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RAPID COMMUNICATION

Influence of iron on the severity of hepatic fibrosis in patients with chronic hepatitis C

Tsung-Jung Lin, Li-Ying Liao, Shyr-Yi Lin, Chih-Lin Lin, Ting-An Chang

Tsung-Jung Lin, Li-Ying Liao, Chih-Lin Lin, Department of Gastroenterology, Ren-Ai Branch, Taipei City Hospital, Taipei, Taiwan, China
Tsung-Jung Lin, Shyr-Yi Lin, Graduate Institute of Medical Sciences, Taipei Medical University, Taipei, Taiwan, China
Ting-An Chang, Department of Pathology, Ren-Ai Branch, Taipei City Hospital, Taipei, Taiwan, China
Supported by grants from the Taipei Institute of Pathology, Taipei, Taiwan, China
Correspondence to: Dr. Li-Ying Liao, Department of Gastroenterology, Ren-Ai Branch, Taipei City Hospital, 5F., No. 52, Lane 240, Guangfu S. Rd., Da-an District Taipei City 106, Taiwan, China. ronlin@aptg.net
Telephone: +886-2-27093600-1157 Fax: +886-2-27047859
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Abstract

AIM: To evaluate the association among hepatic fibrosis, serum iron indices, and hepatic iron stores in patients with Chronic Hepatitis C (CHC).

METHODS: Thirty-two CHC patients were included in our study. The histological degree of fibrosis and inflammation activity was assessed according to the Metavir system. The serum iron indices including ferritin, iron and transferrin saturation were measured. Hepatic iron deposition was graded by Perls' stain.

RESULTS: The CHC patients with severe hepatic fibrosis (n = 16) were significantly older than CHC patients with mild fibrosis (n = 16) (P = 0.024). The serum iron indices, increased serum iron store and positive hepatic iron stain were not significantly different between the two groups. In multivariate logistic regression analysis, the age at biopsy was an independent predictor of severe hepatic fibrosis (Odds ratio = 1.312; P = 0.035). The positive hepatic iron stain was significantly associated with the values of alanine aminotransferase (ALT) (P = 0.017), ferritin (P = 0.008), serum iron (P = 0.019) and transferrin saturation (P = 0.003). The ferritin level showed significant correlation with the value of ALT (r = 0.531; P = 0.003), iron (r = 0.467; P = 0.011) and transferrin saturation (r = 0.556; P = 0.002).

CONCLUSION: Our findings suggest that the severity of hepatitis C virus (HCV)-related liver injury is associated with patient age at biopsy. Both serum iron indices and hepatic iron deposition show correlation with serum indices of chronic liver disease but are not related to grade and stage of liver histology.

Key words: Chronic hepatitis C; Hepatitis C virus; Hepatic iron; Serum iron; Hepatic fibrosis

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INTRODUCTION

Elevations in serum iron, ferritin and transferrin saturation are common in patients with chronic hepatitis C (CHC), as are mild increases in hepatic iron concentration. It has been reported that up to 40%-46% of patients has elevated serum iron, ferritin, or transferrin saturation level[1,2]. Although the degree of iron deposition is usually mild, histological evidence of liver iron accumulation can be observed in 10%-42.1% of patients with CHC[2-4]. Increased amounts of iron in the liver may promote the progression of liver disease by adding oxygen free radicals that increase oxidative stress[5,6]. Iron overload is responsible for liver damage through the generation of reactive oxygen species leading to lipid peroxidation and alteration of the cellular membrane[7]. Therefore, iron overload may play a role in the pathogenesis of some chronic liver diseases, especially when iron is combined with other hepatotoxic factors such as virus, free fatty acid, and alcohol[8]. In addition to the production of oxidative stress, the iron may enhance the rates of viral replication and impair the host immune system[6]. Despite these observations, the exact role of iron overload in patients with CHC remains unclear.

Factors that increase the risk of progression of hepatitis C virus (HCV)-associated hepatic fibrosis include older age at infection, male sex, alcohol abuse, and concurrent viral infection, particularly with human immunodeficiency virus or hepatitis B virus[9]. The influence of viral load and genotype on the pathogenesis of liver disease is not completely resolved. Most studies have reported that HCV RNA level has no relation to the activity of liver disease[10]. There are 6 major HCV genotypes. The most types are type 1a, 1b, 2a, 2b in Taiwan and about 65% of HCV

infections are type 1b[11]. In early studies, HCV genotype 1b was found to be associated with a more severe liver disease[12]. However, the association between genotype 1b and a more severe liver disease had not been found in studies with adjustment for the confounding factors[10,13].

Whether the degree of hepatic iron deposition in patients with CHC affects the natural history of the disease remains to be determined. The aim of this study was to assess the association among hepatic fibrosis and serum iron indices, hepatic iron stores in patients with CHC. This study was also performed to assess the other potential factors related to the severity of hepatic fibrosis in these patients, including age, gender, liver enzyme tests, viral load and genotype of HCV. We had adjusted for the other confounding factors, such as alcohol abuse, obesity, and concurrent human immunodeficiency virus or hepatitis B virus infection.

MATERIALS AND METHODS

CHC patients

The patients with CHC were collected at our outpatient department since October 2003. CHC was diagnosed by alteration in liver enzymes persisting for more than 6 mo associated with positive HCV antibody. Patients with potentially secondary causes of iron overload were excluded, including alcohol abuse (ethanol consumption > 20 g/d), ribavirin therapy, and multiple transfusions. The body mass index (BMI) of the patients was not over 27 kg/m^2. Co-infection with human immunodeficiency virus or hepatitis B virus was also excluded. Serum levels of ceruloplasmin were within normal range. Serological tests for autoimmune hepatitis (anti-nuclear antibody, anti-smooth muscle antibody) and for primary biliary cirrhosis (anti-mitochondrial antibody) were negative.

Serological evaluation

The serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined by using an Olympus 5000 analyzer. The upper limit of normal for ALT is 34 IU/L. HCV antibody was detected by a commercially available enzyme-linked immunosorbent assay (AxSYM. ABBOTT Diagnostic Corporation, USA). The iron status of each patient was evaluated by biochemical tests. Serum iron (normal range, 60-160 μg/dL) was measured by the colorimetry and ferritin (normal range: 18-274 ng/mL in men and 6-283 ng/mL in women) was measured by a commercially available enzyme-linked immunosorbent assay (AxSYM. ABBOTT Diagnostic Corporation, USA). Transferrin saturation was calculated as (the serum iron divided by the TIBC) × 100%. The increased serum iron store was defined by transferrin saturation > 50% and/or ferritin > upper normal limit.

Hepatic fibrosis stage

The hepatic specimens were obtained with the SURECUT needle by ultrasonography-guided biopsy of liver. The degree of fibrosis and inflammation activity was assessed according to the Metavir system[14]. The Metavir system scores both necroinflammatory changes on a 4-point scale of 0 to 3 and fibrosis on a 5-point scale from 0 to 4.

Hepatic iron deposition

The histological assessment of hepatic iron stores was revealed by Perls' stain on liver biopsy specimens[15]. Hepatic iron deposition was graded on a scale from 0 to 4. Perls' stain is also called Prussian Blue reaction. It is used to demonstrate ferric iron and ferritin. This is not a true staining technique rather, it is a histochemical reaction. The protein is split off by the hydrochloric acid, allowing the potassium ferrocyanide to combine with the ferric iron. This forms the ferric ferrocyanide or Prussian Blue.

Viral load of HCV

The viral load of HCV was checked according to the Cobas Amplicor HCV Monitor Test, version 2.0, Roche Diagnostics. It is based on five major processes: specimen preparation; reverse transcription of the target RNA to generate complementary DNA (cDNA); polymerase chain reaction (PCR) amplification of target cDNA using HCV specific complimentary primers; hybridization of the amplified products to oligonucleotide probes specific to the targets; and detection of the probe-bound amplified products by colorimetric determination.

Viral genotype of HCV

The most viral genotypes of HCV are type 1a, 1b, 2a, 2b in Taiwan. We used the method of type-specific PCR to analyze the viral genotype of HCV. Based on variation in nucleotide sequence within restricted regions in the putative C (core) region of HCV, four groups of HCV had been illustrated[16]. They were types 1a, 1b, 2a and 2b. The method depended on the amplification of a C gene sequence by PCR using a universal primer (sense) and a mixture of four type-specific primers (antisense). HCV types were determined by the size of the products specific to each of them. The primers of first round PCR were 5'-CGAAAGGCCTTGTGGTACTG-3' and 5'-ATATACCCCATGAGGTCGGC-3'. The primers of second round PCR were sense primer 104: 5'-AGGAAGACTTCCGAGCGGTC-3' and four antisense primers. They were antisense primer 132: 5'-TGCCTTGGGGATAGGCTGAC-3', antisense primer 133: 5'-GAGCCATCCTGCCCACCCCA-3', antisense primer 134: 5'-CCAAGAGGGACGGGAACCTC-3' and antisense primer 135: 5'-ACCCTCGTTTCCGTACAGAG-3'.

Statistical analysis

Data were summarized as mean ± SD. Data were compared between groups on the basis of hepatic fibrosis stage. Categorical variables were compared with the chi-square test or Fisher's exact test as required. Continuous variables were compared between groups by using the unpaired *t*-test. The Mann-Whitney test was used when it was appropriate. Independent factors related to hepatic fibrosis severity were assessed by using multivariate logistic regression analysis. Correlations among selected variables were assessed by the Spearman correlation coefficient. The $P < 0.05$ was statistically significant.

Table 1 Demographic and laboratory data of 32 patients with CHC

Variable	Total population (n = 32)	Severe fibrosis (n = 16)	Mild fibrosis (n = 16)	P
Age (yr)	56.47 ± 10.92	60.75 ± 6.50	52.19 ± 12.85	0.024
Sex (male:female)	15 : 17	8 : 8	7 : 9	0.723
AST (IU/L)	105.47 ± 56.18	119.31 ± 55.75	91.63 ± 54.82	0.167
ALT (IU/L)	163.03 ± 102.99	167.81 ± 102.15	158.25 ± 106.95	0.798
Iron (μg/dL)	155.07 ± 43.27	149.50 ± 33.00	160.27 ± 51.71	0.513
TIBC (μg/dL)	356.97 ± 48.38	351.50 ± 41.23	362.07 ± 55.18	0.566
Transferrin saturation (%)	43.63 ± 11.69	42.51 ± 7.85	44.68 ± 14.62	0.621
Ferritin (ng/mL)	291.19 ± 213.72	329.41 ± 222.08	255.53 ± 206.72	0.362
Increased serum Iron store	14 (43.75%)	8 (50.00%)	6 (37.50%)	0.476
Positive hepatic Iron stain	4 (12.50%)	1 (6.25%)	3 (18.75%)	0.600
Viral genotype (1:2)	22 : 6	12 : 2	10 : 4	0.648
Viral load ($\times 10^6$ copies/mL)	4.94 ± 6.26	6.39 ± 8.16	3.48 ± 3.22	0.231

CHC: Chronic hepatitis C; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; TIBC: Total iron binding capacity. Data are expressed as mean ± SD or patients number (percentage). Transferrin saturation was calculated as serum iron divided by TIBC × 100%. The increased serum iron store was defined by transferrin saturation > 50% and/or ferritin > upper normal limit.

RESULTS

Thirty-two patients fulfilling inclusion criteria were studied. The demographic and laboratory data of the patients are summarized in Table 1. The mean age of the 32 patients was 56.47 ± 10.92 year-old. Fourteen patients (43.75%) had increased serum iron stores and only four patients (12.5%) had positive hepatic iron stain. In the four patients, three patients were grade one and one patient was grade two on Perls' stain. Of 32 patients, 16 patients showed severe hepatic fibrosis (stages 3 or 4) and 16 patients had mild fibrosis (stages 0, 1 or 2) on histology.

The CHC patients with severe hepatic fibrosis were significantly older than the CHC patients with mild fibrosis (60.75 ± 6.50 *vs* 52.19 ± 12.85 year-old; P = 0.024). The other variables showed in Table 1, including gender, liver enzyme tests, serum iron indices, increased serum iron store, positive hepatic iron stain, viral load and genotype of HCV, were not significantly different between patients with severe and mild hepatic fibrosis. In multivariate logistic regression analysis, the age at biopsy was still an independent predictor of severe hepatic fibrosis (Odds ratio = 1.312; P = 0.035) (Table 2).

We stratified our data according to patient sex because women may have lower serum iron markers than men. All the serum iron indices and hepatic iron stain were not associated with severe hepatic fibrosis in men and women, respectively. Univariate analysis across grades of histological inflammation activity also did not show a significant association between inflammation activity and any of the serum iron indices or the presence of hepatic tissue iron, age, gender, liver enzyme tests, viral load and genotype of HCV (data not shown).

The positive hepatic iron stain was significantly associated with the values of ALT (P = 0.017) and all the three serum iron indices, including ferritin (P = 0.008), iron (P = 0.019) and transferrin saturation (P = 0.003) (Table 3). The ferritin level had significant correlation with the value of ALT, iron and transferrin saturation in Spearman correlation test (P = 0.003, 0.011 and 0.002, respectively). Nonetheless, no significant correlation was found between ferritin and grade of inflammation activity or stage of hepatic fibrosis severity (Table 4).

Table 2 Multivariate logistic regression analysis of independent predictors of severe hepatic fibrosis

Variable	Odds ratio	95% CI	P
Age	1.312	1.020-1.688	0.035
Male gender	14.138	0.835-239.266	0.066
Increased serum iron store	0.834	0.081-8.595	0.879
Positive hepatic iron stain	1.584	0.067-37.349	0.775
Viral load	1.412	0.923-2.161	0.112

CI: Confidence interval; ALT: Alanine aminotransferase. The increased serum iron store was defined by transferrin saturation > 50% and/or ferritin > upper normal limit. The ALT and viral genotype were eliminated in multivariate backward logistic regression analysis. We forcibly add the two factors increased serum iron store and positive hepatic iron stain into the analysis.

Table 3 Univariate analysis of demographic and laboratory data in relation to hepatic iron stain

Variable	Positive hepatic iron stain (n = 4)	Negative hepatic iron stain (n = 28)	P
Age (yr)	52.50 ± 9.04	57.04 ± 11.19	0.446
Sex (male:female)	3 : 1	12 : 16	0.319
AST (IU/L)	141.50 ± 99.94	100.32 ± 47.89	0.473
ALT (IU/L)	275.00 ± 172.18	147.04 ± 82.23	0.017
Iron (μg/dL)	210.50 ± 45.11	146.20 ± 36.55	0.019
Transferrin saturation (%)	62.34 ± 10.71	40.64 ± 8.80	0.003
Ferritin (ng/mL)	591.23 ± 119.70	243.19 ± 184.65	0.008

Data are expressed as mean ± SD. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase. Transferrin saturation was calculated as (the serum iron divided by the TIBC) × 100%.

Table 4 Correlations with ferritin levels among laboratory data, hepatic inflammation and fibrosis scores

Variable	ALT	Iron	Transferrin saturation	Inflammation score	Fibrosis score
Spearman coeff	0.531	0.467	0.556	-0.085	0.317
P	0.003	0.011	0.002	0.659	0.094

ALT: Alanine aminotransferase. Transferrin saturation was calculated as (the serum iron divided by the TIBC) × 100%.

DISCUSSION

It has been recognized for more than 30 years that iron stores may be increased in alcoholic liver disease[17]. In nonalcoholic steatohepatitis, 58% of patients has elevated serum iron indices and in some cases increased hepatic iron stores[18]. In order to prevent the effect of

the potential confounding factors in hepatic iron stores, we carefully excluded patients that had alcohol abuse (ethanol consumption > 20 g/d), BMI over 27 kg/m^2, previous ribavirin therapy and multiple transfusions. The lower rate of positive hepatic iron stain (12.5%) may partly be due to the stringent selection criteria used in our study. In our previous study, we showed that the HFE mutations associated with hereditary hemochromatosis were infrequent in Taiwan and they may not contribute to iron accumulation in CHC patients even when serum iron overload was observed in more than one third of these patients[19]. Therefore, we didn't exclude the few CHC patients with HFE mutations in our study.

Although the iron-related oxidative stress may play a role in the pathogenesis of CHC, the association between serum iron markers, hepatic iron stores, and hepatic fibrosis stage remains controversial. Previous studies had evaluated the potential impact of hepatic iron store on CHC but they had produced discordant results. Three studies had found that hepatic iron tissue deposition was associated with severe hepatic fibrosis in patients with CHC[20-22]. Despite the association, they did not found a correlation between the amount of hepatic iron store and the fibrosis score. The absence of dosing effect suggests that there is a cut-off point at which all patients are more likely to have severe fibrosis, and all patients with values above this level have an equal risk regardless of the quantity of tissue iron concentration[20]. In other words, there is a threshold effect, and once present, increasing hepatic iron does not correlate with increasing fibrosis[22]. The other studies had proposed the discordant conclusions. No association was observed between the presence of hepatic iron deposition and fibrosis score in these reports[23-26]. In our study, significant iron that was detectable histologically was also unrelated to the severity of hepatic fibrosis. It is well established that a heavy iron overload per se can cause hepatic fibrosis, as observed in patients with hereditary hemochromatosis. In a semi-quantitative evaluation of hepatic iron in patients with CHC, most had minimal or mild deposits[25]. Our study had the similar results. In the four patients with positive hepatic iron stain, three patients were grade one and one patient was grade two on Perls' stain. This may be the reason why the hepatic iron stain was not associated with severe hepatic fibrosis in our study. Mild degree of hepatic iron deposition may not reach the threshold at which iron will enforce hepatic injury.

In the present study we did not find any association between serum iron indices or hepatic iron stain and degree of hepatic fibrosis or inflammation activity in patients with CHC. However, our study had showed that hepatic iron stain was associated with altered ALT values and serum iron indices. The ferritin levels also showed correlation with ALT values and the other two serum iron indices. That is, the biochemical injury of liver can be predicted by tissue or serum iron contents but the histological damage can't. This is consistent with the finding that the decline in serum AST and ALT values after phlebotomy is not associated with a change in histological activity of inflammation or fibrosis[27]. The mechanism by which iron accumulates in some patients with CHC is unclear. Whether this iron accumulation is cause or result of liver injury is unknown. Previous studies had reported a positive correlation between serum ferritin concentration and ALT level in patients with CHC[1,28]. Since serum iron index correlated significantly with the value of ALT, it was likely that the excess iron could be related to its release from destroyed hepatocytes as a result of liver injury associated with HCV. This suggested that iron parameters in patients with CHC acted either as markers of the chronic inflammatory state or cytolytic liver activity but did not directly reflect the progression of hepatic fibrosis. Furthermore, the tissue iron contents did associate with the all serum iron indices in our study. In other words, ferritin, iron and tranferrin saturation were all excellent predictors for presence of hepatic iron in patients with CHC.

Our study found that older age at biopsy was associated with severe hepatic fibrosis in patients with CHC. This suggested that hepatitis C infection may somehow become more fibrogenic with advancing host age. This is in accordance with previous studies showing that severity of HCV-related liver injury can be predicted by patient age[23,24,29]. The mechanism underlying this association is unknown. The possible explanations might include immune factors, increased fibrogenesis, or decreased fibrolysis[9]. The ability of hepatocytes to regenerate or the state of activated hepatic lipocytes alters with age and thus gives rise to increased fibrosis[29]. Nonetheless, these speculations are unproven yet. Our data allow a conclusion that CHC will place an increasing burden on health care services in the next decades as the population with CHC ages.

Our study do have a potential limitation. In two large-scale studies, age at onset of infection had been identified as predictive factor of progression in CHC[13,30]. Since the time of onset of infection derived from clinical history may not be reliable, we had omitted this variant in our study.

In conclusion, the severity of HCV-related liver injury is associated with patient age at biopsy. Significant iron deposition in the liver is uncommon in CHC patients. Both serum iron indices and hepatic iron deposition show correlation with serum indices of chronic liver disease but are not related to grade and stage of liver histology. The viral load and genotype of HCV are also not associated with hepatic fibrosis severity and inflammation activity. Our study conclusions suggest that patients with CHC should be treated as early as possible. Our findings do not support the role for iron depletion therapy by phlebotomy in patients with CHC, including those with elevated serum iron indices or positive hepatic iron stain.

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