

Up-regulation of Plasma Hexosylceramide (d18:1/18:1) Contributes to Genotype 2 Virus Replication in Chronic Hepatitis C

A 20-Year Cohort Study

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Abstract: The aim of the present study was to explore the relationship between plasma sphingolipids and hepatitis C virus (HCV) replication in chronic hepatitis C (CHC) patients.

A cohort of 120 treatment-naïve CHC patients was included. Liver biopsies and the Scheuer scoring system were used to assess hepatic inflammatory activity. Blood biochemical indicators, HCV-RNA load, and immunological markers were also measured. Forty-four plasma sphingolipids were identified and quantified using high-performance liquid chromatography–tandem mass spectrometry.

The hexosylceramide (HexCer) (d18:1/18:1) level was significantly different between patients with a low HCV load ($<10^6$ IU/mL) and a high HCV load ($\geq 10^6$ IU/mL), and it was positively correlated with the HCV-RNA load ($r = 0.337$, $P = 0.001$) in CHC patients. Additionally, the plasma HexCer (d18:1/18:1) level (odds ratio 1.302, 95% confidence

interval 1.129–1.502) was an independent factor for a high HCV-RNA load. For patients with hepatic inflammation grade ≤ 2 or HCV genotype 2, HexCer (d18:1/18:1) was independently related to a high HCV-RNA load.

Plasma HexCer (d18:1/18:1) might be involved in the high viral replication level in chronic HCV infection, especially for CHC patients with genotype 2.

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Abbreviations: ALT = alanine aminotransferase, APOB = apolipoprotein B, AST = aspartate aminotransferase, C3 = complement 3, Cer = ceramide, CHC = chronic hepatitis C, CI = confidence interval, HCV = hepatitis C virus, HDV = hepatitis delta virus, HexCer = hexosylceramide, HIV = human immunodeficiency virus, HVL = high viral load, LVL = low viral load, OR = odds ratio, PALB = prealbumin, PLT = platelet, SD = standard deviation, SM = sphingomyelin, TP = total protein.

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INTRODUCTION

Hepatitis C virus (HCV) infection has been recognized as a leading cause of chronic liver disease, since it was discovered in 1989.¹ Approximately 3% of the population worldwide—130 to 170 million people—has an HCV infection.² Approximately 10% to 20% of patients with chronic HCV infection progress to cirrhosis in a period of 20 to 30 years.³ Although many factors are associated with disease progression, persistent HCV replication has been recognized as one of the main determinants of hepatic deterioration.⁴ Suppression of viral replication is an important strategy for preventing the progression of chronic liver disease. Although the combination of pegylated interferon- α and ribavirin or direct-acting antiviral agents could remove the viruses, a few patients suffer from treatment failure, and they require treatment in the future. Therefore, further searches for novel targets that are closely related to the HCV replication process are urgently needed.

Sphingolipids interact with cholesterol and glycosphingolipid during the formation of lipid rafts. These provide a platform for signal transduction and pathogen infection,⁵ and also play crucial roles in the viral life cycle.⁶ For instance, lipid rafts may be the sites for HCV-RNA synthesis.⁷ Recent studies have demonstrated that sphingolipids are closely related to liver disease, especially ceramide metabolites.⁸ Ceramides play an essential role in inhibiting HCV entry.⁹ During HCV infection, the suppression of sphingolipid biosynthesis with a serine palmitoyltransferase inhibitor, which is the first-step enzyme in the ceramide biosynthetic pathway, could suppress viral

replication in HCV-infected chimeric mice and HCV replicon cells.^{10–12} Additionally, HCV infection regulates various aspects of lipid metabolism within infected hepatocytes.^{13–15} For example, HCV upregulates the sphingomyelin and ceramide levels, promoting viral replication in the hepatocytes of humanized chimeric mice.¹⁶ Although multiple reports have indicated that dysregulation of sphingolipid synthesis affects HCV replication in animal models and cells, the relationship of plasma sphingolipids with HCV replication in chronic hepatitis C (CHC) patients remains poorly understood.

Previously, we established mature high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) techniques to quantify sphingolipids.^{17,18} On the basis of a platform that improved quantitative high-throughput lipidomic, we initially confirmed a relationship between the plasma sphingolipids and hepatic inflammation in CHC.¹⁹ Subsequently, we demonstrated that glycosphingolipid and sphingomyelin were closely related to hepatic steatosis and fibrosis in CHC patients, respectively.^{20,21} On the basis of the previous reports and studies, we hypothesized that one or a subset of sphingolipids might be involved in HCV replication.

Therefore, in the present study, with the help of HPLC-MS/MS, liver biopsy, and HCV-RNA quantification, we aimed to evaluate the relationship between plasma sphingolipids and HCV replication among treatment-naïve patients with CHC, and also the subgroup of patients without significant necroinflammation (grade ≤ 2) or with HCV genotype 2.

METHODS

Patients

A cohort of 122 treatment-naïve CHC patients with a clear history of paid plasma donation from 1992 to 1995, from Dingxi City (Gansu Province, China), who were followed between July 2010 to June 2011, were enrolled in the present study.²² The CHC diagnosis was made in accordance with established criteria.^{23,24} Patients were excluded if they were coinfecting with HBV, human immunodeficiency virus (HIV), or hepatitis delta virus (HDV), or if they had a malignant disease, including hepatocellular carcinoma, or there were no biopsy results. Two cases were excluded; one was for ascites, and the other one was because the liver biopsy specimen volume was too small. One hundred twenty patients were eligible for the study. Informed consents were obtained. The study was performed according to the provisions of the Declaration of Helsinki and was approved by the Institutional Review Board of Beijing YouAn Hospital, Capital Medical University, Beijing, China.

Clinical Data Collection

The blood biochemical indicators, HCV-RNA load, and immunological markers were collected as previously described.²² Fasting blood samples were collected at the time of biopsy and stored at -80°C . Routine serological indications were extracted in all patients.

Liver Biopsy

Liver biopsies were performed as described.²² The Scheuer scoring system was used to assess hepatic inflammatory activity.²⁵ We selected patients without significant necroinflammation (grade ≤ 2) for the subgroup statistical analysis.

HPLC-MS/MS

Serum sphingolipids were detected by HPLC-MS/MS as previously described.¹⁷ Sphingolipidomic assays were

performed at the Institute of Materia Medica, Peking Union Medical College (Beijing, China).

Statistical Analysis

Data were expressed as mean \pm standard deviation (SD), unless otherwise specified. Statistical comparisons for continuous variables were performed using the independent-samples *t* test or Mann–Whitney *U* test. Categorical variables were analyzed using the Pearson chi-square test. Correlation analysis between the plasma sphingolipids and HCV-RNA load was made using Spearman rank correlation. After univariate analysis, stepwise logistic regression analysis with a forward selection, and the *P* values of entry and removal were set to 0.05 and 0.1, respectively. Statistical analysis was performed using SPSS version 19.0 (Chicago, IL). A *P* value <0.05 was considered statistically significant.

RESULTS

Clinical Characteristics of Patients

In our cohort, there were 57 (47.5%) male and 63 (52.5%) female patients who had a mean age of 51.33 years. The mean serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) values were 60.42 and 47.94 U/L, respectively; they were mildly elevated at the time of biopsy.

TABLE 1. Demographic and Clinical Characteristics of the Patients With Chronic Hepatitis C (n = 120)

Variables	Value
Age, yrs, mean \pm SD	51.33 \pm 7.33
Male, n (%)	57 (47.50)
ALT, U/L, mean \pm SD	60.42 \pm 70.88
AST, U/L, mean \pm SD	47.94 \pm 44.31
Total bilirubin, $\mu\text{mol/L}$, mean \pm SD	16.51 \pm 7.25
Direct bilirubin, $\mu\text{mol/L}$, mean \pm SD	3.26 \pm 1.36
Total protein, g/L, mean \pm SD	70.93 \pm 4.95
Albumin, g/L, mean \pm SD	43.21 \pm 2.36
Globulin, g/L, mean \pm SD	29.19 \pm 17.28
γ -GGT, U/L, mean \pm SD	22.04 \pm 16.50
Alkaline phosphatase, U/L, mean \pm SD	76.29 \pm 20.91
Cholinesterase, U/L, mean \pm SD	6957.09 \pm 1442.44
Prothrombin activity, %, mean \pm SD	92.41 \pm 9.11
Scheuer inflammation grade, n (%)	
Grade 0	1 (0.83)
Grade 1	16 (13.33)
Grade 2	67 (55.83)
Grade 3	34 (28.33)
Grade 4	2 (1.67)
HCV-RNA load, IU/mL, n (%)	
$<10^6$	72 (60.00)
$\geq 10^6$	48 (40.00)
HCV genotype, n (%)	
1b	44 (36.67)
2	53 (44.17)
Unknown	23 (19.17)

Data are presented as mean \pm standard deviation (SD) or number (%).

ALT = alanine aminotransferase, AST = aspartate aminotransferase, GGT = gamma-glutamyl transferase, HCV = hepatitis C virus, SD = standard deviation.

According to the Scheuer inflammation score, grade 2 was present in 67 patients, accounting for the largest proportion (55.8%). This was followed by grades 3 (28.3%, 34/120) and 1 (13.3%, 16/120). Grades 0 and 4 were found in 0.8% (1/120) and 1.7% (2/120) of the patients, respectively. The number of patients presenting with an HCV-RNA load less than 10⁶ IU/mL was 72 (60%); 48 (40%) patients had more than 10⁶ IU/mL. In terms of genotypes, 44.2% (53/120) of patients had HCV genotype 2, which was closely followed by HCV genotype

1 (36.7%, 44/120). The remainder did not belong to either genotype 1 or genotype 2 (19.2%) (Table 1).

Relationship Between Plasma Sphingolipids and HCV-RNA Load in CHC Patients

Plasma sphingolipids in the 120 patients were detected by HPLC-MS/MS. Forty-four sphingolipids were identified and quantified. Twenty-two plasma sphingolipids showed a significant difference between patients with a low viral load (LVL,

TABLE 2. Plasma Sphingolipid Profile of Untreated Chronic Hepatitis C Patients According to HCV-RNA Load

Variables, pmol/mL	<10 ⁶ IU/mL (n = 72)	≥10 ⁶ IU/mL (n = 48)	P*
Sphingosine-1-P	153.49 ± 34.30	138.11 ± 41.25	0.03
Cer (d18:1/16:0)-1-P	24.21 ± 11.06	29.05 ± 9.17	0.004
Cer (d18:1/18:1)-1-P	17.60 ± 11.82	15.37 ± 15.40	0.03
Cer (d18:1/18:0)-1-P	210.48 ± 75.83	219.56 ± 85.32	0.85
Cer (d18:1/20:0)-1-P	19.10 ± 10.89	23.43 ± 12.46	0.05
Cer (d18:1/24:0)-1-P	14.33 ± 6.09	14.22 ± 4.67	0.81
Cer (d18:1/16:0)	126.30 ± 33.13	151.18 ± 41.78	<0.001
Cer (d18:1/18:1)	3.28 ± 1.19	3.53 ± 1.48	0.51
Cer (d18:1/18:0)	25.74 ± 11.54	30.17 ± 15.98	0.16
Cer (d18:1/20:0)	21.71 ± 8.01	20.42 ± 9.59	0.20
Cer (d18:1/22:0)	150.07 ± 57.63	203.56 ± 96.88	<0.001
Cer (d18:1/24:1)	378.50 ± 121.03	447.00 ± 163.28	0.03
Cer (d18:1/24:0)	546.36 ± 117.24	626.18 ± 92.86	0.12
Cer (d18:1/26:0)	4.29 ± 1.96	4.98 ± 2.25	0.07
dhSphingosine	3.28 ± 1.92	2.76 ± 1.94	0.05
dhSphingosine-1-P	25.48 ± 5.78	25.60 ± 6.72	0.92
HexCer (d18:1/6:0)	5.05 ± 5.24	3.88 ± 2.01	0.64
HexCer (d18:1/12:0)	17.34 ± 11.84	16.28 ± 18.26	0.03
HexCer (d18:1/14:0)	8.05 ± 3.22	6.40 ± 2.41	0.003
HexCer (d18:1/16:0)	1338.46 ± 558.33	1235.59 ± 395.82	0.44
HexCer (d18:1/18:1)	10.45 ± 5.69	15.59 ± 6.00	<0.001
HexCer (d18:1/18:0)	9.67 ± 5.98	12.95 ± 6.08	0.001
HexCer (d18:1/20:0)	17.18 ± 11.24	23.49 ± 12.77	0.02
HexCer (d18:1/22:0)	291.23 ± 84.02	312.68 ± 81.17	0.14
HexCer (d18:1/24:1)	456.06 ± 184.07	484.62 ± 151.11	0.18
HexCer (d18:1/24:0)	355.75 ± 101.52	383.48 ± 100.79	0.13
dhCer (d18:0/16:0)	7.64 ± 2.38	8.76 ± 2.73	0.02
dhCer (d18:0/18:0)	7.24 ± 2.60	7.75 ± 3.16	0.48
dhCer (d18:0/20:0)	36.01 ± 14.08	38.66 ± 19.88	0.48
dhCer (d18:0/22:0)	117.47 ± 55.80	153.17 ± 84.82	0.03
dhCer (d18:0/24:1)	45.47 ± 25.18	49.71 ± 21.96	0.18
dhCer (d18:0/24:0)	110.82 ± 48.27	133.17 ± 64.90	0.08
dhCer (d18:0/26:1)	6.23 ± 3.93	6.72 ± 4.38	0.37
dhCer (d18:0/26:0)	9.11 ± 4.11	11.01 ± 5.17	0.02
Lyso-SM (d18:1)	5.08 ± 1.70	5.75 ± 1.92	0.05
SM (d18:1/12:0)	10.22 ± 2.85	10.12 ± 2.96	>0.99
SM (d18:1/14:0)	4.67 ± 1.10	5.00 ± 0.90	0.02
SM (d18:1/16:0)	3329.00 ± 392.23	3507.84 ± 347.10	0.005
SM (d18:1/18:1)	757.25 ± 217.79	645.18 ± 171.88	0.005
SM (d18:1/18:0)	1467.92 ± 210.89	1563.03 ± 214.39	0.01
SM (d18:1/20:0)	17.81 ± 2.65	19.29 ± 2.97	0.007
SM (d18:1/22:0)	25.74 ± 4.41	28.14 ± 4.33	0.001
SM (d18:1/24:1)	2758.56 ± 631.99	3280.42 ± 647.29	<0.001
SM (d18:1/24:0)	1552.46 ± 246.28	1659.26 ± 235.79	0.02

Data are expressed as mean ± standard deviation.

Cer = ceramide, dhCer = dihydroceramide, dhSphingosine = dihydrosphingosine, HexCer = hexosylceramide, SM = sphingomyelin.

*P values were calculated by the independent-sample *t* test or the nonparametric Mann-Whitney *U* test. Bold values indicated that *P* < 0.05.

HCV-RNA load $<10^6$ IU/mL) and a high viral load (HVL, HCV-RNA load $\geq 10^6$ IU/mL) ($P < 0.05$) (Table 2). To further analyze the correlation between these differentially expressed sphingolipids and HCV-RNA load, Spearman correlation analysis was performed. The results of the correlation analysis revealed that 9 sphingolipids were correlated with the HCV-RNA load (Table 3).

Routine blood indicators, such as the platelet (PLT), prealbumin (PALB), apolipoprotein B (APOB), and $CD4^+$ T-cell ratio, and $CD8^+$ T-cell ratio, had significant differences between LVL and HVL patients ($P < 0.05$). Then, PLT, PALB, APOB, $CD4^+$ T-cell ratio, $CD8^+$ T-cell ratio, sphingosine-1-P, ceramide (Cer) (d18:1/16:0)-1-P, Cer (d18:1/16:0), Cer (d18:1/22:0), hexosylceramide (HexCer) (d18:1/18:1), HexCer (d18:1/18:0), sphingomyelin (SM) (d18:1/18:1), SM (d18:1/22:0), and SM (d18:1/24:1) were initially included in the multivariate analysis. The odds ratio (OR) for HexCer (d18:1/18:1) and HexCer (d18:1/18:0) reached 1.302 (95% confidence interval [CI] 1.129–1.502) and 0.868 (95% CI 0.757–0.995), respectively (Table 4).

Identification of HCV Replication in the Subgroup of CHC Patients Without Significant Necroinflammation (Grade ≤ 2)

Hepatic necroinflammatory activity is closely related to the rapid disease progression in CHC patients,²⁶ and hepatic

TABLE 3. The Correlation Analysis between Differentially Expressed Sphingolipids and HCV-RNA Load in Patients With Chronic Hepatitis C

Variables, pmol/mL	Correlation Coefficient (n = 100)*	P†
Sphingosine-1-P	-0.212	0.03
Cer (d18:1/16:0)-1-P	0.206	0.04
Cer (d18:1/18:1)-1-P	-0.190	0.06
Cer (d18:1/16:0)	0.201	0.05
Cer (d18:1/22:0)	0.248	0.01
Cer (d18:1/24:1)	0.140	0.16
HexCer (d18:1/12:0)	-0.140	0.17
HexCer (d18:1/14:0)	-0.229	0.02
HexCer (d18:1/18:1)	0.337	0.001
HexCer (d18:1/18:0)	0.279	0.005
HexCer (d18:1/20:0)	0.193	0.06
dhCer (d18:0/16:0)	0.081	0.42
dhCer (d18:0/22:0)	0.047	0.64
dhCer (d18:0/26:0)	0.071	0.48
SM (d18:1/14:0)	0.115	0.25
SM (d18:1/16:0)	0.175	0.08
SM (d18:1/18:1)	-0.256	0.01
SM (d18:1/18:0)	0.171	0.09
SM (d18:1/20:0)	0.105	0.30
SM (d18:1/22:0)	0.211	0.04
SM (d18:1/24:1)	-0.318	0.001
SM (d18:1/24:0)	0.097	0.34

Cer = ceramide, dhCer = dihydroceramide, dhSphingosine = dihydro-sphingosine, HexCer = hexosylceramide, SM = sphingomyelin.

*Twenty patients' viral loads were below the detection limit.

†P values were calculated by Spearman correlation test. Bold values indicated that $P < 0.05$.

inflammation caused by T cells contributes to HCV replication.^{27–29} To reduce the impact of hepatic inflammation and the possible accompanying cytotoxic T-lymphocyte response on HCV replication, we evaluated the association of sphingolipids with the HCV-RNA load in the subgroup of CHC patients without significant necroinflammation (grade ≤ 2) after adjusting for the immunological index. Routine blood indicators PLT, PALB, complement 3 (C3), and the $CD8^+$ T-cell ratio were significantly different between LVL and HVL patients ($P < 0.05$). Cer (d18:1/16:0)-1-P, Cer (d18:1/22:0), HexCer (d18:1/14:0), HexCer (d18:1/18:0), HexCer (d18:1/18:1), Lyso-SM(d18:1), SM (d18:1/18:1), SM (d18:1/20:0), and SM (d18:1/24:1) showed significant differences between LVL and HVL patients ($P < 0.05$) for all plasma sphingolipids (Tables S1 and S2, <http://links.lww.com/MD/B7>). For the logistic regression analysis, PLT, PALB, C3, $CD8^+$ T-cell ratio, Cer (d18:1/16:0)-1-P, Cer (d18:1/22:0), HexCer (d18:1/14:0), HexCer (d18:1/18:0), HexCer (d18:1/18:1), Lyso-SM(d18:1), SM (d18:1/18:1), SM (d18:1/20:0), and SM (d18:1/24:1) were included. The final results showed that the OR for HexCer (d18:1/18:1) reached 1.186 (95% CI 1.085–1.296) (Table 5).

Relationship Between Plasma Sphingolipids and HCV-RNA Load in CHC Patients With HCV Genotype 2

The relationship between plasma sphingolipids and the HCV-RNA load in 53 patients with HCV genotype 2 was analyzed. Of all plasma sphingolipids, Cer(d18:1/24:0), HexCer (d18:1/18:1), HexCer (d18:1/18:0), SM (d18:1/18:1), SM (d18:1/22:0), and SM (d18:1/24:1) were significantly different between LVL and HVL patients ($P < 0.05$) in univariate analysis (Tables S3 and S4, <http://links.lww.com/MD/B7>). Routine blood indicators, such as the total protein (TP), globulin, total bile acid, PALB, APOB, and IgG, showed significant differences between LVL and HVL patients ($P < 0.05$). In plasma sphingolipids, Cer (d18:1/24:0) (OR 1.009, 95% CI 1.001–1.017) and HexCer (d18:1/18:1) (OR 1.173, 95% CI 1.031–1.334) were independently linked to the HCV-RNA load (LVL vs HVL), after adjusting for other factors (Table 6).

DISCUSSION

In this study, a cohort of antiviral treatment naïve, non-diabetic, and nonobese CHC patients were enrolled. After eliminating the influence of antiviral treatment, we revealed the true relationship between plasma sphingolipids and HCV replication. For the first time, we found that the altered plasma sphingolipids were closely associated with HCV replication in chronic HCV infection, especially for CHC patients with genotype 2.

Sphingolipids have recently been found to be involved in liver disease; for example, they play an important role in hepatitis C virus entry,⁹ HCV infection, virion maturation,³⁰ liver fibrosis progression, and antiviral therapy responsiveness.³¹ To the best of our knowledge, the role of plasma sphingolipids in HCV replication of CHC patients is currently unknown. In our study, although several sphingolipids correlate with HCV replication, only HexCer (d18:1/18:1) always performed outstandingly in the subsequent analysis and subgroup analysis.

In the correlation analysis, HexCer (d18:1/18:1) was positively correlated with the HCV-RNA load, which reflects the viral replication level. Additionally, alterations in plasma HexCer (d18:1/18:1) were independently related to HVL after

TABLE 4. Indicators Related to HCV-RNA Load in Chronic Hepatitis C Patients in Univariate and Multivariate Analyses

Variables	<10 ⁶ IU/mL (n = 72)	≥10 ⁶ IU/mL (n = 48)	P*	OR (95% CI)	P [†]
Age, yrs	50.76 ± 7.57	52.17 ± 6.95	0.31		
Male, n (%)	32 (44.44%)	25 (52.08%)	0.41		
ALT, U/L	58.86 ± 58.30	62.76 ± 87.04	0.88		
AST, U/L	46.33 ± 34.58	50.36 ± 56.16	>0.99		
TBIL, μmol/L	15.99 ± 7.48	17.28 ± 6.86	0.20		
DBIL, μmol/L	3.26 ± 1.50	3.26 ± 1.12	0.56		
TP, g/L	71.63 ± 5.24	69.89 ± 4.33	0.06		
Albumin, g/L	43.27 ± 2.50	43.12 ± 2.17	0.74		
Globulin, g/L	28.21 ± 5.20	30.67 ± 26.68	0.09		
ALP, U/L	74.48 ± 22.48	79.00 ± 18.19	0.13		
PLT, 10 ⁹ /L	181.51 ± 51.30	156.13 ± 52.88	0.01	0.988 (0.980–0.997)	0.009
PALB, g/L	164.93 ± 36.61	219.88 ± 274.59	0.006		
APOA1, g/L	118.77 ± 30.42	129.60 ± 31.16	0.06		
APOB, g/L	59.39 ± 12.81	62.88 ± 14.20	0.02		
CD4 ⁺ T-cell ratio, %	41.68 ± 9.32	46.93 ± 13.06	0.02		
CD4 ⁺ T-cell count, /μL	730.26 ± 239.06	646.30 ± 220.34	0.06		
CD8 ⁺ T-cell ratio, %	26.32 ± 11.04	30.51 ± 9.09	0.007		
CD8 ⁺ T-cell count, /μL	463.85 ± 222.84	426.23 ± 183.78	0.45		
Sphingosine-1-P, pmol/mL	153.49 ± 34.30	138.11 ± 41.25	0.03		
Cer (d18:1/16:0)-1-P, pmol/mL	24.21 ± 11.06	29.05 ± 9.17	0.004		
Cer (d18:1/22:0), pmol/mL	150.07 ± 57.63	203.56 ± 96.88	<0.001		
HexCer (d18:1/14:0), pmol/mL	8.05 ± 3.22	6.40 ± 2.41	0.003		
HexCer (d18:1/18:1), pmol/mL	10.45 ± 5.69	15.59 ± 6.00	<0.001	1.302 (1.129–1.502)	<0.001
HexCer (d18:1/18:0), pmol/mL	9.67 ± 5.98	12.95 ± 6.08	0.001	0.868 (0.757–0.995)	0.04
SM (d18:1/18:1), pmol/mL	757.25 ± 217.79	645.18 ± 171.88	0.005		
SM (d18:1/22:0), pmol/mL	25.74 ± 4.41	28.14 ± 4.33	0.001		
SM (d18:1/24:1), pmol/mL	2758.56 ± 631.99	3280.42 ± 647.29	<0.001		

Data are means ± SD or number of patients (%).

ALP = alkaline phosphatase, ALT = alanine aminotransferase, APO = apolipoprotein, AST = aspartate aminotransferase, CI = confidence interval, DBIL = direct bilirubin, OR = odds ratio, PALB = prealbumin, PLT = platelet, TBIL = total bilirubin, TP = total protein.

*P values are acquired by univariate analyses.

†P values are acquired by multivariate analyses.

TABLE 5. Indicators Related to Sphingolipids in Chronic Hepatitis C Patients With Hepatic Inflammation Grade ≤2 in Univariate and Multivariate Analyses

Variables	<10 ⁶ IU/mL (n = 52)	≥10 ⁶ IU/mL (n = 32)	P*	OR (95% CI)	P [†]
Age, yrs	50.27 ± 7.54	51.63 ± 6.96	0.41		
Male, n (%)	21 (40.38%)	17 (53.13%)	0.26		
ALT, U/L	50.27 ± 35.42	44.96 ± 26.17	0.44		
AST, U/L	38.18 ± 20.49	37.01 ± 13.61	0.88		
TBIL, μmol/L	15.42 ± 6.73	17.78 ± 7.45	0.14		
DBIL, μmol/L	3.09 ± 1.27	3.27 ± 1.12	0.42		
TP, g/L	71.58 ± 5.25	69.93 ± 4.45	0.14		
Albumin, g/L	43.57 ± 2.49	43.18 ± 2.02	0.46		
Globulin, g/L	27.80 ± 5.37	32.54 ± 32.61	0.27		
ALP, U/L	72.38 ± 19.04	74.36 ± 14.67	0.62		
PLT, 10 ⁹ /L	191.12 ± 51.60	166.81 ± 53.71	0.04		
PALB, g/L	170.83 ± 37.78	247.73 ± 333.61	0.005		
C3, g/L	1.03 ± 0.13	0.96 ± 0.16	0.03	0.005 (0.000–0.246)	0.008
C4, g/L	0.22 ± 0.06	0.19 ± 0.07	0.08		
CD4 ⁺ T-cell ratio, %	42.13 ± 9.76	45.70 ± 12.58	0.33		
CD4 ⁺ T-cell count, μ/L	727.21 ± 254.97	624.81 ± 208.75	0.06		
CD8 ⁺ T-cell ratio, %	25.75 ± 10.99	32.10 ± 8.53	0.007		
CD8 ⁺ T-cell count, μ/L	438.54 ± 191.58	434.55 ± 144.43	0.92		
Cer (d18:1/16:0)-1-P, pmol/mL	23.38 ± 9.51	30.24 ± 9.41	0.002		

Variables	<10 ⁶ IU/mL (n = 52)	≥10 ⁶ IU/mL (n = 32)	P*	OR (95% CI)	P [†]
Cer (d18:1/22:0), pmol/mL	152.72 ± 57.12	211.22 ± 92.10	0.001		
HexCer (d18:1/14:0), pmol/mL	8.12 ± 3.23	5.93 ± 2.30	0.001		
HexCer (d18:1/18:1), pmol/mL	10.48 ± 5.88	16.37 ± 5.76	<0.001	1.186 (1.085–1.296)	<0.001
HexCer (d18:1/18:0), pmol/mL	9.73 ± 6.46	12.97 ± 5.57	0.002		
Lyso-SM (d18:1), pmol/mL	4.98 ± 1.66	5.90 ± 1.94	0.022		
SM (d18:1/18:1), pmol/mL	780.76 ± 207.07	623.39 ± 156.48	<0.001		
SM (d18:1/22:0), pmol/mL	25.74 ± 4.51	28.42 ± 4.13	0.002		
SM (d18:1/24:1), pmol/mL	2728.52 ± 661.08	3366.74 ± 595.99	<0.001		

Data are means ± SD or number of patients (%).

ALP = alkaline phosphatase, ALT = alanine aminotransferase, AST = aspartate aminotransferase, C = Complement, CD = cluster differentiation, Cer = ceramide, CI = confidence interval, DBIL = direct bilirubin, HexCer = hexosylceramide, OR = odds ratio, PALB = prealbumin, PLT = platelet, TBIL = total bilirubin, TP = total protein.

*P values are acquired by univariate analyses.

†P values are acquired by multivariate analyses.

TABLE 6. Indicators Related to Sphingolipids in Chronic Hepatitis C Patients With HCV Genotype 2 in Univariate and Multivariate Analyses

Variables	<10 ⁶ IU/mL (n = 28)	≥10 ⁶ IU/mL (n = 25)	P*	OR (95% CI)	P [†]
Age, yrs	51.79 ± 6.48	52.84 ± 6.22	0.55		
Male, n (%)	13 (46.43%)	12 (48.00%)	0.91		
ALT, U/L	61.04 ± 50.27	44.22 ± 28.82	0.11		
AST, U/L	52.83 ± 38.56	38.25 ± 14.64	0.14		
TBIL, μmol/L	18.74 ± 9.54	16.59 ± 5.77	0.65		
DBIL, μmol/L	3.72 ± 1.86	3.12 ± 0.86	0.52		
TP, g/L	73.29 ± 5.40	69.86 ± 4.25	0.01		
Albumin, g/L	43.11 ± 2.31	43.56 ± 2.23	0.47		
Globulin, g/L	30.21 ± 4.78	33.81 ± 36.90	0.004		
TBA, μmol/L	27.09 ± 96.02	5.50 ± 3.92	0.01		
PLT, 10 ⁹ /L	160.57 ± 43.34	152.48 ± 48.12	0.52		
PALB, g/L	157.33 ± 31.83	189.73 ± 33.60	0.001		
APOA1, g/L	121.18 ± 36.88	135.80 ± 30.35	0.12		
APOB, g/L	58.52 ± 12.20	66.69 ± 10.71	0.003		
LgA, g/L	2.11 ± 0.83	1.83 ± 0.62	0.18		
IgM, g/L	1.26 ± 0.38	1.48 ± 0.68	0.31		
IgG, g/L	18.12 ± 4.00	14.96 ± 2.85	<0.001	0.723 (0.535–0.978)	0.04
Cer (d18:1/24:0), pmol/mL	562.01 ± 101.73	659.10 ± 83.67	<0.001	1.009 (1.001–1.017)	0.03
HexCer (d18:1/18:1), pmol/mL	10.24 ± 5.73	17.35 ± 6.04	<0.001	1.173 (1.031–1.334)	0.02
HexCer (d18:1/18:0), pmol/mL	9.28 ± 6.26	14.28 ± 6.22	0.001		
SM (d18:1/18:1), pmol/mL	780.94 ± 242.74	657.76 ± 167.60	0.04		
SM (d18:1/22:0), pmol/mL	25.88 ± 4.32	29.22 ± 4.56	0.009		
SM (d18:1/24:1), pmol/mL	2737.27 ± 719.91	3348.29 ± 621.63	0.001		

Data are means ± SD or number of patients (%).

ALT = alanine aminotransferase, APO = apolipoprotein, AST = aspartate aminotransferase, Cer = ceramide, CI = confidence interval, DBIL = direct bilirubin, HexCer = hexosylceramide, Ig = immune globulin, OR = odds ratio, PALB = prealbumin, TBA = total bile acid, TBIL = total bilirubin, TP = total protein.

*P values are acquired by univariate analyses.

†P values are acquired by multivariate analyses.

adjusting for confounding variables. Moreover, an in vitro study reported that glycosphingolipids play a crucial role in the HCV life cycle and that they share a link with phosphatidylinositol 4-phosphate in HCV replication.³² However, the detailed underlying mechanisms responsible for the association of HexCer (d18:1/18:1) with HCV replication is unclear.

Our previous studies indicated that plasma sphingolipids are related to hepatic inflammation in CHC, especially hepatic

necroinflammation (grade >2).^{18,19} To eliminate the impact of necroinflammation on plasma sphingolipids and the possibly accompanying cytotoxic T-lymphocyte response on HCV replication, we analyzed the relationship between plasma sphingolipids and HCV replication in CHC patients without hepatic necroinflammation (grade ≤2). Remarkably, the plasma HexCer (d18:1/18:1) level was also independently related to HCV replication in this subgroup analysis. However, another sphingolipid,

HexCer (d18:1/18:0), as an independent factor for HCV replication in CHC patients, was not associated with viral replication in CHC patients who had hepatic inflammation grade ≤ 2 .

In our cohort, HCV genotypes 1b and 2 were the major types, which were consistent with the HCV genotype prevalent in China, where genotypes 1b and 2 account for nearly 80% of HCV infection patients.^{33,34} In CHC patients with genotype 2, the plasma HexCer (d18:1/18:1) level was also independently related to HCV replication after adjusting for the confounding factor in the multivariate analysis. However, Cer (d18:1/24:0), which was an independent factor for viral replication in genotype 2 CHC patients, was not associated with HCV replication in any of the 120 CHC cohort patients. We also explored the role of sphingolipids in CHC patients with genotype 1b. Regrettably, no association was found between these sphingolipids and HCV replication in CHC patients with genotype 1b (data not shown).

Although our study did not reveal the mechanisms for plasma HexCer (d18:1/18:1) in HCV replication on the basis of the present data, we speculated that elevated HexCer (d18:1/18:1) might contribute to the pathogenesis of viral replication in CHC patients, especially for genotype 2 CHC patients. The sample size in this cohort was not sufficiently large, and patients with other genotypes were not analyzed in this study. Therefore, it would make sense to verify the role of HexCer (d18:1/18:1) in large-scale clinical studies in the future.

In conclusion, our results suggest, for the first time, the crucial role of plasma HexCer (d18:1/18:1) in HCV replication. Additionally, HexCer has potential as a novel therapeutic target for antiviral therapy in CHC, especially in patients with genotype 2. These findings will provide new insights into the molecular mechanism of HCV replication in CHC. Further studies are necessary to determine the detailed mechanisms responsible for these associations.

REFERENCES

- Mohd Hanafiah K, Groeger J, Flaxman AD, et al. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. *Hepatology*. 2013;57:1333–1342.
- Hajarizadeh B, Grebely J, Dore GJ. Epidemiology and natural history of HCV infection. *Nature Rev Gastroenterol Hepatol*. 2013;10:553–562.
- Westbrook RH, Dusheiko G. Natural history of hepatitis C. *J Hepatol*. 2014;61(1 Suppl):S58–S68.
- Almasio PL, Venezia G, Craxi A. The impact of antiviral therapy on the course of chronic HCV infection. A systematic review. *Panminerva Medica*. 2003;45:175–182.
- van der Meer-Janssen YP, van Galen J, Batenburg JJ, et al. Lipids in host-pathogen interactions: pathogens exploit the complexity of the host cell lipidome. *Prog Lipid Res*. 2010;49:1–26.
- Negro F. Abnormalities of lipid metabolism in hepatitis C virus infection. *Gut*. 2010;59:1279–1287.
- Aizaki H, Lee KJ, Sung VM, et al. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. *Virology*. 2004;324:450–461.
- Mari M, Fernandez-Checa JC. Sphingolipid signalling and liver diseases. *Liver Int*. 2007;27:440–450.
- Voisset C, Lavie M, Helle F, et al. Ceramide enrichment of the plasma membrane induces CD81 internalization and inhibits hepatitis C virus entry. *Cell Microbiol*. 2008;10:606–617.
- Umehara T, Sudoh M, Yasui F, et al. Serine palmitoyltransferase inhibitor suppresses HCV replication in a mouse model. *Biochem Biophys Res Commun*. 2006;346:67–73.
- Sakamoto H, Okamoto K, Aoki M, et al. Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. *Nature Chem Biol*. 2005;1:333–337.
- Katsume A, Tokunaga Y, Hirata Y, et al. A serine palmitoyltransferase inhibitor blocks hepatitis C virus replication in human hepatocytes. *Gastroenterology*. 2013;145:865–873.
- Kapadia SB, Chisari FV. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. *Proc Natl Acad Sci USA*. 2005;102:2561–2566.
- Su AI, Pezacki JP, Wodicka L, et al. Genomic analysis of the host response to hepatitis C virus infection. *Proc Natl Acad Sci USA*. 2002;99:15669–15674.
- Takano T, Tsukiyama-Kohara K, Hayashi M, et al. Augmentation of DHCR24 expression by hepatitis C virus infection facilitates viral replication in hepatocytes. *J Hepatol*. 2011;55:512–521.
- Hirata Y, Ikeda K, Sudoh M, et al. Self-enhancement of hepatitis C virus replication by promotion of specific sphingolipid biosynthesis. *PLoS Pathog*. 2012;8:e1002860.
- Qu F, Wu CS, Hou JF, et al. Sphingolipids as new biomarkers for assessment of delayed-type hypersensitivity and response to triptolide. *PLoS One*. 2012;7:e52454.
- Li JF, Qu F, Zheng SJ, et al. Plasma sphingolipids as potential indicators of hepatic necroinflammation in patients with chronic hepatitis C and normal alanine aminotransferase level. *PLoS One*. 2014;9:e95095.
- Qu F, Zheng SJ, Wu CS, et al. Lipidomic profiling of plasma in patients with chronic hepatitis C infection. *Anal Bioanal Chem*. 2014;406:555–564.
- Li JF, Qu F, Zheng SJ, et al. Elevated plasma sphingomyelin (d18:1/22:0) is closely related to hepatic steatosis in patients with chronic hepatitis C virus infection. *Eur J Clin Microbiol Infect Dis*. 2014;33:1725–1732.
- Li JF, Qu F, Zheng SJ, et al. Plasma sphingolipids: potential biomarkers for severe hepatic fibrosis in chronic hepatitis C. *Molec Med Rep*. 2015;12:323–330.
- Li JF, Liu S, Ren F, et al. Fibrosis progression in interferon treatment-naïve Chinese plasma donors with chronic hepatitis C for 20 years: a cohort study. *Int J Infect Dis*. 2014;27:49–53.
- Ghany MG, Strader DB, Thomas DL, et al. American Association for the Study of Liver D. Diagnosis, management, and treatment of hepatitis C: an update. *Hepatology*. 2009;49:1335–1374.
- Hepatotomy Branch I. Parasitology branch CMA. [Guideline of prevention and treatment of hepatitis C]. *Chin J Prevent Med*. 2004;38:210–215.
- Scheuer PJ. Classification of chronic viral hepatitis: a need for reassessment. *J Hepatol*. 1991;13:372–374.
- Ghany MG, Kleiner DE, Alter H, et al. Progression of fibrosis in chronic hepatitis C. *Gastroenterology*. 2003;124:97–104.
- Helbig KJ, Ruzsiewicz A, Semendric L, et al. Expression of the CXCR3 ligand I-TAC by hepatocytes in chronic hepatitis C and its correlation with hepatic inflammation. *Hepatology*. 2004;39:1220–1229.
- He XS, Greenberg HB. CD8+ T-cell response against hepatitis C virus. *Viral Immunol*. 2002;15:121–131.
- Freeman AJ, Pan Y, Harvey CE, et al. The presence of an intrahepatic cytotoxic T lymphocyte response is associated with low viral load in patients with chronic hepatitis C virus infection. *J Hepatol*. 2003;38:349–356.
- Suzuki T, Masaki T, Aizaki H. [Involvement of nonstructural protein 5A and lipids on production of hepatitis C virus particles]. *Uirusu*. 2008;58:199–205.

31. Georgios Grammatikos NF, Dimitra B, Stephanie S, et al. Variations in serum sphingolipid levels associate with liver fibrosis progression and poor treatment outcome in hepatitis C virus but not hepatitis B virus infection. *Hepatology*. 2015;61:812–822.
32. Khan I, Katikaneni DS, Han Q, et al. Modulation of hepatitis C virus genome replication by glycosphingolipids and four-phosphate adaptor protein 2. *J Virol*. 2014;88:12276–12295.
33. Cui Y, Jia J. Update on epidemiology of hepatitis B and C in China. *J Gastroenterol Hepatol*. 2013;28(Suppl 1):7–10.
34. Rao H, Wei L, Lopez-Talavera JC, et al. Distribution and clinical correlates of viral and host genotypes in Chinese patients with chronic hepatitis C virus infection. *J Gastroenterol Hepatol*. 2014;29:545–553.