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 CCl4 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...
INTRODUCTION

Complement complex 1 subunit α (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. Serum C1q levels increase with age from 45 to 75 years old, irrespective of sex differences in healthy individuals, and increase in a variety of pathological conditions, including infectious and inflammatory diseases. In liver diseases, human serum C1q levels were found to be increased in liver cirrhosis (LC), chronic hepatitis (CH) caused by infection with hepatitis C virus (HCV), and alcohol-associated liver disease. Furthermore, C1q-mediated ethanol-induced liver injury in mice and hepatic insulin resistance in mice fed a high-fat diet 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 and cancer promotion. C1q also binds to frizzled receptors (FZDs), subsequently activating canonical Wnt signaling and promoting aging-associated decline in tissue regeneration. 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. Therefore, a comprehensive understanding of HSC activation and mechanistic activators is essential in the development of new diagnostic and therapeutic approaches. The canonical Wnt positive-signaling loop, which includes Wnt, FZDs, nuclear β-catenin, and stearyl-coenzyme A, is critical to the progression of liver fibrosis through HSC activation. 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.

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). In the process of osteogenic differentiation, β-catenin-independent Wnt signaling pathway activation identified C1q as a ligand for FZDs, which are expressed in liver parenchyma (hepatocytes) and nonparenchymal cells (HSCs, hepatic sinusoidal endothelial cells, and Kupffer cells). 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. Briefly, Wister rats aged 6 weeks (n = 21) were orally administered CCl4 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 CCl4 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 CCl4 administration, whereas the deceased group contained rats that died within 21 weeks of CCl4 administration. The control designation indicated advanced fibrotic rats 5 weeks after CCl4 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 disgorge 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

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.
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 ethane-sulfonic acid (HEPES; Gibco), sodium pyruvate (Gibco), and penicillin and streptomycin (Gibco) at 37°C in a 5% CO₂ 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 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,
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 CCl₄ 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...
(A) \( \text{human Ctq (active form)} \rightarrow 48 \text{h} \rightarrow \text{Check cell morphology and gene expression} \)

(B) Control, Ctq, TGF-β

(C) Control, Ctq, TGF-β

(D) Relative expression levels for different conditions

(E) Relative expression levels for different conditions

(F) Diagram illustrating various signaling pathways

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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 C1q 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.

**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 CCl4 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; CCl4, 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.

**Figure 3** Liver C1q levels were associated 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, 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), PIINP (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.


**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 | 0.88 | 0.70 | 0.89 | 0.72 | 0.78 | 0.85 |
|    | P < 0.0001 | P < 0.0001 | P < 0.0001 | P = 0.0001 | P = 0.0003 | P = 0.0001 | P = 0.0001 |
| C1qb| 0.86 | 0.95 | 0.77 | 0.85 | 0.75 | 0.85 | 0.78 |
|    | P < 0.0001 | P < 0.0001 | P < 0.0001 | P < 0.0001 | P < 0.0001 | P < 0.0001 | P < 0.0001 |
| C1qc| 0.88 | 0.95 | 0.87 | 0.87 | 0.75 | 0.87 | 0.89 |
|    | P < 0.0001 | P < 0.0001 | P < 0.0001 | P < 0.0001 | P < 0.0001 | P < 0.0001 | 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.
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 $\geq$ 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
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. CTGF production has been identified in active HSCs through the Hippo signaling pathway, including YAP/TAZ, 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. 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, and CTGF accelerates in vivo TGF-β-mediated fibrogenic actions. 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. 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, HAS1, and CD44. Increased ELF measurement along with TIMP-1, HAS, and CD44 levels have been reported in advanced liver diseases, 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. 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 (%).

| 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/ALH-PBC/ | 4/23/6/2/3 | 1/19/5/2/0 |
| 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 |
| 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 |
| T-bil (mg/dl) | 0.8 ± 0.5 | 1.2 ± 0.7* |
| PLT (>10⁵ cells/μl) | 17.4 ± 6.2 | 11.8 ± 5.9*** |
| eGFR (ml/minute/1.73 m²) | 78.8 ± 17.1 | 70.4 ± 23.9 |
| HA (ng/ml) | 149.8 ± 197.6 | 496.6 ± 511.8**** |
| PIINP (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, γ-glutamyltransferase; HA, hyaluronic acid; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; Na, sodium; NASH, nonalcoholic steatohepatitis; PBC, primary biliary cholangitis; PIINP, 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 < 0.1; ***p < 0.001; ****p < 0.0001.
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, Kazushige 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.

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CONFLICT OF INTEREST
The authors state no conflict of interest.

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