Biopsy Proven Fibrosis in Non-Alcoholic Fatty Liver Disease: An Analysis of Risk Factors

Kaumudee Pattnaik^{*}, Pallavi Bhuyan^{*}, Ayaskant Singh[†], Shivaram P. Singh[‡], Preetam Nath[§], Sanjib Kar[¶],
Bijay Misra^{**}, Jayashree Rath^{††}

^{*}Department of Pathology, SCB Medical College, Cuttack, Odisha, India, [†]Department of Gastroenterology, IMs and SUM Hospital, Bhubaneswar, Odisha, India, [‡]Department of Gastroenterology, SCB Medical College, Cuttack, Odisha, India, [§]Department of Gastroenterology, KIMs, Bhubaneswar, India, [¶]Gastro Liver Care, Cuttack, India, ^{**}Institute of Gastro and Kidney Care Hospital, Bhubaneswar, India and ^{††}Department of Pathology, KIMs, Bhubaneswar, India

Background: Non-alcoholic fatty liver disease (NAFLD) is emerging as an important cause of liver disease in India. NAFLD is characterized by hepatic steatosis in absence of a significant alcohol use or other known liver disease. Non-alcoholic steatohepatitis (NASH) is a progressive form of NAFLD which deserves particular attention because it is more prone for development of fibrosis. Liver biopsy is the gold standard for diagnosis of NASH by evaluating necroinflammatory activity and stages of fibrosis. The aim of the study was to analyze liver biopsy specimens and identify risk factors associated with fibrosis in patients of NAFLD in eastern coastal India. **Methods:** A total of 216 subjects with fatty liver in ultrasonography (USG) were selected for needle biopsy. Those NAFLD cases showing fibrosis in biopsy were analyzed for risk factors association. **Results:** Definite NASH was diagnosed in 50 (23.14%), borderline NASH in 66 (30.55%) and not NASH in 100 (46.39%) of cases. Those patients with fibrosis (22%) were taken as cases and those without fibrosis (78%) were taken as controls for risk factor analysis. Age > 40 [odds ratio (OR) 2.01 (1.09–4.04)], female gender [OR 2.74 (1.24–6.05)], body mass index (BMI) > 23 [OR 15.36 (4.59–51.37)] and moderate fatty change in USG [OR 1.89 (1.01–3.62)] were observed as risk factors for progression to fibrosis in NAFLD cases. **Conclusion:** Older age, females, obesity and moderate fatty liver on USG are risk factors for development of fibrosis in patients with NAFLD. Patients with these risk factors should be selected for liver biopsy and to be kept for close follow-up. (J CLIN EXP HEPATOL 2018;8:367–374)

Non-alcoholic fatty liver disease (NAFLD) is an entity similar to alcoholic-like histologic liver disease but observed in patients without excessive alcohol intake < 20 g/week or absolutely no history of alcohol intake. NAFLD (steatosis of liver) is highly prevalent in western countries. With the introduction of westernized lifestyle and the increasing frequency of obesity in the Asia-Pacific region, the prevalence of NAFLD has increased over the past two decades. Studies from different regions of India have shown that NAFLD is very common in Indians.^{1–3} NAFLD is a spectrum that ranges from simple benign steatosis to steatohepatitis (NASH) and cirrhosis and may progress to end stage liver

disease or hepatocellular carcinoma.^{4,5} Once NASH develops the disease process is no more stable as with increasing fibrosis, it progresses to cirrhosis in 20% of cases.⁶ Metabolic syndrome with insulin resistance have been identified to play vital role in development of NAFLD and its progression. NAFLD, as conveniently recognized, a metabolic disorder, was largely confined to residents of affluent industrialized Western countries. In few decades, obesity and insulin resistance are not restricted to the West as witnessed by their increasing universal distribution. In particular, there has been upsurge in metabolic syndrome in the Asia-Pacific region, although there are critical difference in the extent of adiposity between Eastern and Western population. India has largest number of people with diabetes in the world.⁷ Moreover, Asian- Indians are more prone to insulin resistance and have increased visceral fat. Hence, NAFLD is soon going to emerge as the commonest liver ailment in India as in the West. But there is very limited published data on NAFLD and risk factors associated with its severe forms on liver histology from the developing world, especially India. Ultrasound is unable to diagnose steatosis in the liver when this is less than 20–30% on liver biopsy. Unfortunately there is no good non-invasive markers which separates steatosis from steatohepatitis. Some markers of liver fibrosis and imaging techniques aimed at measuring liver stiffness are under investigation as tools to determine severity of liver fibrosis

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Address for correspondence: Kaumudee Pattnaik, Assistant Prof Pathology, SCB Medical College, Cuttack, India.

E-mail: scb_gastro_dept@hotmail.com

Abbreviations: ALK: alkaline phosphatase; ALT: alanine transferase; AST: aspartate transferase; BMI: body mass index; CI: confidence interval; FBS: fasting blood sugar; H&E: Hematoxylin & Eosin; HDL: high density cholesterol; IR: insulin resistance; LDL: low density cholesterol; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; NAS: NAFLD Activity Score; OR: odds ratio; PPBS: post-prandial blood sugar; Tc: total cholesterol; Tg: triglyceride; USG: ultrasonography
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in patients who have NAFLD, but none of them yet can replace liver biopsy.^{8,9} The documentation of NAFLD in liver biopsy is characterized by macrovesicular steatosis in at least 5% of hepatocytes. Moreover, the only method to reach at a diagnosis of NASH is biopsy which can also depict fibrosis if it is evident. Only a single study showed 14.7% prevalence of NAFLD by liver biopsy without any highlights on disease progression.¹⁰ Amarpurkar et al. have noticed a lack in data about impact of NAFLD causing serious significant chronic liver disease in the form of fibrosis and raised emphasis on a prospective cooperative study with risk factor association.⁵ The metabolic syndrome related liver disease needs awareness in the community of eastern India to know the significant factors for disease burden, hence we have carried out a case-control study on fibrosis of NAFLD origin all being proved by biopsy. Three issues require consideration by liver biopsy in cases of ultrasonographically diagnosed fatty liver: (1) the frequency of NASH, i.e. the progressive form of disease and (2) the frequency of advanced fibrosis in NAFLD (3) to identify the strongly associated risk factors in fibrosis of NAFLD origin so that early intervention may prevent further progression of the disease process.

MATERIAL AND METHODS

It was a case control study done on consecutive patients of NAFLD from eastern coastal region of India. They were enrolled after obtaining informed consent for liver biopsy between the period from January 2011 to January 2017. The study was approved in institutional ethics committee. Subjects were excluded if they showed evidence of known liver disease [viral hepatitis, autoimmune disease and Wilson's disease], hepatotoxic drug intake or known alcohol consumption (>20 g/day). Viral markers including hepatitis B virus surface antigen (HBsAg), antibodies to hepatitis C virus (anti-HCV, third generation), and autoimmune markers (antinuclear antibody [ANA], anti-smooth muscle antibody [ASMA], anti-liver kidney microsomal antibody [LKM], antimitochondrial antibody [AMA]) were negative in all patients. They also had normal serum ceruloplasmin levels. Liver function test including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) along with fasting blood sugar (FBS), 2 h post-prandial blood sugar (PPBS), serum insulin with calculated insulin resistance ($\text{FBS (mg/dl)} \times \text{fasting insulin } (\mu\text{ unit/ml})/405$), lipid profile including total cholesterol (Tc)/high density cholesterol (HDL) ratio were measured enzymatically.

Trans-abdominal ultrasonographic grading of fatty liver was defined according to the standard criteria accepted by the American Gastroenterology Association,¹¹ i.e.

- Grade 1 (Mild): An increase in hepatic echogenicity with renal echogenicity as a reference.

- Grade 2 (Moderate): The presence of enhancement and lack of differentiation in periportal intensity and the vascular wall due to great hyperechogenicity of the parenchyma.
- Grade 3 (Severe): When the echogenic liver obscures the diaphragmatic outline.

All of them were subjected to liver biopsy after obtaining informed consent. Liver biopsy was done using a true cut needle (liver biopsy gun 16 Fr BARD) through the intercostal approach.

Anthropometric measurements were noted and body mass index (BMI) was calculated. Any co morbid condition like hypertension, diabetes and dyslipidemia were recorded in the register. Needle biopsy tissue of around 2 cm length was formalin-fixed and paraffin embedded. Serial sections were stained Hematoxylin and Eosin (H&E) and Masson trichome stains. Liver tissue containing at least 11 portal tracts were selected for evaluation. The major features were macrovesicular steatosis, ballooning degeneration, lobular inflammation and fibrosis. Zone 3 steatosis was looked for. Ballooning degeneration was identified by scattered or cluster (patch) of slight to moderate enlarged hepatocytes with characteristic flocculent or irregularly clumped reticulated appearance of cytoplasm and rounding of margins (normal hepatocytes are cuboidal to polygonal in shape). Inflammation is prominent in lobules than in zone 3 or portal area and this lobular inflammation is characterized by lymphocytes, but may include neutrophils, eosinophils and/or activated Kupffer cells. Based on the original histopathologic criteria (necroinflammatory grading) of Brunt et al., each case was categorized as Mild (Grade 1), Moderate (Grade 2) or Severe (Grade 3) NASH.¹² Subsequently for clinical evaluation and follow up, the severity of the disease was assessed as per NAFLD Activity Score (NAS) taking into the account of constellation of the above major features.^{13,14} NAS is the summation of semiquantitative evaluation of steatosis, ballooning degeneration, lobular inflammation and fibrosis. NAFLD cases were histologically categorized as: (I) not NASH; (II) borderline or possible NASH, and (III) definite or diagnostic of NASH. NAS with more than equal to 5 was correlated with diagnosis of NASH and scores of less than 3 were diagnosed as 'not NASH'.

Data were analyzed by using statistical package SPSS version 17.0. Fibrosis was treated as a categorical variable either as absent/mild (F0/F1) or portal/bridging (F2/F3). The results were expressed as median (range) for continuous variables and as frequencies (proportions) for categorical variables. Factors suspected to influence the severity of fibrosis were tested in univariate analysis. 95% confidence interval (CI) and *P* value of <0.05 were considered as statistically significant.

RESULT

Biopsy and clinical data from 216 adults with ultrasonographically fatty liver were evaluated. Out of 216 patients,

Table 1 Baseline Characteristics of Biopsy Proven NAFLD Patients.

Variables	Number of cases	Percentage
Obese BMI > 23	128/216	59.26
Mod. fatty change	78/216	36.11
Transaminitis	163/216	75.46
Dyslipidemia	200/216	92.59
Diabetes mellitus	49/216	22.69
Metabolic syndrome	96/216	44.44
Hypertension	43/216	19.91
Insulin resistance	28/52	53.85
USG fatty liver grade		
Mild	138	63.9
Moderate	72	33.3
Severe	6	2.8

183 were male and 33 female. On evaluation of metabolic profile, two-third (59%) patients were obese (BMI > 23), 75% of them had transaminitis, 93% had dyslipidemia, 23% were diabetic and 20% were hypertensive (Table 1). In spite of these metabolic derangements, only 36% had moderate fatty change on ultrasonography (USG) rest were mild. Metabolic syndrome was present in only 45% of patients.

Histopathology of NAFLD patients revealed mild steatosis in 96 (44.4%) cases, moderate steatosis in 101 (46.8%) and severe steatosis in 19 (8.8%) cases (Figure 1). Necroinflammatory activity as measured by lobular inflammation (score 1, 2 and 3) was seen in 83 (38.4%) patients and ballooning degeneration (score 1, 2) was seen in 138 (63.9%) patients (Figure 2). Fatty change alone (not NASH) was noted in 27.3% cases, borderline NASH in 36.6%, while 36.1% had definite NASH on liver biopsy of NAFLD patients (Table 2). Fibrosis (stage 1 and 2) was noted in 21.8% of cases and advanced fibrosis (stage 3) was seen in only one patient (Figure 3).

Out of 79 cases, 10 cases of borderline NASH and out of 78 cases, 38 cases of definite NASH showed evidence of

Figure 1 Different grades of steatosis (Hematoxylin & Eosin stain).

Figure 2 Ballooned hepatocytes (A); lobular inflammation (B) (Hematoxylin & Eosin stain).

Table 2 Histopathological Grading (Necroinflammatory Scoring) of NAFLD Patients.

Necroinflammatory grading of Brunt et al.	NAFLD Activity Score (NAS) of Kleiner et al.	No. of cases (%)
Mild, Grade 1	Not NASH (score 1, 2)	59 (27.3%)
Moderate, Grade 2	Borderline NASH (score 3, 4)	79 (36.6%)
Severe, Grade 3	NASH (score 5–8)	78 (36.1%)
	NAS (NAFLD Activity Score)	3.5 ± 1.7

fibrosis. All those borderline cases were in stage 1a fibrosis. The breakup of 38 definite NASH cases revealed that 31 cases were in stage 1, 6 cases in stage 2 and a single case in stage 3 fibrosis. No cirrhotic stage was observed. Out of those cases in stage 1 fibrosis, majority was in 1a and 1b and surprisingly there was no case was found in stage 1c, i.e. portal inflammation.

Those patients with fibrosis (22%) were taken as group A and those without fibrosis (78%) were taken as group B for risk factor analysis. As shown in Table 3, factors associated with fibrosis were higher age, i.e. age > 40 years [CI 1.09–4.04, OR 2.11, $P = 0.02$], female gender [CI 1.24–6.05, OR 2.74, $P = 0.01$], high BMI > 23 [CI 4.59–51.37, OR 15.36, $P < 0.001$] and moderate fatty change on USG

Figure 3 Different stages of fibrosis (Masson trichome stain).

Table 3 Association of Anthropometric and Biochemical Parameters with Fibrosis (n = 48).

Variables	OR	95% CI	P value
Female	2.74	1.24–6.05	0.01
Age > 40	2.11	1.09–4.04	0.02
BMI > 23	15.36	4.59–51.37	<0.001
Moderate fatty change in ultrasonography	1.89	1.01–3.62	0.05
Transaminitis	1.30	0.60–2.84	0.49
Diabetes	1.41	0.66–3.02	0.36
AST/ALT > 1	1.85	0.90–3.81	0.09
Dyslipidemia	0.34	0.26–2.75	0.78
Metabolic syndrome	1.48	0.78–2.82	0.22
Chol > 200 mg/dl	0.72	0.34–1.51	0.38
Tg > 150	1.66	0.80–3.44	0.16
HDL < 40	0.94	0.49–1.79	0.85
LDL > 100	0.55	0.28–1.07	0.08
Hypertension	0.90	0.40–2.05	0.81
TC/HDL	0.50	0.24–1.06	0.07
IR	1.29	0.41–4.03	0.65

IR = insulin resistance.

Table 4 Correlation of Histological Severity with Fibrosis.

Histologic variables	With fibrosis N = 48 (%)	Without fibrosis N = 168 (%)	Correlation coefficient with fibrosis	P value
Steatosis	44 (91.67)	77 (45.83)	0.450	<0.001
Lobular inflammation	47 (97.92)	129 (76.79)	0.295	<0.001
Ballooning degeneration	41 (85.42)	97 (57.74)	0.268	<0.001
NAS	5.08 ± 1.23	3.35 ± 1.48	0.455	<0.001

NAS = NAFLD Activity Score.

[CI 0.98–3.62, OR 1.89, $P = 0.05$]. Univariate analysis of AST, ALT, AST/ALT > 1, FBS, PGBS, triglyceride, cholesterol, LDL, Tc/HDL > 3.5, hypertension, and insulin resistance did not reach statistically significant value with fibrosis as compared to cases without fibrosis.

Table 4 shows that development of fibrosis depends on the magnitude of steatosis and necroinflammation and ballooning degeneration. The higher the NAS the greater is the chance for developing fibrosis.

DISCUSSION

Fatty change alone (Not NASH) was noted in 27.3% cases, borderline NASH in 36.6%, while 36.6% had definite NASH on liver biopsy of NAFLD patients. Fibrosis (stage 1–3) was noted in only 21.8% of cases. 168 cases (77.78%) did not show fibrosis (stage 0). All the ‘not NASH’ categories were in stage 0. None of our patients had cirrhosis on the index biopsy and out of all fibrosis cases, one patient had advanced (stage 3) fibrosis. That single case of stage 3 fibrosis was considered as advanced fibrosis, was kept in close follow up for treatment planning and a paired liver biopsy to evaluate its response to treatment. That particular case was a 45 years female with high BMI of 30 kg/m² and had NAS 7/8 necroinflammatory scoring. She was clinically diagnosed with metabolic syndrome and had a high value of insulin resistance of 17 (normal cut off being 2.5). However most (>90%) of the NAFLD cases had minimal or no fibrosis on presentation. Similar mild histology among NAFLD patients has been reported in other studies from India as well as in a study from Australia.^{15–17} This is in contrast to the reported series from France, where advanced fibrosis or cirrhosis has been found to be present in up to 30% of NAFLD cohorts.¹⁸

The risk factors found to be the predictors of fibrosis in our study group of 216 cases were older age group, female gender, obesity and moderate fatty change on USG. No other known factors were found associated with fibrosis. The phenotype of our patients with fatty liver is perhaps distinctly different from that of Western population as no such relationship with liver fibrosis was found in this study. The lack of association might be due to smaller number of patients in the group of severe fibrosis, as only one patient had advanced fibrosis. The milder histological

disease in the present cohort may also represent a genetic predisposition in our patients, just like their genetic predisposition to develop metabolic syndrome. Whether these patients with mild fibrosis continue to have a mild disease or progress to a stage of cirrhosis can only be answered once we have an adequate period of follow up on them. The NAFLD patients have milder disease has been shown in a study from North India. This study by Duseja et al. had concluded that the extreme form of obesity, diabetes mellitus, hypertension, metabolic syndrome, iron abnormalities, and HFE gene mutations are less common in Indians as compared to the West.¹⁹ They also concluded that majority of these patients have mild histological disease at presentation and they most often respond to lifestyle modifications and UDCA.¹⁹

Another difference is that around 50% of the patients were without insulin resistance. A previous study by Singh et al. had previously shown that nearly half of the NAFLD population from coastal Eastern India were without IR.²⁰ Despite the absence of IR, a significant proportion, about a third of these patients had high grades of fibrosis. The authors believed that insulin resistance is probably not the sole factor, but it is possibly a heterogeneous disorder, with a distinct group of NAFLD patients without IR. Factors like gut microflora, H Pylori and genetic factors may play a role in the pathogenesis apart from insulin resistance.

We had another case control study (diabetics vs. non-diabetics) from 515 patients diagnosed with fatty liver in USG and 240 of them were selected for biopsy. Fibrosis was documented in 31.4% in diabetics and 27% in non-diabetics. The present study also reflects similar figure with regards to fibrosis range.²¹ Another study from India on 80 liver biopsy specimens showed that a proportion of patients with NAFLD show only hepatic steatosis and an increasing grade of steatosis is associated with greater inflammation, hepatocyte injury and acinar fibrosis.²² Similar results has been reflected in this study. A similar study was carried out by Williams et al. where 29.9% of ultrasound positive fatty liver cases showed NASH on liver biopsy but fibrosis analysis was not done.²³

A Delhi based study, where 71 patients with histological diagnosis of NASH were retrieved and their clinical and anthropometric analysis was done. Out of 38 NASH cases

12 biopsies were in stage 0. Majority of cases 31 (62%) showed stage 1 fibrosis and the prevalence of fibrosis in different stages is comparable to our study.²⁴ However, the necroinflammatory activity showed quite a high score as the authors have selected only the NASH cases whereas the present study population was incidentally detected NAFLD cases on USG.

An Asian study conducted in China analyzed liver biopsies from those patients who had transaminitis and fatty liver in USG.²⁵ They found that majority of cases were in stage 1 and 10% of cases in stage 3 fibrosis. The explanation given by the authors was due to high percentage of body fat and visceral fat. Asians have high percentage of visceral body fat compared to white Caucasians and black despite low average BMI. Thus Asia pacific criteria recommended for BMI are overweight at BMI 23–24.9 and obese BMI ≥ 25 kg/m². NAFLD lesions are more prevalent in overweight persons with BMI of 23–25 in contrast to the western population where the lesions are found with BMI beyond 30 kg/m².^{26,27}

Some of the above mentioned studies by different authors have taken small number of patients as liver biopsy is an invasive procedure and causes inconvenience to patients. Here a large sample size of 216 patients was analyzed and biopsy is the only reliable mode to reach at a diagnosis of NASH and fibrosis. None of the non-invasive imaging techniques have sufficient sensitivity and specificity to stage NAFLD related fibrosis and cannot distinguish simple steatosis from NASH with or without fibrosis. The advantage of biopsy is that it can even identify stage 1 fibrosis.

On evaluation of metabolic profile, two-third (59%) patients were overweight and obese (BMI > 23). NAFLD is proposed to be the hepatic manifestations of metabolic syndrome. Madan et al. has shown that triglyceride level, transaminitis and metabolic syndrome are strongly associated with high necroinflammatory score but the association with fibrosis are not statistically significant, similar to our observation.¹⁵

Insulin resistance and metabolic syndrome play a central role in the development of NAFLD, this study reveals their evidence in 54% and 45% of cases respectively. Further prospective study on association of insulin resistance with NAFLD fibrosis is required. Our study had few limitations. Being a retrospective study, there may be some recall bias. Overall risk assessment for insulin resistance was not possible in this small group of patients (in 22% of subjects who had fibrosis) which may also a drawback.

CONCLUSION

In this eastern coastal region of India, although the NAFLD patients have a histologically milder disease as compared to their Western counterparts. Older age, females, obesity and moderate fatty liver on USG are risk

factors for development of fibrosis in patients with NAFLD. Furthermore, there was a positive correlation between higher grades of histological features such as steatosis, lobular inflammation and ballooning degeneration with higher stages of fibrosis. Awareness of the risk factors, namely, obese and or moderate fatty change in USG, should be selected as a cohort for evaluation of NAFLD by liver biopsy.

CONFLICTS OF INTEREST

The authors have none to declare.

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