

# Association between Gene Polymorphisms of Connective Tissue Growth Factor and the Progression of Chronic Liver Disease Associated with Hepatitis C

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## Abstract

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**Objective** Fibrogenic cytokines, such as transforming growth factor-beta 1 play a central role in the progression of liver fibrosis. Recently, functional gene polymorphisms in these cytokines have been identified, and some reports have validated the presence of associations between these polymorphisms and disease progression. Connective tissue growth factor (CTGF) is a stimulating factor for fibroblast proliferation and matrix production. This study aimed to examine the relationship between CTGF gene polymorphisms and the progression of hepatitis C virus (HCV)-related chronic liver disease, as well as the incidence and prognosis of hepatocellular carcinoma (HCC).

**Methods** A review was conducted among 235 HCV patients (117 patients with chronic hepatitis (CH) and 118 patients with liver cirrhosis (LC)). The CTGF gene polymorphism (rs6918698; -945 G/C) was identified according to the chimeric cycling probe method. The rate of liver fibrosis progression was measured using two liver fibrosis prediction formulas, the Forns index and the FibroIndex. All HCC patients were followed regularly every month.

**Results** The frequency of the -945 C allele was higher among the LC patients than the CH patients. Regarding the rate of liver fibrosis progression over five years, C homozygotes tended to exhibit a faster rate than G carriers, although the difference was not significant. Among the LC patients, the C homozygotes demonstrated lower prothrombin times, higher rates of indocyanine green retention and higher Child-Pugh scores than the G carriers. There were no significant tendencies in the genotype distribution, irrespective of the status of HCC. However, the prognosis of HCC was poorer for the C homozygotes than for the G carriers.

**Conclusion** A CTGF -945 C homozygote status is a significant risk factor for the progression of HCV-related chronic liver disease, including HCC.

**Key words:** connective tissue growth factor (CTGF), liver fibrosis, genotype, HCV, hepatocellular carcinoma (HCC)

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## Introduction

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Hepatitis C virus (HCV)-related chronic liver disease is one of the most important health issues worldwide. Approximately 170 million people, or 3% of the world's population, are chronically infected with HCV (1). Chronic HCV infection commonly induces reactive immune inflammation,

which results in continuous liver tissue damage and the progression of liver fibrosis to cirrhosis. Liver fibrosis is a highly dynamic process, and the rate at which fibrosis develops exhibits substantial individual variation. Previous reports have shown that individual features, such as a male gender, older age, infection and excessive alcohol consumption, are significantly associated with the progression of liver fibrosis (2). However, factors promoting the progres-

sion of HCV-related liver fibrosis remain poorly understood.

Transforming growth factor-beta 1 (TGF- $\beta$ 1) is the central cytokine responsible for hepatic fibrogenesis (3). Connective tissue growth factor (CTGF; also named CCN2) is a representative TGF- $\beta$  downstream modulator that functions as a profibrotic cytokine (4). CTGF is produced by hepatocytes, hepatic stellate cells (HSCs: Ito cells), myofibroblasts and cholangiocytes (5, 6). CTGF is associated with key fibrosis functions, including the transdifferentiation of HSCs into myofibroblasts, proliferation of fibroblasts, production of the extracellular matrix (ECM) and formation of adhesion and granulation tissue (7). CTGF also contributes to the development of fibrosis by acting in synergy with various profibrogenic growth factors, including platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) (7).

Numerous functional gene polymorphisms of ECM metabolism-related factors have been identified, and previous reports have demonstrated possible relationships between these gene polymorphisms and the progression of fibrosis of chronic liver disease (8). We previously studied the relationships between HCV-related chronic liver disease progression and the presence of functional single-nucleotide polymorphisms (SNPs) of inflammatory cytokines (interleukin-1 beta (IL-1 $\beta$ ), an IL-1 receptor antagonist and IL-10), TGF- $\beta$ , matrix metalloproteinases (MMP-1, -2, -3, -7 and -9) and tissue inhibitors of MMPs (TIMP-1 and -2). Many of these SNPs are associated with liver fibrosis and the development and prognosis of HCC, at least in part (9-13). Recent reports have shown that various fibrosis-related cytokines and MMPs interact with CTGF. For example, IL-10 and TGF- $\beta$  are upstream modulators of CTGF (14). Furthermore, IL-1 $\beta$  and MMP-3 exhibit facilitative relationships with CTGF (15). Therefore, we hypothesized that CTGF gene polymorphisms affect the development and/or progression of HCV-related liver fibrosis. The CTGF gene contains regions of SNPs. A putative SNP located in the promoter region (rs6918698; -945 G/C) has recently been studied in relation to the severity of various chronic inflammatory diseases (16). In general, the G allele exhibits a higher transcriptional activity than the C allele (17). However, there are few reports confirming a relationship between CTGF -945 G/C and the progression of fibrosis in patients with chronic liver disease. In the present study, we examined the possible relationship between the CTGF -945 G/C polymorphism and the progression of chronic HCV liver disease. In addition, the degree of liver fibrosis has been shown to be a strong predictor of HCC development (18). Therefore, we additionally examined the association between the CTGF -945 G/C polymorphism and the incidence and prognosis of HCC in Japanese patients.

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## Materials and Methods

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### Subjects

All patients with chronic HCV infection treated at Tottori University Hospital were recruited for this study. Overall, 235 patients with HCV-related chronic liver disease were enrolled. All subjects were Japanese and positive for serum HCV-RNA. Patients were excluded if they had chronic hepatitis B infection, autoimmune hepatitis, primary biliary cirrhosis and/or alcoholism or the habitual ingestion of >150 g ethanol per day. Twenty patients had received interferon treatment prior to enrollment in our study, none of whom had achieved a sustained virologic response. The diagnoses of chronic hepatitis (CH) and liver cirrhosis (LC) were confirmed based on clinical features and the results of histological examinations, laboratory tests and/or imaging studies (computed tomography (CT) and magnetic resonance imaging (MRI) (19)). All of the study subjects received routine blood liver function tests every three months at our department. The liver function was observed over five years in 63 CH patients.

The diagnosis of HCC was defined based on the results of imaging studies, including angiography, dynamic contrast-enhanced CT and MRI. Forty-five patients also underwent liver histological examinations. The grade of HCC histologic differentiation was determined according to the classification of Edmondson and Steiner. Clinicopathological features, including the tumor diameter and morphology, presence of tumor thrombosis, the levels of serum tumor markers [alpha-fetoprotein (AFP) and protein induced by the lack of vitamin K or antagonists-II (PIVKA-II)] and the CLIP (Cancer of the Liver Italian Program; a prognostic scoring system for HCC patients) score, were referred to the first diagnosis of HCC. Among patients with multiple HCC tumors, the largest lesion was evaluated as the HCC diameter. Following the first diagnosis of HCC, all HCC patients received monotherapy or combination therapy consisting of surgical resection, microwave coagulation therapy (MCT), transcatheter arterial chemoembolization (TACE), percutaneous ethanol injection (PEI), percutaneous acetic acid injection (PAI), and radiofrequency ablation (RFA) and followed up regularly every month. The mean follow-up period in the HCC patients was 3.9 $\pm$ 2.5 years.

This study was approved by the Committee for the Ethics of Medical Experiments on Human Subjects of the Medical Faculty of Tottori University. Written informed consent was obtained from each subject prior to blood collection.

### DNA extraction

Genomic DNA was extracted from peripheral white blood cells using a DNA extraction kit (DNA Quick II; Dainippon Pharmaceutical, Osaka, Japan) according to the manufacturer's instructions.

**Table 1.** Allele Frequencies and Genotype Distribution of CTGF-945 G/C in CH (n=117) and LC (n=118) Patients

CTGF-945	CH patients (n = 117)			LC patients (n = 118)			p value
	CC	CG	GG	CC	CG	GG	
	27	55	35	39	54	25	0.15
	(23%)	(47%)	(30%)	(33%)	(46%)	(21%)	
Genotype	C homo		G carrier	C homo		G carrier	
	27		90	39		79	0.09
	(23%)		(77%)	(33%)		(67%)	
Allele frequencies	C		G	C		G	0.04*
	0.47		0.53	0.56		0.44	

CTGF: connective tissue growth factor, CH: chronic hepatitis, LC: liver cirrhosis

The  $\chi^2$  test was used to examine differences in the distributions of alleles and genotypes between CH patients and LC patients. \* =  $p < 0.05$

### Analysis of the CTGF -945 G/C polymorphism

The CTGF -945 G/C polymorphism was identified according to the cycling probe method using Cycleave PCR<sup>®</sup> (Takara Bio, Ootsu, Japan) and DNA/RNA chimeric probe-adapted real-time polymerase chain reaction (PCR) (20). The sequences of the primer set and probes were as follows: PCR forward primer, 5'-TTGATCATTTGTCACCTTGAGG-3'; PCR reverse primer, 5'-CTTTGCATCCCAGTGTTC-3'; G allele probe, 5'Eclipse-AGGGACgAG-FAM3'; C allele probe, 5' Eclipse-AAGGGAGgAG-FAM 3' (italic lower case letters represent RNA). PCR was carried out at a final volume of 20  $\mu$ L, including 2  $\mu$ L (10 ng) of genomic DNA, 0.4  $\mu$ L of both primers, 0.8  $\mu$ L of the cycling probe, 10  $\mu$ L of Cycleave PCR<sup>®</sup> Reaction Mix (Takara Bio), and 6.4  $\mu$ L of sterile H<sub>2</sub>O. The PCR conditions were as follows: 95°C for 25 seconds, followed by 45 cycles of denaturation at 95°C for 5 seconds, primer annealing at 55°C for 10 seconds and extension and fluorescence emission at 72°C for 15 seconds. Fluorescent signals were assessed using a LightCycler 1.5 (Roche, Mannheim, Germany).

### Analysis of the rate of liver fibrosis progression

We examined the rate of liver fibrosis progression based on long-term observations of the CH patients. The paired liver biopsy test is the gold standard for estimating the degree of liver fibrosis progression. However, liver biopsies are associated with complications, such as hemorrhage, other organ injury and infection, with consequent possible death (21). Therefore, we evaluated the rate of liver fibrosis progression noninvasively using two liver fibrosis predicting formulas based on routine laboratory tests: the Forns index (22) and the FibroIndex (23). We were able to follow 63 CH patients over five years with routine blood tests. There were nine patients in whom we were unable to collect gamma globulin values, a factor of the FibroIndex.

The formulas for the Forns index and FibroIndex are as follows:

$$\text{Forns index} = 7.811 - 3.131 \times \ln(\text{platelet count}) + 0.781 \times \ln(\text{GGT}) + 3.467 \times \ln(\text{age}) - 0.014 \times (\text{cholesterol})$$

$$\text{FibroIndex} = 1.738 - 0.064 (\text{platelet count} \times 10^4 / \text{mm}^3)$$

$$+ 0.005 (\text{AST [IU/L]}) + 0.463 (\text{gamma globulin [g/dL]})$$

The changes in the index values were calculated by subtracting the patient's values obtained at the time of study enrollment from the values obtained at the completion of the five-year follow-up period and comparing the mean value with the incidence of the CTGF -945 G/C genotype. The patients were divided into the following two groups based on the presence/absence of progression of liver fibrosis those exhibiting an index change of  $>0$  after five years were defined as demonstrating progression, while those with an index change of  $<0$  were defined as not demonstrating progression.

### Statistical analysis

The values are expressed as the mean  $\pm$  SD. Differences in the genotype distribution were determined using the chi-square test. Differences in data between the two groups were assessed using Student's t-test, the chi-square test and Fisher's exact test, as appropriate. We used a logistic regression analysis to adjust for aging, and the log-rank test to examine differences in the overall survival rate obtained according to the Kaplan-Meier method. A p value of  $<0.05$  was considered to be statistically significant.

## Results

The genotype distributions and allele frequencies of CTGF -945 G/C among the Japanese patients with chronic HCV infection are shown in Table 1. The CTGF -945 G/C genotype was distributed in accordance with the Hardy-Weinberg equation ( $p=0.78$  and  $p=0.45$  for CH patients and LC patients, respectively) (24). The C allele frequency of CTGF -945 G/C was significantly higher in the LC patients than in the CH patients ( $p=0.04$ ), although there were no significant differences in genotype between the two groups.

Regarding the relationship between the genotype and the rate of liver fibrosis progression in the CH patients, the CTGF -945 C homozygotes tended exhibit a faster rate of progression of fibrosis than the G carriers, as calculated using both the Forns index and FibroIndex. However, the difference was not significant (Table 2).

**Table 2. Liver Fibrosis Progression Rates over a 5-year Follow-up Period in CH Patients; Assessed by Forns Index and FibroIndex**

		CTGF -945 G/C		p value
		G carrier	C homo	
Forns index difference (Mean ± SD)		0.24 ± 1.10	0.59 ± 1.21	0.35
Change in Forns index (n=63)	Unchanged	21 (40%)	2 (18%)	0.30
	Progressed	31 (60%)	9 (82%)	
FibroIndex difference (Mean ± SD)		-0.07 ± 0.34	0.13 ± 0.44	0.14
Change in FibroIndex (n=54)	Unchanged	26 (59%)	4 (40%)	0.31
	Progressed	18 (41%)	6 (60%)	

Forns index =  $7.811 - 3.131 \times \ln(\text{platelet count}) + 0.781 \times \ln(\text{GGT}) + 3.467 \times \ln(\text{age}) - 0.014 \times (\text{cholesterol})$

FibroIndex =  $1.738 + 0.064 \times (\text{platelet} \times [10^4/\text{mm}^3]) + 0.005 \times (\text{AST [IU/L]}) + 0.463 \times (\text{gamma globulin [g/dL]})$

unchanged = (Forns index value at 5 years after study enrollment) - (Forns index value at time of study enrollment)  $\leq 0$

progressed = (Forns index value at 5 years after study enrollment) - (Forns index value at time of study enrollment)  $> 0$

There were 9 patients in whom we were not able to collect gamma globulin values.

**Table 3. Clinical Findings according to CTGF -945 G/C Genotypes in LC Patients**

	CTGF -945 G/C		p value
	G carrier	C homo	
Age at diagnosis	66 ± 9	67 ± 9	0.39
Gender (male / female)	45 / 34	16 / 23	0.10
Platelet ( $\times 10^4$ )	9.8 ± 4.3	11.7 ± 15.5	0.33
Albumin (g/dL)	3.4 ± 0.5	3.3 ± 0.5	0.25
Bilirubin (mg/dL)	1.2 ± 0.8	1.3 ± 0.5	0.98
Prothrombin time (%)	77.4 ± 14.4	69.4 ± 14.8	0.007*
Child-Pugh score	5.9 ± 1.0	6.6 ± 1.0	0.003*
ICG-R (%)	28.7 ± 15.3	35.2 ± 16.3	0.045*
Hyaluronic acid (ng/mL)	271.8 ± 158.6	351.5 ± 125.9	0.22
Type IV collagen (ng/mL)	8.3 ± 2.2	10.1 ± 3.1	0.13

CTGF: connective tissue growth factor, LC: liver cirrhosis, ICG-R: indocyanine green retention-rates, \* =  $p < 0.05$

In order to examine the effects of the CTGF -945 G/C genotype on the patients with advanced liver fibrosis, we compared clinical liver function parameters, as shown in Table 3. Among the LC patients, the CTGF -945 C homozygotes had significantly lower prothrombin times ( $p=0.007$ ), higher Child-Pugh scores ( $p=0.003$ ) and higher indocyanine green retention rates (ICG-R) at 15 minutes ( $p=0.045$ ) than the G carriers.

In order to investigate the relationship between the CTGF -945 G/C genotype and the incidence of HCV-related HCC, we compared the CTGF -945 G/C genotype distribution and allele frequency between the patients with ( $n=91$ ) and without ( $n=144$ ) HCC, as shown in Table 4. The proportion of C homozygotes and frequency of the C allele tended to be higher among the HCC patients than non-HCC patients, although the trend was not significant ( $p=0.054$  and  $p=0.15$ , respectively). We compared clinical liver function param-

eters in order to examine the background characteristics of the patients with and without HCC, as shown in Table 5. The HCC patients exhibited significantly lower levels of platelets ( $p=0.025$ ) and albumin ( $p=0.0001$ ) and higher values of ICG-R ( $p=0.04$ ), hyaluronic acid ( $p=0.00001$ ) and Type IV collagen ( $p=0.002$ ) than the patients without HCC. In order to adjust for aging, we used a logistic regression analysis. Ultimately, only the hyaluronic acid levels were found to be significantly different between the patients with and without HCC.

Next, we analyzed the clinical parameters of the HCC patients at the first diagnosis of HCC. The CTGF -945 C homozygotes had higher Child-Pugh scores ( $p=0.03$ ). On the other hand, the tumor diameter, number of tumors, degree of tumor tissue differentiation, presence of tumor thrombosis, CLIP score, initial treatment of HCC and HCC recurrence rate, which may affect the HCC prognosis, did not differ be-

**Table 4. CTGF -945 G/C Genotype Distributions between Non HCC Patients and HCC Patients with HCV Related Chronic Liver Disease**

	non HCC (n = 144)		HCC (n = 91)			p value	
	CC	CG	GG	CC	CG		GG
Genotype	34 (24%)	72 (50%)	38 (26%)	32 (35%)	37 (41%)	22 (24%)	0.15
	C homo		G carrier		C homo		G carrier
	34 (24%)		110 (76%)	32 (35%)		59 (65%)	0.054
Allele frequencies	C 0.49		G 0.51	C 0.55		G 0.45	0.15

HCC: hepatocellular carcinoma, HCV: hepatitis C virus

**Table 5. (A) Clinical Findings between Non HCC Patients and HCC Patients with HCV Related Chronic Liver Disease. (B) Multivariate Logistic Regression Model Aimed to Evaluate Independent Factor**

A				
	non HCC (n = 144)	HCC (n = 91)	p value	
Age at diagnosis		63.3 ± 10.6	67.0 ± 8.1	0.005*
Gender (male / female)		75 / 69	54 / 37	0.28
Platelet (×10 <sup>4</sup> )		14.2 ± 7.1	11.7 ± 9.9	0.025*
Albumin (g/dL)		3.8 ± 0.5	3.5 ± 0.5	0.0001*
Bilirubin (mg/dL)		0.9 ± 0.6	0.9 ± 0.6	0.74
Prothrombin time (%)		83.1 ± 19.5	79.7 ± 13.1	0.16
Child-Pugh score		6.2 ± 1.0	5.9 ± 1.1	0.09
ICG-R (%)		23.1 ± 14.9	28.0 ± 15.2	0.04*
Hyaluronic acid (ng/mL)		122.0 ± 115.0	309.2 ± 140.8	0.00001*
Type IV collagen (ng/mL)		6.3 ± 2.5	8.8 ± 2.9	0.002*
AFP (ng/mL)		145 ± 828	1,162 ± 7,588	0.13
PIVKA- II (mAU/mL)		1,532 ± 8,309	4,623 ± 27,751	0.33
B				
	Odds ratio	95% CI		p value
Age at diagnosis	1.07	0.95 - 1.21		0.28
Platelet	1.10	0.90 - 1.34		0.37
Albumin	10.4	0.13 - 811.0		0.29
ICG-R	1.16	0.95 - 1.40		0.14
Hyaluronic acid	1.02	1.00 - 1.05		0.04
Type IV collage	0.71	0.38 - 1.33		0.28

HCC: hepatocellular carcinoma, HCV: hepatitis C virus, ICG-R: indocyanine green retention rates, AFP: alfa fetoproteins, PIVKA- II: protein induced by Vitamin K absence or antagonists- II, CI: confidence interval, \* = p &lt; 0.05

tween the -945 C homozygotes and the G carriers (Table 6). We also examined the prognosis of the HCC patients divided into groups of C homozygotes and G carriers. As determined according to the Kaplan-Meier analysis, the C homozygotes had a significantly poorer prognosis than the G carriers (p=0.01, Figure), and more HCC patients died due to HCC (Table 7). In addition, there were no significant differences in the cause of death between the C homozygotes and the G carriers (p=0.20, Table 7).

## Discussion

In this study, we examined the association between the CTGF -945 G/C polymorphism and the progression of liver fibrosis in Japanese patients with chronic HCV infection. To the best of our knowledge, this is the first study to investigate the possible association between CTGF -945 G/C and HCV-related chronic liver disease progression, including the development and prognosis of HCC in a Japanese popula-

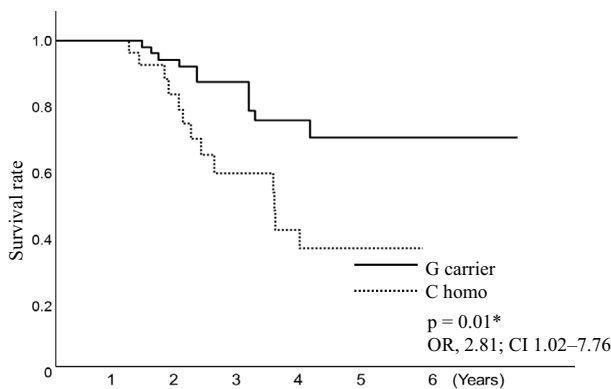
tion.

The CTGF -945 gene is located at chromosome 9q23. A total of 33 SNPs associated with this gene with a minor allele frequency of >20% have been identified, and seven sites of linkage disequilibrium have been evaluated using the Haploview software program (Broad Institute, Cambridge, USA; available at <http://www.broadinstitute.org/mpg/haploview>) (25). Among these polymorphisms, the CTGF -945 G/C polymorphism exhibits a high frequency of minor alleles in a broad range of ethnicities, including the Japanese population. Moreover, CTGF -945 G/C is a functional SNP located in the putative transcriptional element region. The G allele has a higher transcriptional activity than the C allele (17). Several studies have examined the relationship between CTGF polymorphisms and chronic inflammatory diseases (26, 27). Most of these studies have reported that the CTGF -945 high transcriptional G allele is associated with susceptibility to various chronic inflammatory diseases, such as systemic sclerosis and cardiovascular events related to

**Table 6. Clinical Findings according to CTGF -945 G/C Genotypes in HCC Patients**

CTGF -945 G/C	G carrier	C homo	p value
Age at diagnosis	66 ± 8	68 ± 9	0.25
Gender (male / female)	37 / 22	17 / 15	0.50
Positive history of interferon therapy	24%	24%	>0.99
Habitual alcohol consumption cases	41%	40%	>0.99
HCV genotype (1 / 2)	10 / 5	16 / 2	0.20
HCV-RNA (KIU/mL)	292 ± 248	180 ± 214	0.20
AFP (ng/mL)	324 ± 121	12,659 ± 2,273	0.16
PIVKA- II (mAU/mL)	245 ± 579	3,209 ± 12,804	0.14
Child-Pugh score	5.7 ± 1.0	6.2 ± 1.0	0.03*
HCC size (cm)	2.5 ± 1.2	3.0 ± 1.6	0.09
Tumor formation (mono / multi / massive)	29 / 21 / 1	14 / 14 / 1	0.73
Tumor differentiation (well / moderate / poor)	20 / 10 / 0	6 / 8 / 1	0.12
Tumor thrombosis (+ / -)	1 / 52	0 / 30	-
CLIP score	1.0 ± 0.9	1.1 ± 0.9	0.62
Initial treatment (operation / local therapy / TAE / BSC)	8 / 44 / 6 / 1	5 / 23 / 4 / 0	0.87
HCC recurrence	91%	93.8%	0.66

CTGF: connective tissue growth factor, HCC: hepatocellular carcinoma, HCV: hepatitis C virus, AFP: alfa fetoproteins, PIVKA-II: protein induced by Vitamin K absence or antagonists-II, CLIP: Cancer of Liver Italian Program, TAE: transcatheter arterial embolization, BSC: best supportive care, \* = p < 0.05



**Figure.** Prognosis of the HCC patients (G carriers versus C homozygotes) according to the Kaplan-Meier analysis. OR: odds ratio; CI: confidence interval \* <0.05

atherosclerosis. The present study indicated different findings, in that the higher transcriptional -945 G allele appears to play a protective role against chronic liver disease progression. Similar to our results, Dessein et al. reported that the rs6918698 (-945) C homozygote is associated with advanced liver fibrosis in schistosome-infected patients (25).

Indeed, the serum CTGF level has been observed to be increased in patients with chronic liver disease (28), and the HCV core protein upregulates CTGF production in HSCs (29). However, the physiological/pathological function of CTGF in liver tissue appears to differ in other organs and remains under discussion (30). For example, the liver is likely to play a major role as a clearance organ for serum CTGF (30). CTGF binds to low-density lipoprotein receptor-related proteins (LRPs), which are abundantly expressed on hepatocytes and activated HSC membranes (30, 31). CTGF

**Table 7. The Cause of Death in HCC Patients**

	G carrier	C homo
HCC	8 (80%)	6 (67%)
Varix rupture	0 (0%)	1 (11%)
Liver failure	1 (10%)	2 (22%)
Other	1 (10%)	0 (0%)

HCC: hepatocellular carcinoma

There were 4 patients in whom we were not able to confirm the cause of death.

bound with LRP is rapidly internalized and degraded in the endosomal/lysosomal compartment. The LRP is a co-receptor between Wnt and CTGF, and it has been demonstrated that CTGF interferes with the Wnt signaling pathway (31, 32). The aberrant upregulation of the Wnt-beta-catenin signal pathway enhances the activation and survival of HSCs, resulting in the progression of liver fibrosis. CTGF has also been reported to promote tumor development and growth (33). However, our results suggest that lower transcriptional allele C homozygotes are at higher risk for HCC development than G allele carriers. As mentioned above, CTGF regulates the function of the Wnt signal pathway in a competitive manner. It has also been suggested that a higher CTGF expression induced by the -945 allele G suppresses fibrosis, carcinogenesis and tumor growth in liver tissue via the downregulation of the Wnt signal pathway in hepatocytes.

Furthermore, CTGF binds to VEGF<sub>165</sub>, a VEGF isoform, and suppresses the angiogenic activity (34). The VEGF expression level is correlated with sinusoidal capillarization, which results in hepatocyte hypoxia associated with liver dysfunction and disruption of the portal circulation (35). There is a possibility that CTGF provides protection against abnormal angioarchitecture remodeling, distinctive of cirrho-

sis, by inhibiting VEGF. Furthermore it has been well demonstrated that HCC is one of the most vascular solid tumors, in which angiogenesis plays an important role in the development, progression and metastasis of HCC. Therefore, it is possible that the low transcriptional allele of CTGF is associated with a high rate of carcinogenesis of HCC.

The present study is associated with various limitations. For example, the sample size was relatively small and the statistical power was poor. In addition, there may be some potential biases, such as the selection of patients who were able to visit our university hospital continuously for medical tests. In addition, five years is still too short a duration to measure significant differences in the rate of liver fibrosis progression, as, in patients with HCV-related CH, the median rate of fibrosis progression per year is approximately 0.1 fibrosis units according to the METAVIR classification (36). Future studies would be strengthened by increasing the sample size as well as following the patients longitudinally.

In conclusion, we herein demonstrated that the CTGF -945 G/C gene polymorphism influences fibrosis progression in patients with HCV-related chronic liver disease. In this study, patients with the lower transcription CTGF -945 C allele exhibited an accelerated rate of liver fibrosis progression. In addition, the CTGF -945 C homozygotes demonstrated shorter a survival time compared to the HCV-related HCC patients. Further studies using larger sample sizes are needed in order to confirm the association between the CTGF -945 G/C polymorphism and the progression of chronic liver disease.

**The authors state that they have no Conflict of Interest (COI).**

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#### References

- Marcellin P, Lau GK, Zeuzem S, et al. Comparing the safety, tolerability and quality of life in patients with chronic hepatitis B vs chronic hepatitis C treated with peginterferon alpha-2a. *Liver Int* **28**: 477-485, 2008.
- Heydtmann M, Adams DH. Chemokines in the immunopathogenesis of hepatitis C infection. *Hepatology* **49**: 676-688, 2009.
- Gressner OA, Rizk MS, Kovalenko E, Weiskirchen R, Gressner AM. Changing the pathogenetic roadmap of liver fibrosis? Where did it start; where will it go? *J Gastroenterol Hepatol* **23**: 1024-1035, 2008.
- Leask A, Abraham DJ. The role of connective tissue growth factor, a multifunctional matricellular protein, in fibroblast biology. *Biochem Cell Biol* **81**: 355-363, 2003.
- Kobayashi H, Hayashi N, Hayashi K, Yamataka A, Lane GJ, Miyano T. Connective tissue growth factor and progressive fibrosis in biliary atresia. *Pediatr Surg Int* **21**: 12-16, 2005.
- Gressner OA, Lahme B, Demirci I, Gressner AM, Weiskirchen R. Differential effects of TGF- $\beta$  on connective tissue growth factor (CTGF/CCN<sub>2</sub>) expression in hepatic stellate cells and hepatocytes. *J Hepatol* **47**: 699-710, 2007.
- Leask A, Abraham DJ. All in the CCN family: essential matricellular signaling modulators emerge from the bunker. *J Cell Sci* **119**: 4803-4810, 2006.
- Promrat K, McDermott DH, Gonzalez CM, et al. Associations of chemokine system polymorphisms with clinical outcomes and treatment responses of chronic hepatitis C. *Gastroenterology* **124**: 352-360, 2003.
- Okamoto K, Mimura K, Murawaki Y, Yuasa I. Association of functional gene polymorphisms of matrix metalloproteinase (MMP)-1, MMP-3 and MMP-9 with the progression of chronic liver disease. *J Gastroenterol Hepatol* **20**: 1102-1108, 2005.
- Okamoto K, Mandai M, Mimura K, Murawaki Y, Yuasa I. The association of MMP-1, -3 and -9 genotypes with the prognosis of HCV-related hepatocellular carcinoma patients. *Res Commun Mol Pathol Pharmacol* **117-118**: 77-89, 2005.
- Okamoto K, Ishida C, Ikebuchi Y, et al. The genotypes of IL-1 beta and MMP-3 are associated with the prognosis of HCV-related hepatocellular carcinoma. *Intern Med* **49**: 887-895, 2010.
- Ishida C, Ikebuchi Y, Okamoto K, Murawaki Y. Functional gene polymorphisms of interleukin-10 are associated with liver disease progression in Japanese patients with hepatitis C virus infection. *Intern Med* **50**: 659-666, 2011.
- Ikebuchi Y, Ishida C, Okamoto K, Murawaki Y. Association of TIMP-1 and TIMP-2 gene polymorphisms with progression of liver fibrosis in patients with type C chronic liver disease. *Biochem Genet* **51**: 564-574, 2013.
- Verma V, Chakravarti A, Kar P. Cytokine levels of TGF- $\beta$ , IL-10, and sTNF $\alpha$ RII in type C chronic liver disease. *Dig Dis Sci* **53**: 2233-2237, 2008.
- Karger A, Fitzner B, Brock P, et al. Molecular insights into connective tissue growth factor action in rat pancreatic stellate cells. *Cell Signal* **20**: 1865-1872, 2008.
- Rueda B, Simeon C, Hesselstrand R, et al. A large multicentre analysis of CTGF -945 promoter polymorphism does not confirm association with systemic sclerosis susceptibility or phenotype. *Ann Rheum Dis* **68**: 1618-1620, 2009.
- Fonseca A, Lindahl GE, Ponticos M, et al. A polymorphism in the CTGF promoter region associated with systemic sclerosis. *N Engl J Med* **357**: 1210-1220, 2007.
- Yoshida H, Shiratori Y, Moriyama M, et al. Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. *Ann Intern Med* **131**: 174-181, 1999.
- Zuern C, Heimrich J, Kaufmann R, et al. Down-regulation of MTUS1 in human colon tumors. *Oncol Rep* **23**: 183-189, 2010.
- Bekkaoui F, Poisson I, Crosby W, Cloney L, Duck P. Cycling probe technology with RNase H attached to an oligonucleotide. *Biotechniques* **20**: 240-248, 1996.
- Bravo AA, Sheth SG, Chopra S. Liver biopsy. *N Engl J Med* **344**: 495-500, 2001.
- Forns X, Ampurdanès S, Llovet JM, et al. Identification of chronic hepatitis C patients without hepatic fibrosis by a simple predictive model. *Hepatology* **36**: 986-992, 2002.
- Koda M, Matunaga Y, Kawakami M, Kishimoto Y, Suou T, Murawaki Y. FibroIndex, a practical index for predicting significant fibrosis in patients with chronic hepatitis C. *Hepatology* **45**: 297-306, 2007.
- Crow JF. Eighty years ago: the beginnings of population genetics. *Genetics* **119**: 473-476, 1988.
- Dessein A, Chevillard C, Arnaud V, et al. Variants of CTGF are associated with hepatic fibrosis in Chinese, Sudanese, and Brazilians infected with schistosomes. *J Exp Med* **206**: 2321-2328, 2009.
- Zhang X, Nie S, Si X, Luo Y, Tang W. Association between the CTGF -945C/G polymorphism and systemic sclerosis: a meta-

- analysis. *Gene* **509**: 1-6, 2012.
27. Cozzolino M, Biondi ML, Banfi E, et al. CCN2 (CTGF) gene polymorphism is a novel prognostic risk factor for cardiovascular outcomes in hemodialysis patients. *Blood Purif* **30**: 272-276, 2010.
  28. Kovalenko E, Tacke F, Gressner OA, et al. Validation of connective tissue growth factor (CTGF/CCN2) and its gene polymorphisms as noninvasive biomarkers for the assessment of liver fibrosis. *J Viral Hepat* **16**: 612-620, 2009.
  29. Shin JY, Hur W, Wang JS, et al. HCV core protein promotes liver fibrogenesis via up-regulation of CTGF with TGF- $\beta$ 1. *Exp Mol Med* **37**: 138-145, 2005.
  30. Gressner OA, Gressner AM. Connective tissue growth factor: a fibrogenic master switch in fibrotic liver diseases. *Liver Int* **28**: 1065-1079, 2008.
  31. Cheng JH, She H, Han YP, et al. Wnt antagonism inhibits hepatic stellate cell activation and liver fibrosis. *Am J Physiol Gastrointest Liver Physiol* **294**: G39-G49, 2008.
  32. Guo Y, Xiao L, Sun L, Liu F. Wnt/ $\beta$ -catenin signaling: a promising new target for fibrosis diseases. *Physiol Res* **61**: 337-346, 2012.
  33. Brigstock DR. The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. *Endocr Rev* **20**: 189-206, 1999.
  34. Inoki I, Shiomi T, Hashimoto G, et al. Connective tissue growth factor binds vascular endothelial growth factor (VEGF) and inhibits VEGF-induced angiogenesis. *FASEB J* **16**: 219-221, 2002.
  35. Rosmorduc O, Wendum D, Corpechot C, et al. Hepatocellular hypoxia-induced vascular endothelial growth factor expression and angiogenesis in experimental biliary cirrhosis. *Am J Pathol* **155**: 1065-1073, 1999.
  36. Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. *Lancet* **349**: 825-832, 1997.