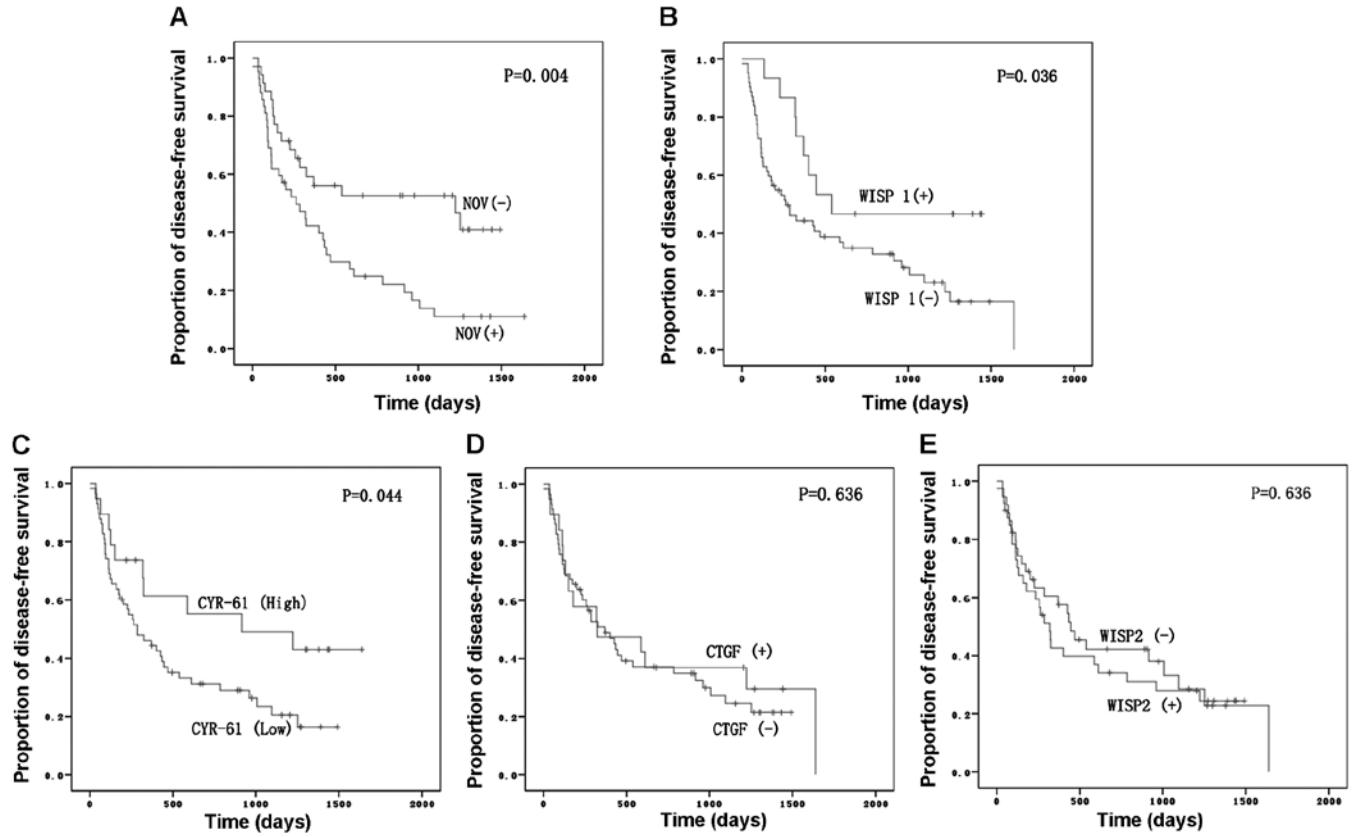

Figure 4. Disease-free survival curves of HCC patients subgrouped according to expression levels of the CCN family: NOV (A), WISP1 (B), CYR61 (C), CTGF (D) and WISP2 (E). HCC, hepatocellular carcinoma; CYR61, cysteine-rich 61; CTGF, connective tissue growth factor; NOV, nephroblastoma overexpressed protein; WISP, Wnt-induced protein.

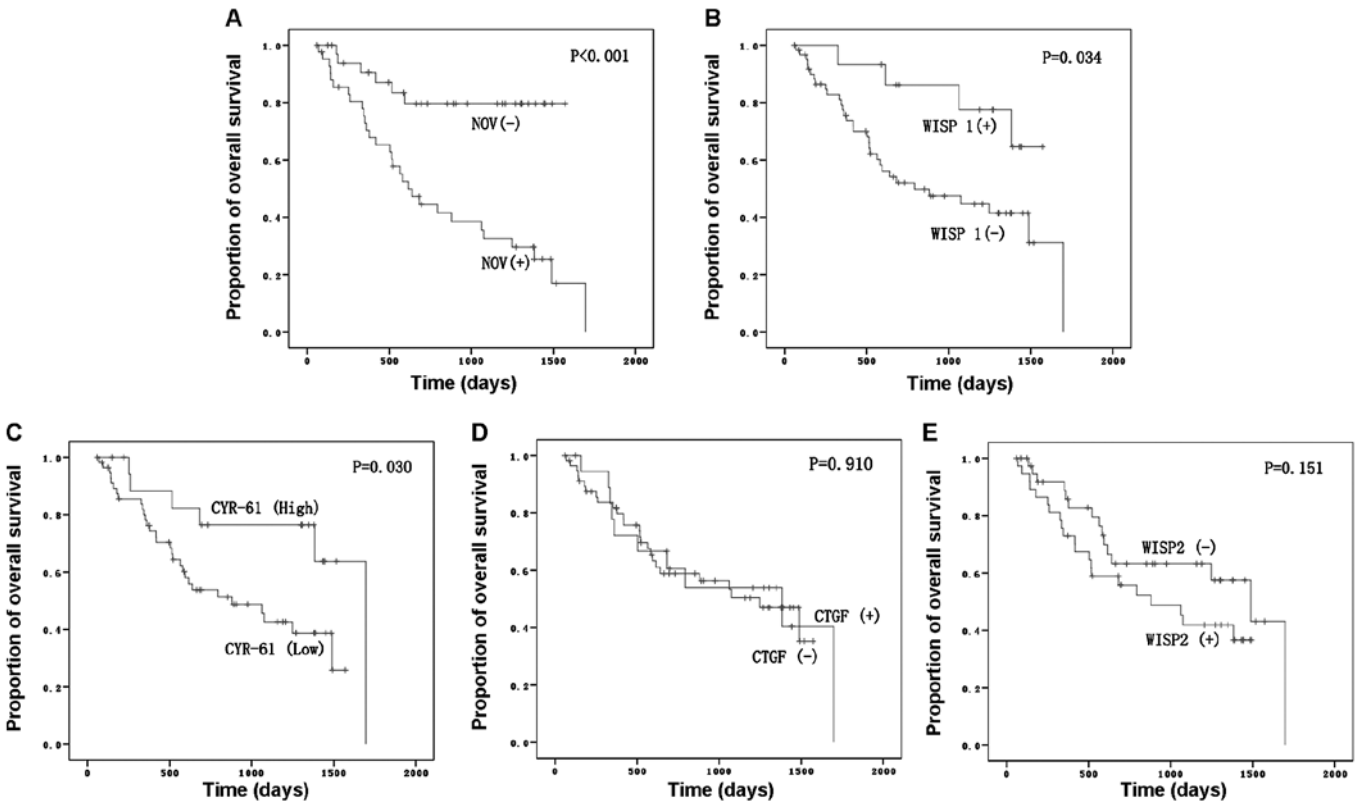

Figure 5. Overall survival curves of HCC patients subgrouped according to expression levels of the CCN family: NOV (A), WISP1 (B), CYR61 (C), CTGF (D) and WISP2 (E). HCC, hepatocellular carcinoma; CYR61, cysteine-rich 61; CTGF, connective tissue growth factor; NOV, nephroblastoma overexpressed protein; WISP, Wnt-induced protein.

Table II. Relationships between the expression levels of NOV, WISP1, CRY61, CTGF and WISP2 and the clinical features of the HCC patients.

A, Relationships between the expression levels of NOV and WISP1 and the patient clinical features

Clinical characteristics	NOV expression			WISP1 expression		
	Negative (n=36)	Positive (n=44)	P-value	Negative (n=65)	Positive (n=15)	P-value
Age (years)	48.2±11.1	51.4±10.5	0.187[a]	51.3±11.7	46.3±7.5	0.120[a]
Gender			0.234[b]			0.229[c]
Male	31	42		61	12	
Female	5	2		4	3	
Tumor size (cm)	7.2±4.7	8.8±3.5	0.092[a]	8.8±4.0	5.0±3.1	0.001[a,e]
AFP			0.246[d]			0.336[d]
Negative	16	14		26	4	
Positive	20	30		39	11	
HBsAg			0.927[d]			0.467[c]
Negative	6	7		12	1	
Positive	30	37		53	14	
Direct liver invasion			0.486[d]			0.010[d,e]
Negative	15	15		20	10	
Positive	21	29		45	5	
Bile duct invasion						0.090[c]
Negative	32	33	0.113[d]	50	15	
Positive	4	11		15	0	
Venous invasion			0.021[d,e]			0.018[c,e]
Negative	16	9		16	9	
Positive	20	35		49	6	
Tumor microsatellite instability			0.271[d]			0.019[d,e]
Negative	24	24		35	13	
Positive	12	20		30	2	
Cellular differentiation			0.036[d,e]			0.091[d]
I/II	24	19		32	11	
III/IV	12	25		33	4	
pTNM stage			<0.001[d,e]			<0.001[d,e]
I/II	24	12		23	13	
III/IV	12	32		42	2	

B, Relationships between expression level of CRY61, CTGF, WISP2 and the patient clinical features

Clinical characteristics	CYR61 expression			CTGF expression			WISP2 expression		
	Low (n=61)	High (n=19)	P-value	Negative (n=60)	Positive (n=20)	P-value	Negative (n=42)	Positive (n=38)	P-value
Age (years)	51.0±11.0	46.8±9.8	0.144[a]	49.5±12.1	49.5±10.4	0.542[a]	49.8±12.2	50.2±9.2	0.879[a]
Gender			1.000[b]			0.493[c]			1.000[b]
Male	17	56		56	17		38	35	
Female	2	5		4	3		4	3	
Tumor size (cm)	8.3±4.1	7.3±4.2	0.351[a]	8.5±4.4	6.9±3.2	0.141[a]	8.7±4.5	7.4±3.6	0.170[a]
AFP			0.542[d]			0.424[d]			0.298[d]
Negative	24	6		21	9		18	12	
Positive	37	13		39	11		24	26	

Table II. Continued.

Clinical characteristics	CYR61 expression			CTGF expression			WISP2 expression		
	Low (n=61)	High (n=19)	P-value	Negative (n=60)	Positive (n=20)	P-value	Negative (n=42)	Positive (n=38)	P-value
HBsAg			0.769[b]			0.115[c]			0.617[d]
Negative	9	4		7	6		6	7	
Positive	52	15		53	14		36	31	
Direct liver invasion			0.946[d]			0.424[d]			0.563[d]
Negative	23	7		21	9		17	13	
Positive	38	12		39	11		25	25	
Bile duct invasion			0.101[d]			0.408[c]			0.943[d]
Negative	9	6		47	18		34	31	
Positive	52	13		13	2		8	7	
Venous invasion			0.547[d]			0.889[c]			0.587[d]
Negative	18	7		19	6		12	13	
Positive	43	12		41	14		30	25	
Tumor microsatellite instability			0.391[d]			0.292[d]		0.927[d]	
Negative	26	6		34	14		25	23	
Positive	35	13		26	6		17	15	
Cellular differentiation			0.678[d]			0.518[d]			0.108[d]
I/II	32	11		28	11		19	24	
III/IV	29	8		32	9		23	14	
pTNM stage			0.444[d]			0.604[d]			0.685[d]
I/II	26	10		26	10		18	18	
III/IV	35	9		34	10		24	20	

[a]Independent-sample t-test; [b]Fisher's exact test; [c]continuity correction test; [d]Pearson's chi-squared test; [e]Statistically significant values ($p<0.05$); HCC, hepatocellular carcinoma; CYR61, cysteine-rich 61; CTGF, connective tissue growth factor; WISP, Wnt-induced secreted protein; NOV, nephroblastoma overexpressed protein.

While controlling for clinical details (age, gender, tumor size, AFP, HBsAg status, direct liver invasion, bile duct invasion, venous invasion, tumor microsatellite instability, cellular differentiation, pTNM stage and tumor relapse), Cox regression for multivariate survival analysis was performed to estimate the possible effects of the 5 genes on cancer prognosis. With the Enter method in Cox regression for survival, NOV was the most significant independent predictor for relative risk of death (RR=0.252, P=0.002) (Table V). Stepwise Cox regression for multivariate survival analysis using the method of Forward Stepwise Likelihood Ratio was used to evaluate the prognostic co-variates. The results showed that the best model for the HCC prognosis in the present study contained 2 covariates: NOV and CYR61 (Table V). The RR of NOV was 0.216, meaning that the survival probability for patients with positive NOV expression may be decreased to 21.6% of that of patients with negative NOV expression. While the RR of CYR61 was 2.77, which meant that the survival probability of patients whose tumors had enhanced CYR61 expression was 2.77-fold higher compared to those whose tumors showed decreased expression of CYR61.

Discussion

Previous studies have demonstrated that the CCN family of genes plays an important role in enhancing the tumorigenesis of many types of cancer. Inhibition of their expression in these tumors slows tumor growth. In contrast, several of these CCN proteins are expressed at lower levels in selected cancers, and by forcing their expression, tumor growth may be slowed or halted (10-12). In the present study, high expression levels of NOV and low expression levels of CYR61 and WISP1 appear to have profound effects on the behavior of HCC, and may influence disease-free and overall survival.

NOV was first reported as an overexpressed gene in nephroblastomas induced by the myeloblastosis virus (17). Previous studies have suggested that NOV has a conflicting role in different types of cancers. For example, NOV had an inhibitory effect on cell growth in the C6 glioma cell line (18) and normal endothelial cells (19). Other investigators have shown that the amino-truncation of the NOV protein induced morphological transformation (16,20) and oncogenic activation (21,22). The NOV protein also promoted bone

Table III. Correlations between overall survival and expression of NOV, WISP1, CYR61, CTGF and WISP2 in HCC patients.

Factors	Alive (n=41)	Deceased (n=36)	Mean survival (days)	SE	P-value
Age (years)	49.83±12.1	49.56±8.3			0.880[a]
Gender					0.720[b]
Male	36	34	1,054.7	80.4	
Female	5	2	996.6	243.4	
Tumor size (cm)	7.1±4.6	9.1±3.5			0.024[a,c]
AFP					0.663[b]
Negative	17	12	1,032.5	109.6	
Positive	24	24	1,046.7	99.1	
HBsAg					0.870[b]
Negative	7	5	989.7	165.2	
Positive	34	31	1,060.4	84.2	
Direct liver invasion					0.033[b,c]
Negative	18	10	1,295.1	115.9	
Positive	23	26	882.3	86.4	
Bile duct invasion					
Negative	37	25	1,097.4	72.5	0.018[b,c]
Positive	4	11	705.5	180.1	
Venous invasion					<0.001[b,c]
Negative	21	4	1,294.8	67.2	
Positive	30	32	862.5	93.0	
Tumor microsatellite instability					0.001[b,c]
Negative	31	16	1,266.3	94.3	
Positive	10	20	741.1	101.4	
Cellular differentiation					0.155[b]
I/II	24	16	1,111.1	91.1	
III/IV	17	20	928.4	113.0	
pTNM stage					<0.001[b,c]
I/II	28	7	1,338.4	78.5	
III/IV	13	29	789.0	98.1	
NOV					<0.001[b,c]
Negative	29	6	1,329.4	89.0	
Positive	13	30	828.9	93.6	
WISP1					0.034[b,c]
Negative	30	32	968.4	88.6	
Positive	11	4	1,351.9	103.4	
CYR61					0.030[b,c]
Low expression	28	30	924.33	79.9	
High expression	13	6	1,358.2	144.8	
CTGF					0.910[b]
Negative	32	26	1,011.2	80.9	
Positive	9	10	1079.1	156.1	
WISP2					0.151[b]
Negative	25	15	1,177.7	109.8	
Positive	16	21	897.3	92.1	

[a]Cox regression model test for continuous variable; [b]log-rank test. [c]P<0.05 are set for statistically significant; HCC, hepatocellular carcinoma; CYR61, cysteine-rich 61; CTGF, connective tissue growth factor; NOV, nephroblastoma overexpressed protein; WISP, Wnt-induced protein.

Table IV. Correlations among the expression levels of NOV, WISP1, CYR61, CTGF and WISP2 in the HCC sample.

Genes		Expression status of CCN						
A	B	$A^- B^-$	$A^- B^+$	$A^+ B^-$	$A^+ B^+$	κ-value	SE	95% CI
NOV	WISP-1	28	8	37	7	-0.059	0.083	-0.222, 0.104
NOV	CYR-61	32	4	39	5	0.002	0.065	-0.125, 0.129
NOV	CTGF	29	7	31	13	0.095	0.09	-0.081, 0.271
NOV	WISP-2	23	13	19	25	0.204	0.108	-0.00768, 0.416
WISP-1	CYR-61	60	5	11	4	0.224	0.135	-0.0406, 0.489
WISP-2	CTGF	52	13	8	7	0.236	0.123	-0.00508, 0.477
WISP-3	WISP-2	37	28	5	10	0.148	0.09	-0.0284, 0.324
CYR-61	CTGF	55	16	5	4	0.143	0.116	-0.0844, 0.370
CYR-61	WISP-2	40	31	2	7	0.142	0.074	-0.00304, 0.287
CTGF	WISP-2	37	23	5	15	0.282	0.096	0.0938, 0.470

CI, confidence interval; HCC, hepatocellular carcinoma; CYR61, cysteine-rich 61; CTGF, connective tissue growth factor; NOV, nephroblastoma overexpressed protein; WISP, Wnt-induced protein.

Table V. Cox regression for multivariate overall survival analysis of NOV, WISP1, CYR61, CTGF, and WISP2 for the HCC patients.

A, Method = Enter

Gene	N	Wald	RR	95% CI	P-value
NOV	77	9.244	0.252	0.104-0.613	0.002
WISP1	77	3.506	2.84	0.952-8.468	0.061
CYR61	77	3.196	2.521	0.915-6.950	0.074
CTGF	77	0.192	0.827	0.354-1.931	0.661
WISP2	77	1.486	0.637	0.308-1.316	0.223

B, Method = Forward Stepwise Likelihood Ratio

Step	Gene	B	SE	Wald	RR	95% CI	P-value
1	NOV	-1.534	0.449	11.68	0.216	0.089-0.52	0.001
2	CYR61	1.01019	0.489	4.341	2.77	1.062-7.226	0.037
	NOV	-1.534	0.449	11.663	0.216	0.089-0.52	0.001

CI, confidence interval; HCC, hepatocellular carcinoma; CYR61, cysteine-rich 61; CTGF, connective tissue growth factor; NOV, nephroblastoma overexpressed protein; WISP, Wnt-induced protein.

metastasis in prostate cancer through the RANKL-dependent pathway (23). To our knowledge, this represents the first major analysis of NOV in tissue samples from HCC patients. In the present study, 44 of 80 (55%) HCC samples overexpressed NOV, and the expression levels were significantly upregulated in HCC samples compared to the matched non-cancerous tissues. Furthermore, the expression levels were significantly associated with venous invasion, cellular differentiation and pTNM stage in HCC patients, as well as disease-free and overall survival of HCC patients. These conclusions suggest that NOV may play an important role in the progression of HCC, which merits further investigation.

WISP1 was first identified as an upregulated gene in C57 MG mouse mammary epithelial cells transformed with Wnt-1 (8). Additionally, WISP1 has been suggested to either inhibit or enhance tumor development. For example, the expression of WISP1 was inversely correlated with the proliferation and metastatic growth of melanoma cells (24,25). In contrast, human breast cancers overexpressed this protein (8). Overexpression of WISP1 promotes the growth of normal kidney fibroblasts in rats and induces tumor formation in nude mice (9). In the present study, we found that WISP1 was significantly downregulated in HCC samples. The expression level was inversely associated with tumor size, direct liver invasion, venous invasion, tumor microsatellite instability, pTNM stage, disease-free and overall survival.

The CYR61 protein was the first described member of the CCN family and has a rich cysteine motif. Previous studies have shown that CYR61 is overexpressed and tumorigenic in breast cancers (26), colon adenocarcinomas, and bladder papillomas (19). In contrast, forced expression of CYR61 in lung cancers slowed the growth of cancerous cells (27), Similarly, studies have shown that CYR61 is downregulated in HCC samples. Forced expression of CYR61 in HCC cell lines suppressed cell proliferation, whereas downregulation by siRNA increased cell proliferation, mainly though the DNA damage response and p53 pathways (28). In the present study, 71 of 80 (88%) HCC samples had CYR61 expression levels significantly lower than that in the matched non-cancerous tissues. Furthermore, low expression levels of CYR61 were significantly associated with shorter disease-free and shorter overall survival of the HCC patients.

CTGF was discovered in the conditioned medium of human umbilical vein endothelial cells. Previous studies have shown that CTGF plays an oncogenic role in the development of human HCC (29). Downregulation of CTGF by inhibition of TGF-β blocked the tumor-stroma crosstalk and tumor progression in HCC (30). Furthermore, CTGF was found to

be involved in the development of liver fibrosis, mainly by hepatic stellate cells (31,32), and indeed, fibrotic liver tissues express high levels of CTGF (33-36). Gressner *et al* reported that the serum CTGF-concentrations were higher in cirrhotic patients than in HCC patients with or without cirrhosis (37). The present study found that levels of CTGF were higher in non-cancerous tissues than that in matched HCC samples, most likely because many non-cancerous tissues were from patients with liver cirrhosis.

WISP2 was found to be upregulated in central nervous tissues in GM3-only mice (38) and is an oncogenic factor in human breast cancers (39,40). In the present study, the expression levels of WISP2 in primary HCC showed no difference from levels in the paired non-cancerous tissues. Additionally, no significant relationship was found between the expression of WISP2 and the clinical features. These findings suggest that WISP2 may not be involved in the development of HCC.

In conclusion, our results revealed that the enhanced expression of NOV and decreased expression of WISP1 and CYR61 correlate statistically with the overall prognosis of HCC patients, as well as their pathological and clinical features. The data suggest that NOV appears to play an oncogenic role, while WISP1 and CYR61 may have suppressive roles in either the development or progression of HCC. These findings are the first steps in exploring the functional mechanisms by which the CCN protein family members participate in HCC, which may include determining the signals that control the expression of these CCN proteins and promote the transformation and/or progression of HCC. Understanding these mechanisms will prove useful for the diagnosis, prognosis and therapy of HCC.

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