HEPATOSTEATOSIS IS A SIGNIFICANT INDEPENDENT RISK FACTOR FOR PORTAL FIBROSIS IN HCV INFECTED PATIENTS WITH GENOTYPE 1B

HCV GENOTİP 1B İLE İNFEKTE HASTALARDA HEPATOSTEATOZ PORTAL FİBROZİS İÇİN BAĞIMSIZ BİR RİSK FAKTÖRÜDÜR

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ABSTRACT

Purpose: *We studied patients with chronic hepatitis C virus (HCV) infection for the presence of liver steatosis, and examined relationships between steatosis and host factors, viral factors, and portal fibrosis.* ***Methods:*** *Twenty subjects with confirmed chronic HCV infection were enrolled in the study. The exclusion criteria included history of alcohol abuse, serological evidence of hepatitis B virus and/or human immunodeficiency virus, interferon or immunosuppressive treatment, obesity (body mass index >32 kg/m²), concommitant liver disease, or presence of systemic disease. The host parameters studied were age, gender, body mass index, and serum levels of cholesterol, triglyceride, and ferritin. We also investigated viral factors of HCV genotype and serum HCV viral load. Liver biopsy specimens were examined and graded for steatosis and portal fibrosis. Data were analyzed according to presence or absence of steatosis, and on the basis of the various grades of fatty degeneration and fibrotic change.* ***Results:*** *Twelve of the patients were men, and the mean patient age was 46.25 ± 11.34 years (range, 20-61 years). All patients were of HCV genotype 1b. Histopathological examination revealed that five patients (20%) had no signs of fatty degeneration in the liver. All these individuals were men. The severity of steatosis was grade 1 in 6, grade 2 in 4, and grade 3 in 5 patients. Portal fibrosis was grade 1 in 10, grade 2 in 5 and grade 3 in 5 patients. Steatosis was more severe in the female subjects ($p<0.05$). Serum ferritin level was strongly correlated with grade of fibrosis. There were significant associations between steatosis and age, gender, serum ferritin level, and serum HCV viral load levels. Regression analysis and kappa statistics showed that steatosis is an independent predictor of portal fibrosis.* ***Conclusion:*** *The study indicated that steatosis, and probably also iron, play important roles in the development of portal fibrosis in chronic HCV infection.*

Key Words: *Chronic Hepatitis C Virus Infection, Steatosis, Portal Fibrosis.*

ÖZET

Amaç: *Kronik hepatit C infeksiyonlu olgularda karaciğer steatozu varlığı çalışıldı ve steatoz ile hasta faktörleri, viral faktörler ve portal fibrozis ilişkisi incelendi.* ***Metod:*** *Kronik hepatit C virüs infeksiyon varlığı verifiye edilmiş 20 hasta çalışmaya dahil edildi. Alkol kullanım öyküsü, hepatit B virüs, human immunodeficiency virus varlığı yönünden pozitif seroloji, interferon veya immunsupresif tedavi öyküsü, obesite (vücut kitle indeksi >32 kg/m2), eşlik eden karaciğer hastalığı veya sistemik bir hastalığın olması çalışmadan dışlanma kriterleridir. Hastaya ait parametreler; yaş, cinsiyet, vücut kitle indeksi, serum kolesterol, trigliserid ve ferritin düzeyleridir. Biz ayrıca hepatit C virus genotip ve heaptit C viral yük gibi viral faktörleri de inceledik. Veriler, steatoz varlığına veya yokluğuna, yağlı dejenerasyon ve fibrotik değişikliğin çeşitli derecelerine bağlı olarak değerlendirildi.* ***Sonuçlar:*** *Hastaların 12'si erkek olup, ortalama hasta yaşı 46.25 ± 11.34 (aralık, 20-61) yıldı. Bütün hastalarda heaptit C virus genotip 1b saptandı. Histopatolojik inceleme ile 5 hastada (%20) karaciğerde yağlı değişim yokdu. Bu olguların tümü erkek cinsiyetinde idi. Steatozun derecesi 6 hastada grade 1, 4 hastada grade 2, 5 hastada grade 3 idi. Portal fibroz 10 hastada grade 1, 5 hastada grade 2, 5 hastada grade 3 olarak bulundu. Steatoz kadın olgularda daha ciddi olarak saptandı ($p<0.05$). Serum ferritin düzeyi fibrozis derecesi ile anlamlı olarak korelasyon göstermekte idi. Steatoz ile yaş, cinsiyet, serum ferritin ve serum hepatit C viral yük düzeyleri arasında istatistiksel olarak ciddi bir ilişki mevcut idi. Regresyon analizi ve kappa istatistiği, steatozun portal fibrozis için bağımsız bir gösterge olduğunu ortaya koymuştur.* ***Yorum:*** *Çalışmamız; steatoz ve olasılıkla demirin kronik hepatit C virus infeksiyonu gelişiminde önemli bir rol oynadığını göstermektedir.*

Anahtar Kelimeler: *Kronik Hepatitis C Virus İnfeksiyonu, Steatoz, Portal Fibroz.*

INTRODUCTION

The pathogenesis of hepatitis C virus (HCV)-induced liver damage is still not understood. Focal distribution of lobular inflammation in association with HCV infection suggests a cytopathic effect of the virus itself (1). One of the proposed direct or indirect consequences of this effect is hepatic steatosis, which is a characteristic histopathologic finding in chronic HCV infection; however, the mechanisms of this fatty degeneration and its consequences in chronic HCV infection are not clear. According to some authors, hepatosteatosis and increased lipid peroxidation are consecutive events which ultimately promote increased collagen production by Ito cells (2, 3). There is a growing body of evidence that the lipid peroxidation links steatosis to necroinflammation and fibrosis in patients with chronic HCV infection (3, 4).

In the context of these proposed mechanisms, we sought to investigate the presence of hepatosteatosis in chronic HCV infection, to examine the host and viral factors that affect the degree of steatosis, and to clarify the link between steatosis and portal fibrosis in patients with chronic HCV.

PATIENTS AND METHODS

We studied 20 patients who had chronic HCV infection. Each enrolled subject had elevated alanine aminotransferase (ALT) levels (1.5 times upper normal) for at least 6 months, and had tested positive for anti-HCV antibody on second-generation enzyme-linked immunosorbent assay. We also confirmed HCV infection in each, based on detection of circulating HCV RNA with the polymerase chain reaction (PCR). Commercial radioimmunoassays were used to test serum for hepatitis B virus antigens, and individuals who tested positive for hepatitis B or had a clinical history of alcohol abuse were excluded. Each subject in the study also met the following exclusion criteria: having previous interferon or immunosuppressive treatment, presence of other liver disease, diabetes mellitus, obesity, chronic renal failure, and hepatocellular carcinoma. Also, none of the patients were taking medication that could affect lipid metabolism or induce steatosis.

In each patient, we calculated body mass index (BMI), recorded prothrombin time (PT), and measured serum levels of liver enzymes, albumin, cholesterol, triglyceride, and ferritin.

PCR amplification of the HCV noncoding region, and HCV genotyping:

We amplified the 5' noncoding region (NCR) of HCV RNA using nested primer sets 209/940 (outer) and 211/939 (inner). cDNA was synthesized from 10 µl of RNA, and then the first round of amplification was done with the outer set of primers using RT-PCR technique, and under the conditions recommended by the supplier (Calypso RT-PCR system, DNAmp Ltd., UK). All reactions were performed in a thermal cycler (DNA Engine, MJR, USA). The settings used were as follows: one cycle of 50 °C for 30 min, 94 °C for 2 min, followed by 30 cycles of 94 °C for 20 s, 55 °C for 35 s, and 68 °C for 1 min. Two µl of the first-round RT-PCR product was then amplified with the inner set of primers. We analyzed the second-round amplification product, which contained the 251 bp NCR, by restriction fragment length polymorphism, and performed genotyping as described by Abacou H et al (5).

Quantification of HCV RNA:

Quantification of HCV RNA was done using the AcuGen RT-Amplisensor (Biotronics Tech. Corp. Lowell, MA, USA) assay, according to the manufacturer's instructions. Each HCV run included a dilution series of 10-10,000 copies/ml of RNA transcript together with positive and negative control serum samples. We assessed the analytic sensitivity of the 5'NCR amplifications by diluting the same RNA transcript. HCV 5'NCR positive RNA samples were included in the quantity determination assays (6).

All patients underwent percutaneous liver biopsy. The specimens were fixed in formalin, embedded in paraffin, stained with hematoxylin and eosin, and stained with silver to identify reticulin fibers. The slides were all examined by the same experienced pathologist, who scored the histopathological findings of inflammation and fibrosis according to Knodell's classification. The same examiner also semiquantitatively assessed the histological findings typical of HCV hepatitis, including steatosis, bile duct damage, and lymphoid aggregates. Severity of steatosis was graded as absent, 1 (<30% of hepatocytes affected), 2 (30-70% of hepatocytes affected), or 3 (>70% of hepatocytes affected). Architectural changes reflective of the degree of portal fibrosis

were graded as absent, mild (stage 1, periportal fibrous expansion), moderate (stage 2, portal-portal septa), severe (stage 3, bridging fibrosis), and cirrhosis.

Statistical analysis: Analysis of variance was used to test differences among the mean values for the continuous variables (age; levels of cholesterol, liver enzymes, and albumin; body mass index; viral titer; PT) with patients grouped according to degree of steatosis or portal fibrosis. Differences in median values for serum ferritin levels and differences in gender distribution in the various groupings for steatosis and fibrosis were analyzed using the Kruskal-Wallis and Chi-square tests, respectively. Multiple regression analysis and correlation testing were done to identify relationships between portal fibrosis or steatosis and other parameters such as age, gender, viral load, serum ferritin, serum cholesterol, and body mass index. We also reevaluated the relationship between degree of portal fibrosis and severity of steatosis using kappa statistics.

RESULTS

Twelve of the patients were men, and the mean patient age was 46.25 ± 11.34 years (range, 20-61 years). Body mass index ranged from 15.27-31.81, with a mean of 26.20 ± 4.72. HCV genotype analysis revealed that all 20 patients were of genotype 1b. The serum HCV RNA levels ranged from $1.5x10^3$-$5.99x10^6$ copies/ml, with a logarithmic mean of 2,235,595 ± 2,113,108. The median serum ferritin level was 61.8 ng/ml, and the range was 4-702 ng/ml. Serum cholesterol levels ranged from 101 to 198 mg/dL, with a mean value of 162.3 ± 23.96 mg/dL. The mean serum triglyceride level was 115.35 ± 46.13 mg/dL, and the range was 52-210 mg/dL.

Histopathological examination revealed that five patients (20%) had no signs of fatty degeneration in the liver (Table 1). All these remaining patients (80%) exhibited typical non-zonal hepatosteatosis (Fig. 1 and 2). Severity of steatosis was recorded as grade 1 in 6 patients, grade 2 in 4 patients, and grade 3 in 5 patients. Fibrosis was grade 1 in 10 patients, grade 2 in 5 patients, and grade 3 in 5 patients. The characteristics and laboratory findings for the patients included in each grade of steatosis and portal fibrosis are listed in Table 2 and 3. None of the biopsies showed hepatocellular carcinoma. Proliferation of bile canaliculi was observed in 14 cases (70%), but only 3 of these patients had significant bile duct damage. Lymphoid aggregates and follicles in the portal tracts were

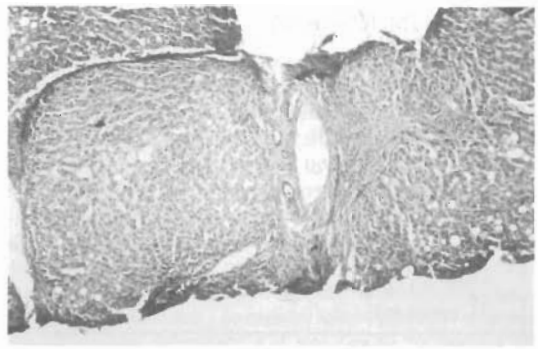

Fig. 1: This section of liver tissue shows moderate steatosis with a high degree of portal fibrosis extending into parenchyma (stained green with trichrome).

Table 1: Demographic and laboratory characteristics of the HCV-infected study group.

PATIENTS	With steatosis	Without Steatosis	p value
Age (years)	49±9.3	38±13.8	>0.05
Gender (Male/Female)	7/8	5/0	0.054
BMI (kg/m^2)	26.7±4.20	24.5±6.26	>0.05
Viral load (median in copies/ml)	34.5	84.5	>0.05
Ferritin (median in ng/ml)	1730 000	1930000	>0.05
Cholesterol (mg/dL)	162.4±26.2	161.8±17.8	>0.05
Triglyceride (mg/dL)	115.4±50.6	115.2±33.5	>0.05
ALT (U/L)	111.6±71.7	85.4±25.3	>0.05
ALP (U/L)	168.7±79.7	106.8±45.3	>0.05
GGT (median in U/L)	53	25	>0.05
Albumin (g/dL)	4.1± 0.4	4.5±0.1	<0.05
PT (s)	12.8 ± 2.1	12.5±1.09	>0.05

noted in 17 patients (85%). These were arranged either in simple aggregations or as well-formed follicles with germinal centers.

There was a statistically significant difference in the number of female and male subjects in the groups with grades 0 and 3 steatosis ($p<0.05$, Table 4). Analysis of age distribution relative to severity of steatosis showed that older individuals had higher grades of steatosis (Table 2); however, there were no significant differences in mean age when patients were grouped according to grade of steatosis. Similarly, there were no statistical differences among the various steatosis group means for BMI, prothrombin time, serum viral load or serum levels of ferritin, cholesterol, triglyceride, ALT, alkaline phosphatase (ALP), gammaglutamyl transpeptidase (GGT), or albumin. However, multiple regression analysis

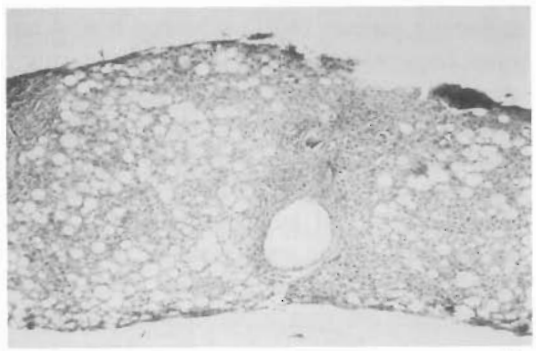

Fig. 2: Histopathological examination of a liver specimen showed severe steatosis with portal fibrosis, lymphocytic infiltration, and interlobular bile duct damage.

Table 2: The patients' demographic and laboratory parameters listed according to severity of steatosis.

	STEATOSIS				
	Absent N:5	**Grade 1 N:6**	**Grade 2 N:4**	**Grade 3 N:5**	**p value**
Age (years)	38±13.8	43.5±9	49±9.4	55.6 ±6.1	>0,05
Gender (Male/Female)	5/0	4/2	1/2	2/4	>0,05
BMI (kg/m²)	24.51±6.27	25.7±4.3	26.3±3.8	28.4±4.75	>0,05
Viral load (median in copies/ml)	1.9×10^6	1.8×10^4	434×10^3	3.6×10^5	>0,05
Ferritin (median in ng/ml)	34.5	51.6	123	188	>0,05
Cholesterol (mg/dL)	161.8±17.88	172.1±20.8	168.7±26.2	145±28.6	>0,05
Triglyceride (mg/dL)	115.2±33.51	97±42.2	127.25±58	128±58.5	>0,05
ALT (U/L)	71.4±15.4	75.3±19.9	212.5±68.7	74.4±9.9	>0,05
ALP (U/L)	106.8±45.3	128.8±64.1	187.5±90.4	202±82.8	>0,05
GGT (median in U/L)	25	26	72.5	73	>0,05
Albumin (g/dL)	4.50±0.14	4.3±0.35	4.2±0.1	3.9±0.7	>0,05
PT times (s)	12.58±1.10	12.1±0.56	12.1±0,9	14.3±3.2	>0,05

Table 3: The relationships between grade of portal fibrosis and the various demographic and laboratory parameters.

	PORTAL FIBROSIS			
	Grade 1 (n:10)	**Grade 2 (n:5)**	**Grade 3 (n:5)**	**p value**
Age (years)	42±12.9	50±6.04	51±10.2	>0,05
Gender (Male/Female)	8/2	2/3	2/3	>0,05
BMI (kg/m²)	25.3±5.2	25.6±3.6	28.3±4.7	>0,05
Viral load (median in copies/ml)	2.1×10^6	0.69×10^5	1.5×10^6	>0,05
Ferritin (median in ng/ml)	58	82	188	>0,05
Cholesterol (mg/dL)	167.1±17.3	165.6±24.2	149.4± 34.4	>0,05
Triglyceride (mg/dL)	115.4± 32.5	84.8±27.3	145.8±68.16	>0,05
ALT (U/L)	117.4±42.3	139±81.7	118.2±91.8	>0,05
ALP (U/L)	128.8±64.1	130.6±67.8	247.6±65.3	>0,05
GGT (median) (U/L)	29.5	24	73	>0,05
Albumin (g/dl)	4.4± 0.7	4.24±0.2	3.9±0.70	>0,05
PT times (s)	12.43± 0.8	12.2± 0.9	14±3.4	>0,05

Table 4: Degree of steatosis listed according to gender in the HCV-infected patients.

	STEATOSIS			
Sex	**Absent (n:5)**	**Grade 1 (n:6)**	**Grade 2 (n:3)**	**Grade 3 (n:6)**
Female	0% (n:0)	33,3 % (n:2)	50% (n:2)	80% (n:4)
Male	100% (n:5)	66,7% (n:4)	50% (n:2)	20% (n:1)
	p<0.05	p>0.05	p>0.05	p< 0.05

Appendix

Log linear model of regression analysis.

	Df	Change in likelihood ratio	P value
Fibrosis*fat	6	15.195	0.019
Fat*viral load	3	28.117	0.000
Fat*age	3	12.227	0.005
Fat*ferritin	3	31.869	0.000
Fat *gender	3	27.061	0.000

showed meaningful relations between steatosis and age, gender, serum ferritin level, and viral load (p:0.000).

We did the same statistical testing for portal fibrosis in relation to the various parameters. The results showed that the three groups with different grades of portal fibrosis were statistically similar in terms of age distribution, gender distribution, PT, BMI, viral load, and serum levels of liver enzymes, albumin, cholesterol, triglyceride, and serum ferritin. Multiple regression analysis showed that steatosis was the only parameter statistically associated with portal fibrosis (p:0.000).

Correlation analysis demonstrated that grade of steatosis, level of serum albumin, and level of ferritin were correlated with grade of fibrosis (r:0.76, r: - 0.46, and r:0. 61, respectively). Kappa statistics showed 60% (Cohen kappa value K: 0.60) agreement between grade of hepatosteatosis and grade of portal fibrosis.

DISCUSSION

We investigated patients who had no known risk factors for fatty liver apart from HCV infection. After confounding variables such as age and gender were identified and excluded based on regression analysis, our most important finding was that steatosis is independently related to portal fibrosis. The results indicate that severity of steatosis is a good predictor of severity of portal fibrosis, since kappa statistics revealed 60% agreement between severity of fibrosis and steatosis in our patients. These results are in accord with the current literature, which has reported that HCV-related hepatosteatosis is a risk factor for stellate cell activation, a process that leads to excess production of collagen type 1 and abnormal extracellular matrix protein synthesis (7).

Our work also examined various host factors that might potentially affect the presence and severity of hepatosteatosis in HCV-infected patients. The findings revealed that patients with steatosis were not more hyperlipidemic or overweight than those without steatosis. Further, we found that mean BMI was higher in patients with steatosis than in individuals without fatty liver, but the difference was not statistically significant. Other published reports have stated that BMI is a significant independent parameter determining the degree of steatosis in patients with and without HCV infection (7,8). One study demonstrated that HCV patients with high serum cholesterol and triglyceride levels were at greater risk for developing steatosis (8). The reason for the discrepancy between our results and those of other reports may be the relatively small number of HCV patients we studied. Results from a larger series of HCV-infected patients may give a more accurate picture of the importance of these parameters (BMI in particular) in steatosis development.

Regarding other factors, we found no significant difference in serum viral load in the HCV-infected patients with and without fatty

liver. This suggests that the finding of steatosis in association with HCV may not reflect the cytopathogenicity of the virus itself. However, as generally known, serum HCV levels do not correlate with liver tissue HCV RNA levels. A previous report stated that intrahepatic HCV RNA levels are strongly correlated with severity of hepatosteatosis (9). The authors claimed that these tissue HCV RNA levels reflect the quantity of HCV core protein in the liver, and that viral cytopathogenicity is the underlying cause of HCV-related steatosis. In our analysis, although the log means and median measurements of serum viral load were similar in patients with and without hepatic steatosis, regression analysis revealed an association between steatosis and viral load. The latter may be evidence that viral factors do play a role in the development of steatosis.

We were unable to test for genotype-dependent differences in steatosis development because all our patients were of HCV genotype 1b. One group of researchers reported that certain HCV genotypes, particularly type 3a, are at greater risk for developing fatty liver (10). Other resarches found that the grade of steatosis in genotype 3a was correlated with levels of intrahepatic HCV replication. However, they indicated that HCV genotype 1b is more strongly associated with BMI (11).

Serum ferritin level and free-iron concentration in the liver are reportedly higher in HCV patients than in healthy controls or individuals infected with hepatitis B virus. However, one study has indicated that hepatic iron levels and histochemically stainable iron in the liver are similar in HCV patients with and without steatosis (10). Our findings concurred, in that we found no statistical difference in the serum ferritin levels of patients with and without fatty liver. Since all five individuals with elevated serum ferritin also had high ALT levels, it is possible that elevated ferritin was the result of increased release from dying hepatocytes. However, our findings indicated that serum ferritin level is strongly correlated with grade of portal fibrosis, and is also associated with severity of steatosis. These results are in line with those of Farinati et al., who documented elevated serum ferritin concentrations in HCV patients (4). These authors also detected elevated liver iron levels, and suggested that this was an indication of the global effect of HCV on hepatocytes. In addition, they found elevated lipid peroxides in patients with HCV infection.

These researchers and others have suggested that hepatocellular mitochondrial alterations in HCV patients lead to inhibition of free fatty acid oxidation and steatosis, which has been labeled the "first hit" in the process of necroinflammation and fibrosis (12). It has already been proven that the mere presence of oxidizable fat within the liver is enough to induce lipid peroxidation (13). However, lipid peroxidation requires other factors as well (a combination which comprises the "second hit" in inflammatory and fibrotic degeneration), and some investigators believe that free iron may be part of this second phase (14). In biological environments, free iron is a source of hydroxyl radicals, which are capable of inducing oxidative stress (15). Further evidence for the role of iron in lipid peroxidation in HCV patients is that these individuals have decreased systemic glutathione (GSH) levels in addition to decreased plasma and hepatocyte GSH levels (12). Iron is known to be a cofactor in the reactions that lead to oxidization of reduced GSH (12). It has also been discovered that, in HCV patients, lipid peroxides are mainly found in CD68-positive macrophages located in the portal tracts (16) These cells stain positive for iron. After the second hit, the increased levels of free-radicals produced as a result of the depletion of systemic GSH, promote lipid peroxidation. This increases collagen gene transcription in Ito cells, the principal collagen-producing cells in the liver, and fibrosis ensues (2, 12).

Three-quarters of our patients exhibited hepatic steatosis. One case was the microvesicular form of fatty liver, and the remaining patients had the non-zonal macrovesicular form. The patient with microvesicular type fatty change exhibited severe necroinflammation and grade 3 fibrosis. As in the remainder of the patients, this individual had no known risk factors for hepatosteatosis other than HCV infection. Her liver histopathology was similar to that noted in the patients with macrovesicular steatosis. She also shared the same viral genotype and had a statistically similar viral load and serum ferritin level to those in the other patients. We were unable to determine why

this individual developed microvesicular as opposed to macrovesicular steatosis. Further, there is nothing in the literature to explain the development of these two different forms of steatosis in HCV infection. More in-depth investigation of viral and host factors may help answer this question in the future.

In conclusion, our results indicate that development of steatosis in the setting of HCV infection contributes to portal fibrosis. We also believe that serum iron kinetics contribute to the lipid peroxidation and portal fibrosis that occur in HCV infection. The role of host factors, particularly BMI, in portal fibrosis progression needs to be more clearly defined in a larger number of HCV-infected patients. In our opinion, it is important to refer to previous authors' conclusions that lean body mass and lower serum iron levels may help prevent the progression of fibrosis in these patients. Also, the pathophysiological importance of the type of steatosis (micro- versus macrovesicular) needs to be investigated in HCV-infected patients.

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