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Original Article

Serum N-terminal DDR1: A Novel Diagnostic Marker of Liver Fibrosis Severity

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Abstract

Background and Aims: The expression of discoidin domain receptor 1 (DDR1) is commonly up-regulated and undergoes collagen-induced ectodomain (N-terminal) shedding during the progression of liver fibrosis. This study aimed to evaluate the clinical utility of N-terminal DDR1 as a diagnostic biomarker for liver fibrosis. ***Methods:*** N-terminal DDR1 shedding was evaluated using cell lines, liver fibrosis mouse models, clinical data of 298 patients collected from February 2019 to June 2020. The clinical data were divided into test and validation cohorts to evaluate the diagnostic performance of serum N-terminal DDR1. ***Results:*** Time- and dosage-dependent N-terminal DDR1 shedding stimulated by collagen I was observed in a hepatocyte cell line model. The type I collagen deposition and serum N-terminal DDR1 levels concurrently increased in the development of liver fibrosis in mouse models. Clinical data demonstrated a significant diagnostic power of serum N-terminal DDR1 levels as an accurate biomarker of liver fibrosis and cirrhosis. The diagnostic performance was further increased when applying N-DDR1/albumin ratio, achieving area under the curve of 0.790, 0.802, 0.879, and 0.865 for detecting histological fibrosis stages F ≥2, F ≥3, F 4 with liver biopsy as a reference method, and cirrhosis according to imaging techniques, respectively. With a cut-off of 55.6, a sensitivity, specificity, positive predictive value, and negative predictive value of 82.7%,76.6%, 67.4%, and 88.3% were achieved for the detection of cirrhosis. ***Conclusions:*** Serum N-terminal DDR1 appears to be a novel diagnostic marker for liver fibrosis.

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Introduction

Liver fibrosis (LF) denotes a series of dynamic pathophysiological changes characterized by hepatocyte degeneration and necrosis due to chronic liver injury, including hepatotoxic drug injury, cholestasis, etc. LF has been the most crucial predictor of liver-related morbidity and mortality in liver diseases.[1] However, studies have revealed that early-stage LF and even liver cirrhosis can be reversed.[2] Moreover, detecting advanced LF and cirrhosis is crucial for choosing adequate support treatment, determining surveillance intervals, and predicting clinical outcomes.[3,4] Although liver biopsy is still the gold standard for clinical diagnosis, its inherent shortcomings, such as sampling error of biopsy specimen caused by different operators,[5] expensive operation cost,[6] and complications such as bleeding and bile fistula caused by the invasive operation, all contribute to its poor tolerance and low repeatability.[7] These problems have led to the question of its applicability as a reference standard for LF.[8,9] Different serological biomarkers combined with clinical parameters, such as body mass index (BMI) and age, were gradually developed into a non-invasive, low-cost, and repeatable alternative method compared with liver biopsy. These markers have been widely studied in nonalcoholic fatty liver disease and viral hepatitis, in which they are mainly used to detect significant fibrosis (F ≥2) or advanced fibrosis (F ≥3). At present, a variety of widely reported markers, including the aspartate to platelet ratio index (commonly referred to as APRI),[10] enhanced liver fibrosis (commonly referred to as ELF) test,[11] Fibrosis 4 (commonly referred to as FIB4)-index[12] or FibroTest[13] are receiving much attention.

The discoidin domain receptors (DDRs), including DDR1 and DDR2, are a unique receptor tyrosine kinase (RTK) family containing a discoidin homology domain in their extracellular region. DDRs are the only collagen-activated RTKs,

Keywords: Liver fibrosis; Serum biomarker; DDR1; FIB-4.
Abbreviations: DDR1, discoidin domain receptor 1; LF, liver fibrosis; ECM, extracellular matrix.
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showing different structural and functional homologies. DDR1 is mainly expressed in epithelial cells. Studies have confirmed that DDR1 plays an essential role in tissue fibrosis, and it is considered an attractive antifibrotic target.[14,15] Several studies have demonstrated that DDR1 expression and function are associated with fibrotic diseases, such as atherosclerosis, arthritis, and many types of cancers.[16–19] In pulmonary fibrosis and chronic kidney disease, activation of DDR1 stimulates inflammatory pathways, including cytokine synthesis.[16,17] Proinflammatory cytokines further enhance inflammatory cell infiltration, extracellular matrix (ECM) synthesis and DDR1 expression, to form a positive feedback loop. The combination of collagen and DDR1 leads to the activation and phosphorylation of DDR1, which transmits various collagen signals from epithelial cells.[20,21] The clustering and activation of DDR1 mediate tractional collagen remodeling, which is vital for mechanical compaction and reorganization of collagen in the ECM during fibrosis.[22] Collagen has been reported to activate DDR1 aggregation, enhancing its binding to collagen, and DDR1 allows collagen remodeling to promote fibrosis.[22,23] Upon collagen binding, shedding of N-terminal DDR1 can be induced by membrane-anchored collagenase, membrane type (MT) 1-, MT2-, and MT3 matrix metalloproteinases (commonly known as MMPs).[14,15,24,25] Collagen specifically stimulates the N-terminal DDR1 shedding but does not affect other transmembrane proteins.[24] Collagen-stimulated shedding of the N-terminal DDR1 has been confirmed in many studies;[14,15,24–26] however, the expression and diagnostic value of serum N-terminal DDR1 in LF remain unclear. This study aims to explore the phenomenological relationship between LF and N-terminal DDR1 shedding.

Methods

Cell culture

Human embryonic kidney (HEK) 293 cells and HL-7702 cells were cultured in Dulbecco's modified Eagle's medium/F12 nutrient mixture (commonly known as DMEM/F12; Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum. AML12 medium was also used and supplemented with 2 mM L-glutamine, and 100 U/mL penicillin, and 100 mg/mL streptomycin. All cells were cultured at 37°C and in an atmosphere of 5% CO_2.

Western blot analysis

The serum-free medium's supernatant was collected and added with an equal amount of methanol and 1/4 volume of chloroform. The supernatant protein was obtained by centrifugation of 17,000×*g* for 15 m. After removing the transparent aqueous phase, the white layer on top of the chloroform phase was left (composed of protein). Methanol was added into the remaining liquid, which was centrifuged at 17,000×*g* for 15 m. After decanting the liquid phase, the protein remained at the bottom.

Equal amounts of protein per sample were separated by polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membranes were blocked in 5% skim milk, followed by incubation with the primary antibodies overnight at 4°C and secondary antibodies 1 h at 25°C. The immunoreactive bands were visualized using an enhanced chemiluminescence detection system and standard autoradiography.

The following antibodies were used: anti-DDR1 (C-20) (sc-532; Santa Cruz Biotechnology, Dallas, TX, USA); DDR1 ECD antibody (AF2396; R&D Systems, Minneapolis, MN, USA); type I collagen immunohistochemistry antibody (ab34710; Abcam, Cambridge, UK); and DDR1 immunohistochemistry antibody (cst5583; Cell Signaling Technology, Danvers, MA, USA).

LF mouse models

The male C57Bl/6 mice were used to construct an LF model via bile duct ligation (BDL) (6–8 weeks). Mice were anesthetized with 4% chloral hydrate. Following abdominal midline skin incision through the musculature, laparotomy ligation was performed with two ligatures close to the liver hilum immediately below the bifurcation and one ligature around the cystic duct. The abdominal incision was closed with absorbable suture, and the skin incision with normal suture. The entire surgical procedures were performed under sterile conditions. The animals were sacrificed at 0, 5, 10, and 15 days after surgery. Liver tissue and serum were harvested. Six control animals were included at each time point.

Carbon tetrachloride (CCl4) was diluted with peanut oil to a concentration of 0.1% and injected intraperitoneally at a dose of 1 mL/100 g twice a week. The mice were sacrificed at the 6th, 9th, and 12th weeks to collect liver tissue and serum. All experimental animals were sacrificed by the carbon dioxide method.

Enzyme-linked immunosorbent assay (ELISA)

According to the manufacturer's protocol, the serum levels of DDR1 were measured using a commercial ELISA kit (CHE0283; Beijing 4A Biotech Co., Ltd., Beijing, China). The OD_{450} value of the detection solution was measured immediately after mixing.

Statistical analyses

All statistical analyses were performed using SPSS v.23.0 (IBM Corp., Armonk, NY, USA). Summary statistics for normally distributed continuous variables were expressed as means and standard deviation, whereas quantitative variables without a normal distribution were expressed as the median and interquartile range (IQR). The means of continuous variables were compared using the Mann-Whitney *U*-test. Categorical data were presented as numbers and frequencies (%) and were compared using the chi-squared test or Fisher's exact test. The Pearson and Spearman correlation tests were used to analyze the relationship between the serum DDR1 levels and various clinical characteristics or categorical data. The receiver operating characteristic (commonly referred to as ROC) curve analysis was performed to assess the diagnostic accuracy. Area under the curve (AUC) was used to identify optimal sensitivity and specificity levels. All *p*-values were two-sided, and differences with *p*-values <0.05 were considered statistically significant.

Ethics approval

All animal experiments were approved by the Ethics Committee of Tongji Hospital, Huazhong University of Science and Technology (HUST). The study was conducted according to the Declaration of Helsinki, and reviewed by the Ethics Committee of the Tongji Hospital (TJ-IRB20190706).

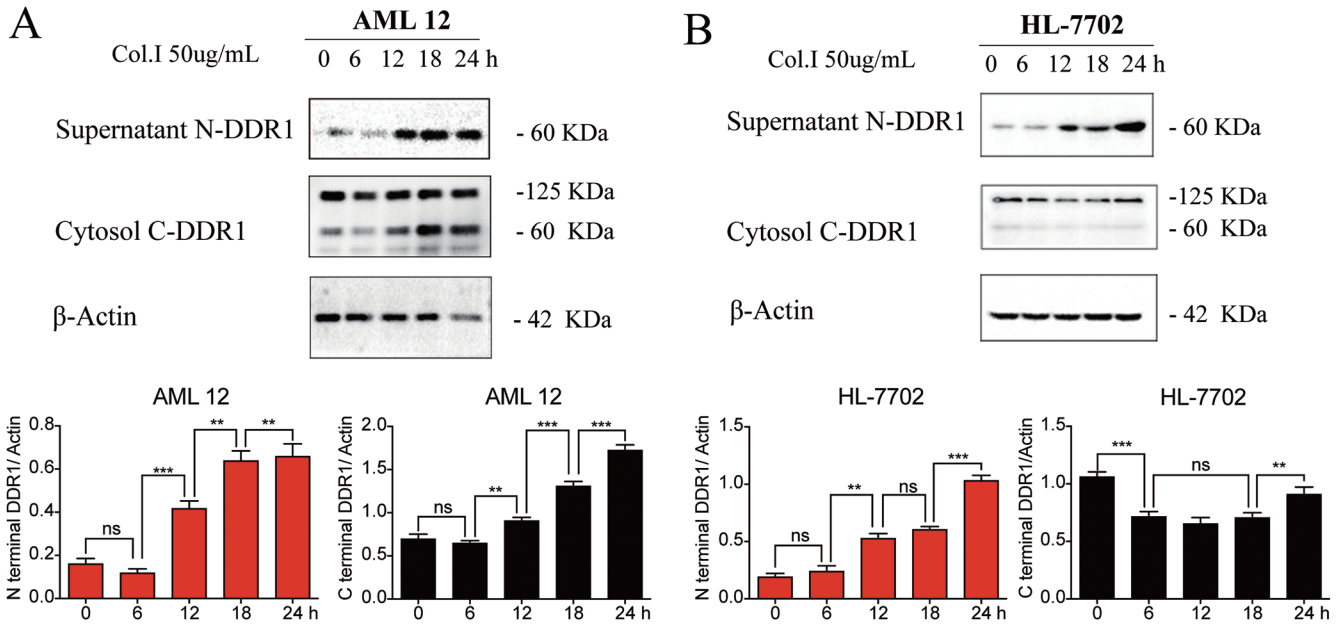

Fig. 1. Type I collagen promotes the N-terminal DDR1 shedding in hepatocytes. (A–F) Western blot analysis of DDR1 shedding at different times in AML12 (A, B) and HL-7702 liver cell line (C–F). Student's *t*-test was applied for statistical analyses, comparing each variant group for multiple comparisons. *p<0.05, **p<0.01, *** p<0.001, **** p<0.0001.

Results

Type I collagen promotes N-terminal DDR1 shedding in a time- and concentration-dependent manner

Collagen is known to promote the shedding of N-terminal DDR1.[25] To quantify type I collagen-induced DDR1 shedding at different times and concentrations, we transiently transfected HEK293 cells with a DDR1 expression vector and then incubated with serum-free DMEM containing 10 μg/mL type I collagen, and ectodomain shedding was analyzed at 0, 2, 4, 6, and 8 h. The level of N terminal-DDR1 with a molecular size of 60 kDa in the culture supernatant was assayed by immunoblotting (Supplementary Fig. 1A). Upon treatment, the concentration of the N-terminal DDR1 in the culture supernatant increased, which was consistent with previous studies.[14,15,24,26]

Further, we tested N-terminal DDR1 shedding at different collagen concentrations (0, 25, 50, and 75 μg/mL) (Supplementary Fig. 1B). N-terminal DDR1 shedding increased significantly as the collagen concentration increased. This trend, however, was reversed at a collagen concentration of 100 μg/mL. This phenomenon may be explained by the saturation of stimulus that has been reported in A431 cells.[24] Nevertheless, the results clearly showed that N-terminal DDR1 shedding due to type I collagen stimulation is time- and concentration-dependent.

Type I collagen stimulated the shedding of N-terminal DDR1 in hepatocytes

To further investigate if the collagen-induced DDR1 shedding is consistent in hepatocytes expressing endogenous DDR1, AML12 and HL-7702 cells were cultured. We then used 50 μg/mL collagen I as the optimal concentration to treat normal hepatocytes for 0, 6, 12, 18, and 24 h, respectively. Similar findings of DDR1 shedding were found in the hepatocyte cell lines. Notably, HL-7702 cells quantitatively exhibited small amounts of shedding, even in the absence of collagen. Furthermore, the shedding level progressively increased over time. This phenomenon may indicate that N-terminal DDR1 undergoes minor spontaneous shedding at a basal level, while the addition of collagen significantly increases shedding. After collagen stimulation for 12 h, AML12 cells also exhibited DDR1 shedding (Fig. 1A, B). These results thus confirmed collagen-induced endogenous DDR1 shedding in normal liver cell lines.

Serum levels of N-terminal DDR1 were associated with the severity of LF

To further confirm the relationship between type I collagen deposition and LF, the LF models were generated using CCl4 treatment and BDL in C57BL/6 mice. To confirm the successful induction of LF, hematoxylin and eosin, Sirius red, and collagen staining was performed in liver tissues (Fig. 2A, B). In the CCL4-induced mouse model, a small number of fibrous septa and fibrous tissue hyperplasia were observed at the 6th and 9th weeks after model induction. At the 12th week, many fibrous septa and fiber deposition caused structural disorder of the liver tissue (Fig. 2A). In the BDL model mice, on the 5th day after treatment, the fibrous tissue around the vessel proliferated. On the 10th day, the hyperplasia increased with small areas of necrosis. On the 15th day, the liver tissue showed widespread necrosis and apparent fibrous tissue hyperplasia (Fig. 2B). Taken together, type I collagen deposition was increased concurrently with the increased degree of fibrosis *in vivo*.

To verify the correlation between the degree of LF in the mouse model and the N-terminal DDR1 levels in the serum, a commercial ELISA kit was used to measure the serum levels of N-terminal DDR1. The results suggested that the mean serum level was significantly higher than in the control group and increased with the degree of fibrosis (Fig. 2C,

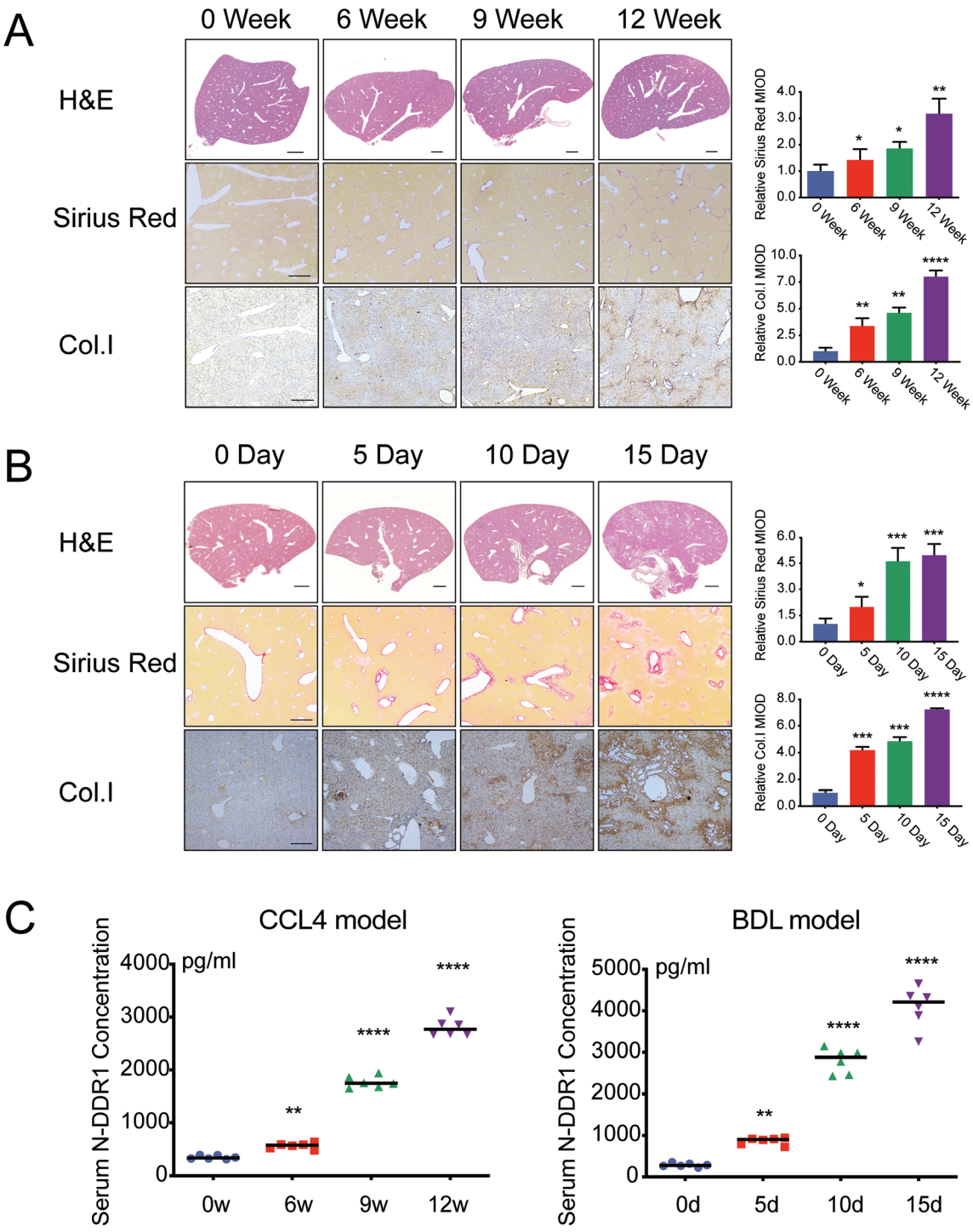

Fig. 2. Type I collagen deposition and serum N-terminal DDR1 in the CCl4-induced and BDL mouse model. (A. B) Representative images of hematoxylin and eosin (HE), Sirius red, and collagen I staining performed the mouse model liver tissue. (C) ELISA was used to measure the serum levels of N-terminal DDR1 in CCL4 and BDL models at 0, 6, 9, and 12 weeks, or 0, 5, 10, 15 days and compared with healthy controls, respectively. (HE, 4×, scale bar: 500 μm; Sirius red and collagen I staining, 40×, scale bar: 500 μm)

Supplementary Fig. 1C, D).

To further investigate the association of serum levels of N-terminal DDR1 and the severity of LF in patients, a total of 298 patients (median age: 53 years; male sex: 54.3%) were included from February 2019 to June 2020, and liver biopsy was performed. We strictly screened patient data, excluded tumor patients, patients with common kidney and lung diseases, osteoarthritis and atherosclerosis, or patients with a history of the above diseases. The serum N-terminal DDR1 levels were measured by ELISA in

Table 1. Patient characteristics

Characteristics	All patients (*n*=298)	Test cohort (*n*=230)	Validation cohort (*n*=68)	*p*-value
Age in years, median (IQR)	53.0 (46–61)	54.0 (46–62)	50.0 (45–57.5)	0.053
Male sex, % (*n*)	54.3 (162)	54.3 (125)	54.4 (37)	0.993
BMI, median (IQR)	23.5 (20.5–26.6)	23.4 (20.4–26.5)	24.0 (21.1–26.7)	0.206
Laboratory parameter				
AST in U/mL, median (IQR)	26.0 (19.0–39.3)	25.0 (19.0–38.0)	31.5 (21.5–47.5)	0.045
ALT in U/mL, median (IQR)	22 (14–37.25)	20 (14–36)	25.5 (18.0–44.5)	0.028
GGT in U/mL, median (IQR)	48.5 (26.0–104.0)	45.5 (24.0–89.0)	75.0 (30.5–137.0)	0.014
Albumin in mg/dL, median (IQR)	41.1 (38.0–43.8)	41.4 (38.3–44.2)	39.8 (36.8–42.9)	0.014
Total bilirubin in mol/L, median (IQR)	12.7 (9.0–17.5)	12.4 (8.9–17.0)	13.2 (9.5–19.7)	0.259
Platelet count in G/L, median (IQR)	173.5 (122.0–237.0)	171.5 (124.0–233.0)	181.0 (118.5–241.5)	0.690
MELD score, median (IQR)	5.7 (5.5–5.9)	5.7 (5.4–5.9)	5.7 (5.5–5.9)	0.712
HBeAg positivity, % (*n*)	60.1 (179)	58.7 (135)	64.7 (44)	0.374
Healthy controls, % (*n*)	6.7 (20)	8.7 (20)	0.0 (0)	n.d
Cirrhosis according to imaging, % (*n*)	36.9 (110)	36.1 (83)	39.7 (27)	0.587
US only	60.0 (66)	56.6 (47)	70.4 (19)	n.d
US+CT	16.4 (18)	18.1 (15)	11.1 (3)	n.d
US+MRI	12.7 (14)	13.3 (11)	11.1 (3)	n.d
US+CT+MRI	10.9 (12)	12.0 (10)	7.4 (2)	n.d
N-DDR1, % (*n*)	100 (298)	100 (230)	100 (68)	n.d
FIB4, % (*n*)	100 (298)	100 (230)	100 (68)	n.d
FibroScan, % (*n*)	93.3 (278)	91.3 (210)	100 (68)	n.d

ALT, alanine transaminase; AST, aspartate transaminase; CT, computed tomography; GGT, γ-glutamyl transpeptidase; HBeAg, hepatitis B e antigen; MRI, magnetic resonance imaging; n.d., not determined; US, ultrasound.

all 110 patients with liver cirrhosis and 188 patients without liver cirrhosis (according to imaging). A total of 210 cases (Department of Liver Surgery, Tongji Hospital) and 20 health personnel (physical examination centers, Tongji Hospital) were investigated in the test cohort. Then, 68 LF patients in the validation cohort (Infectious Diseases Department of Tongji Hospital) were investigated to confirm our research results. The basic data of the cases are shown in Table 1. Among the total 298 samples, according to the METAVIR classification system, there were 20 F 0, 64 F 1, 52 F 2, 89 F 3 and 73 F 4 cases. The mean serum N-terminal DDR1 levels in patients with significant LF (F ≥2) and advanced fibrosis (F ≥3) were 2,386.4 pg/mL (IQR: 1,595.6; 2,750.6, *n*=214) and 2,501.9 pg/mL (IQR: 1,919.7; 2,891.5, *n*=162). Additionally, histopathological examination and staining on F 1-F 4 fibrosis cases were performed, revealing that collagen deposition increased with the degree of LF (Supplementary Fig. 2). Spearman's correlation coefficients of the N-terminal DDR1 levels with degree of LF was 0.574 ($p \leq 0.001$).

The N-terminal DDR1 levels were increased according to the stage of fibrosis. For the diagnosis of LF, in addition to the gold-standard liver biopsy pathological examination, preoperative imaging is also necessary. Because imaging detection of less advanced fibrosis is challenging, imaging is often used to assess liver cirrhosis and its complications.[27] To distinguish, the present study used F 4 to represent pathological end-stage cirrhosis, while cirrhosis was used to describe the end-stage diagnosis of imaging. According to liver biopsy and imaging results, the serum N-terminal DDR1 levels, N-terminal DDR1/albumin ratio, FIB4 test results, and transient elastography (TE) results of all patients are shown in Figures 3 and Supplementary Figure 3. Overall, the N-terminal DDR1 levels increased according to the stage of fibrosis. N-terminal DDR1 levels were significantly higher in patients with F 4 compared to those with F 0 to F 3 (median N-DDR1: 2,853.8 pg/mL, IQR: 2,497.4; 2,947.2 versus 1,951.5 pg/mL, IQR: 1,287.4; 2,570.8, $p<0.0001$), as well as in patients with cirrhosis according to imaging compared to patients without cirrhosis or healthy controls (median N terminal-DDR1: 2,515.4 pg/mL, IQR 2,312.6; 3,060.0 versus 1,566.7 pg/mL, IQR: 1,155.8; 2,227.9 vs. 1,343.4, IQR: 870.6; 1,778.1). Taken together, these data revealed that N terminal-DDR1 levels do not significantly change during the early stages of hepatic fibrosis (F 0–F 3) and were highly elevated in liver cirrhosis (F 4) compared to advanced fibrosis (F 3).

Comparison of the diagnostic accuracy of serum N terminal-DDR1/albumin ratio, FIB-4, and TE for patients with LF

The AUC values of the N-terminal DDR1 ROC curve for detecting F ≥2, F ≥3, and F 4 compared to healthy control and cirrhosis were 0.764, 0.776, 0.845, and 0.827, respectively. Calculating the N-DDR1/albumin ratio increased the AUC to 0.790, 0.802, 0.879, and 0.865, respectively (Supplementary Fig. 4). Spearman's correlation coefficients of the N-DDR1/albumin ratio with degree of LF was 0.627

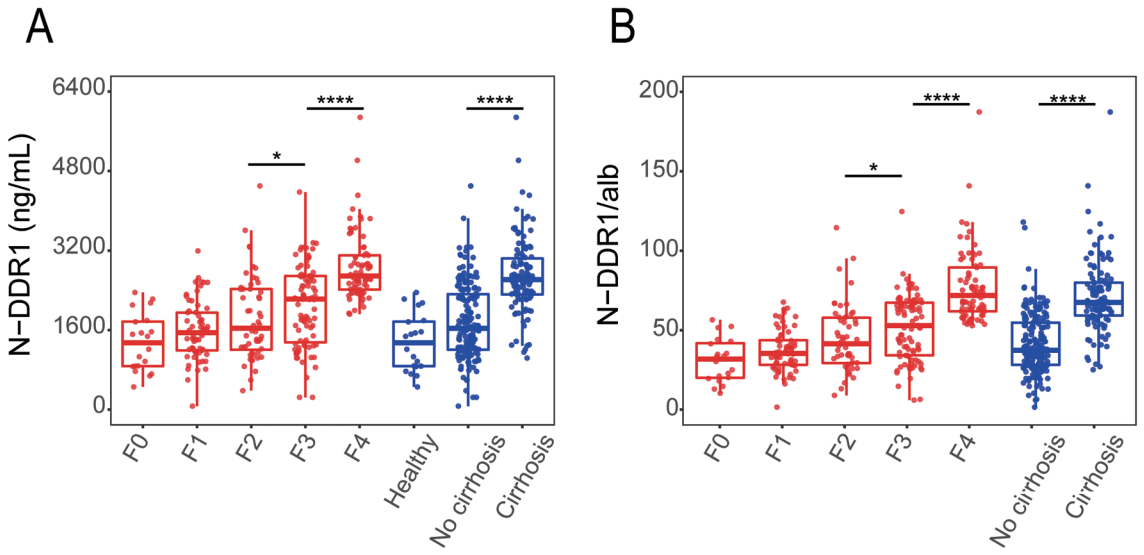

Fig. 3. Serum levels of N-DDR1 (A) and N-DDR1/albumin ratio (B) results stratified according to liver biopsy and the presence of imaging test results.

($p \leq 0.001$). The N-DDR1/albumin ratio, stratified by fibrosis grade and cirrhosis, and the detailed statistical results, are listed in Table 2 and Supplementary Table 1.

The FIB4 test and TE had excellent diagnostic accuracy for the detection of LF and cirrhosis. Data on their diagnostic performance is shown in Supplementary Table 1. In the present study, the N-DDR1/albumin ratio demonstrated acceptable accuracy for detecting F ≥2, F ≥3, F 4, and cirrhosis compared to the FIB4 test (Fig. 4) as well as TE. Spearman's correlation coefficients of the N-terminal

Table 2. Results of non-invasive fibrosis assessment

Fibrosis/Cirrhosis	AUC, 95% CI	Sensitivity, %	Specificity, %	PPV	NPV	Youden's index	Cut-off
N-DDR1							
All patients							
F ≥2	0.764	62.62	85.71	91.8	47.4	0.4833	2,202.44
F ≥3	0.776	70.99	77.21	78.8	69.1	0.4819	2,202.44
F 4	0.845	93.15	66.22	47.2	96.8	0.5937	2,215.5
Cirrhosis	0.827	90.91	65.43	60.6	92.5	0.5633	1,894.492
Hepatitis B virus							
F ≥2	0.788	66.21	91.18	97.0	38.8	0.5738	2,202.44
F ≥3	0.792	73.50	79.03	86.9	61.3	0.5254	2,202.44
F 4	0.830	88.68	69.05	54.7	93.5	0.5773	2,264.6
Cirrhosis	0.827	91.67	66.32	70.6	90.0	0.5798	1,894.492
N-DDR1/albumin							
All patients							
F ≥2	0.790	64.49	84.52	91.4	48.3	0.4901	51.6171
F ≥3	0.802	72.84	78.68	80.3	70.9	0.5152	52.3512
F 4	0.879	98.63	68.00	50.0	99.4	0.6663	52.9793
Cirrhosis	0.865	82.73	76.6	67.4	88.3	0.5932	55.6054
Hepatitis B virus							
F ≥2	0.816	73.79	85.29	95.5	43.3	0.5909	44.2517
F ≥3	0.820	81.2	74.19	85.6	67.6	0.5539	44.3576
F 4	0.860	98.11	64.29	53.6	98.8	0.6340	52.9193
Cirrhosis	0.866	82.14	76.84	75.8	83.0	0.5898	55.6054

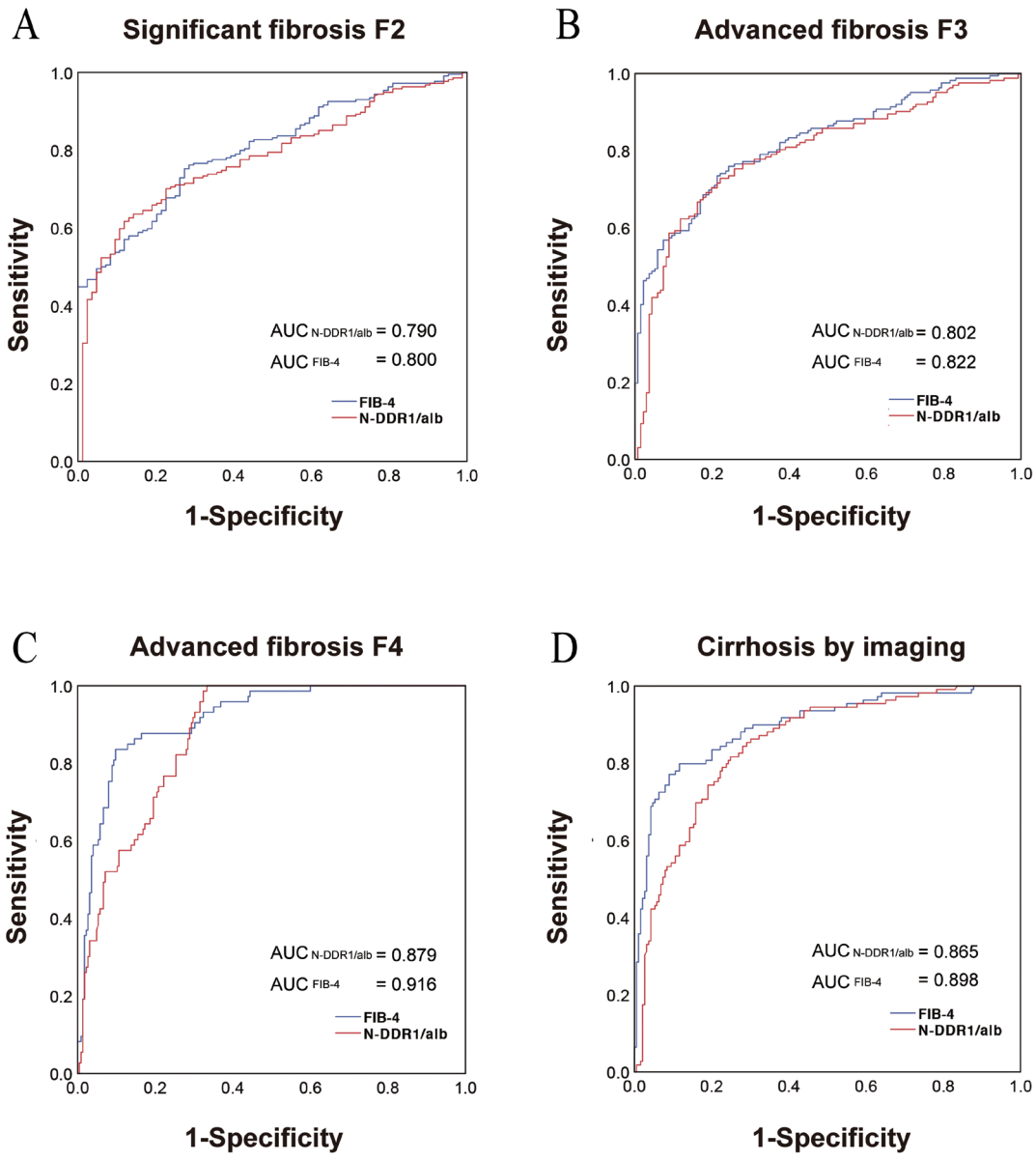

Fig. 4. Comparison of the diagnostic accuracy displayed as the AUC of the N-DDR 1/albumin ratio and FIB4 test for significant fibrosis (F ≥2) (A), advanced fibrosis (F ≥3) (B), fibrosis grade 4 (F 4) (C), and liver cirrhosis (D) according to imaging.

DDR1 levels with N-DDR1/albumin ratio, FIB4 test, and TE were 0.963 ($p \leq 0.001$), 0.380 ($p \leq 0.001$), and 0.453 ($p \leq 0.001$), respectively. The Spearman coefficient for the correlation of the N-DDR1/albumin ratio with the FIB4 test, and TE was 0.442 ($p \leq 0.001$) and 0.516 ($p \leq 0.001$), respectively.

Moreover, serum N-terminal DDR1 levels and N-DDR1/albumin ratio were independent of sex (median N-DDR1 levels [IQR]: male vs. female: 2,029.2 pg/mL [1,320.0; 2,642.2] vs. 2,219.5 pg/mL [1,375.9; 2,219.5], p=0.623; median N-DDR1/albumin ratio [IQR]: 51.6 [33.5; 67.1] versus 52.9 [32.6; 66.4], p=0.819). Additionally, the N-terminal DDR1 levels and N-DDR1/albumin ratio were independent of BMI category. In total, 65.8% (n=196/298) of patients had normal weight (BMI <25), 22.5% (n=67/298) were overweight (25≤BMI≤30), and 11.7% (n=35/298) conformed to the World Health Organization obesity criterion (BMI>30).[28] No significant difference was found among median N-terminal DDR1 levels (i.e. IQR) in normal weight, overweight and obese patients with corresponding values of 2,215.8 pg/mL (1,380.0; 2,688.4), 2,146.4 pg/mL (1,283.9; 2,544.1), and 1,938.6 ng/mL (1,262.9; 2,583.8), respectively (p=0.687). The median N-DDR1/albumin ratio (i.e. IQR) in normal weight, overweight and obese patients was 52.6 (33.5; 67.5), 50.7 (31.9; 63.1), and 51.6 (32.4; 66.5), respectively (p=0.756).

Discussion

DDR1 was identified as an attractive antifibrotic target that plays a vital role in LF.[29] Recent research focused on the diagnostic value and accuracy of DDR1 as a biomarker for

significant, advanced LF and cirrhosis compared to FIB4 and TE. The present study confirmed serum N-terminal DDR1 as an accurate biomarker of liver cirrhosis. Its diagnostic accuracy could be further increased by calculating the DDR1/albumin ratio, achieving an AUC of 0.879 for the diagnosis of F 4. Moreover, an AUC of 0.790, 0.802, 0.879, and 0.865 was achieved corresponding to histological fibrosis stages F ≥2, F ≥3, F 4 with liver biopsy as a reference method, and cirrhosis according to imaging techniques, respectively. With a cut-off of 55.6, a sensitivity, specificity, positive predictive value, and negative predictive value of 82.7%,76.6%, 67.4%, and 88.3% for the detection of cirrhosis was achieved. Notably, the serum N-terminal DDR1 level and DDR1/albumin ratio were independent of sex and BMI.

Transformation from healthy to pathological tissue may cause the ECM to become stiff, promoting myofibroblast activation and collagen deposition.[30] Collagen is the most abundant ECM component in the human body, and type I–V collagens can stimulate DDR1 activation.[20,21] Type I collagen, which is essential for the interstitial matrix structure, is predominantly produced by fibroblasts. According to the literature, the analysis of ECM components in patients with mild to moderate or severe LF showed a significant increase in type I collagen.[31–33] A previous study had shown that type I collagen promoted the shedding of DDR1 and the release of N-terminal fragments.[24] Therefore, type I collagen was used as a trigger to study the phenomenon of DDR1 shedding at the extracellular fragments in this study, which is schematically depicted in the accompanying graphic abstract. Similar to previous studies, the phenomenon of ectodomain shedding was confirmed in hepatocytes. Moreover, the shed fragment was also found in the serum. Indeed, a previous study demonstrated that a low level of DDR1 shedding occurs constitutively.[34] The present study validated collagen-induced DDR1 shedding in HEK293T cells transiently transfected with a DDR1 expression vector. Furthermore, it was found that the concentration of extracellular DDR1 fragments in the culture supernatant increased with the stimulation time after treatment with collagen I at 0, 2, 4, 6 and, 8 h, respectively. Subsequently, the cells for 8 h were 0, 25, 50, 75 μg/mL of collagen I. The results showed that as the collagen concentration was increased to 50 μg/mL, the extracellular DDR1 concentration was also increased. The previously reported saturation of collagen-stimulated DDR1 shedding can explain this phenomenon.[24] However, there was no similar phenomenon in the intracellular fragment of DDR1, which may be related to the strict regulation of the RTK family, because uncontrolled RTK activity leads to tumorigenesis.[24] The ligand-RTK complex is subject to endocytosis on the cell surface, which leads to the dissociation of the RTK from the ligand in the endocytic vesicle.[35–37] The ligand-receptor or both are subsequently degraded inside the cell to effectively terminate signaling.[35,37]

To investigate whether the shed extracellular DDR1 fragments can be detected in the blood, CCl4- and BDL-induced hepatic fibrosis models were established in mice. An obvious deposition of collagen I in the ECM of the liver was found, which is associated with the increase of LF. At the same time, the concentration of DDR1 in the serum of the mice concurrently increased with the degree of fibrosis. An exploratory study of clinical samples using immunohistochemistry also demonstrated the concurrent increase of collagen deposition and the serum levels of N-DDR1 with the aggravation of liver cirrhosis.

Activated fibroblasts are common cellular effectors of excessive fibrous ECM deposition in organs such as the lung, liver, kidney, and skin.[38] Furthermore, the expression of DDR1 is mainly limited to the epithelial cells of the aforementioned organs. Previous studies have shown that DDR1 plays a crucial role in the pathogenesis of renal fibrosis and glomerulosclerosis, which was further confirmed by *in vivo* experiments.[29] Researchers observed increased expression of DDR1 in bronchoalveolar lavage cells of patients with idiopathic pulmonary fibrosis.[39] Recently, single-cell sequencing of bronchial epithelial cells revealed increased expression of DDR1 in idiopathic pulmonary fibrosis patients, and the DDR1 small molecule inhibitor CQ-061was found to have antifibrotic and anti-inflammatory effects in bleomycin-induced idiopathic pulmonary fibrosis mice.[40] Consequently, patients with tumor, common kidney and lung diseases, osteoarthritis and atherosclerosis, or patients with a history of the above diseases were excluded. In this study, according to the medical history and physical examination results, 18 cases of chronic lung disease and 30 chronic kidney disease cases were excluded. The present study included 298 patients with chronic liver disease and 20 normal controls. A test cohort comprising 210 cases and health personnel was first investigated, followed by a validation cohort of 68 cases to confirm our findings. The results showed that the serum N-DDR1 levels and N-DDR1/albumin ratios were positively correlated with FIB4 and TE. The results of Spearman correlation statistical analysis showed that the degree of LF was correlated with serum N-DDR1 and DDR1/Alb ratio. The AUC confirmed that the serum N-DDR1/albumin ration and FIB-4 had similar diagnostic efficacy. Similar to DDR1, the receptor tyrosine kinase sAxl was reported to be a predictor of LF. However, DDR1 had better diagnostic effect than sAxl.[41] Previous studies have shown that TE was greatly affected by body weight, and obesity increased the difficulty of monitoring, affecting the accuracy of diagnosis.[41] However, body weight was not found to affect the serum DDR1/albumin ration in this study. Its repeated applicability and low cost are suitable as a screening parameter for advanced LF and cirrhosis, especially when TE is not available or applicable.

There are still some limitations in the existing research. The process of shedding and its significance are not yet entirely clear;[26] this study only demonstrated a correlation between shedding and serum N-terminal DDR1 levels. DDR1 is widely expressed in epithelial cells of the skin, lung, liver, kidney, intestine, colon, and brain. Regrettably, none of the available models include markers completely specific for LF, instead also reflecting hepatocyte damage or necrotic inflammatory activity and not only fibrosis.[42] Furthermore, due to the low rate of early screening and physical examination, the clinical specimens used in this study were mainly cirrhosis samples. Therefore, similar to the previous studies,[43] the best outcome was observed when differentiating fibrosis levels between patients with minimal or no fibrosis and patients with advanced fibrosis or cirrhosis, but the accuracy of diagnosing intermediate fibrosis was relatively poor. We tried to construct two different LF models to verify collagen-induced DDR1 shedding. However, most of the clinical samples used in this study were affected by hepatitis B virus-induced LF, which is common in Asians. Therefore, larger sample size is still needed for further validation.

In conclusion, this study indicated that serum N-terminal DDR1 levels could be used as a serological marker for diagnosing LF. Compared with invasive liver biopsy, serological markers have the advantages of inducing less damage, while being low cost and easy to repeat measurements. In practice, serum markers are often used in combination with other biomarkers or methods. Overall, serum N-terminal DDR1 may be an innovative serological diagnostic marker for LF.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study conception and design (WZ, ZhaZ, YuxZ), acquisition of data (YuxZ, YujZ), analysis and interpretation of data (YuxZ, ZhuZ, PF), drafting of the manuscript (YuxZ, YujZ), critical revision of the manuscript for important intellectual content (WZ, ZhaZ, HL), administrative, technical, or material support, study supervision (WZ, YC, HL).

Data sharing statement

All data are available upon request.

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