ORIGINAL ARTICLE

AASLD
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THE STUDY OF LIVER DISEASES

Complement complex 1 subunit q-mediated hepatic stellate cell activation with connective tissue growth factor elevation is a prognostic factor for survival in rat and human chronic liver diseases

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Abstract

Complement complex 1 subunit q (C1q) has multiple functions, including cell migration, in addition to its traditional complement-activating effect. Research shows C1q is a ligand for frizzled receptors (FZDs). FZD-induced yes-associated protein (YAP)/transcriptional co-activator with PDZ-binding motif (TAZ) alternate Wnt signaling activation induces connective tissue growth factor (CTGF) production and hepatic stellate cell (HSC) activation. However, no study exists in which C1q directly induces CTGF in HSCs. Here, we investigated the role of C1q in HSC activation. Human HSCs (LX2) were incubated with C1q to assess HSC activation. C1q and fibrotic markers were assessed using immunohistochemistry, immunoblotting, and quantitative reverse-transcription polymerase chain reaction in cirrhotic rats administered CCl_4 for 21 weeks. Serum C1q, liver function, and fibrosis score were measured in 91 patients with chronic liver disease. The correlations between serum C1q and liver function, fibrosis score, and survival prognosis were examined. C1q-activated LX2s showed morphologic changes, up-regulation of *CTGF*, tissue inhibitors of metalloproteinases (*TIMP-1*), and alternate Wnt signal genes *FZD2*, *TAZ*, and cysteine-rich angiogenic inducer 61 (*Cyr61*). Cirrhotic rat liver C1q expression correlated with the Azan-positive area and expression of *CTGF*, *TIMP-1*, hyaluronan synthase (*HAS*)1, *HAS3*, and *CD44*. Expression of C1q protein and *C1q*, *CTGF*, and *TIMP-1* genes were higher in deceased cirrhotic rat livers compared to surviving rats. Human serum C1q levels increased in liver cirrhosis compared to chronic hepatitis and correlated with liver fibrosis and functional markers. Ten patients suffered liver-related death over a 66-month observation period. The C1q cut-off value (11 mg/dl) showed

patients with serum values < 11 mg/dl had longer rates of survival compared to C1q ≥ 11 mg/dl. *Conclusion*: C1q-mediated HSC activation in liver fibrosis is associated with CTGF elevation. Additionally, serum C1q may be diagnostic for survival in human chronic liver diseases.

INTRODUCTION

Complement complex 1 subunit q (C1q) consists of six heterotrimers with each containing the C1qa, C1qb, and C1qc segments. C1q is traditionally recognized as one of the components in the complement system that enhances the ability of antibodies and phagocytic cells to clear microbes and damaged cells from an organism, promote inflammation, and attack the pathogen's cell membrane, in conjunction with other complement factors.[1] Serum C1q levels increase with age from 45 to 75 years old, irrespective of sex differences in healthy individuals,[2,3] and increase in a variety of pathological conditions, including infectious and inflammatory diseases.[4] In liver diseases, human serum C1q levels were found to be increased in liver cirrhosis (LC),[5] chronic hepatitis (CH) caused by infection with hepatitis C virus (HCV),[6] and alcohol-associated liver disease.[7] Furthermore, C1q-mediated ethanol-induced liver injury in mice[8] and hepatic insulin resistance in mice fed a high-fat diet[9] occur through activation of the classical complement pathway. Besides its traditional complement-activating effect, C1q exhibits a variety of complement-independent activities, such as playing a role in cell processes, including migration and remodeling[1] and cancer promotion.[10] C1q also binds to frizzled receptors (FZDs), subsequently activating canonical Wnt signaling and promoting aging-associated decline in tissue regeneration.[11] However, the complement-independent activities of C1q in chronic liver diseases is not well understood.

During chronic liver damage, continuous hepatic stellate cell (HSC) activation occurs through a variety of stimulators, such as transforming growth factor β (TGF-β) and platelet-derived growth factor, which induce excessive matrix deposition and pathological scar tissue formation leading to the progression of liver fibrosis and eventually resulting in cirrhosis, hepatocellular carcinoma (HCC), and liver failure.[12–14] Therefore, a comprehensive understanding of HSC activation and mechanistic activators is essential in the development of new diagnostic and therapeutic approaches.[15] The canonical Wnt positive-signaling loop, which includes Wnt, FZDs, nuclear β-catenin, and stearoyl-coenzyme A, is critical to the progression of liver fibrosis through HSC activation.[16,17] Furthermore, current studies have demonstrated that the yes-associated protein (YAP) signaling pathway is a critical driver of HSC activation that leads to connective tissue growth factor (CTGF) production from activated HSCs.[18]

In other fields of research outside of liver disease, several studies have revealed that CTGF production is induced by the activation of an alternative Wnt signaling pathway involving Wnt–FZD-induced YAP/transcriptional co-activator with PDZ-binding motif (TAZ).[19] In the process of osteogenic differentiation, β-catenin-independent Wnt signaling pathway activation identified C1q as a ligand for FZDs,[11] which are expressed in liver parenchyma (hepatocytes) and nonparenchymal cells (HSCs, hepatic sinusoidal endothelial cells, and Kupffer cells).[20] Based on this evidence, we hypothesized that C1q contributes to HSC activation through FZD-induced YAP/TAZ activation and CTGF production. Here, we investigated the role of C1q in HSC activation and liver fibrosis by using a human HSC cell line, cirrhotic rats, and patients with chronic liver disease. In addition, we examined whether serum C1q levels are a prognostic factor for survival in rats and human patients.

MATERIALS AND METHODS

Animals

Our animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Hokudo Co., Ltd. (Sapporo, Japan). Our cirrhosis rat model has been described in detail.[14] Briefly, Wister rats aged 6 weeks (n = 21) were orally administered CCl_4 at 1.0 ml/kg twice a week for 5 weeks to establish a rat model of advanced fibrosis. To maintain the cirrhotic state, rats received CCl_4 at 0.5 ml/kg orally twice a week for 16 weeks (total 21 weeks). We divided the rats into a survival group and deceased group. The survival group contained rats that survived for 21 weeks after CCl_4 administration, whereas the deceased group contained rats that died within 21 weeks of CCl_4 administration. The control designation indicated advanced fibrotic rats 5 weeks after CCl_4 administration. Blood was collected at 9, 13, 17, and 21 weeks after established cirrhosis and was kept at −80°C. All rats that survived were killed under anesthesia at the termination of treatment. Whole rat blood was collected and disgorged into tubes with or without anticoagulant. For rats that were dying, we checked the rat condition every day, killed them under anesthesia just before death, and collected blood and liver tissue. Liver tissue was fixed in formalin for 24 hours and embedded in paraffin. The remaining

liver tissue was quickly frozen in liquid nitrogen and stored at −80°C. Serum was used for measurement of albumin (Alb), total-bilirubin (T-bil), alanine aminotransferase (ALT), and creatinine (Cr) at Hokudo Co., Ltd. Serum was used for C1q enzyme-linked immunosorbent assay (ELISA; Hycult Biotech, Wayne, PA, USA) according to the manufacturer's instruction.

Liver immunostaining

Liver sections were prepared and stained for C1q (Abcam, Cambridge, UK) by immunohistochemistry using paraffin-embedded samples according to the manufacturer's instructions. All pictures were taken by KEYENCE (BZ-X710) (KEYENCE, Japan).

Immunoblot analysis

For immunoblot analysis, 20 μg of whole-liver lysate was resolved by TGX precast gels and transferred to polyvinylidene fluoride membranes (BioRad, Hercules, CA, USA). Blotted membranes were incubated with anti-C1q antibody (Abcam) or anti-CTGF antibody (Abcam), followed by peroxidase-conjugated secondary antibody (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Protein bands were visualized using enhanced chemiluminescence reagents (Thermo Fisher Scientific) and digitized using a charge-coupled device camera (LAS4000 mini; Fuji film, Japan). Expression intensity was quantified by Multi Gauge (Fuji). Protein loading was verified using β-actin (GeneTex) antibody.

In vitro cell culture studies

LX2 cells were grown and maintained in Dulbecco's modified Eagle's medium (Gibco, Camarillo, CA, USA) supplemented with 10% fetal bovine serum (GE Healthcare Life Sciences), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES; Gibco), sodium pyruvate (Gibco), and penicillin and streptomycin (Gibco) at 37°C in a 5% CO_2 incubator. LX2 cells were cultured in medium without serum for 24 hours, then treated with 100 μg/ml of human C1q purified from human serum (Sigma-Aldrich, Saint Louis, MO, USA), or 5 ng/ml of TGF-β (R&D Systems, Minneapolis, MN, USA) as a positive control for 24 hours or 48 hours. Treated LX2 cells were stained with calcein-AM (Thermo Fisher Scientific) to observe the cell morphology or were collected for RNA extraction. Cell migration was assessed by wound healing. LX2 cells were treated with mitomycin (1 μg/ml) for 2 hours before the scratch. A scratch was performed through the confluent cell monolayer, and detached cells and debris were washed away. Cells were treated with 100 μg/ml of human C1q purified from human serum or 5 ng/ml of TGF-β for 24 hours. Treated LX2 cells were stained with calcein-AM for imaging of migrated cells into the wound. Cell viability was measured by the lactate dehydrogenase (LDH) cytotoxicity assay (Dojindo, Japan) using cell-conditioned media after 48 hours, according to the manufacturer's instructions.

Real-time polymerase chain reaction

Total RNA was isolated from liver tissue by using Trizol (Thermo Fisher Scientific) followed by deoxyribonuclease treatment (Thermo Fisher Scientific) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 500 ng of total RNA by using the cDNA Synthesis kit (Takara, Shiga, Japan). Real-time polymerase chain reaction (qPCR) quantification was performed with two forward and reverse primers and KAPA SYBR FAST qPCR master mix (KAPA Biosystems, Woburn, MA, USA) in a 7300 Real-Time PCR Detection System (Thermo Fisher Scientific). The PCR primers were used to amplify each gene as listed in Table S1. Mean values were normalized to *β2-microglobulin* for messenger RNA (mRNA).

Human samples

All research was conducted in accordance with both the Declarations of Helsinki and Istanbul and was approved by the Clinical Research Ethics Review Committee of Mie University Hospital (H2019-004). This study was performed retrospectively on stored samples, and patients could opt out of their data being used. Written informed consent was obtained from all subjects at the time of blood sampling. From December 2013 to August 2016, patients (n = 91) were recruited by the stage of their chronic liver disease, which was clinically diagnosed by blood tests, imaging by ultrasound or computed tomography, and esophageal varix by endoscopy, as general protocol dictates. Liver biopsy was performed when diagnosis was difficult, using the general protocol. HCC was diagnosed based on histologic findings or typical imaging characteristics. Patients who had other malignancies within the past 3 years, severe hepatic failure (serum T-bil level >5 mg/dl), uncontrollable infection, heart failure greater than the New York Heart Association-defined category of class II, human immunodeficiency virus infection, pregnancy, or psychiatric problems were deemed to be unsuitable for clinical study. Blood samples were collected for measurement of serum C1q, Alb, T-bil, aspartate aminotransferase (AST), ALT, gamma-glutamyltransferase (GGT), platelet count (PLT), hyaluronic acid (HA), tissue inhibitors of metalloproteinases (TIMP-1), and N-terminal procollagen III propeptide (PIIINP). Serum C1q was measured at Medical and Biological Laboratories Co., Ltd. (Japan). Serum was used for CTGF ELISA (Cloud-Clone, Katy,

TX, USA) according to the manufacturer's instructions. The enhanced liver fibrosis (ELF) score was calculated using HA, TIMP-1, and PIIINP. For the analysis of survival, we eliminated four patients who were lost to follow-up within a month.

Statistical analyses

All data are expressed as mean±SEM unless otherwise noted. Data were analyzed using unpaired *t* tests or the Mann-Whitney U test in two groups. Correlation was determined using single regression analysis or the Spearman rank-sum test. For each continuous variable, the optimal cut-off value that maximized the sum of sensitivity and specificity was selected using receiver operating characteristic (ROC) curve analysis for survival. Cumulative survival rates were estimated with the Kaplan-Meier method and compared between groups by the log-rank test. Statistical analyses were performed using GraphPad (GraphPad Software Inc., CA, USA) for comparison of continuous variables. Differences were considered to be significant at $p < 0.05$.

RESULTS

C1q induces CTGF production through the alternative Wnt signaling pathway in LX2 cells

To investigate whether C1q activates HSCs, we added human C1q to LX2 cells (human-cultured HSCs) and examined cell morphology as well as gene expression profiles related to fibrosis and the alternative Wnt signaling pathway (Figure 1A). C1q induced an increase in tube formation (Figure 1B) and nonoriented migration (Figure 1C) after 48 hours of treatment compared to untreated cells. TGF-β was used as a positive control (Figure 1B,C). Cell viability was 92.5% for C1q and 85.5% for TGF-β at 48 hours after addition of C1q (Figure S1A). The addition of C1q induced a significant elevation of *CTGF* mRNA levels ($p < 0.01$) and a slight elevation of *TIMP-1* mRNA levels, but no increase was observed in the level of collagen 1-alpha (*collagen*) mRNA (Figure 1D) at 48 hours after addition. In contrast, TGF-β significantly induced *TIMP-1*
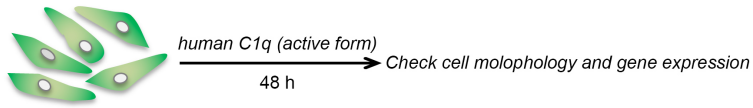
and *collagen* mRNA levels, but no elevation of *CTGF* mRNA level was observed (Figure 1D). These results suggest a different HSC activation mechanism induced by C1q than the HSC activation by TGF-β. Recently, several studies demonstrated that C1q binds to FZD receptors[11] expressed on LX2 cells[16] and primary HSCs.[20] Further, C1q directs YAP activation-induced CTGF production in activated HSCs[18] and drives alternate Wnt-related CTGF production through the FZD–TAZ/YAP signaling pathway.[19] These observations led us to further investigate whether C1q directly induces CTGF elevation through an alternate Wnt signaling pathway in LX2 cells. C1q administration to LX2 cells induced a significant elevation of *FZD2* ($p < 0.05$) and *TAZ* mRNA levels, leading to a significant increase of cysteine-rich angiogenic inducer 61 (*Cyr61*) ($p < 0.01$) as a result of alternative Wnt signaling pathway expression[19] (Figure 1D). In contrast, TGF-β did not induce elevated *TAZ* and *Cyr61* mRNA levels (Figure 1E). The addition of C1q also induced a slight elevation of *CTGF*, *TIMP-1*, *FZD2*, and *TAZ* mRNA levels at 24 hours after addition, but no significant elevation was observed (Figure S1B). These results show that C1q leads to CTGF elevation through an alternative Wnt signaling pathway (Figure 1F).

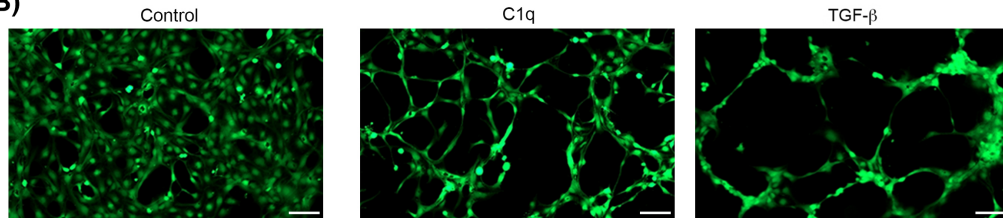### Liver C1q expression correlates with fibrotic genes, including *CTGF*, in cirrhotic rats

We next examined the association between liver C1q and CTGF as well as liver fibrosis-related genes from cirrhotic rats administered CCl_4 for 21 weeks (5 weeks to establish advanced fibrosis and an additional 16 weeks to determine survival percentage); we reported this in a previous study.[21] Liver C1q was expressed in hepatocytes as well as infiltrated macrophages and was increased in cirrhotic rats compared to normal rats; this was assessed using immunohistochemistry probing with a C1q antibody (Figure 2A). Liver C1q expression was strongly correlated with the degree of liver fibrosis assessed by Azan staining ($r = 0.70$, $p < 0.05$) (Figure 2B). Furthermore, liver *C1qc* mRNA levels were strongly and significantly correlated with *CTGF* ($r = 0.87$, $p < 0.0001$) (Figure 2C). These results led us to further investigate whether liver C1q levels were associated with other

FIGURE 1 C1q activated human cultured stellate cells through *CTGF* expression. (A) Schematic illustration of the *in vitro* experiment using LX2 cells. (B) Images of LX2 cell morphology stained by calcein and treated with control (medium), C1q, or TGF-β. Scale bar, 100 μm. (C) Representative micrographs of nonoriented migration, wound healing, and assay of LX2 cells. Scale bar, 100 μm. (D) *CTGF*, *TIMP-1*, and *collagen* genes as measured by qPCR. All gene expression levels were normalized to the housekeeping control *β-actin* and are shown relative to the expression levels of control. (E) *FZD2*, *TAZ*, and *Cyr61* genes as measured by qPCR. All gene expression levels were normalized to the housekeeping control *β-actin* and are shown relative to the expression levels of control. (F) Cartoon of CTGF production by C1q in HSCs. ** $p < 0.01$, * $p < 0.05$. Values are mean±SEM from three biological replicates. *C1q*, complement component 1 subunit q; *Collagen*, collagen 1-alpha; Cont, control; *CTGF*, connective tissue growth factor; *Cyr61*, cysteine-rich angiogenic inducer 61; *FZD2*, frizzled-2; h, hours; HSC, hepatic stellate cell; qPCR, quantitative polymerase chain reaction; *TAZ*, transcriptional co-activator with PDZ-binding motif; TGF-β, transforming growth factor β; *TIMP-1*, tissue inhibitor of metalloproteinases-1.
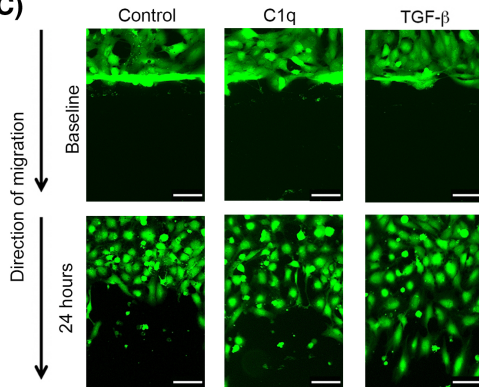
(A)
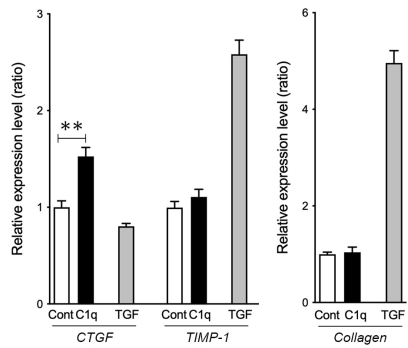

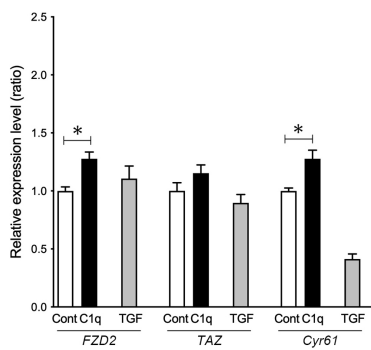
human C1q (active form)

48 h

Check cell molophology and gene expression

(B)

Control | C1q | TGF-β

(C)

Control | C1q | TGF-β

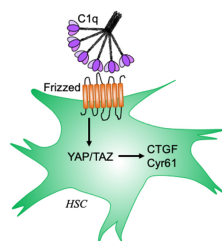Direction of migration

Baseline

24 hours

(D)

(E)

(F)

fibrotic genes, including early to advanced-stage liver fibrosis. Liver *C1qc* mRNA levels were significantly correlated with *TIMP-1* ($r = 0.87$, $p < 0.0001$) (Figure 2D), hyaluronan synthase (*HAS*)1 ($r = 0.75$, $p < 0.001$) (Figure 2E), *HAS3* ($r = 0.87$, $p < 0.0001$) (Figure 2F), and *CD44* as a receptor for HAS ($r = 0.89$, $p < 0.0001$) (Figure 2G), but there was no association with α-smooth muscle actin (α-*SMA*) or *collagen* (data not shown). C1q consists of three subcomponents, C1qa, C1qb, and C1qc. Other C1q subcomponents, namely *C1qa* and *C1qb*, were also strongly and significantly correlated with *CTGF* mRNA levels as well as other fibrotic genes (Table 1). These results indicate that C1q levels are associated with advanced liver fibrosis.

Liver C1q and fibrotic genes are significantly increased in deceased cirrhotic rats

The strong correlation between liver C1q levels and liver fibrosis, including the marker CTGF, led us to further explore whether C1q levels are associated with survival in cirrhotic rats. Liver C1q expression from whole-liver lysates was up-regulated in the deceased group when compared to the survival group as assessed by immunoblotting (Figure 3A). Liver *C1qa*, *C1qb*, and *C1qc* mRNA levels were also significantly increased in the deceased group when compared to the survival group ($p < 0.001$, $p < 0.05$, respectively) (Figure 3B). Liver *CTGF* ($p < 0.001$), *TIMP-1* ($p < 0.05$), *HAS3* ($p < 0.001$), and *CD44* ($p < 0.05$) mRNA levels were also significantly increased in the deceased group (Figure 3C). These mRNA levels were up-regulated from normal rats to advanced fibrotic rats, except HAS3 and deceased cirrhotic rats (Figure 3B,C). Furthermore, these mRNA levels in the survival cirrhotic rat group were decreased by the same levels found in the advanced fibrotic rats (Figure 3B,C). In contrast, liver α-*SMA* and *collagen* mRNA levels were not elevated in the deceased group (Figure S2A). Liver CTGF expression from whole-liver lysates was slightly up-regulated in the deceased group when compared to the survival group as assessed by immunoblotting (Figure S2B). Blood C1q concentration was also increased in the deceased group, although it was not significant (Figure 3D). These results suggest that liver C1q levels are an indicator of overall survival in our cirrhotic model of disease.

Serum C1q levels are correlated with liver function and liver fibrosis score in patients with chronic liver disease

We showed that liver C1q levels are associated with liver fibrosis, including CTGF expression, and rate of survival in cirrhotic rats. To investigate whether serum C1q levels are a prognostic factor for prolonged survival in liver disease, we further examined the association of serum C1q levels with liver fibrosis in human chronic liver diseases. We recruited 91 patients, 40 men and 51 women, with a median ± SD age of 65 ± 11 years. This group comprised 35 patients with LC and 56 patients with CH. The cohort of study patients was admitted to our study due to the following causative agents: five with hepatitis B virus, 44 with HCV, 21 with nonalcoholic steatohepatitis, eight with alcoholism, five with primary biliary cholangitis/autoimmune hepatitis, and eight with unknown disease. Serum C1q levels were significantly increased in patients with LC compared to patients with CH ($p < 0.0001$) (Figure 4A). Because serum C1q levels are known to increase with age from 45 to 75 years,[2] we checked the association between serum C1q levels and age in this cohort and determined that serum C1q levels were not significantly correlated with age (Figure S3). Serum C1q levels were significantly correlated with liver function, including Alb ($r = -0.46$, $p < 0.001$), T-bil ($r = 0.33$, $p < 0.01$), choline esterase (ChE) ($r = -0.42$, $p < 0.001$), and PLT ($r = -0.29$, $p < 0.01$) (Figure 4B). Serum C1q levels were also significantly correlated with the index for liver fibrosis, including HA ($r = 0.44$, $p < 0.0001$), PIIINP ($r = 0.47$, $p < 0.0001$), and TIMP-1 ($r = 0.49$, $p < 0.0001$), resulting in an ELF score ($r = 0.48$, $p < 0.0001$) (Figure 4C). Notably, the strongest correlation with serum C1q measurements was the level of serum CTGF ($r = 0.61$, $p < 0.0001$) (Figure 4D). We also explored the correlation of serum CTGF levels with liver function and fibrosis. Serum CTGF levels were correlated with Alb ($r = -0.36$, $p < 0.01$), T-bil ($r = 0.38$, $p < 0.01$), ChE ($r = -0.29$, $p < 0.05$), and PLT ($r = -0.49$, $p < 0.0001$) (Figure S4A). However, the correlation of serum CTGF levels with fibrotic factors was not strong (serum CTGF vs. HA, PIINP, TIMP-1, and ELF: $r = 0.31-0.38$ and all $p < 0.01$) (Figure S4B) compared to the correlation of C1q with fibrotic factors (Figure 4). These results suggest that serum C1q levels indicate liver fibrosis, including CTGF production.

FIGURE 2 Liver C1q levels were associated with liver fibrosis, including *CTGF* levels. (A) Immunohistochemical staining of liver sections from normal (n = 3) and advanced fibrotic rats specific for C1q at 5 weeks (control) (n = 3) or cirrhotic rats specific for 21 weeks of CCl$_4$ administration (n = 17). Scale bar, 100 μm. (B) Correlation between liver *C1qc* mRNA levels and Azan-positive area. (C–G) Correlation between liver *C1qc* mRNA levels and (C) *CTGF*, (D) *TIMP-1*, (E) *HAS1*, (F) *HAS3*, or (G) *CD44* mRNA levels in cirrhotic rats (n = 17) as measured by qPCR. mRNA levels from normal rats are indicated by a broken line. All gene expression levels were normalized to the housekeeping control β2-microglobulin and are shown relative to the expression levels of control rats. *C1q*, complement component 1 subunit q; CCl$_4$, carbon tetrachloride; *CTGF*, connective tissue growth factor; *HAS*, hyaluronan synthase; mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction; *TIMP-1*, tissue inhibitor of metalloproteinases-1; w, weeks.
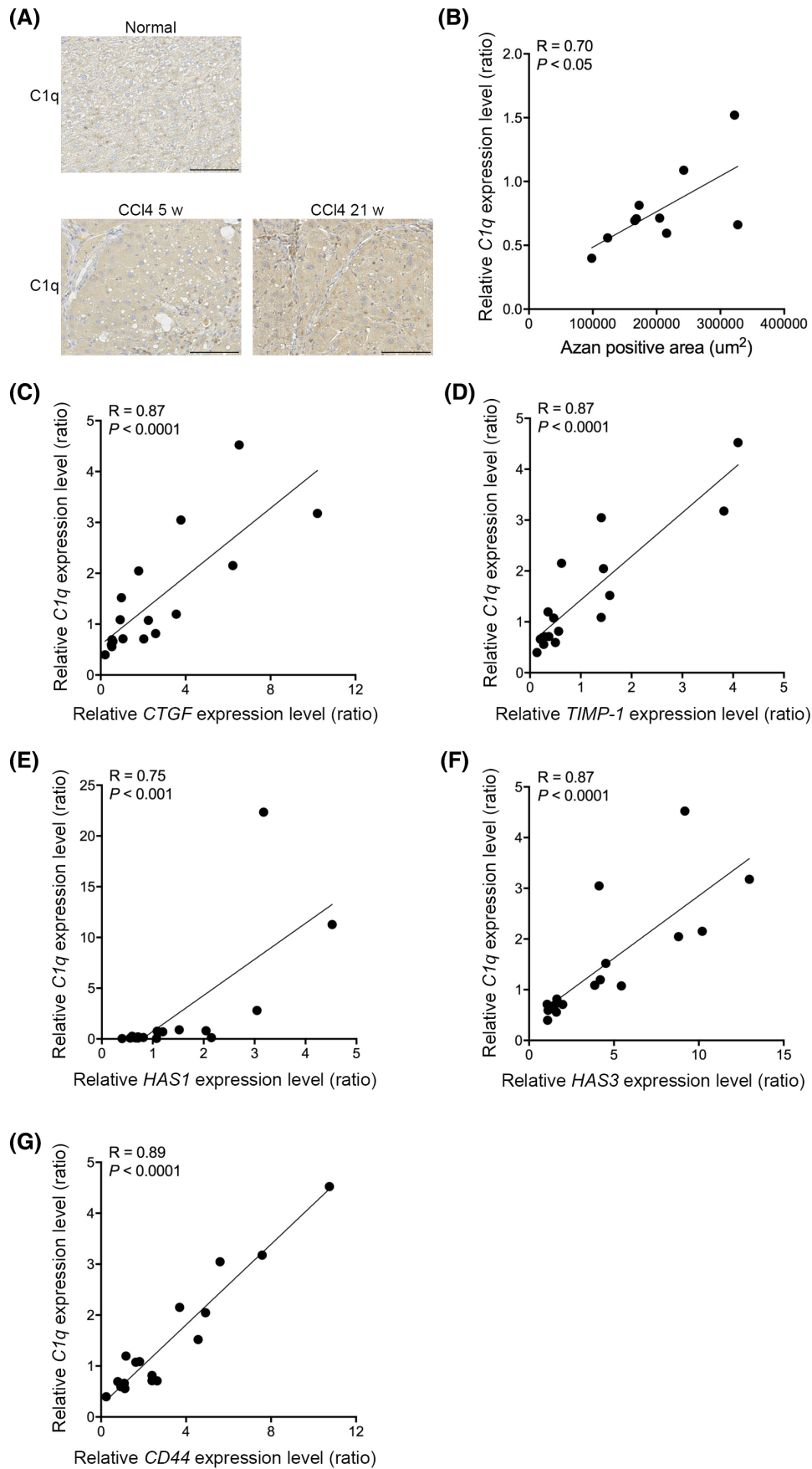
TABLE 1 Association of liver *C1qa*, *C1qb*, and *C1qc* mRNA levels in cirrhotic rats

	C1qa	*C1qb*	*C1qc*	*CTGF*	*TIMP-1*	*HAS1*	*HAS3*	*CD44*
C1qa		0.86 P < 0.0001	0.88 P < 0.0001	0.70 P = 0.0022	0.89 P < 0.0001	0.72 P = 0.0017	0.78 P = 0.0003	0.85 P < 0.0001
C1qb	0.86 P < 0.0001		0.95 P < 0.0001	0.77 P = 0.0005	0.85 P < 0.0001	0.75 P = 0.0008	0.85 P < 0.0001	0.78 P = 0.0004
C1qc	0.88 P < 0.0001	0.95 P < 0.0001		0.87 P < 0.0001	0.87 P < 0.0001	0.75 P = 0.0007	0.87 P < 0.0001	0.89 P < 0.0001

Abbreviations: *C1q*, complement complex 1 subunit q; *CTGF*, connective tissue growth factor; *HAS*, hyaluronan synthase; mRNA, messenger RNA; *TIMP-1*, tissue inhibitor of metalloproteinases-1.
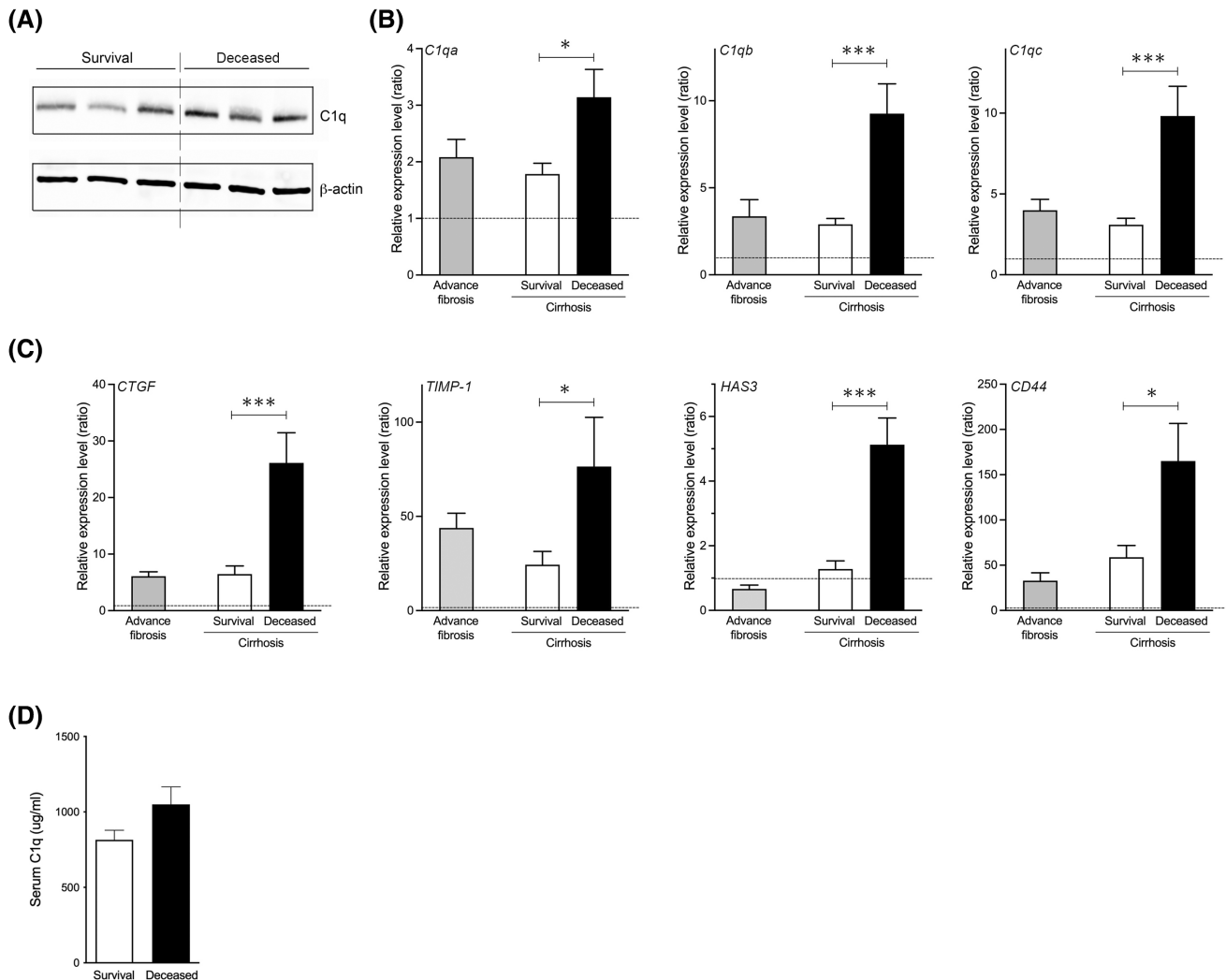

FIGURE 3 Liver C1q and fibrotic genes, including *CTGF*, are elevated in deceased cirrhotic rats. (A) Protein expression of C1q and β-actin in whole liver using immunoblotting. (B) *C1q* gene in the advanced fibrotic rats (n = 3) and survival (n = 10) and deceased (n = 7) groups of cirrhotic rats as measured by qPCR. All gene expression levels were normalized to the housekeeping control *β2-microglobulin* and are shown relative to the expression levels of normal rats (n = 3). The normal baseline of gene expression in normal rats is shown as a broken line. (C) *CTGF, TIMP-1, HAS1, HAS3,* and *CD44* genes in the advanced fibrotic rats and surviving and deceased cirrhotic rats as measured by qPCR. All gene expression levels were normalized to the housekeeping control *β2-microglobulin* and are shown relative to the expression levels of normal rats. The normal baseline of gene expression in normal rats is shown as a broken line. (D) Serum C1q levels in the surviving and deceased cirrhotic rats. ***$p < 0.001$, *$p < 0.05$. Values are mean±SEM. *C1q*, complement component 1 subunit q; *CTGF*, connective tissue growth factor; *HAS*, hyaluronan synthase; qPCR, quantitative polymerase chain reaction; *TIMP-1*, tissue inhibitor of metalloproteinases-1.
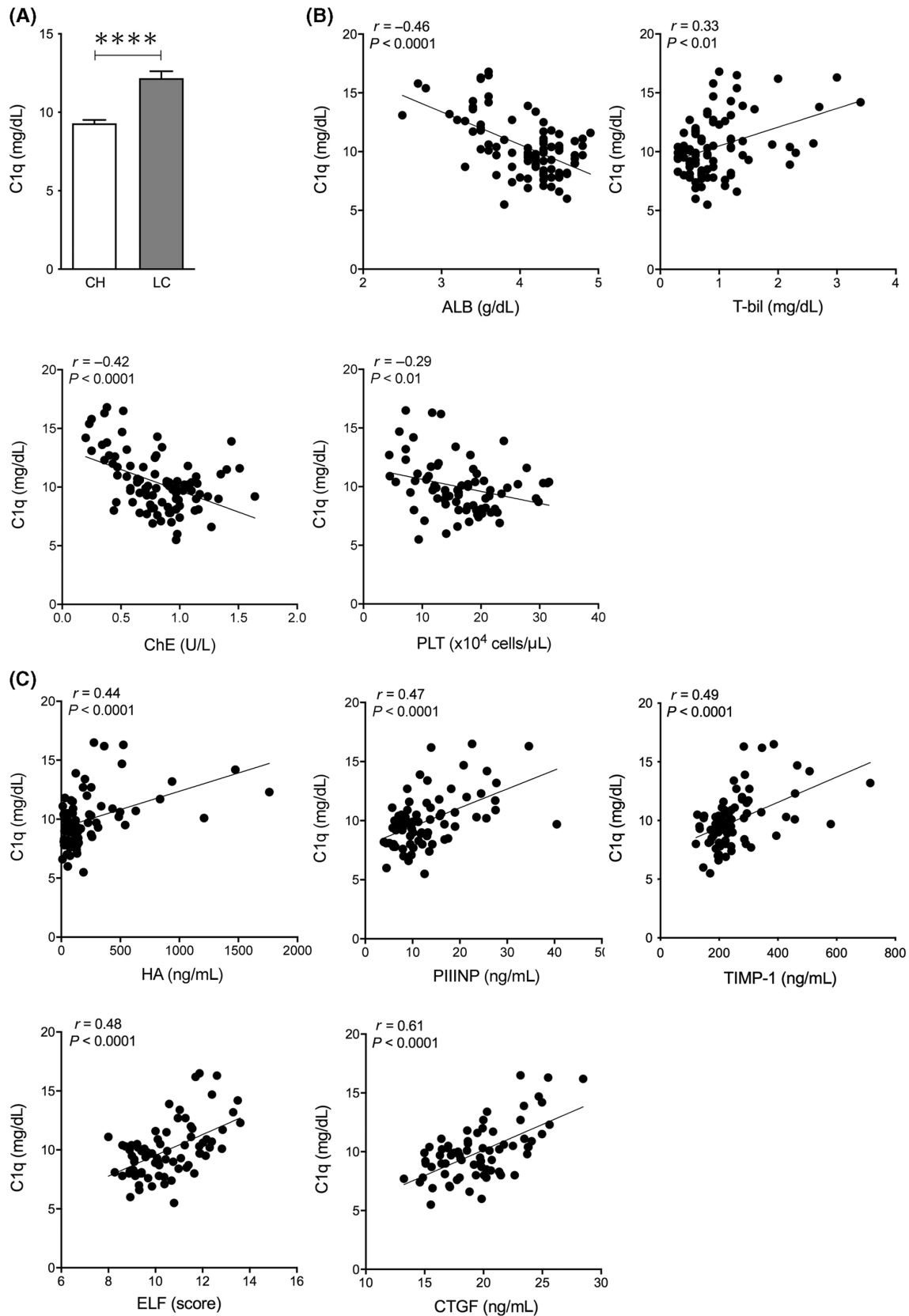
FIGURE 4 Serum C1q levels are correlated with liver function and fibrotic factors in human chronic liver diseases. (A) Serum C1q levels in patients with CH and LC (n = 91). (B) Correlation between serum C1q levels and Alb, T-bil, ChE, or PLT. (C) Correlation between serum C1q levels and fibrotic factors, including HA, PIIINP, TIMP, ELF, or CTGF. ****$p < 0.0001$. Values are mean±SEM. Alb, albumin; CH, chronic hepatitis; ChE, choline esterase; CTGF, connective tissue growth factor; ELF, enhanced liver fibrosis; HA, hyaluronic acid; LC, liver cirrhosis; PIIINP, N-terminal procollagen III propeptide; PLT, platelet; T-bil, total bilirubin; TIMP-1, tissue inhibitor of metalloproteinases-1.
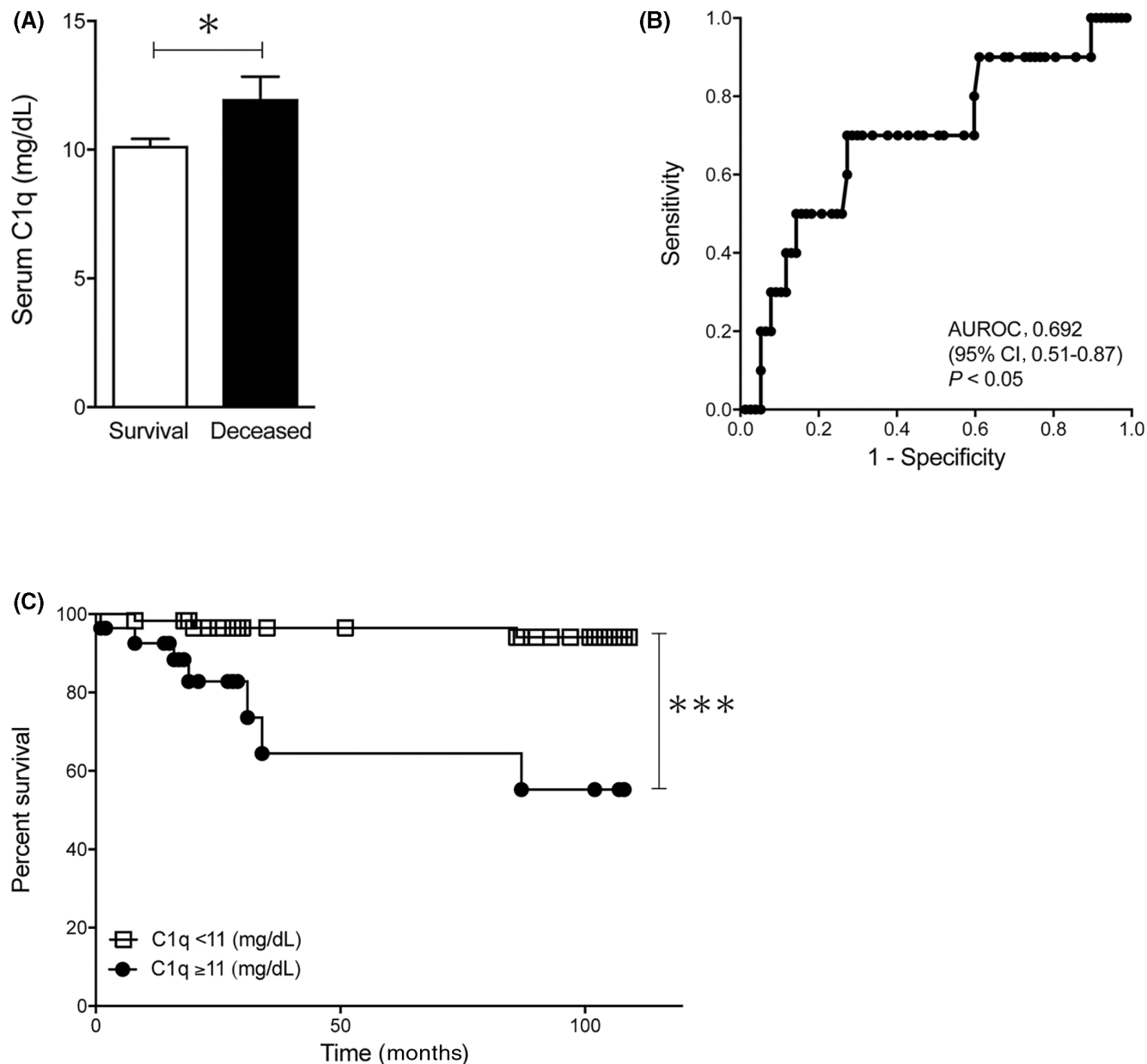
FIGURE 5 Survival was increased in human chronic liver diseases guided by serum C1q levels. (A) Serum C1q levels in surviving or deceased patients with chronic liver disease (n = 87). (B) AUROC of serum C1q levels. (C) Survival curve in human chronic liver diseases with serum C1q levels. Values are mean±SEM. ***$p < 0.001$, *$p < 0.05$. AUROC, area under the receiver operating characteristic curve; C1q, complement component 1 subunit q.

Serum C1q level is a prognostic factor for survival in patients with chronic liver disease

Finally, we explored whether serum C1q levels could be used for the prognosis of survival in patients with chronic liver disease. In our cohort, 10 out of 87 patients were deceased in the average follow-up period of 65.8 months (median, 90 months). This total excludes four patients (initial total of 91) due to no patient follow-up. Thus, all causes of death were considered liver related. Serum C1q levels were significantly elevated in patients who died compared to surviving patients

($p < 0.05$) (Figure 5A). ROC analysis showed an area of 0.692, sensitivity of 0.7, and specificity of 0.727 and led to the cut-off value of serum C1q levels at 11 mg/dl (Figure 5B). A significant decrease of Alb, PLT, and PT as well as significant increases in T-bil, blood urea nitrogen (BUN), age, presence of LC, and presence of HCC were observed in patients with C1q≥11 mg/dl when compared to patients with C1q<11 mg/dl. However, there was no difference in these patients with respect to other factors, such as incidence of HCC and other liver and kidney function tests (Table 2). Notably, liver fibrotic factors, including HA, PIIINP, TIMP-1, ELF, and CTGF, were significantly increased in patients with

TABLE 2 Patient characterization separated by serum C1q levels at 11 mg/dl

	C1q < 11	C1q ≥ 11
Patient number	59	28
Sex (male/female)	23/36	13/15
Age	62.4 ± 10.7	68.9 ± 10.6[***]
HBV/HCV/NASH/AL/AIH-PBC/others	4/23/20/6/3/3	1/19/5/1/2/0
CH/LC	48/11	6/22[****]
HCC (+/−)	5/54	17/11[****]
Alb (g/dl)	4.3 ± 0.3	3.7 ± 0.6[****]
AST (IU/L)	39 ± 24	50 ± 28
ALT (IU/L)	35 ± 20	38 ± 30
GGT (IU/L)	68.5 ± 113	75.1 ± 71.5
T-bil (mg/dl)	0.8 ± 0.5	1.2 ± 0.7[*]
PLT (×10^4 cells/μl)	17.4 ± 6.2	11.8 ± 5.9[***]
PT (%)	93.7 ± 15.1	74.1 ± 13.8[****]
BUN (mg/dl)	14.1 ± 3.9	16.8 ± 5.3[**]
Cr (mg/dl)	0.70 ± 0.19	0.80 ± 0.28
eGFR (ml/minute/1.73 m^2)	78.8 ± 17.1	70.4 ± 23.9
HA (ng/ml)	149.8 ± 197.6	496.6 ± 511.8[****]
PIIINP (ng/ml)	11.0 ± 6.1	18.8 ± 7.7[****]
TIMP-1 (ng/ml)	227 ± 82	343 ± 129[****]
ELF	10.1 ± 1.1	11.6 ± 1.4[****]
CTGF (ng/ml)	18.7 ± 2.7	22.7 ± 3.1[****]

Note: Values are mean ± SD.

Abbreviations: AIH, autoimmune hepatitis; AL, alcoholic liver disease; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; C1q, complement complex 1 subunit q; Cr, creatinine; CTGF, connective tissue growth factor; eGFR, estimated glomerular filtration rates; ELF, enhanced liver fibrosis panel; GGT, gamma-glutamyltransferase; HA, hyaluronic acid; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; Na, sodium; NASH, nonalcoholic steatohepatitis; PBC, primary biliary cholangitis; PIIINP, procollagen III amino-terminal peptide; PLT, platelet count; PT, prothrombin time; T-bil, total bilirubin; TIMP-1, tissue metalloprotease inhibitor 1.

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

C1q ≥ 11 mg/dl compared to patients with C1q < 11 mg/dl (Table 2). The survival rate was significantly decreased in patients with C1q ≥ 11 mg/dl compared to patients with C1q < 11 mg/dl ($p < 0.001$) (Figure 5C). Our findings warrant larger clinical studies to further investigate the utility of monitoring serum C1q as a prognostic factor of survival in chronic diseases of the liver.

DISCUSSION

In the present study, we demonstrated that C1q mediates CTGF production through an alternative Wnt signaling pathway in LX2 cells and is associated with liver fibrosis in cirrhotic rats and patients with chronic liver disease. We also revealed that serum C1q levels can be used as a prognostic factor for survival in human chronic liver disease.

C1q-mediated LX2 activation was found to be associated with cellular morphologic changes and *CTGF* increase through the up-regulation of *FZD2*, *TAZ*, and *Cyr61* as an alternative Wnt signaling pathway.[19] CTGF production has been identified in active HSCs through the Hippo signaling pathway, including YAP/TAZ,[18] suggesting that C1q may be binding to FZD upstream of the YAP/TAZ signaling pathway. TGF-β is also a well-known key mediator of HSC activation through the mothers against decapentaplegic homolog 3 (Smad3) signaling pathway.[22] Indeed, TGF-β-activated LX2 cells, with consequent cellular morphologic changes and paired elevation of *collagen* and *α-SMA* levels, had no up-regulation of *FZD2* and *Cyr61* levels, meaning that the HSC activation mechanism was different depending on the stimulating factor. Several groups have reported that CTGF enhances TGF-β activities. CTGF binds directly to TGF-β, resulting in increased binding to TGF-β receptors,[23] and CTGF accelerates *in vivo* TGF-β-mediated fibrogenic actions.[24] These observations suggest that HSC activation by C1q induces CTGF production followed by an increase in TGF-β activities and thus a positive-feedback loop of liver fibrosis.

In cirrhotic rats, liver *C1q* mRNA levels were significantly associated with liver fibrosis as assessed by Azan staining and were significantly correlated with mRNA levels of liver *CTGF*, *TIMP-1*, *HAS1* and *HAS3*, and *CD44* as a receptor for HAS, the levels of which increased in advanced liver disease.[25] The association pattern with liver fibrosis was similar among *C1qa*, *C1qb*, and *C1qc* mRNA levels, which is reasonable based on the C1q protein structure consisting of C1qa, C1qb, and C1qc in equal amounts. Although liver *C1q* mRNA levels were not associated with liver *α-SMA* and *collagen* mRNA levels, which are highly expressed in myofibroblasts at early stage liver fibrosis, liver *C1q* mRNA was determined to be a reasonable measure of advanced fibrosis in our cirrhotic rats. Furthermore, an increase in liver C1q level was observed in deceased cirrhotic rats, much the same as other fibrotic genes, including *CTGF*, *TIMP-1*, *HAS3*, and *CD44*. Increased ELF measurement along with TIMP-1, HAS, and CD44 levels have been reported in advanced liver diseases,[25,26] meaning that liver C1q levels will be an indicator of patient survival in advanced liver diseases. In patients with chronic liver disease, serum C1q levels were significantly correlated with measures of liver fibrosis, such as an elevated ELF test score and CTGF levels, which have been identified as indicators of advanced fibrosis.[27] We also showed for the first time that serum C1q levels can be used as a prognostic marker for survival in human chronic liver diseases. Patients presenting with C1q ≥ 11 mg/dl have a higher risk of death associated with lower Alb, PLT, and PT (%)

as well as higher ELF test measurements and CTGF levels. Several reports have illustrated that serum C1q levels are gradually increased due to aging between 45 and 75 years old in healthy individuals[2,3]; however, we found no significant association between serum C1q levels and age in human chronic liver diseases. This suggests that the association between serum C1q levels and liver fibrosis is superior to that of patient age in a cohort of patients with chronic liver disease.

C1q is one of the key regulators of CTGF production, which is a protein associated with advanced liver disease. This points to the potential for serum C1q values to be used as a prognostic tool in the determination of patient survival, although we need further studies to validate our results, using a larger cohort as part of a multiple center study. In contrast, C1q is not a great therapeutic target for liver fibrosis due to its role as an essential molecule in the complement pathway. FZDs have been recognized as potential therapeutic targets in human cancers,[28] and inhibitory approaches targeted to FZD using a selective peptide inhibitor[29] or a synthetic anti-FZD antibody[30] have already been developed. Further studies are required to explore the role of FZD in advanced liver diseases associated with CTGF production stimulated by C1q in other types of liver cells, such as hepatocytes, because FZDs are also expressed outside the hepatic parenchyma.[20]

AUTHOR CONTRIBUTIONS

Akiko Eguchi and Motoh Iwasa: concept and study design. Akiko Eguchi, Ryosuke Sugimoto, and Mina Tempaku: performed experiments. Ryosuke Sugimoto, Kyoko Yoshikawa, Naohiko Yoshizawa, Kazushi Sugimoto, Hiroshi Hasegawa, Yoshiyuki Takei, and Hayato Nakagawa: human resources collection. Akiko Eguchi, Motoh Iwasa, and Hiroshi Hasegawa: data curation and formal analysis. Akiko Eguchi: wrote the manuscript. Motoh Iwasa and Hayato Nakagawa: edited the manuscript. All authors read and revised the manuscript.

FUNDING INFORMATION

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

1. Nayak A, Ferluga J, Tsolaki AG, Kishore U. The non-classical functions of the classical complement pathway recognition subcomponent C1q. Immunol Lett. 2010;131:139–50.
2. Yonemasu K, Kitajima H, Tanabe S, Ochi T, Shinkai H. Effect of age on C1q and C3 levels in human serum and their presence in colostrum. Immunology. 1978;35:523–30.
3. Willems E, Alkema W, Keizer-Garritsen J, Suppers A, van der Flier M, Philipsen R, et al. Biosynthetic homeostasis and resilience of the complement system in health and infectious disease. EBioMedicine. 2019;45:303–13.
4. van de Bovenkamp FS, Dijkstra DJ, van Kooten C, Gelderman KA, Trouw LA. Circulating C1q levels in health and disease, more than just a biomarker. Mol Immunol. 2021;140:206–16.
5. Yoshida H, Sato M, Watanabe S, Nishimaki T, Morito T, Kasukawa R, et al. C1q and immune complexes in liver cirrhosis sera. Tohoku J Exp Med. 1983;140:73–9.
6. Dumestre-Perard C, Ponard D, Drouet C, Leroy V, Zarski JP, Dutertre N, et al. Complement C4 monitoring in the follow-up of chronic hepatitis C treatment. Clin Exp Immunol. 2002;127:131–6.
7. Santiesteban-Lores LE, Carneiro MC, Isaac L, Bavia L. Complement system in alcohol-associated liver disease. Immunol Lett. 2021;236:37–50.
8. Cohen JI, Roychowdhury S, McMullen MR, Stavitsky AB, Nagy LE. Complement and alcoholic liver disease: role of C1q in the pathogenesis of ethanol-induced liver injury in mice. Gastroenterology. 2010;139:664–74.e1.
9. Hillian AD, McMullen MR, Sebastian BM, Roychowdhury S, Kashyap SR, Schauer PR, et al. Mice lacking C1q are protected from high fat diet-induced hepatic insulin resistance and impaired glucose homeostasis. J Biol Chem. 2013;288:22565–75. Erratum in: J Biol Chem. 2013;288:28308.
10. **Bulla R**, **Tripodo C**, **Rami D**, **Ling GS**, Agostinis C, Guarnotta C, et al. C1q acts in the tumour microenvironment as a cancer-promoting factor independently of complement activation. Nat Commun. 2016;7:10346.
11. Naito AT, Sumida T, Nomura S, Liu ML, Higo T, Nakagawa A, et al. Complement C1q activates canonical Wnt signaling and promotes aging-related phenotypes. Cell. 2012;149:1298–313. Erratum in: Cell. 2012;150:659–60.
12. Kisseleva T, Brenner D. Molecular and cellular mechanisms of liver fibrosis and its regression. Nat Rev Gastroenterol Hepatol. 2021;18:151–66.
13. Yang YM, Kim SY, Seki E. Inflammation and liver cancer: molecular mechanisms and therapeutic targets. Semin Liver Dis. 2019;39:26–42.
14. Filliol A, Schwabe RF. Contributions of fibroblasts, extracellular matrix, stiffness, and mechanosensing to hepatocarcinogenesis. Semin Liver Dis. 2019;39:315–33.
15. Patel K, Sebastiani G. Limitations of non-invasive tests for assessment of liver fibrosis. JHEP Rep. 2020;2:100067.
16. Cheng JH, She H, Han YP, Wang J, Xiong S, Asahina K, et al. Wnt antagonism inhibits hepatic stellate cell activation and liver fibrosis. Am J Physiol Gastrointest Liver Physiol. 2008;294:G39–49.
17. **Lai KKY**, **Kweon SM**, **Chi F**, Hwang E, Kabe Y, Higashiyama R, et al. Stearoyl-CoA desaturase promotes liver fibrosis and tumor development in mice via a Wnt positive-signaling loop by stabilization of low-density lipoprotein-receptor-related proteins 5 and 6. Gastroenterology. 2017;152:1477–91.
18. **Mannaerts I**, **Leite SB**, Verhulst S, Claerhout S, Eysackers N, Thoen LF, et al. The Hippo pathway effector YAP controls mouse hepatic stellate cell activation. J Hepatol. 2015;63:679–88.
19. Park HW, Kim YC, Yu B, Moroishi T, Mo JS, Plouffe SW, et al. Alternative Wnt signaling activates YAP/TAZ. Cell. 2015;162:780–94.

20. Zeng G, Awan F, Otruba W, Muller P, Apte U, Tan X, et al. Wnt'er in liver: expression of Wnt and frizzled genes in mouse. Hepatology. 2007;45:195–204.

21. Iwasa M, Kobayashi Y, Mifuji-Moroka R, Hara N, Miyachi H, Sugimoto R, et al. Branched-chain amino acid supplementation reduces oxidative stress and prolongs survival in rats with advanced liver cirrhosis. PLoS One. 2013;8:e70309.

22. Massague J, Blain SW, Lo RS. TGFbeta signaling in growth control, cancer, and heritable disorders. Cell. 2000;103:295–309.

23. Abreu JG, Ketpura NI, Reversade B, De Robertis EM. Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-beta. Nat Cell Biol. 2002;4:599–604.

24. Mori T, Kawara S, Shinozaki M, Hayashi N, Kakinuma T, Igarashi A, et al. Role and interaction of connective tissue growth factor with transforming growth factor-beta in persistent fibrosis: a mouse fibrosis model. J Cell Physiol. 1999;181:153–9.

25. Nanji AA, Tahan SR, Khwaja S, Yacoub LK, Sadrzadeh SM. Elevated plasma levels of hyaluronic acid indicate endothelial cell dysfunction in the initial stages of alcoholic liver disease in the rat. J Hepatol. 1996;24:368–74.

26. Parkes J, Roderick P, Harris S, Day C, Mutimer D, Collier J, et al. Enhanced liver fibrosis test can predict clinical outcomes in patients with chronic liver disease. Gut. 2010;59:1245–51.

27. Kovalenko E, Tacke F, Gressner OA, Zimmermann HW, Lahme B, Janetzko A, 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. 2009;16:612–20.

28. Zeng CM, Chen Z, Fu L. Frizzled receptors as potential therapeutic targets in human cancers. Int J Mol Sci. 2018;19:1543.

29. Nile AH, de Sousa E Melo F, Mukund S, Piskol R, Hansen S, Zhou L, et al. A selective peptide inhibitor of Frizzled 7 receptors disrupts intestinal stem cells. Nat Chem Biol. 2018;14:582–90. Erratum in: Nat Chem Biol. 2018;14:902.

30. Pavlovic Z, Adams JJ, Blazer LL, Gakhal AK, Jarvik N, Steinhart Z, et al. A synthetic anti-Frizzled antibody engineered for broadened specificity exhibits enhanced anti-tumor properties. MAbs. 2018;10:1157–67.

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