

Nonalcoholic steatohepatitis in Asian Indians is neither associated with iron overload nor with HFE gene mutations

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Abstract

AIM: The pathogenesis of occurrence of liver inflammation and fibrosis in patients with nonalcoholic steatohepatitis (NASH) is not completely understood. Other than insulin resistance, iron abnormalities have been thought to be one of the triggering factors. Therefore, our aim was to study the role of iron abnormalities and HFE gene mutations in patients with NASH.

METHODS: Thirty-one patients of NASH diagnosed on the basis of clinical examination, biochemistry, ultrasonography and liver biopsy ($n = 14$) were included in the study. Serum iron parameters ($n = 23$) (iron, ferritin, total iron-binding capacity and transferrin saturation), Perls' iron staining on liver biopsies ($n = 14$) and HFE gene mutations (C282Y and H63D) ($n = 16$) were studied in these patients. The association between iron staining, necroinflammatory activity and fibrosis stage on liver biopsies was also determined.

RESULTS: Elevated serum iron, ferritin and transferrin saturation above 55% were observed in 4.3% of patients. On histology, 71% of the patients had negative iron staining, 21.4% had 1+ staining, 7.2% had 2+ staining and none had 3+ or 4+ staining. There was no association between the degree of iron staining and necroinflammatory activity ($P = 0.55$) and fibrosis stage ($P = 0.09$) on histology. None of the patients had C282Y HFE gene mutation and four patients (25%) were found to be heterozygotes for H63D gene mutation.

CONCLUSION: Our study does not favor iron overload and HFE gene mutations as major factors in the pathogenesis of NASH in Asian Indians.

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Key words: Nonalcoholic steatohepatitis; Iron overload; HFE gene; Mutation

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INTRODUCTION

Non-alcoholic steatohepatitis (NASH) is a clinicopathological entity that occurs in non alcoholics but histologically simulates alcoholic hepatitis^[1]. Mechanisms leading to inflammation, fibrosis, and cirrhosis from a relatively benign stage of only steatosis are poorly understood^[2]. For this purpose, NASH is considered a two-hit process^[3]. Initial hit leads to excessive fat accumulation in the liver parenchyma and the second hit initiated by an oxidative stress may lead to lipid peroxidation, thus damaging the cells and producing a variety of proinflammatory and fibrogenic byproducts^[2,3]. This second hit may be initiated by various factors including insulin resistance and hepatic iron with or without HFE gene mutation of hemochromatosis^[2,4].

The prevalence of HFE gene mutations and iron abnormalities in NASH patients have been linked to the ethnicity^[5]. There is no study from India^[6,7] on the role of iron abnormalities and HFE gene mutations in patients with NASH. We retrospectively studied 31 patients of NASH and analyzed the serum and liver iron abnormalities in them and also determined the mutations in the HFE gene.

MATERIALS AND METHODS

Thirty-one patients with NASH attending liver clinic of our Institute over a period of two years (April 2001- March 2003) were diagnosed on the basis of history, clinical examination, serum biochemistry, ultrasonography, upper gastrointestinal (GI) endoscopy, and were included in the study. Since liver biopsy in patients with NASH is controversial, it was carried out only in 14 patients who failed to respond to a treatment protocol. All patients gave an informed consent and the Institute's Ethics Committee approved the study. None of the patients consumed more than 20 g/d of alcohol, which was confirmed by at least two family members or relatives. All these patients had over two times elevated transaminases for more than six mo and had a hyperechoic liver on ultrasound examination. They were all negative for hepatitis B virus surface antigen (HBsAg-Monolisa Ag HBs plus, Biorad), antibodies to hepatitis C virus (anti HCV -3rd generation, LG HCD 3.0) and autoimmune markers including anti-nuclear antibody (ANA), anti-smooth muscle antibody (ASMA), anti-liver kidney microsomal antibody (LKM1), antimitochondrial antibody (AMA). They all had normal serum ceruloplasmin levels and no evidence of Kayser-Fleischer rings on slit lamp examination.

Overnight fasting samples were collected in iron free tubes from 23 patients to perform serum iron, total iron-binding capacity (TIBC) and transferrin saturation (TS)^[8]. Serum ferritin was performed using ELISA kits (Orgentec, USA). Normal values of serum iron parameters are mentioned in Table 1. For studying HFE gene mutations (C282Y and H63D), genomic DNA was extracted from peripheral blood leucocytes of 16 patients and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed by using the primers as described by Feder *et al*^[9]. Grading, staging and Perls Prussian blue staining for iron were done on liver biopsy specimens from 14 patients as described by Brunt *et al*^[10].

All patients underwent anthropometric measurements to calculate the body mass index (BMI) (body weight in Kg/height

in meter²) while waist hip ratio (W/H) for the central obesity was determined in 13 patients. Overweight, obesity, and abnormal waist hip ratio were defined as per the international criteria^[11,12] (Table 1). A fasting plasma glucose ≥ 126 mg/dL on more than one occasion or a random plasma glucose of more than 200 mg/dL in a symptomatic patient or a two-hour plasma glucose more than 200 mg/dL on glucose tolerance test (GTT) was defined as diabetes^[13]. Impaired glucose tolerance (2 h plasma glucose after 75 g of oral glucose between 140-200 mg/dL) and impaired fasting glucose (fasting plasma glucose between 110-126 mg/dL) were also defined^[13]. Dose and duration of drugs were recorded in patients who were known diabetics for a long duration. Lipid profiles including serum cholesterol, high-density lipoprotein (HDL), low-density-lipoprotein (LDL) and serum triglycerides were done in all patients (Table 1).

Table 1 Anthropometric, biochemical, serum iron parameters, liver iron staining and HFE gene mutations in 31 patients with NASH

Parameter (n=31)	Reference value	Abnormal-n (%)
1 BMI	<25 kg/m ²	23 (74.2)
I Overweight	25-29	20 (64.5)
II Obesity	≥ 30	3 (9.7)
2 W-H ratio (n = 13)		12 (92)
I. Males (n = 10)	<0.9	10 (100)
II. Females (n = 3)	<0.85	2 (67)
3 S cholesterol	130-200 mg/dL	13 (41.9)
4 HDL	35-60 mg/dL	8 (25.8)
5 LDL	<130 mg/dL	13 (41.9)
6 S Triglycerides	30-200 mg/dL	10 (32.3)
7 Diabetes		3 (9.7)
8 Impaired glucose tolerance		7 (22.6)
Serum iron parameters (n = 23)		
9 Serum iron	65-170 μ g/dL	1 (4.3)
10 Transferrin saturation	<55%	1 (4.3)
11 Serum Ferritin	20-250 ng/mL	1 (4.3)
Perls Prussian blue staining on liver biopsy (n = 14)		
12 Iron staining positivity	0	10 (71.4)
	1+	3 (21.4)
	2+	1 (7.2)
	3+	0
	4+	0
13 HFE gene mutations (n = 16)		
I. Normal/normal		12 (75)
II. C282Y/C282Y		0
III. C282Y/H63D		0
IV. H63D/H63D		0
V. H63D/Normal		4 (25)

Statistical analysis

Chi square test was used to determine the association between the grade of iron staining and necroinflammatory grade and the stage of fibrosis on liver biopsies. $P < 0.05$ was considered statistically significant.

RESULTS

There were 31 patients (M-25, F-6) with a mean age of 38.4 years (Range 19-70 years). Their clinical presentations included asymptomatic transaminases detected incidentally either during work up for other non-hepatic diseases (17 patients), generalized weakness and body aches (8 patients), and non-specific upper abdominal discomfort (6 patients). The rest of liver function tests were normal and upper GI endoscopy did not show varices in any patient. Results of the lipid profile, presence of diabetes and impaired glucose tolerance (IGT) are shown in Table 1. Abnormalities found in serum iron studies and on Perls Prussian blue staining of liver biopsies are also shown in Table 1. None of the 16 patients studied for HFE gene mutations revealed C282Y

mutations, while four (25%) patients were found to be heterozygotes for H63D mutations. The relation between the grading, staging, and iron positivity on Perls staining of liver biopsies is shown in Table 2. There was no association between the iron staining and degree of inflammation ($P = 0.55$) and fibrosis stage ($P = 0.09$).

Table 2 Grade of iron staining, necroinflammatory grade and stage of fibrosis in liver biopsies of 14 patients with NASH

Iron grade	Necroinflammatory grade	Fibrosis stage
0	Mild	2
1	Mild	3
0	Mild	3
0	Mild	2
0	Moderate	2
0	Mild	2
0	Mild	2
0	Mild	2
1	Mild	3
0	Mild	0
2	Mild	1
0	Mild	1
0	Mild	2
1	Moderate	2

No association between iron grade and necroinflammatory grade ($P = 0.55$) and stage of fibrosis ($P = 0.09$).

DISCUSSION

We did not find significant abnormalities of serum and liver iron in our patients with NASH. Only one patient had a high serum iron level, one having a high ferritin level and one having a high TS level above 55%, but the values were not high enough to either suggest hereditary hemochromatosis (HHC) or an iron overload state. Though we did not measure the hepatic iron concentration (HIC) and did not calculate the hepatic iron index (HII = $HIC \div \text{age}$), no significant positivity for Perls stain was found on liver histology (Table 1). Moreover, Perls staining on liver biopsies had no association with the degree of necroinflammatory activity (grade) and fibrosis (stage) (Table 2).

The two-hit hypothesis^[3] in the pathogenesis of NASH includes deposition of excessive fat in hepatocytes (first hit) and development of oxidative stress and lipid peroxidation (second hit). In addition to other factors like insulin resistance,^[14] oxidative stress may be initiated by excessive hepatic iron. With the saturation of mitochondrial β -oxidation by excess free fatty acids in the liver, peroxisomal β -oxidation generates hydrogen peroxide. In the presence of excess iron, hydrogen peroxide is converted to hydroxyl radicals, thus leading to oxidative stress and release of various proinflammatory and fibrogenic products^[15]. There are studies both favoring and refuting the role of iron in the pathogenesis of NASH^[4,16]. Fifty-eight percent patients in a series by Bacon *et al*^[17] had elevated iron indices, while some patients had stainable iron in their liver biopsy specimens and an elevated hepatic iron concentration. Another study^[18] showed the presence of hyperferritinemia with normal transferrin saturation in patients with NASH and suggested that a simultaneous disorder of iron and glucose and/or lipid metabolism in most cases associated with insulin resistance was responsible for persistent hyperferritinemia. On the other hand, Younossi *et al*^[19] studied 15 patients with NAFLD including NASH and found that all patients had a normal HII and there was no correlation between the degree of iron accumulation and different degrees of clinically aggressive forms of NAFLD and NASH. The relationship between the iron overload and NASH was further complicated by not finding changes of NASH in patients who were homozygotes for hereditary hemochromatosis

and had massive iron accumulation^[20]. Iron abnormalities in patients with NASH may occur as a secondary phenomenon to insulin resistance syndrome and non-hereditary hemochromatosis iron overload may be one of the components of insulin resistance syndrome or as suggested by Mendler *et al*^[21] the “insulin resistance-associated hepatic iron overload” syndrome.

Earlier studies have shown the prevalence of homozygosity and heterozygosity for either of the C282Y or H63D mutations to be significantly higher (69%) in patients with NASH than controls (40%)^[22]. In one study, the presence of C282Y mutation in patients with NASH was significantly associated with the degree of iron staining, HIC and the degree of fibrosis on liver biopsy specimens^[16]. Since the C282Y HFE gene mutation was observed almost exclusively among Caucasian subjects of Northern European (Anglo-Celtic) ancestry, its influence on the progression of disease was dependent on the population studied. H63D gene mutation on the other hand is more widely distributed but has a minor role in the pathogenesis of iron overload. As expected, none of our patients was heterozygotes for the C282Y gene mutation and the finding that four patients were heterozygotes for H63D mutation might not be significant. Chitturi *et al*^[5] addressed this issue of ethnicity in studying the iron abnormalities and HFE mutation in patients with NASH. They found that the frequency of C282Y heterozygosity was increased only in Anglo-Celtic patients with NASH compared to ethnic blood donors. There were no C282Y homozygotes in NASH group and the frequency of compound C282Y/H63D and H63D heterozygotes were not increased in NASH. In addition, they found that hepatic iron was not a factor linked to the hepatic fibrogenesis in patients with NASH^[5]. We studied 58 normal controls and 154 individuals with β thalassemia trait and found that no subject was either heterozygous or homozygous for the C282Y mutation in the past few years^[23]. However, the prevalence of H63D mutation was 16.5% in these 212 subjects, with an allele frequency of 8.73% with two individuals being homozygous for the mutation. Even in patients with hereditary hemochromatosis, both of these mutations were negative in eight patients and one patient was heterozygous for H63D mutation^[23] suggesting that ethnic background has influences on the frequency of C282Y HFE gene mutation and H63D mutation can be found in normal individuals and non-liver disease patients.

It seems that iron contributes to the pathogenesis of NASH only in individuals with excess serum and hepatic iron. In patients with NASH, who had no serum and hepatic iron abnormalities, other factors might play a part in the pathogenesis of NASH. Since C282Y HFE gene mutation has been found almost exclusively in Caucasians of North-European descent, it is not an important contributor in patients with NASH in other populations including Asian Indians. On the other hand, H63D HFE gene mutation is less specific and its presence in patients with NASH may not be significant.

In conclusion, our study does not favor iron overload and HFE gene mutations as major factors in the pathogenesis of NASH in Asian Indians, but would require further studies to confirm the findings.

REFERENCES

- Ludwig J, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 1980; **55**: 434-438
- Chitturi S, Farrell GC. Etiopathogenesis of nonalcoholic steatohepatitis. *Semin Liver Dis* 2001; **21**: 27-41
- Day CP, James OF. Steatohepatitis: a tale of two “hits”? (editorial). *Gastroenterology* 1998; **114**: 842-845
- Blendis L, Oren R, Halpern Z. NASH: Can we iron out the pathogenesis? *Gastroenterology* 2000; **118**: 981-983
- Chitturi S, Weltman M, Farrell GC, McDonald D, Kench J, Liddle C, Samarasinghe D, Lin R, Abeygunasekera S, George J. HFE mutations, hepatic iron, and fibrosis: ethnic specific association of NASH with C282Y but not with fibrotic severity. *Hepatology* 2002; **36**: 142-149
- Amarapurkar DN, Amarapurkar AD. Nonalcoholic steatohepatitis: clinicopathological profile. *J Assoc Physicians India* 2000; **48**: 311-313
- Agarwal SR, Malhotra V, Sakhuja P, Sarin SK. Clinical, biochemical and histological profile of nonalcoholic steatohepatitis. *Indian J Gastroenterol* 2001; **20**: 183-186
- Dacie JV, Lewis SM, Hoffbrand AV. Investigation of megaloblastic and iron deficiency anemias. In Dacie JV, Lewis SM (eds): *Practical Hematology* Churchill Livingstone: Singapore 1984: 313-336
- Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, Dormishian F, Domingo R, Ellis MC, Fullan A, Hinton LM, Jones NL, Kimmel BE, Kronmal GS, Lauer P, Lee VK, Loeb DB, Mapa FA, McClelland E, Meyer NC, Mintier GA, Moeller N, Moore T, Morikang E, Prass CE, Quintana L, Starnes SM, Schatzman RC, Brunke KJ, Drayna DT, Risch NJ, Bacon BR, Wolff RK. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nat Genet* 1996; **13**: 399-408
- Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol* 1999; **94**: 2467-2474
- Willett WC, Dietz WH, Colditz GA. Guidelines for healthy weight. *N Engl J Med* 1999; **341**: 427-434
- World Health Organisation. Definition, diagnosis and classification of diabetes mellitus and its complications-Part 1: Diagnosis and classification of diabetes mellitus. Geneva: *World Health Organisation* 1999: 31-33
- Diabetes care: report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 2002; **25**: S5-S20
- Chitturi S, Abeygunasekera S, Farrell GC, Holmes-Walker J, Hui JM, Fung C, Karim R, Lin R, Samarasinghe D, Liddle C, Weltman M, George J. NASH and insulin resistance: Insulin hypersecretion and specific association with the insulin resistance syndrome. *Hepatology* 2002; **35**: 373-379
- James OF, Day CP. Non-alcoholic steatohepatitis (NASH): a disease of emerging identity and importance. *J Hepatol* 1998; **29**: 495-501
- George DK, Goldwurm S, MacDonald GA, Cowley LL, Walker NI, Ward PJ, Jazwinska EC, Powell LW. Increased hepatic iron concentration in nonalcoholic steatohepatitis is associated with increased fibrosis. *Gastroenterology* 1998; **114**: 311-318
- Bacon BR, Farahvash MJ, Janney CG, Neuschwander-Tetri BA. Nonalcoholic steatohepatitis: an expanded clinical entity. *Gastroenterology* 1994; **107**: 1103-1109
- Fargion S, Mattioli M, Fracanzani AL, Sampietro M, Tavazzi D, Fociani P, Taioli E, Valenti L, Fiorelli G. Hyperferritinemia, iron overload, and multiple metabolic alterations identify patients at risk for nonalcoholic steatohepatitis. *Am J Gastroenterol* 2001; **96**: 2448-2455
- Younossi ZM, Gramlich T, Bacon BR, Matteoni CA, Boparai N, O'Neill R, McCullough AJ. Hepatic iron and nonalcoholic fatty liver disease. *Hepatology* 1999; **30**: 847-850
- Bacon BR. Hemochromatosis: diagnosis and management. *Gastroenterology* 2001; **120**: 718-725
- Mendler MH, Turlin B, Moirand R, Jouanolle AM, Sapey T, Guyader D, Le Gall JY, Brissot P, David V, Deugnier Y. Insulin resistance-associated hepatic iron overload. *Gastroenterology* 1999; **117**: 1155-1163
- Bonkovsky HL, Jawaid Q, Tortorelli K, LeClair P, Cobb J, Lambrecht RW, Banner BF. Non-alcoholic steatohepatitis and iron: increased prevalence of mutations of the HFE gene in non-alcoholic steatohepatitis. *J Hepatol* 1999; **31**: 421-429
- Garewal G, Das R, Kaur J, Chawla Y. H63D mutation of the HFE gene in beta thalassemia traits does not cause iron overload and the hereditary haemochromatosis in North India is of the non-HFE gene type. *Blood* 2002; **100**: 46