ORIGINAL ARTICLE

Association of Genetic Non-alcoholic Fatty Liver Disease with Insulin Resistance-Are we Different?

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Abstract

Introduction: Metabolic risk factors such as obesity, insulin resistance, type 2 diabetes mellitus and dyslipidemia are associated with non-alcoholic fatty liver disease (NAFLD). In the development and progression of NAFLD genetic mutations also play a significant role. NAFLD associated with the rs 738409 polymorphism of patatin-like phospholipase domain containing 3 gene (PNPLA3) G allele does not feature the typical metabolic abnormalities of NAFLD, including insulin resistance. In the light of rising epidemic of metaobesity in our population this study aimed to evaluate the relation of PNPLA3 polymorphism with insulin resistance.

Methods: In this case control hospital based study, 100 patients of NAFLD were recruited based on ultrasound findings of hepatic steatosis. Healthy subjects age and gender matched(*n* = 100) from the institute who volunteered to be part of the study were recruited as controls based on the sole criteria of the absence of fatty liver on ultrasonography and normal alanine and aspartate transaminases (ALT and AST) levels. Anthropometry, biochemical profiles and insulin resistance by homeostatic model assessment of insulin resistance (HOMA-IR) were assessed.

Results: A higher frequency of CG and GG genotypes of rs738409 polymorphism of PNPLA3 was observed in patients with NAFLD than controls. These patients with G allele had increased ALT, dyslipidemia and insulin resistance. The polymorphism had positive correlation with severity of hepatic steatosis.

Conclusion: The presence of the PNPLA3 G allele is associated with a risk of NAFLD. Our study shows that subjects with variant PNPLA3 are not only at increased risk for the development and progression of NAFLD, but also have increased insulin resistance.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is defined as hepatic fat accumulation (steatosis) exceeding 5% in the absence of excessive ethanol consumption, drugs, toxins, infectious diseases or any other specific etiologic factors of liver disease.[1] NAFLD embraces a morphological spectrum ranging from non alcoholic fatty liver (NAFL) to non alcoholic steatohepatitis (NASH) in which hepatic inflammation and fibrosis co-exist and NASH can progress towards cirrhosis and even hepatocellular carcinoma.[2] NAFLD has been recognized as the leading cause of chronic liver disease, with a prevalence up to 20%–30% in the general population.[3]

NAFLD shares common pathophysiology and frequently associated with features of the metabolic syndrome that predisposes individuals to type 2 diabetes and cardiovascular disease (CVD) or to NASH and cirrhosis.[4] Genetic and environmental factors have important roles in the development of NAFLD.[5,6]

According to genome-wide association study of NAFLD a single nucleotide polymorphism (rs738409, encoding I148M) in the patatin-like phospholipase domain-containing protein 3 (PNPLA3) gene on chromosome 22 conferred susceptibility to NAFLD in a population that included Hispanic, African-American and European-American individuals.[7,8] Another systematic review showed that rs738409 GG genotype was associated not only with liver fat accumulation but also with susceptibility to more aggressive disease.[9]

NAFLD often coexists with metabolic syndrome has been incriminated as a risk factor of future cardiovascular events [10] but the common genetic forms of NAFLD, especially those associated with variation in the genes PNPLA3 are not associated with insulin resistance, features of the metabolic syndrome or an increased risk of type 2 diabetes or CVD.[11,12]

In view of rising epidemic of obesity and metabolic syndrome in our country, which are the root causes of NAFLD, we need to study the genetic aspects of this common disease in our own population and to also assess its propensity towards cirrhosis or metabolic syndrome. In the present study we aimed to find out prevalence of PNPLA3 I148M variant in patients with NAFLD and its association with insulin resistance and other metabolic traits.

Material and Methods

The present study was carried out in a medical college hospital situated in North India as a case

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Table 1: Baseline characteristics of patients with NAFLD and controls

Variables	Patients with NAFLD (n=100)	Controls (n=100)	P value
Age(years)	45±8.2	46±7	0.4
Male Gender	46(46%)	47(47%)	0.12
Waist circumference (cm)	93±12	81±14	0.001
Hypertension	32(32%)	12(12%)	0.001
Dyslipidemia	21(21%)	11(11%)	0.02
Prediabetes	12(12%)	6(6%)	0.01

Data is expressed as n(%) or mean±SD

control study, which was conducted between January 2015 to June 2016. All consecutive patients >18 years old with hepatomegaly detected on clinical and/or ultrasonographic examination attending the outpatient department or admitted in medical wards of the of Era's Lucknow Medical College and diagnosed to have hepatic steatosis were included after screening of inclusion criteria. The patients were diagnosed to have NAFLD on the basis of presence of hepatic steatosis as bright/echogenic liver on abdominal ultrasound. Ultrasonography was performed by a high resolution B-mode scanner of General Electric Logic 5 with a 3.5 MHz convex-array probe. Hepatic steatosis was defined as diffuse increase in fine echoes in liver parenchyma with impaired visualization of intrahepatic vessels and the diaphragm. Exclusion Criteria comprised of pregnancy, alcohol consumption (> 20 gm/day), Positive hepatic markers of viral hepatitis, autoimmune hepatitis, Wilson's disease, Hemochromatosis, drugs known to cause fatty liver (methotrexate, estrogens amiodarone, tamoxifen).

NAFLD was defined as the presence of an ultrasonographic pattern consistent with the following criteria: liver-kidney echo discrepancy, attenuated echo penetration, visibility of diaphragm, and obscure hepatic vessel structures. The aforementioned ultrasonographic pattern was scored as described by Chan et al.[13] The subjects were categorized to have mild, moderate, or severe steatosis if the overall score was 1–3, 4–6, or 7–9, respectively.

The controls were age and sex matched healthy individuals who had ultrasonographically normal liver echogenecity and normal liver function tests enrolled from the hospital staff and their relatives. The study protocol was approved by the Institutional Ethics Committee and written informed consent was obtained from all study participants.

Table 2: Frequency of genotypes among study participants

Genotype	Patients with NAFLD (n=100)	Controls (n=100)	P value
CC	20	51	0.03
CG	55	32	0.001
GG	25	17	0.01

Determination of PNPLA3 genotype

PNPLA3 rs738409 was genotyped by the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA, USA).Five milliliters of peripheral blood were collected from all the subjects in 0.5M EDTA tubes. Genomic DNA was isolated from whole blood using the standard phenol-chloroform extraction method. The quantity and quality of DNA was checked by spectrophotometry and gel electrophoresis. DNA was stored at -20°C. A simple PCR-RFLP based method was used for the assay of PNPLA3 rs738409. The sequence of sense primer is 5'- TGGGCCTGAAGTCCGAGGGT-3' and that of anti sense primer was 5'-CCGACACCAGTGCCCTGCAG-3'. PCR mixture consist of 1X PCR buffer (Tris-HCl 10 mM, KCl 50 mM, $MgCl_2$ 2 mM), 0.2 mM each dNTP, 1 mM each primer, 100 ng genomic DNA and 1.5 U Taq DNA polymerase in a total volume of 50 µL.The amplification program was as follow: Initial denaturation at 94°C for 2 min, denaturation at 94°C for 30 sec, annealing at 66°C for 30 sec and synthesis at 72°C for 30 sec. Final synthesis was carried out at 72°C for 5 min. PCR product was digested with 4U of BtsCI (Biolabs inc., New England, U.S.) at 50°C for 4 hr. Digested PCR product was electrophoresed in 2% agarose gel and visualized on a UV transilluminator. A detailed history, thorough physical examination was performed, and anthropometric measurements were recorded. The metabolic syndrome was defined according to International Diabetes Federation (IDF) criteria. [14] Biochemical parameters including liver transaminases, alanine transaminase (ALT), and aspartate transaminase (AST) fasting blood glucose, lipid profile and insulin levels were measured. Homeostasis model assessment (HOMA) method for insulin resistance was calculated by the formula: Fasting serum insulin(microunits/ml)x fasting serum glucose(millimoles per litre)/22.5. [15]

Statistical analysis

The Statistical Package for the Social Sciences version17 (SPSS, Inc., Chicago, IL USA) was used for data analysis. To ensure the normal distribution of variables, Kolmogorov-Smirnov test was applied. Nonparametric tests (chi-squared test, Fisher exact probability test, and Mann-Whitney U test) were used to compare the characteristics of the groups.

We used Pearson's correlation coefficient to assess the relationships and $P < 0.05$ was considered statistically significant.

Results

The present study was conducted to investigate association of PNPLA3 gene polymorphism with insulin resistance in patients with nonalcoholic fatty liver disease. Hundred patients fulfilling the inclusion criteria for NAFLD were enrolled in the study as cases and equal number of (n=100) age and gender matched healthy individuals were recruited as Controls having ultrasonographically normal liver echogenicity and normal liver function tests. Table 1 is showing base line characteristics of cases and controls. Age of subjects ranged from 20-70 years and mean age was 45±8.2 years and 46±7 years. The anthropometric parameters including waist circumference, waist-hip ratio and BMI were higher in patients with NAFLD than controls. Significantly higher proportion of patients with NAFLD as compared to controls had dyslipidemia, hypertension and prediabetes as shown in Table 2.

PNPLA3 rs738409 genotype assay was performed and genotype frequencies were summarized according to 3 possible genotypes (CC, CG and GG) among study participants.

It was observed that PNPLA3 rs738409 polymorphism occurred at a higher frequency in patients with NAFLD than controls as described in Table 3. G allele containing genotypes(CG and GG) were present in 64% in patients with NAFLD and 37% in controls.

Anthropometric parameters

Table 3: Association of Genotypes with metabolic parameters

Variables	CC (n=71) Mean ± SD	CG (n=87) Mean ± SD	GG (n=42) Mean ± SD	F
Waist circumference	24.43 ± 2.85	26.64 ± 3.13	25.50 ± 3.42	9.993
Fasting blood glucose (mg/dl)	110.96 ± 18.37	135.07 ± 28.22	127.33 ± 21.85	20.326
Fasting Insulin (IU/L)	5.08 ± 2.28	6.85 ± 2.84	6.50 ± 2.38	9.826
HOMA-IR	2.37 ± 0.44	3.52 ± 0.47	2.89 ± 0.38	131.370
Triglyceride (mg/dl)	117.37 ± 19.73	155.37 ± 17.90	148.63 ± 15.99	90.527
Total Cholesterol (mg/dl)	113.48 ± 20.40	160.07 ± 24.98	136.50 ± 22.92	80.341
HDL Cholesterol (mg/dl)	40.43 ± 4.96	40.20 ± 4.86	39.34 ± 3.93	0.732
LDL Cholesterol (mg/dl)	119.68 ± 23.66	135.02 ± 24.04	136.81 ± 25.48	9.985
AST (IU/L)	39.95 ± 9.98	63.37 ± 12.47	66.60 ± 13.00	108.459
ALT (IU/L)	42.85 ± 10.66	65.48 ± 12.38	70.4 ± 10.58	121.951
P value: 0.02				

Table 4: Association of G allele genotypes with components of metabolic syndrome

Variables	Total (N=200)	CC (n=71) No. (%)	CG+GG (n=129) No. (%)	Statistical significance	
				χ^2	p
Obesity	102	34 (47.9)	68 (52.7)	0.427	0.514
Raised Triglycerides (>150 mg/dl)	81	3 (4.2)	78 (60.5)	60.108	0.001
Low HDL (<40/<50 males; females)	147	47 (66.2)	100 (77.5)	3.014	0.083
Hypertension	36	8 (11.3)	28 (21.7)	3.380	0.066
Blood glucose (>100 mg/dl)	159	53 (74.6)	106 (82.2)	1.590	0.207
HOMA-IR	2.98± 0.67	1.58±0.44	3.31±0.53	't'=12.624; p=0.002	

Table 5: Association of genotypes and severity of hepatic steatosis (n=100)

Grade	Total (N=100)	CC (n=20) No. (%)	CG (n=55) No. (%)	GG (n=25) No. (%)
Mild	51	19 (95)	22 (40)	10 (40)
Moderate	38	1 (5)	24 (43.6)	13 (52)
Severe	11	0	9 (16.4)	2 (8)
χ^2=20.814 (df=4); p=*0.001*				

Table 6: Correlation of HOMA-IR, fasting glucose and fasting insulin with different genotypes

Genotype	HOMA-IR		Fasting Glucose		Fasting insulin	
	r	p	r	p	r	P
CC	0.122	0.312	0.172	0.153	-0.020	0.868
CG	0.18	0.06	0.239	0.026	0.054	0.618
GG	0.56	0.001	0.38	0.01	0.43	0.01

including waist circumference and waist-hip ratio were significantly higher in patients with G allele. Similarly, a significantly higher proportion of subjects with genotype CG and GG as compared to CC had raised triglycerides and LDL although total cholesterol, HDL and VLDL levels of subjects with CC, CG & GG genotypes were not different as depicted in Table 4. Raised transaminase levels were found among significantly higher proportion of subjects with CG and GG genotypes as compared to CC genotype. HOMA-IR of subjects with CG & GG genotypes was significantly higher as compared to CC genotype (3.31±0.53 vs 1.58±0.44, p=0.01).

We analysed the association of components of metabolic syndrome with the presence of CC, CG & GG genotypes and it was observed that triglycerides and fasting glucose levels were significantly higher in GG genotype. They also had higher HOMA-IR than CC genotype.

Table 5 has shown the impact of genotypes on grades of hepatic steatosis. The presence of G allele was associated with severity of NAFLD. Majority of subjects of CC genotype had mild hepatic steatosis while majority of subjects with CG and GG genotypes had moderate to severe steatosis.

In the present study it was observed that there was positive correlation of GG genotype with HOMA – IR (r = 0.56, P=0.001), fasting insulin (r = 0.43, P =0.01) and fasting glucose levels (r = 0.38,P =0.01) as depicted in Table 6.

Discussion

NAFLD is the most common chronic liver disease whose prevalence has reached global epidemic proportions. Although the disease is relatively benign in the early stages, but may progress to severe forms, including cirrhosis and even hepatocellular carcinoma. [16] A growing body of evidence indicates that NAFLD develops from a complex process in which many factors, including genetic susceptibility and environmental insults, are involved.

The prevalence of NAFLD in the Indian population is estimated to be around 25%-30%. [17],[18] NAFLD is the hepatic manifestation of metabolic syndrome and is associated with obesity, dyslipidemia hypertension, insulin resistance and cardiovascular disease. [19] associated with the PNPLA3 G allele is characterized by an increase in hepatic fat and does not feature the typical metabolic abnormalities of NAFLD, or inflammation in adipose tissue and may be a cause of increased incidence of lean NAFLD.[20]

The present study did not show any gender predisposition for NAFLD. On comparing the anthropometric and clinical profile of NAFLD patients with controls, we found a significant difference between two groups with respect to hypertension, obesity and lipid levels. All these factors are metabolic implications of NAFLD.

In the present study we observed higher frequency of the GG genotype of PNPLA3 gene polymorphism in NAFLD patients than controls. Similar to the findings of present study, Lin et al in a set of obese population with and without NAFLD also found prevalence of G allele to be 44.8% in NAFLD patients as compared to 33.6% in controls.[21] Many studies of this polymorphism have been reported. In addition, the rs738409-GG genotype was associated with a higher risk of liver fibrosis, cirrhosis, and HCC.[22-24]

In present study, we also assessed the metabolic traits including anthropometric parameters of central obesity, lipid profile and liver transaminase levels in patients with NAFLD and controls and found that those with presence of genotype GG and CG, had significantly higher waist circumference, waist-hip ratio, triglycerides and ALT and AST levels. They were more insulin resistant.

In an Indian study by Alam et al, frequency of G allele was significantly higher (62.6%) in NAFLD than in healthy controls.[25] The GG genotype had 6.53 times odds of having NASH. Regression analysis revealed that G allele odds of having cirrhosis was 3.9 times compared to C. The G allele was also significantly associated with steatosis, lobular inflammation, NAFLD activity score, and fibrosis. Patients with NASH had higher HOMA-IR levels.

In another Indian study by Bhatt et al a higher frequency of CG and GG genotypes of the rs738409 polymorphism was

obtained in cases as compared to controls.[26] In this study, the G allele was associated with significantly higher fasting insulin HOMA-IR, ALT and AST values in cases than controls. However, other studies from different populations did not confirm our results. A study by Peng *et al* on evaluating the association of CG/GG genotype with obesity, lipids and raised plasma glucose levels, did not find any significant association for the above variables.[27] Lin et al also did not find a significant association for any of the variables In concordance with our study, though s also found higher AST and ALT levels in CG and GG genotypes as compared to that in CC genotype.[21] This shows a possible deviation dependent on the ethnic variability. Role of ethnic differences affecting the genotypic distribution and their impact on prevalence of anthropometry, liver functions and lipid levels for PNPLA3 variants have also been reported in a study by Bale *et al*. comparing South Indian and north-East Indian population.[28]

The present study also indicated a significant association between GG/CG polymorphism with grades of fatty liver disease. Kantartzis *et al* too in their study showed a significant difference in total liver fat in different polymorphic forms of PNPLA3 variant rs738409. [11] Akuta et al similar to present study also derived a similar significant association between stage of fatty liver disease and rs738409 polymorphism with higher prevalence of those with CG/GG genotype as compared to those having CC genotype.[29]

In previous studies PNPLA3 polymorphisms have been shown to have strong association with the risk for and severity of NAFLD, cirrhosis and hepatocellular carcinoma. In present study, we observed that it had strong relation with not only the severity of grades of NAFLD but carries a higher risk of dyslipidemia, obesity and insulin resistance. So there is a possibility that it can play an evolving role in diagnosis and treatment decisions in patients who have NAFLD.[30]

In the present study our aim was to look for the association of PNPLA3 I148M variant and with insulin resistance in patients with NAFLD. The patients with genetic susceptibility of NAFLD have increased the risk of advanced liver disease but our study showed that they had association with insulin resistance as well.

Considering the high prevalence of carriers PNPLA3 I148M variant and the obesity epidemic, many of our young patients and might develop early onset and severe NAFLD. In these patients, the progression from simple steatosis to steatohepatitis seems to be accelerated and will also be contributed by degree of insulin resistance. Our patients have 'double trouble', i.e. carry both a genetic risk factor and have the metabolic syndrome though larger prospective studies in our population are needed to validate our results.

Limitations of our study were small sample size. Although liver biopsy is considered to be the gold standard for identifying NAFLD and NASH, absence of indication for asymptomatic individuals, the costs involved, risk of complications and ethical concerns were major deterrents. We did not follow the patients to observe the outcome in form of cirrhosis or cardio vascular events. Another major limitation was that patients were not subjected for liver biopsy or fibro scan to assess the severity of fibrosis.

We recognize the fact that our study should be considered exploratory and that the findings should be interpreted with caution because a larger study would be required to be more conclusive. In future, the PNPLA3 gene may be a potential target for therapy in NAFLD. Prospective data with large number of patients are now needed to further understand the association of PNPLA3 polymorphisms and insulin resistance particularly related to metabolic traits of NAFLD and for prediction of final outcome in form of end stage liver disease or cardiovascular disease.

Conclusion

The presence of the *PNPLA3* G allele is associated with a risk of NAFLD. Our study shows that subjects with variant *PNPLA3* are not only at increased risk for the development and progression of NAFLD, but also have increased insulin resistance. Therefore it is very important to identify individuals with genetic susceptibility of hepatic steatosis at an early stage so that appropriate interventions can be planned to curtail progression to higher stages.

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