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The Predictive Role of Tenascin-C and Cellular Communication Network Factor 3 (CCN3) in Post Hepatectomy Liver Failure in a Rat Model and 50 Patients Following Partial Hepatectomy

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Manuscript Preparation E
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Background: Matricellular proteins of the extracellular matrix (ECM) include tenascin-C (TNC) and cellular communication network factor 3 (CCN3). This study aimed to investigate the role of TNC and CCN3 as prognostic factors for post hepatectomy liver failure (PHLF) in a rat model of partial hepatectomy and 50 patients following partial hepatectomy.

Material/Methods: Sprague-Dawley rats underwent 85% (n=53) or 90% hepatectomy (n=53) in the partial hepatectomy (PHx) model. TNC and CCN3 mRNA expression in residual liver tissue was evaluated using quantitative reverse transcription-polymerase chain reaction (qRT-PCR), and enzyme-linked immunoassay (ELISA) determined the serum levels of TNC and CCN3. In 50 patients who underwent partial hepatectomy, TNC and CCN3 serum levels were measured on postoperative day 1 and day 3.

Results: In the rat partial hepatectomy model, mRNA and serum levels of TNC and CCN3 were significantly increased within the first 24 h, and were higher in the 90% PHx group compared with the 85% PHx group. Fifty patients who underwent partial hepatectomy, included patients with PHLF (n=12) and patients without PHLF (n=38). Multivariate analysis confirmed that serum levels on postoperative day 3 $TNC^{high}+CCN3^{high}$ was a significant predictor of PHLF, which was associated with more than twice the risk of severe morbidity when compared with the low-risk patients (80% vs. 30%) and a significantly longer hospital stay (17 days vs. 8 days).

Conclusions: Further studies are needed to evaluate the potential role of the matricellular proteins, TNC and CCN3 as early clinical predictors for PHLF.

MeSH Keywords: **Hepatectomy • Liver Failure • Nephroblastoma Overexpressed Protein • Rats, Sprague-Dawley • Tenascin**

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Background

Postoperative morbidity and mortality are significant concerns after liver resection, even though surgical techniques and perioperative patient care have recently undergone improvements. Post hepatectomy liver failure (PHLF) is a common cause of mortality following hepatectomy and remains clinically challenging [1–3]. In 2011, the multicenter International Study Group for Liver Surgery (ISGLS) developed clinical guidelines for stratification of the severity of PHLF that were intended to be widely applicable [4]. Prediction and prevention of PHLF can be improved by the use of standardized preoperative assessments of liver function and liver fibrosis [5]. However, patients who have a preoperative assessment that indicates low risk, still develop PHLF. Therefore, the assessment parameters used during the early period following hepatectomy may need to be refined.

Matricellular proteins of the extracellular matrix (ECM) are extracellular proteins that are not directly involved in the process of forming structural elements but can modulate cell-matrix interactions and cell functions [6]. Matricellular proteins have high expression levels during embryonic development and in response to injury, and induce loss of cell adhesion, which differs from the adhesion-promoting effect of most ECM proteins [7]. We have previously reported the findings from mRNA expression profile microarray studies in rats, which has shown increased expression of matricellular proteins, including thrombospondin 1 (TSP-1), serpin family E member 1 (Serpine1), tenascin-C (TNC), cellular communication network factor 3 (CCN3), and cellular communication network factor 2 (CCN2), in liver failure after hepatectomy (data not shown).

In 2012, Hayashi et al. showed that TSP-1 was a critical negative regulator in a murine model of liver regeneration, which exerted its effects by a transforming growth factor- β 1 (TGF- β 1)-dependent mechanism [8]. Starlinger et al. reported that serum levels of TSP-1 might be a helpful clinical marker in predicting postoperative liver dysfunction, even as early as the first postoperative day [9]. Plasminogen activator inhibitor type 1 (PAI-1) inhibits the plasminogen activators urokinase (uPA) and tissue plasminogen activator (tPA) and plasmin to reduce fibrinolysis in hemostasis [10]. Also, PAI-1 and uPA have been shown to participate in the regulation of hepatocyte proliferation after partial hepatectomy [11,12]. Chang et al. [13] showed that the chemokine (C-C motif) ligand 2 (CCL2) regulated the activation of hepatocytes during the regeneration of liver tissue following liver resection, and CCL2 expression increased by up-regulation of the connective tissue growth factor (CTGF) gene.

The expression of TNC in adult tissues is normally suppressed, and TNC is only transiently increased following tissue injury [14,15]. TNC mediate the inflammatory and fibrotic responses required for effective tissue repair [16]. For example,

TNC is expressed in conditions that include heart disease and has been shown to participate in ventricular remodeling by releasing cardiomyocytes from adherence to the ECM [17]. CCN3 has several effects in tissue repair and disease processes, including cell proliferation, adhesion, migration, differentiation, and survival [18–21]. As an integrin receptor ligand, CCN3 may have a role in cutaneous wound healing, as shown by the adhesion of skin fibroblasts and the induction of fibroblast chemotaxis [19]. Previous studies have also shown that CCN3 can inhibit the proliferation of pancreatic islet beta cells and vascular smooth muscle cells [22,23]. However, the relationship between TNC, CCN3, and PHLF remains unknown.

Therefore, this study aimed to investigate the role of TNC and CCN3 as prognostic factors for post hepatectomy liver failure (PHLF) in a rat model of partial hepatectomy. The study also aimed to evaluate the predictive validity of the combination of the two parameters, TNC and CCN3, in 50 patients following partial hepatectomy.

Material and Methods

Animal model of partial hepatectomy

Male Sprague-Dawley rats (6–8 weeks old) were maintained in temperature-controlled cages with standard laboratory chow and water and a 12-hourly light and dark cycle. The rats were separated into three groups that included a sham operation group (n=7), an 85% partial hepatectomy (PHx) group (n=53), and a 90% PHx group (n=53). In the 85% PHx model, the right superior lobe and paracaval liver were retained, and the median, left lateral, caudate, and right inferior lobes were resected. In the 90% PHx model, only the caudate lobes and paracaval liver were retained.

The surgical procedures involved in preparing the rat model were as follows. The rats fasted for 12 h before anesthesia with a continuous 1.5 vol% isoflurane/oxygen inhalation with the surgical procedures performed using the techniques described by Madrahimov et al. [24]. After opening the abdomen in the midline, the liver lobes were exposed and freed from the surrounding ligaments. Glisson's sheath that formed the capsule of each hepatic lobe was ligated with 6-0 silk at the position of the hepatic parenchyma near the pedicle, and each lobe was ligated with 4-0 silk by penetrating the liver parenchyma near the inferior vena cava. The right superior or median lobe was ligated in two parts. Then, the ligated lobes were removed with scissors. The abdominal cavity was washed with warm saline and then closed in a simple continuous manner. In the rats in the sham group, the abdomen was kept open for 30 minutes and then closed. After recovery from anesthesia, a 10% glucose solution was provided to all rats.

All experimental animal procedures were performed according to the Animal Care and Use Training Resources from the National Institutes of Health (NIH) and the Laboratory Animal Ethics Committee of the Chinese PLA General Hospital, who approved this study (Reg. No. 2017-X13-65).

Experimental design

Survival was assessed in the rats in the three study groups. In the 85% PHx group and 90% PHx group, seven rats were assigned to each time point at 6 h, 12 h, and 24 h after surgery. A further seven rats were included in the sham group. Immediately after the rats were euthanized by exsanguination, blood was collected from the rats via the vena cava, and the serum was stored at -80°C . Samples of liver tissue were frozen with liquid nitrogen, and other liver samples were fixed with 10% buffered formalin and then embedded in paraffin wax and sectioned for light microscopy.

Real-time quantitative polymerase chain reaction (qRT-PCR)

The RNA simple Total RNA Kit (DP419) (Tiangen, Beijing, China) was used to isolate the total RNA from the liver samples. A RevertAid First Strand cDNA Synthesis Kit (K1622) (ThermoFisher Scientific, Waltham, MA, USA) was used to reverse transcribe 4 μg of total RNA into cDNA following the manufacturer's instructions. TB Green Premix ExTaqII reagent (RR820A) (Takara, Dalian, China) was used to perform the quantitative reverse transcription-polymerase chain reaction (qRT-PCR) cycles on an ABI StepOne Plus Real-Time PCR Detection system (Applied Biosystems, Foster City, CA, USA). The online site of the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) was used to search for specific rat gene sequences, and the corresponding primers were designed using Primer 3 and the NCBI Basic Local Alignment Search Tool (BLAST) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). HPRT was used as the housekeeping gene. The following primer sequences were used in the real-time PCR analysis:
TNC forward: 5'-GACTGCTCAGGTGGAGAGTTT-3';
TNC reverse: 5'-ATGCCAAGGCTTCTGAGTC-3';
CCN3 forward: 5'-GGAAGTGCAATTCGTTGAGGC-3';
CCN3 reverse: 5'-AAGCAAGTACCCCTAAGCC-3';
HPRT forward: 5'-GCTGAAGATTTGGAAAAGGTG-3';
HPRT reverse: 5'-AATCCAGCAGGTGAGCAAAG-3'.

The primers were also evaluated by the amplification curve and melting curve obtained using an ABI StepOne Plus Real-Time PCR Detection System (Applied Biosystems, Foster City, CA, USA). In each sample, the expression of the target gene was defined by normalizing the ΔCT ($\text{CT}_{\text{target}} - \text{CT}_{\text{HPRT}}$) of the sample against the average ΔCT of the tissues from the sham group [25].

Immunohistochemistry

Formalin-fixed paraffin-embedded liver tissue sections were used to detect the expression of Ki67 in one liver tissue section from each rat using immunohistochemical staining with 5 $\mu\text{g}/\text{ml}$ of murine anti-human Ki67 antibody (Cat. No. 550609) (BD Pharmingen, San Diego, CA, USA). Immunostaining was visualized with 3,3-diaminobenzidine (DAB), and the sections were then counterstained with hematoxylin. The Ki67-positive hepatocytes were manually counted in ten random visual fields to determine the Ki67 proliferation index.

Enzyme-linked immunosorbent assay (ELISA)

Serum levels of tenascin-C (TNC) and cellular communication network factor 3 (CCN3) were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits with antibodies to TNC (Cat. No. 27767) (IBL, Fujioka, Japan) and CCN3 (Cat. No. ab205570) (Abcam, Cambridge, MA, USA) according to the kit instructions. Briefly, a standard or sample was added to the appropriate wells of an antibody-coated microplate. After incubation with the primary antibody at room temperature, the wells were washed, and the secondary antibodies were added and incubated at room temperature. Each well was washed, and 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added to each well, which was incubated at room temperature in the dark. Finally, the samples were washed, and the reaction stopped and the results were measured at 450 nm using a microplate reader.

Clinical data from 50 patients who underwent partial hepatectomy

This study also included a prospective observational study of 50 patients who were scheduled to undergo partial hepatectomy for benign or malignant hepatobiliary diseases between April 2017 and February 2018 at the Chinese PLA General Hospital. All patients underwent a careful preoperative assessment. Study approval was obtained from the institutional Ethics Committee. All included study subjects provided written informed consent.

Partial hepatectomy was performed clinically using selective hepatic blood flow blockade, methylene blue staining, intraoperative ultrasound localization, and indocyanine green (ICG) fluorescence imaging, for accurate liver segment resection. The degree of liver resection was graded as minor (<3 segments) or major (≥ 3 segments), according to the Brisbane 2000 nomenclature from the International Hepato-Pancreato-Biliary Association (IHPBA) [26]. Liver failure was defined as reduced coagulation function and concomitant hyperbilirubinemia on or after postoperative day 5, as recommended by the International Group of Hepatic Surgery Research (ISGLS) [4].

The patients were followed for 90 days continuously after the surgery, and postoperative morbidity was prospectively recorded and graded as I–V, based on the postoperative intervention procedures described in the Clavien-Dindo classification of surgical complications [27]. If multiple complications were found in one patient, the most serious complication was included in the analysis.

Analysis of blood and serum from 50 patients who underwent partial hepatectomy

Blood was collected before surgery and on the first and third days after the surgery. Serum levels of TNC and CCN3 were measured with ELISA kits with antibodies to TNC (Cat. No. 27767) (IBL, Fujioka, Japan) and CCN3 (Cat. No. ab193710) (Abcam, Cambridge, MA, USA) strictly in accordance with the manufacturer's instructions. Liver function tests were performed for serum levels of alanine aminotransferase (ALT), total bilirubin (TB), aspartate aminotransferase (AST), albumin (ALB), platelet count (PLT), the prothrombin time (PT), γ -glutamyl transpeptidase (γ -GT), and alkaline phosphatase (ALP), using routine clinical laboratory methods.

Statistical analysis

Data were analyzed using SPSS version 22 software (IBM, Chicago, IL, USA). Numerical data were presented at the mean \pm standard deviation (SD), or the median and range. Student's t-test was used for the parametric tests and Wilcoxon's rank-sum test for the nonparametric tests. The chi-squared (χ^2) test was used to analyze the nominal data. Survival analysis was performed with the log-rank test, which generated Kaplan-Meier curves. The receiver operating characteristic (ROC) curve was analyzed to assess the specificity and sensitivity of the serum levels of TNC and CCN3 in predicting PHLF. Multivariate analysis included all parameters with $p < 0.100$ in the univariate analyses. This study applied binary logistic regression analysis. Line charts were used to describe the trends in the molecular changes. Outliers and extreme values were not included in the box plot illustrations for a better resolution of the interquartile ranges. A p -value < 0.05 was considered to be statistically significant.

Results

The rat model of partial hepatectomy and survival following surgery

All rats in the 85% post hepatectomy (PHx) group ($n=53$) survived 72 h after hepatectomy. However, in the 90% PHx group ($n=53$), only 18 (34%) rats survived 72 h after hepatectomy, and 36 h was the median time to death (Figure 1A). The mean serum total bilirubin (TB) level in the 90% PHx group 12 h and

24 h following the surgery was significantly higher than that in the 85% PHx group (12 h: 6.1 ± 1.7 $\mu\text{mol/L}$ vs. 22.7 ± 8.5 $\mu\text{mol/L}$; $p < 0.001$) (24 h: 4.5 ± 2.1 $\mu\text{mol/L}$ vs. 20.2 ± 11.6 $\mu\text{mol/L}$; $p < 0.001$) (Figure 1B). In the residual liver tissues in the 85% PHx group, the percentage of Ki67-positive cells (proliferation index) was significantly higher than that in the 90% PHx group 24 h after hepatectomy (12 h: $0.05 \pm 0.04\%$ vs. $0.04 \pm 0.04\%$; $p = 0.979$) (24 h: $0.31 \pm 0.05\%$ vs. $0.19 \pm 0.12\%$; $p = 0.003$) (Figure 1C, 1D). Therefore, the 85% resection model was used as the extreme hepatic resection model, and the 90% resection model was used as the post hepatectomy liver failure (PHLF) model.

Expression of hepatic tenascin-C (TNC) and cellular communication network factor 3 (CCN3) mRNA

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to study the expression of hepatic tenascin-C (TNC) and cellular communication network factor 3 (CCN3) mRNA in liver tissue. In the 85% PHx group, TNC mRNA expression showed minor changes within 24 h after partial hepatectomy. In the 90% PHx group, TNC mRNA expression gradually increased during 6 h, 12 h, and 24 h (6 h: 50.4 ± 39.9 vs. 115.1 ± 84.4 ; $p = 0.011$) (12 h: 36.2 ± 31.5 vs. 284.9 ± 134.6 ; $p < 0.001$) (24 h: 62.1 ± 13.6 vs. 523.9 ± 359.5 ; $p = 0.002$) (Figure 2A). In the 90% PHx group, the mRNA level of hepatic TNC was 8.4 times greater than that in the 85% PHx group 24 h after hepatectomy. In the 85% PHx group, CCN3 mRNA expression peaked at 6 h after PHx, which was seven times the initial value found in the sham group and then decreased at 12 h. In the 90% PHx group, CCN3 mRNA expression increased by up to 18 times the initial value 6 h after partial hepatectomy surgery and was maintained until 12 h after partial hepatectomy. In the 90% PHx group, the mRNA level of hepatic CCN3 was 9.2 and 6 times greater than that in the 85% PHx group at 12 h and 24 h, respectively (6 h: 8.5 ± 3.3 vs. 21.0 ± 9.5 ; $p < 0.001$) (12 h: 2.3 ± 0.2 vs. 21.0 ± 11.5 ; $p < 0.001$) (24 h: 2.2 ± 1.5 vs. 13.0 ± 7.0 ; $p < 0.001$) (Figure 2B).

Expression levels of serum TNC and CCN3 protein

An increase in serum TNC was found immediately after partial hepatectomy in both the 85% and 90% PHx groups, and in the 90% PHx group, this increase was maintained. However, in the 85% PHx group, the serum TNC level was significantly reduced 12 h after the surgery (6 h: 3579.5 ± 453.3 ng/mL vs. 3503.4 ± 1036.4 ng/mL; $p = 0.803$) (12 h: 1361.5 ± 461.8 ng/mL vs. 2680.2 ± 612.3 ng/mL; $p < 0.001$) (24 h: 1593.0 ± 817.6 ng/mL vs. 2186.6 ± 730.0 ng/mL; $p = 0.062$) (Figure 3A). Also, the serum level of CCN3 in the 90% PHx group was significantly higher than that in the 85% PHx group at 6 h and 12 h after PHx (6 h: 9.0 ± 1.2 ng/mL vs. 24.3 ± 6.6 ng/mL; $p < 0.001$) (12 h: 8.4 ± 3.1 ng/mL vs. 20.6 ± 8.3 ng/mL; $p = 0.004$) (24 h: 5.8 ± 2.4 ng/mL vs. 8.4 ± 3.2 ng/mL; $p = 0.139$) (Figure 3B).

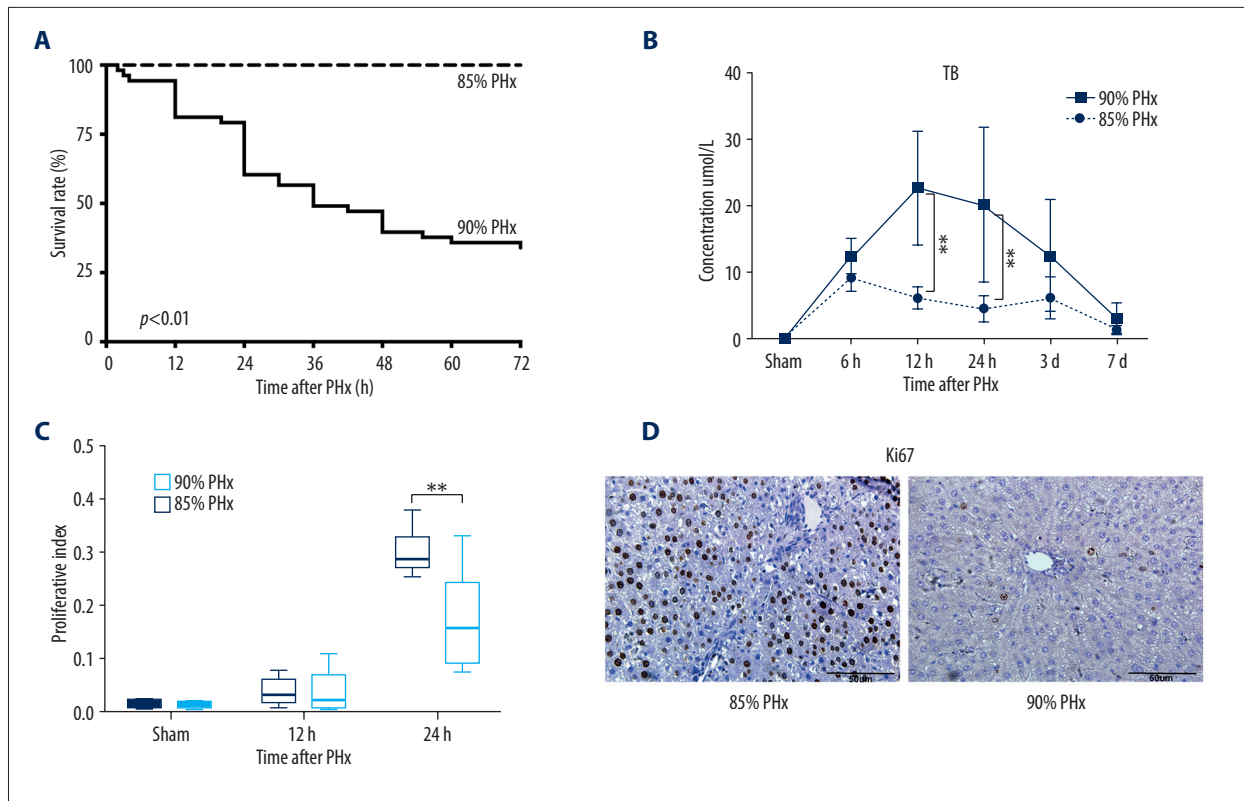


Figure 1. Comparison of the survival rates, serum total bilirubin (TB) level, and proliferative capacity of the residual liver in the 85% partial hepatectomy (PHx) rat group and the 90% PHx rat group. The results of the survival analysis from the 85% partial hepatectomy (PHx) group (broken line) and the 90% PHx group (solid line) ($p < 0.01$) are shown in (A). Perioperative serum total bilirubin (TB) level (B) and proliferative index (C) at various time points in the two groups are shown. Representative images of Ki67 immunohistochemistry in the residual liver in the 85% partial hepatectomy group and the 90% partial hepatectomy group, 24 h after hepatectomy, are shown in (D). ** $p < 0.01$.

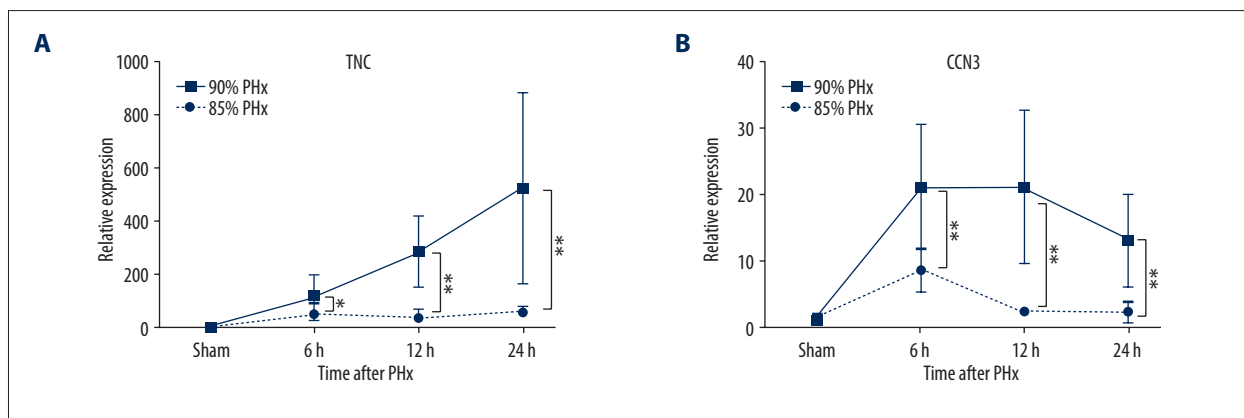


Figure 2. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) for tenascin-C (TNC) and cellular communication network factor 3 (CCN3) in the 85% partial hepatectomy (PHx) rat group and the 90% PHx rat group. (A) The quantitative reverse transcription-polymerase chain reaction (qRT-PCR) for hepatic TNC mRNA in the 85% PHx group (broken line) and the 90% PHx group (solid line). (B) The qRT-PCR for CCN3 mRNA in the 85% PHx group (broken line) and the 90% PHx group (solid line). * $p < 0.05$, ** $p < 0.01$.

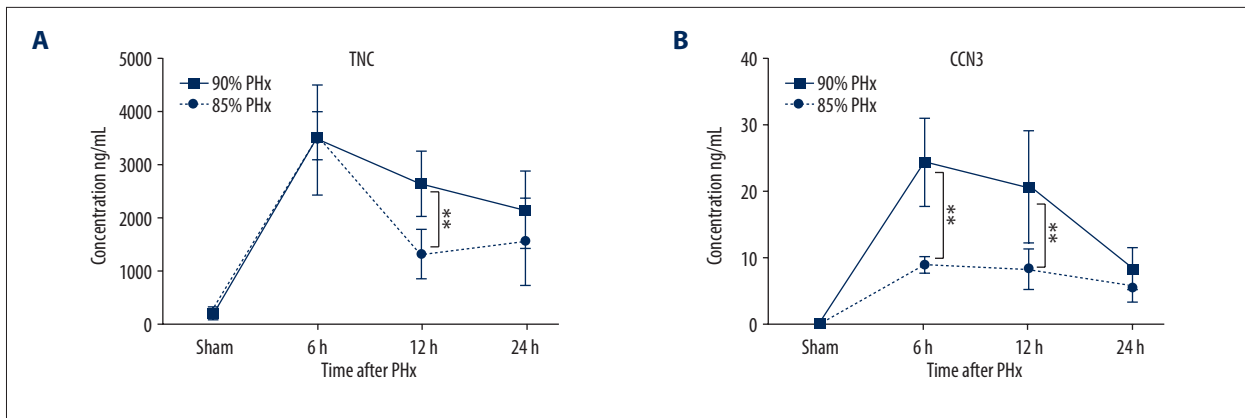


Figure 3. Quantification of serum levels of tenascin-C (TNC) and cellular communication network factor 3 (CCN3) in the 85% partial hepatectomy (PHx) rat group and the 90% PHx rat group. **(A)** Serum TNC levels in the 85% PHx group (broken line) and the 90% PHx group (solid line). **(B)** Serum CCN3 levels in the 85% PHx group (broken line) and the 90% PHx group (solid line). ** $p < 0.01$.

Clinical data and patient demographics in 50 patients following partial hepatectomy

Of the 50 patients who underwent partial hepatectomy, 12 patients had PHLF, and 38 patients did not have PHLF. The clinical and demographic data from these 50 patients are shown in Table 1 and include age, gender, height, body weight, alcohol intake, hepatitis B virus (HBV) surface antigen (HBsAg) positivity, ascites, cirrhosis, disease type, preoperative parameters, extent of liver resection, duration of surgery, and intraoperative blood loss. Twenty-four patients underwent partial hepatectomy for hepatocellular carcinoma (HCC), 15 patients underwent partial hepatectomy for cholangiocarcinoma, six patients underwent partial hepatectomy for non-neoplastic diseases, and five patients underwent partial hepatectomy for other diseases that required liver resection.

There were 35 patients who underwent major liver resection, and a significant difference was observed in the extent of resection between the two groups, who had PHLF and who did not have PHLF ($p = 0.025$). All patients who had liver tumors had the tumors confirmed histologically as complete of R0 resections. Also, compared with the patients in non-PHLF group, in the PHLF group, the duration of surgery was longer, and the intraoperative blood loss was greater ($p = 0.007$, for both). The other indicators, including preoperative liver functions and disease type, were comparable between the two groups. There were no postoperative deaths. The median postoperative hospital stay was 9 days (range, 3–80 days).

Serum TNC and CCN3 levels increased in patients following partial hepatectomy

Serum TNC and CCN3 levels were measured before and after partial hepatectomy. Serum levels of TNC and CCN3 both

increased on postoperative day 1. Postoperative serum levels of TNC increased further on day 3, whereas CCN3 remained at the same level on day 3. Compared with the median preoperative serum TNC level of 742.15 ng/mL (range, 275.55–2101.23 ng/mL), the median serum TNC level on postoperative day 1 was 1168.85 ng/mL (range, 526.35–5506.33 ng/mL; $p < 0.001$). Compared with the preoperative serum TNC, the median serum TNC on postoperative day 3 was 1704.10 ng/mL (range, 670.06–4655.63; $p < 0.001$), with a significant difference between the serum levels of TNC between postoperative day 1 and day 3 ($p = 0.008$). Compared with the median preoperative serum CCN3 level of 2.90 ng/mL (range, 1.30–12.63 ng/mL), the median serum CCN3 level on postoperative day 1 was 5.27 ng/mL (range, 2.37–16.07 ng/mL; $p < 0.001$). Compared with the preoperative serum CCN3, the median serum CCN3 on postoperative day 3 was 5.04 ng/mL (range, 2.28–39.35; $p < 0.001$), with no significant difference between the serum levels of CCN3 between postoperative day 1 and day 3 ($p = 0.061$) (Figure 4A).

No significant difference was detected in the preoperative serum TNC or CCN3 levels between the patients with and without PHLF. However, on postoperative day 3, the patients with PHLF had significantly higher serum levels of TNC than the patients without PHLF. The median preoperative serum level of TNC in non-PHLF was 702.35 ng/mL (range, 275.55–2813.19 ng/mL) compared with a median serum TNC level in PHLF of 988.95 ng/mL (range, 413.63–1293.75 ng/mL; $p = 0.437$) (Figure 4B).

The median serum level of TNC at postoperative day 1 in non-PHLF was 1170.26 ng/mL (range, 526.35–4403.14 ng/mL) compared with a median serum TNC level in PHLF of 1520.45 ng/mL (range, 652.31–5506.33 ng/mL; $p = 0.048$). The median serum level of TNC at postoperative day 3 in non-PHLF was 1570.41 ng/mL (range, 695.43–3518.32 ng/mL) compared with

Table 1. Patient demographics.

Characteristic	Total (n=50)	Group		p Value
		No LF (n=38)	LF (n=12)	
Age* (y)	52 (25–70)	51 (25–70)	56 (33–66)	0.143
Sex				0.769
Male	33	26	7	
Female	17	12	5	
Height** (cm)	167.2±7.2	167.5±7.7	166.1±5.4	0.556
Weight** (kg)	66.2±12.0	66.7±13.2	64.6±6.9	0.480
Alcohol intake#	17 (34)	12 (31.6)	5 (41.7)	0.769
HBsAg (+)#	23 (46)	18 (47.4)	5 (41.7)	0.730
Ascites#	6 (12)	3 (7.9)	3 (25)	0.280
Cirrhosis#	22 (44)	18 (47.4)	4 (33.3)	0.393
Diseases#				
HCC	24 (48)	20 (52.6)	4 (33.3)	0.243
CCC	15 (30)	9 (23.7)	6 (50)	0.17
Non-neoplastic	6 (12)	5 (13.2)	1 (8.3)	0.951
Others	5 (10)	4 (10.5)	1 (8.3)	0.741
Preoperative parameters				
PLT** (×10 ⁹ /L)	215.3±93.1	219.8±92.4	200.9±98.0	0.546
PTA** (%)	90.5±13.3	90±11.5	92±18.7	0.743
ALT* (U/L)	25.5 (6–349)	24.4 (6–349)	38.3 (13–224)	0.340
AST* (U/L)	27.8 (11.7–404)	25.95 (11.7–404)	40.1 (18–179.7)	0.056
ALB** (g/L)	40.6±4.4	41.1±4.2	38.9±4.7	0.121
TB** (μmol/L)	15±7.6	14±7	18.3±8.8	0.091
ALP** (U/L)	103.9±55.9	95.7±53.3	130±58.4	0.063
GGT* (U/L)	73.1 (11–424.1)	66.7 (11–303)	146.3 (46.6–424.1)	0.318
Resection (major)#	35 (70)	23 (60.5)	12 (100)	0.025
Surgery time** (min)	228.2±106.2	197.5±76.5	325.5±130.3	0.007
Blood loss* (mL)	600 (20–8000)	250 (20–4700)	750 (100–8000)	0.007

* Median (range); ** mean±SD; # n (%). LF – liver failure; HCC – hepatocellular carcinoma; CCC – cholangiocarcinoma; PLT – platelet count; PTA – prothrombin time activity; ALT – alanine aminotransferase; AST – aspartate aminotransferase; ALB – serum albumin; TB – serum total bilirubin; ALP – alkaline phosphatase; GGT – γ -glutamyl transpeptidase.

a median serum TNC level in PHLF of 2901.90 ng/mL (range, 670.06–4655.63 ng/mL; p=0.001) (Figure 4C).

The postoperative serum levels of CCN3 in patients with PHLF were significantly higher than those in the patients without PHLF. The median preoperative serum level of CCN3 in non-PHLF

was 2.33 ng/mL (range, 1.30–12.63 ng/mL) compared with a median serum CCN3 level in PHLF of 3.30 ng/mL (range, 2.19–12.45 ng/mL; p=0.292). The median serum level of CCN3 at postoperative day 1 in non-PHLF was 4.48 ng/mL (range, 2.37–12.33 ng/mL) compared with a median serum CCN3 level in PHLF of 7.38 ng/mL (range, 3.99–16.07 ng/mL; p=0.002).

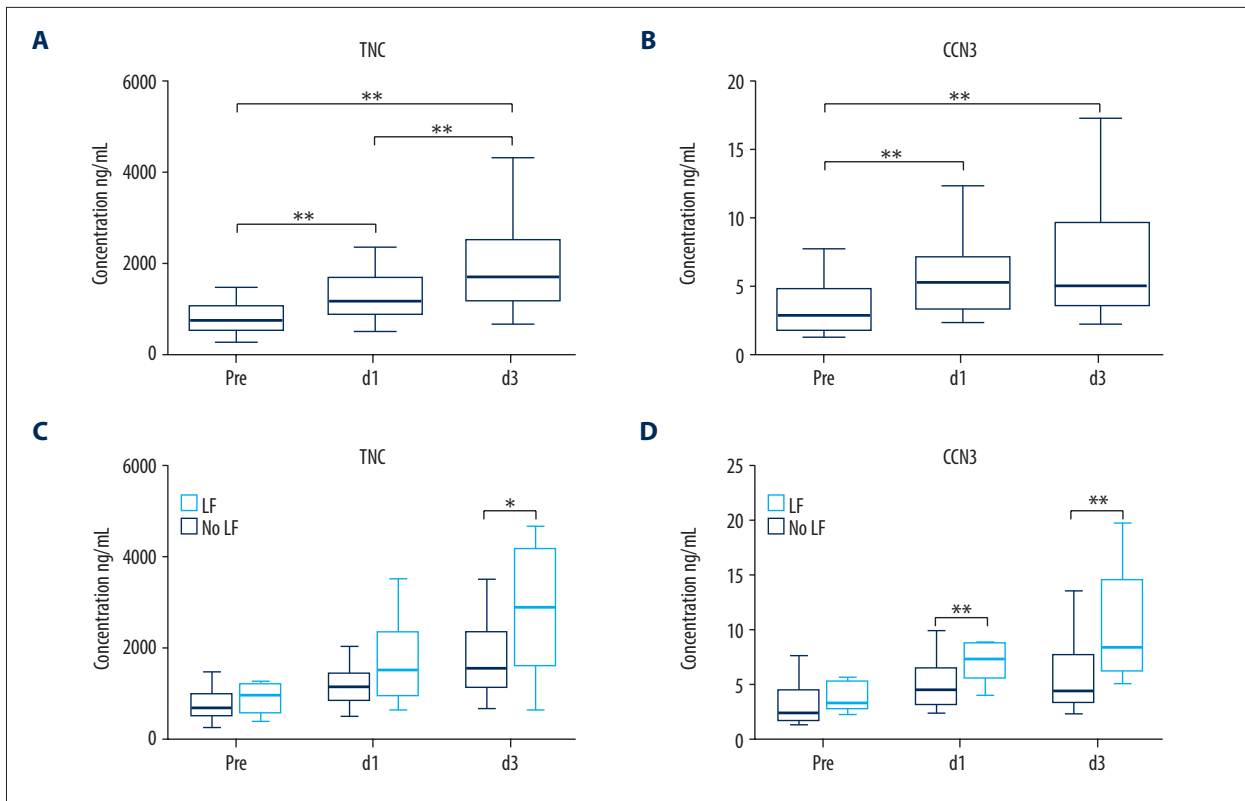


Figure 4. Perioperative tenascin-C (TNC) and cellular communication network factor 3 (CCN3) levels in 50 patients with post-hepatectomy liver failure (PHLF) at increasing time points. **(A)** Perioperative TNC levels before (Pre), and day 1 (d1), and at day 3 (d3) after liver resection. **(B)** Perioperative CCN3 levels before (Pre), and at day 1 (d1), and day 3 (d3) after liver resection. **(C)** and **(D)** show the patients stratified as those with and without post-hepatectomy liver failure (PHLF). * $p < 0.05$, ** $p < 0.01$.

The median serum level of CCN3 at postoperative day 3 in non-PHLF was 4.38 ng/mL (range, 2.28–17.16 ng/mL) compared with a median serum CCN3 level in PHLF of 8.41 ng/mL (range, 5.04–39.35 ng/mL; $p = 0.003$) (Figure 4D).

Postoperative serum levels of TNC and CCN3 levels predictive for PHLF

Based on the above data, we found that the changes in the serum levels of TNC and CCN3 during the early postoperative period were closely associated with the occurrence of PHLF. Serum levels of TNC and CCN3 on day 4 following partial hepatectomy were predictive biomarkers of PHLF using receiver operating curve (ROC) analysis. A significant association between the TNC and CCN3 levels on day 3 and PHLF was found after calculating the areas under the curve (AUC) for TNC=0.75 ($p = 0.008$) and CCN3=0.80 ($p = 0.002$) (Figure 5A, 5B).

We further determined the cutoff values that could detect PHLF from the maximum Youden index for TNC of 2,790 ng/mL (58.3% sensitivity and 89.5% specificity) and serum CCN3 of 5 ng/mL (100% sensitivity and 64.9% specificity). The combination of

high serum TNC and high serum CCN3 (TNC >2790 ng/mL and CCN3 >5 ng/mL) was a new variable that identified the group at high-risk for PHLF. Multivariate logistic regression analysis for the predictive potential of the combination of high serum TNC and high serum CCN3 with other significant factors or factors of interest was close to significance ($p < 0.1$) and the TNC^{high}+CCN3^{high} combination was an independent risk factor for PHLF with an odds ratio (OR) of 26.583 ($p = 0.008$) (Table 2).

Patients in the high-risk group for PHLF had increased postoperative morbidity and increased the length of stay in hospital

In patients who underwent partial hepatectomy, the incidence of PHLF in the high-risk group was 70%, which was significantly higher than the low-risk group at 12.5% ($p < 0.001$) (Figure 6A). The patients in the high-risk group had an increased incidence of Clavien-Dindo [27] grade II–IV postoperative complications (80%) compared with the patients in the low-risk group (30%) ($p = 0.012$) (Figure 6B). Also, the length of hospital stay following surgery was significantly increased due to the high-risk patient profile, with a median postoperative hospital stay in the PHLF

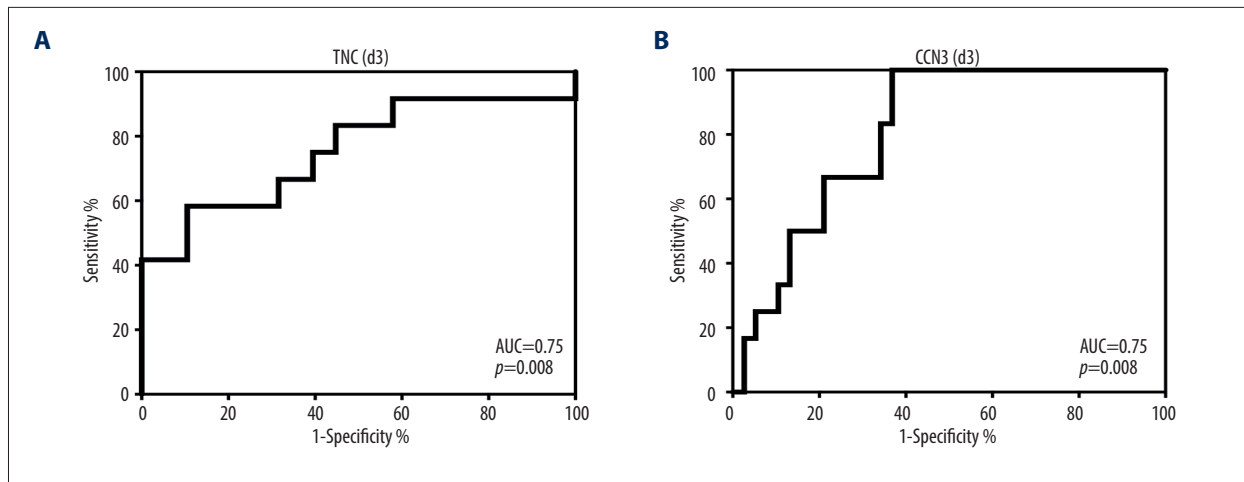


Figure 5. Receiver operating characteristic (ROC) curves and area under the curve (AUC) for serum measurement of tenascin-C (TNC) and cellular communication network factor 3 (CCN3) for the diagnosis of post hepatectomy liver failure (PHLF). **(A)** ROC curve analysis for TNC levels for the diagnosis of PHLF at postoperative day 3. **(B)** ROC curve analysis for CCN3 levels for the diagnosis of PHLF at postoperative day 3.

Table 2. Multivariate logistic regression analysis of the risk of post hepatectomy liver failure (PHLF).

Characteristic	Total (n=50)	Group		OR	95% CI	p Value
		No LF (n=38)	LF (n=12)			
TNC ^{high} +CCN3 ^{high} #	10 (20)	3 (7.9)	7 (58.3)	26.583	2.398–294.634	0.008
AST* (U/L)	27.8 (11.7–404)	25.95(11.7–404)	40.1 (18–179.7)	1.006	0.992–1.021	0.385
TB** (μmol/L)	15±7.6	14±7	18.3±8.8	1.075	0.965–1.197	0.188
ALP** (U/L)	103.9±55.9	95.7±53.3	130±58.4	0.994	0.976–1.012	0.498
Resection (major)#	35 (70)	23 (60.5)	12 (100)	1.720	0.099–29.945	0.710
Surgery time** (min)	228.2±106.2	197.5±76.5	325.5±130.3	1.011	0.999–1.023	0.079
Blood loss* (mL)	600 (20–8000)	250 (20–4700)	750 (100–8000)	1.000	1.000–1.001	0.550

Individual TNC or CCN3 level was not used in the multivariate analysis because they were combined to define the high-risk group (TNC^{high}+CCN3^{high}) and accordingly depended on each other. * Median (range); ** mean±SD; # n (%). LF – liver failure; OR – odds ratio; CI – confidence interval; TNC – tenascin-C; CCN3 – Cellular Communication Network Factor 3; AST – aspartate aminotransferase; TB – serum total bilirubin; ALP – alkaline phosphatase.

group, of 17 days (range, 7–80 days) compared with the non-PHLF group, of 8 days (range, 3–64 days) (p=0.001) (Figure 6C).

Discussion

Currently, several clinical tools are available to evaluate the preoperative hepatic functional reserve of patients, including serum biochemical markers, and comprehensive scoring systems, including the model of end-stage liver disease (MELD) scoring system and the Child-Pugh scoring system, liver function quantitative tests, such as the indocyanine green (ICG) excretion test, and imaging to evaluate the liver parenchyma

and vascular lesions, and liver volume measurements [5]. Also, the development of postoperative liver failure in patients who have undergone partial hepatectomy can depend on the skill of the surgical team and the level of perioperative management, which are factors that cannot be controlled to prevent post hepatectomy liver failure (PHLF) [28]. A combination of early postoperative predictors may be more reliable to predict postoperative risk and improve prognosis. This study evaluated the role of two matricellular proteins, tenascin-C (TNC) and cellular communication network factor 3 (CCN3), as prognostic factors for PHLF in a rat model of partial hepatectomy and in 50 patients following partial hepatectomy.

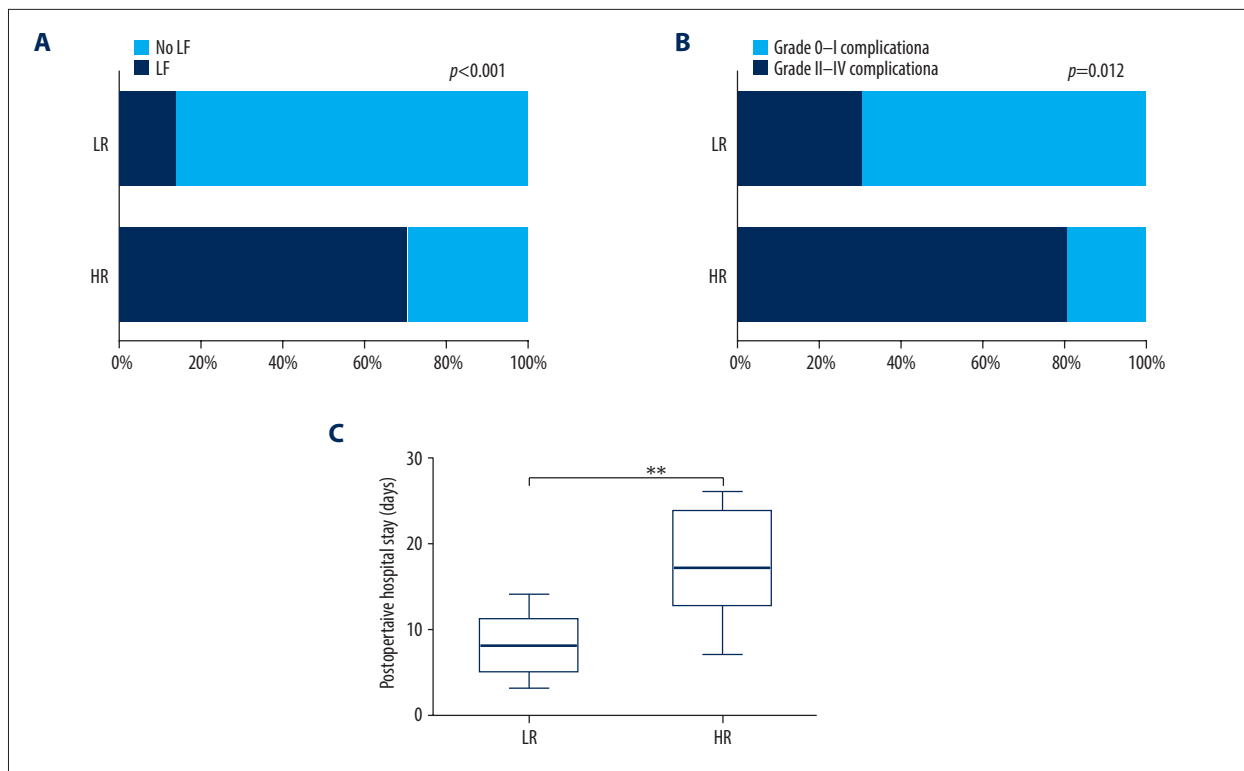


Figure 6. Comparison of the incidence of post hepatectomy liver failure (PHLF), patient morbidity, and postoperative hospitalization in the high-risk and low-risk patient groups. **(A)** Comparison of the incidence of PHLF between the high-risk patient group and the low-risk patient group. **(B)** Comparison of the incidence of morbidity between the high-risk patient group and the low-risk patient group. **(C)** Comparison of the incidence of postoperative hospitalization between the high-risk patient group and the low-risk patient group. $** p < 0.01$.

Ideally, a clinical model that is closely related to the real-world clinical setting is required to investigate the correlation between the matricellular proteins, TNC and CCN3, and liver failure after extensive liver resection. In this study, a safe but extensive hepatectomy was performed in rats by adapting the method described by Madrahimov et al. [24]. All rats that underwent 85% partial hepatectomy survived. However, more than half of the rats that underwent 90% partial hepatectomy died within 72 h after surgery, and the surviving rats showed features of post-hepatectomy liver dysfunction, including persistent hyperbilirubinemia and reduced liver proliferative capacity. Therefore, the 85% resection, or the 85% PHx rat model, was used as the extreme hepatic resection model, and 90% resection was used as the PHLF model in this study.

Matricellular proteins play key roles in tissue remodeling, inflammation, and immune responses, and their levels change in various disease states, including after hepatectomy [6]. Matricellular proteins are soluble proteins that are present in the circulation and serve as biological indicators for the prediction of disease progression [6]. For example, several recent studies have shown that thrombospondin 1 (TSP-1), which is mainly released by activated platelets, inhibits liver

regeneration following partial hepatectomy by activating transforming growth factor- β 1 (TGF- β 1) in a rodent model [8], and the association between TSP-1 and PHLF has been validated in clinical studies [9,29].

TNC is a large extracellular matrix (ECM) glycoprotein that is highly expressed during embryonic development and has a highly regulated spatiotemporal distribution with negligible TNC levels found in most healthy adult tissues [14]. In adults, increased expression of TNC is associated with inflammation, tissue regeneration, and cancer [30,31]. For example, TNC is expressed in heart disease and has been used as a biomarker of patient prognosis and disease progression [17]. Tanaka et al. found that serum TNC levels were closely associated with the histological stage and the degree of inflammation in chronic hepatitis [32].

CCN3 belongs to the CCN family of regulatory proteins, which is the only member of this protein family that inhibits the progression of the cell cycle [33]. In 2008, van Roeyen et al. reported a negative correlation between glomerular cell proliferation and CCN3 in anti-Thy1-induced glomerulonephritis, whereas CCN1 and CCN2 expression is found to increase with cell numbers [34].

However, in contrast to TNC, CCN3 possesses anti-inflammatory activities by negatively regulating nuclear factor kappa B (NF- κ B) activity and inhibiting monocyte adhesion to endothelial cells [35]. Currently, the expression profiles of both TNC and CCN3 after hepatectomy have not previously been reported.

The findings from this study showed that increased expression levels of TNC and CCN3 were found in the rat model after partial hepatectomy, and the expression levels of both proteins in the PHLF model was significantly higher than that in extreme hepatic resection model. Regardless of the significantly increased liver mRNA and serum level of TNC, the mRNA values were not directly correlated with the serum levels following over time. The following assumptions may explain this phenomenon. Firstly, the increase in serum levels of TNC that occurred six hours following partial hepatectomy might not be the result of *de novo* synthesis of the protein but due to exocytosis or degranulation. As protein release decreases, protein synthesis gradually increased more rapidly in the 85% partial hepatectomy group. Secondly, there are many types of TNC isomers, which can be generally divided into low molecular weight isomers and high molecular weight isomers. The enzyme-linked immunoassay (ELISA) kit used in this study mainly detected high molecular weight isomers. This finding suggests a future need to determine the composition of TNC protein that occurs in the serum after hepatectomy, which might have led to inconsistent results.

In the present study, preoperative and postoperative serum samples were collected from patients undergoing partial hepatectomy, and serum levels of TNC and CCN3 were measured in a real-world clinical context. The preoperative clinical findings and liver function tests were compared between the patients with and those without PHLF, and the TNC and CCN3 profiles were similar to those in the animal experiments. These results indicated that TNC and CCN3 might be potential markers of PHLF. Furthermore, the receiver operating characteristic (ROC) curve analysis showed that the TNC and CCN3 levels on postoperative day 3 have a potential role as biomarkers for the prediction of PHLF. Multivariate analysis showed that high serum levels of TNC and CCN3 on postoperative day 3 after hepatectomy was an independent prognostic factor for PHLF. Univariate analysis showed that the degree of complexity of the surgery, including blood loss and duration of surgery, also resulted in PHLF, but this association was not confirmed by multivariate analysis. This study supported the clinical relevance of the high expression pattern of these matricellular proteins, as patients with high serum levels of TNC and CCN3 on postoperative day 3 showed a significantly increased risk of PHLF, Clavien-Dindo grade II-IV postoperative complications, and duration of hospitalization.

Previous studies have shown that portal hyperperfusion of the residual liver after hepatectomy, which leads to a significant

increase in the shear stress of blood flow in the hepatic sinus and portal vein, is the initial cause of PHLF [36,37]. Mechanical stress has previously been shown to increase TNC expression [17,38], which may be an important factor in the subsequent inflammatory response. Previous studies have shown that TNC expression is induced at sites of inflammation [16]. In extensive hepatectomy, the excessive inflammation caused by the surgery can result in damage to healthy liver tissues [39]. These findings shed light on the mechanism of action of TNC in PHLF.

In the present study, increased expression of CCN3 was also found after partial hepatectomy, particularly in patients with liver failure. Therefore, the proinflammatory effect of TNC and the anti-proliferative effect of CCN3 may predominate during the development of PHLF. However, due to their diverse biological functions, the precise mechanism by which these matricellular proteins affect PHLF requires further study. To our knowledge, this preliminary study is the first to show that serum levels of TNC and CCN3 serum levels may be potential biomarkers of liver failure in patients following liver resection.

This study had several limitations. Selection bias may have been caused by the small clinical sample of 50 patients studied. Also, the clinical finding may have been affected by preexisting liver disease and neoplastic diseases, which are known to affect the expression of matricellular proteins [6]. Although these factors were balanced between the study groups, the lack of significant differences may have been caused by the small sample size. The mechanisms involved in the effects of TNC and CCN3 in liver regeneration were not investigated, but further studies should be undertaken to determine the mechanisms involved in the role of these matricellular agents in liver regeneration.

Conclusions

This study aimed to investigate the role of tenascin-C (TNC) and cellular communication network factor 3 (CCN3), which are matricellular proteins of the extracellular matrix (ECM), as prognostic factors for post hepatectomy liver failure (PHLF) in a rat model of partial hepatectomy and 50 patients following partial hepatectomy. In the rat partial hepatectomy model, mRNA and serum levels of TNC and CCN3 were significantly increased within the first 24 hours, and were higher in the 90% partial hepatectomy group compared with the 85% partial hepatectomy group. Multivariate analysis confirmed that increased serum levels of TNC and CCN3 on postoperative day 3 was a significant predictor of PHLF, which was associated with more than twice the risk of severe morbidity when compared with the low-risk patients (80% vs. 30%) and a significantly longer hospital stay (17 days vs. 8 days). Further studies are needed to evaluate the potential role of the matricellular proteins, TNC and CCN3 as early clinical predictors for PHLF.

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