Regional differences in genetic susceptibility to non-alcoholic liver disease in two distinct Indian ethnicities

Govardhan Bale, Avanthi Urmila Steffie, Vishnubhotla Venkata Ravi Kanth, Padaki Nagaraja Rao, Mithun Sharma, Mitnala Sasikala, Duvvur Nageshwar Reddy

AIM
To validate the association of variants in PNPLA3 (rs2281135) and TM6SF2 (rs58542926) genes with ultrasound detected non-alcoholic fatty liver disease (NAFLD).

METHODS
A total of 503 individuals with and without fatty infiltration were recruited. Fatty infiltration was confirmed based on ultrasound findings. Anthropometric data and blood samples were collected from the study group. DNA was isolated from peripheral blood, quality and quantity was assessed by gel electrophoresis and spectrophotometer respectively. Genotyping of the variants in PNPLA3 and TM6SF2 genes was carried out by employing tagman probes (C_15875080_10 for PNPLA3 and C_8946351_10 for TM6SF2 SNP) on real time PCR (Stepone-Lifetechnologies). Genotype data was tested for deviations from Hardy-Weinberg
Distinct genetic susceptibility for NAFLD in Indian ethnicities

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) describes a range of liver conditions beginning with fatty infiltration (accumulation of fat in the liver) that progresses to non-alcoholic steatohepatitis (NASH; fat accumulation along with inflammation and scarring) and cirrhosis (scar tissue replaces hepatic cells)\(^[1]\), that may finally lead to hepatocellular carcinoma (HCC)\(^[2]\). While conditions up to NASH are reversible\(^[3]\), progression beyond NASH to cirrhosis is irreversible\(^[4]\). Therefore it is very important to identify individuals with genetic susceptibility to fat accumulation at an early stage so that appropriate interventions can be planned to curtail/avoid progression to higher stages. Environmental factors including intake of calories\(^[5]\), processed food\(^[6]\) and sedentary lifestyles\(^[7]\) have an impact on the predisposition of an individual to fatty liver and progression. Apart from environmental factors various studies have now confirmed the role of genetics in conferring susceptibility to the disease. Diseases with complex traits including NAFLD result from interactions between environment and polygenic genetic susceptibility made up of many independent modifiers\(^[8]\). Family aggregation, studies on twins and differences in susceptibility and progression suggest a significant heritable component to NAFLD that may be classified under “common disease-common variant” hypothesis\(^[9]\).

The first Genome wide association study for NAFLD identified a SNP in PNPLA3 gene (rs738409; c.444 C > G, p.1148M). Carrier of the minor allele and 148M was associated with a twofold increase in HTGC (Hepatic triglyceride content)\(^[10]\). Subsequent to this, the SNP was replicated in almost all the ethnicities successfully\(^[8]\). Further, two exome wide association studies\(^[11,12]\) carried out independently in African-American and Norwegian ethnicities identified that a variant rs58542926 (p.E167K) in TM6SF2 gene was associated with susceptibility to NAFLD, influencing total cholesterol levels and enhanced risk of myocardial infarction. Subsequently, functional studies identified TM6SF2 as a regulator of liver fat metabolism influencing secretion of triglycerides and lipid droplet content in the liver\(^[13]\). A recent review suggested that male sex, PNPLA3 I148M, TM6SF2 E167K and low birth weight as important predictors of adult NAFLD\(^[14]\) reiterating the importance.
of variants in both PNPLA3 and TM6SF2 genes.

Our earlier pilot study identified variants in PNPLA3 (rs738409), PARVB (rs2073080), SAMM50 (rs2143571) and PZP (rs6487679) genes to be associated with a higher risk of fatty infiltration in individuals of NEI ethnicity. In the present study we replicated variants namely rs58542926 in TM6SF2 and rs2281135 in PNPLA3 genes, identified earlier to confer susceptibility to NAFLD in two distinct ethnicities. While one ethnicity belonged to South India, the other belonged to the North-Eastern region of the country. An earlier study on South Indians has reported that the genomic affinity is proportionate to caste rank—the upper castes being most similar to Europeans, while the lower castes are more similar to Asians. However, the Northeast region’s population results from ancient and continuous flows of migrations from Indo-Gangetic India, Tibet, the Himalayas, present day Bangladesh and Myanmar.

MATERIALS AND METHODS

A total of 503 individuals were recruited for the present study from the Hepatology clinics of Asian Institute of Gastroenterology. Although liver biopsy is considered to be the gold standard for identifying NAFLD, risk of complications, costs involved and ethical concerns limit its use, hence, patients with fatty infiltration were recruited based on ultrasound findings. Ethnicity, age and sex matched healthy subjects who volunteered to be part of the study were recruited as controls based on the sole criteria of the absence of liver fat on ultrasonography with normal liver function tests and negative for other viral indications. Written informed consent was obtained from individuals and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review (Scientific) Board (AIG/AHF IRB: 16/2014). Demographic and anthropometric details (height, weight, BMI and waist circumference) were collected. Whole blood (3 mL) was collected in pre coated EDTA containers from the study group and stored at -20 °C until further analysis. Biochemical investigations like ALT, viral markers and lipid profiles were estimated as per standard methods.

Genotyping

DNA was isolated from blood using a commercial kit (Bioserve Biotechnologies, Hyderabad) following manufacturers protocol. DNA with high molecular weight on agarose gel and A260/280 ratios between 1.8-2.0 were included for genotyping analyses. All the samples were genotyped for SNPs namely rs2281135 in PNPLA3 and rs58542926 in TM6SF2 genes using Taqman single nucleotide genotyping assay (Life Technologies, United States) on the Realtime polymerase chain reaction (PCR) platform. PCR for genotyping consisted of 5 μL of 2 × Taqman genotyping master mix, 0.5 μL of 1 × assay mix (C_15875080_10 for PNPLA3 and C_8946351_10 for TM6SF2 SNP) and 4.5 μL consisting of 8-10 ng of DNA in a final volume of 10 μL. PCR was performed on Step One Realtime PCR (Life technologies, United States) with the following cycling conditions: 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min with fluorescence read after each cycle for a total of 40 cycles. Genotyping calls were made using the allelic discrimination software (Life Technologies, United States) and only auto calls made by the software were considered for further analysis. A known heterozygous and homozygous variant sample was replicated across all the plates and these known genotypes were verified manually during analysis in all the plates.

Statistical analysis

Data was entered in to MS-EXCEL and edited for consistency. Continuous variables were expressed as mean (95%CI) and categorical variables as proportions. Patient characteristics were compared using Student’s t test for continuous variables and χ² test for categorical variables. χ² goodness-of-fit was used to confirm the agreement of the observed genotype frequencies with those of expected (Hardy-Weinberg equilibrium). χ² test was used to analyze the statistical significance of the difference in genotypic distribution of the studied SNPs in patients and controls. The association of the studied SNPs with the disease and various clinical parameters was expressed as odds ratio (95%CI). For transheterozygoisty analysis chi-square test was applied to compare the number of variant carriers in both the genes between patients and controls. A two-tailed P value of ≤ 0.05 was considered statistically significant. The analyses were carried out using Med cal C package.

RESULTS

Although categorization of the study group based on the ultrasound findings yielded two groups, ethnicity was identified as a major confounder for further analysis. Samples were therefore sorted based on ancestry and classified in to Ancestral South Indians (ASI; n = 231; Controls-138 and patients-93) and North-East Indians (NEI; n = 272; controls-109 and patients-163). All the clinical characteristics as shown in Table 1 namely waist circumference, hip circumference, waist/hip ratio, BMI, ALT, AST, Triglycerides were significantly different between the cohorts from both the ethnicities. Further, there was significant difference in the HDL levels only in the NEI group but not in the ASI group.

Genotyping and association with clinical traits

While rs58542926 in TM6SF2 gene was significantly associated (P = 0.0004) with a 2.7 fold higher risk of fatty infiltration in ASI ethnicity, rs2281135 in PNPLA3
gene was associated with 1.9 fold higher risk in the NEI ethnicity (Table 2). rs58542926 in TM6SF2 gene was associated with higher ALT, AST levels in the ASI ethnicity and higher BMI in NEI ethnicity. rs2281135 in PNPLA3 gene was associated with ALT, AST levels in ASI ethnicity and higher BMI in NEI ethnicity. rs58542926 in TM6SF2 rs58542926 and PNPLA3 variants in the two ethnicities studies.

Transheterozygosity analysis
On transheterozygosity analysis ($\chi^2$ test), it was seen that there was a significant difference in individuals who carried variants in both the genes in the patient group as compared to control group in ASI ethnicity ($P=0.009$), but not NEI ethnicity ($P=0.26$) and increased the risk of the disease by 3 fold (OR = 3.11, 95%CI: 1.20-8.04) in the ASI ethnicity. Further, there were significantly higher proportion of individuals with variants in both the genes in the patient group in ASI (patients - 14/93 and controls - 7/138; Z proportion test $P=0.009$) and NEI ethnicities (patients - 17/163 and controls - 7/109; Z proportion test $P=0.06$).

Comparison of controls and patients within the ethnicities
There were significant differences in BMI (higher) AST, ALT and HDL levels (lower levels) in ASI controls as compared to NEI controls. While patients of ASI ethnicity had higher hip circumference, BMI and lower HDL levels patients of NEI ethnicity had higher waist-hip ratios, ALT and AST levels. Likewise, there were significant differences in hip circumference, BMI (higher levels in ASI as compared to NEI patients), waist-hip ratio, ALT, AST levels (higher levels in NEI patients as compared to ASI patients). It was also interesting to note that the HDL levels were significantly lower in the ASI patients (Table 4).

DISCUSSION
In a cohort of 503 individuals comprising individuals with and without NAFLD belonging to two distinct Indian ethnicities, we show here that rs58542926 in TM6SF2 in South Indian and rs2281135 in PNPLA3 in North-East Indian ethnicities confer higher susceptibility to ultrasound measured NAFLD. Further, there were a significant proportion of individuals with variants in both the genes in the patient group as compared to controls, in both the ethnicities, suggesting that although individually the variants may not confer susceptibility in the ethnicity, however carrying an additional variant might compound the risk of the disease. Our earlier pooled genetic association study in a predominantly North-East Indian ethnicity identified that rs738409 in PNPLA3 gene was associated with higher risk of NAFLD apart from variants in PARVB, SAMM50 and PZP.
The first Genome wide association study for NAFLD identified rs738409 in PNPLA3 gene conferring susceptibility to NAFLD\(^\text{10}\). Subsequent to this, the variant

### Table 3  Association of variants with clinical data

| Characteristics | Controls | Patients |
|-----------------|----------|----------|
|                 | Ancestral South Indians | North-East Indians |
|                 | CC | CT | TT | GG | GA | AA | \(\chi^2\) | \(P\) value | CC | CT | TT | GG | GA | AA | \(\chi^2\) | \(P\) value |
| TM6SF2 rs58542926 BMI < 22.9 | 49 | 12 | 2 | 0.03 | 0.98 | 75 | 18 | 5 | 8.24 | 0.01 | 110 | 28 | 4 | 94 | 47 | 2 |
| > 22.9 | 38 | 23 | 2 | 0.59 | 0.74 | 48 | 37 | 10 | 0.077 | 0.96 | 82 | 52 | 8 | 71 | 58 | 14 |
| PNPLA3 rs2281135 BMI < 22.9 | 89 | 17 | 1 | 6.52 | 0.038 | 65 | 20 | 1 | 2.45 | 0.29 | 36 | 19 | 5 | 108 | 42 | 7 |
| > 22.9 | 42 | 33 | 5 | 1.3 | 0.52 | 54 | 28 | 4 | 10.27 | 0.005 | 66 | 71 | 19 | 66 | 69 | 16 |
| TM6SF2 rs58542926 ALT < 30 | 64 | 39 | 4 | 10.19 | 0.006 | 68 | 20 | 3 | 0.96 | 0.61 | 90 | 18 | 0 | 105 | 42 | 5 |
| > 30 | 41 | 32 | 6 | 2.89 | 0.23 | 54 | 30 | 7 | 5.55 | 0.06 | 65 | 40 | 3 | 66 | 69 | 16 |
| PNPLA3 rs2281135 ALT < 30 | 54 | 13 | 4 | 1.64 | 0.43 | 58 | 25 | 6 | 3.89 | 0.14 | 33 | 12 | 1 | 60 | 29 | 1 |
| > 30 | 42 | 33 | 6 | 2.03 | 0.36 | 49 | 22 | 3 | 0.05 | 0.97 | 41 | 32 | 6 | 66 | 69 | 16 |
| TM6SF2 rs58542926 TG < 150 | 54 | 13 | 4 | 71 | 17 | 2 | 0.06 | 0.98 | 3.47 | 0.04 | 33 | 12 | 1 | 60 | 29 | 1 |
| > 150 | 42 | 25 | 8 | 0.29 | 0.86 | 49 | 32 | 8 | 2.49 | 0.28 | 29 | 14 | 3 | 39 | 40 | 11 |
| PNPLA3 rs2281135 TG < 150 | 42 | 25 | 8 | 0.29 | 0.86 | 49 | 32 | 8 | 2.49 | 0.28 | 29 | 14 | 3 | 39 | 40 | 11 |
| > 150 | 26 | 5 | 0 | 0.12 | 0.73 | 49 | 22 | 3 | 0.05 | 0.97 | 57 | 18 | 3 | 59 | 27 | 3 |
| TM6SF2 rs58542926 HDL > 40 | 20 | 11 | 0 | 2.52 | 0.28 | 39 | 28 | 7 | 0.5 | 0.77 | 20 | 11 | 0 | 39 | 28 | 7 |
| < 40 | 46 | 26 | 6 | 0.02 | 0.88 | 39 | 28 | 7 | 0.5 | 0.77 | 71 | 58 | 14 | 71 | 58 | 14 |

\(\chi^2\) test. BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TG: Triglycerides; HDL: High density lipoprotein.

### Table 4  Comparison of clinical data within the ethnicities

| Characteristics | Controls | Patients |
|-----------------|----------|----------|
|                 | Ancestral South Indians (\(n = 138\)) | North-East Indians (\(n = 109\)) | P value | Ancestral South Indians (\(n = 93\)) | North-East Indians (\(n = 163\)) | P value |
| Age (yr) | 34.2 ± 11.9 | 38.5 ± 12.7 | 0.006 | 35.3 ± 8.0 | 36.5 ± 9.2 | 0.29 |
| Gender male/female (\(n\)) | 95/43 | 72/37 | 0.01 | 87/6 | 150/13 | - |
| Waist circumference (cm) | 83.3 ± 9.4 | 81.1 ± 10.7 | 0.27 | 94.7 ± 10.2 | 93.8 ± 10.1 | 0.54 |
| Hip circumference (cm) | 93.0 ± 7.1 | 91.2 ± 6.9 | 0.23 | 100.5 ± 8.6 | 95.1 ± 8.5 | 0.01 |
| Waist/hip ratio | 0.89 ± 0.06 | 0.89 ± 0.07 | 0.01 | 0.95 ± 0.13 | 0.99 ± 0.12 | 0.03 |
| BMI (kg/m\(^2\)) | 23.2 ± 4.0 | 22.1 ± 3.5 | 0.02 | 27.7 ± 4.1 | 25.7 ± 4.0 | 0.003 |
| ALT (IU/L) | 19.8 ± 7.6 | 24.6 ± 7.9 | 0.01 | 88.1 ± 49.5 | 119.3 ± 68.3 | 0.01 |
| AST (IU/L) | 21.2 ± 5.4 | 24.6 ± 6.9 | 0.01 | 553.2 ± 25.6 | 72.3 ± 39.8 | 0.01 |
| Triglycerides (mg/dL) | 134.8 ± 72.6 | 131.4 ± 60.8 | 0.79 | 169.7 ± 82.1 | 180.3 ± 93.7 | 0.44 |
| HDL (mg/dL) | 38.7 ± 8.5 | 47.9 ± 28.7 | 0.02 | 36.3 ± 6.9 | 40.5 ± 13.8 | 0.01 |

\(^1\)Unpaired students t test (two tailed). ASI: Ancestral South Indians; NEI: North-East Indians; BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TG: Triglycerides; HDL: High density lipoprotein.
was found to be associated with the disease in various ethnicities across the world including our own and other studies from India[8,15,18]. PNPLA3 is a 481-residue protein, exhibiting lipase activity against triglycerides in hepatocytes and a missense variant (I148M; rs738409-C>G) results in loss of function promoting hepatic steatosis by limiting triglyceride hydrolysis[19]. Further, another variant (rs2281135) in PNPLA3 gene was identified, that conferred higher risk for NAFLD[11]. rs2281135 is an intronic variant and is known to be in tight linkage disequilibrium with rs738409 in ethnicities including African, Caucasian, Mexican Americans and East African (HapMap data). Apart from variants in PNPLA3, recent research has identified rs58542926 in TM6SF2 gene to be associated with NAFLD. Recombinant protein expression in cultured hepatocytes confirmed that 50% less Glu167Lys TM6SF2 protein was produced relative to wild-type TM6SF2[21]. Further a study identified that TM6SF2 regulates liver fat metabolism and influences triglyceride secretion and lipid droplet content[13]. There is compelling evidence by now that variants in PNPLA3 and TM6SF2 genes are associated with progressive fatty infiltration (steatosis and cirrhosis) and further have a higher risk of progressing to HCC. It is therefore very important to understand the genetic susceptibility an ethnicity carries, so that appropriate lifestyle interventions can be planned to minimize the risk of progression, more so in the absence of reversing the genetic defect.

The intronic SNP (rs2281135) in PNPLA3 gene was associated with a higher risk of fatty infiltration only in NEI ethnicity but not ASI. In an earlier study with a predominant NEI ethnicity we identified that rs738409 in PNPLA3 conferring a higher susceptibility to fatty infiltration. It is known in literature that rs2281135, an intronic variant and rs738409 a functional variant are in tight LD in ethnicities including African, Caucasian, Mexican Americans and East African (HapMap data).

Although the general characteristics between patients and controls were significantly different as expected, it was interesting to note ethnicity based differences in the patient cohorts that could be predictive of higher susceptibility to NAFLD. While, higher hip circumference, BMI, and lower HDL levels could be predictive of a higher risk for NAFLD in the SI ethnicity, higher Waist-Hip ratio could be predictive in NEI ethnicity. Further, higher BMI and lower HDL levels were seen in the controls of SI ethnicity and higher ALT and AST levels were seen in the controls of NEI ethnicity suggesting cohort based differences and cutoffs in the clinical characteristics. Further, interestingly there were higher ALT and AST levels in the NEI ethnicity as compared to ASI ethnicity both between control and patient cohorts suggesting a higher necroinflammatory state in the patients of NEI ethnicity. Earlier genome wide studies have ascribed higher levels to genetic predisposition apart from other influencing factors including demographic such as age, sex, ethnicity, anthropometric features (waist circumference, BMI) and diurnal variation[20].

The genotype data in general did not deviate from Hardy-Weinberg equilibrium. However, it was interesting to note that there was a significant difference (P = 0.02) in the observed and expected genotype frequencies from the patient cohort of ASI ethnicity. Although the samples were represented in sufficient numbers, genotypes visually checked and manually re-scored, non-random mating and population structure excluded, the deviation persisted suggesting that the variant may contribute to disease risk in this ethnicity.

The genotyping data from this study suggests that while TM6SF2 variant was significantly associated with susceptibility to fatty infiltration in the ASI ethnicity, PNPLA3 variant was associated in the NEI ethnicity. However, it was interesting to see that there were a higher proportion of individuals in the patient group who were transheterozygous for PNPLA3 and TM6SF2 variants as compared to the control group suggesting that although there might be individual susceptibility in the two ethnicities, it is important to genotype the individuals for both the variants as there might be additive risk in the presence of the other risk allele. A recent study from Chinese ethnicity corroborated the same[21].

In conclusion, our study has identified distinct genetic susceptibility for ultrasound detected NAFLD in the two ethnicities. However, it is suggested that both the variants have to be genotyped for assessing the risk of the disease, as transheterozygosity of the studied variants seems to confer a higher risk in the population.

**COMMENTS**

**Background**

Non-alcoholic fatty liver disease (NAFLD) with an incidence of 25%-30% is an epidemic that is on the rise globally. There are significant differences in the prevalence, severity and outcome of the disease in various ethnicities that suggests a genetic background to it. Approximately 26%-35% of NAFLD may be contributed by genetic susceptibility according to a study. Therefore it is important to identify genetic susceptibility an individual carries for better management of the disease.

**Research frontiers**

Understanding and identifying ethnicity based variants that confer higher risk of disease will aid in imparting lifestyle and nutrient based recommendations to an individual with fatty infiltration for better management of the disease.

**Innovations and breakthroughs**

The authors have identified distinct genetic susceptibility for NAFLD in the two ethnicities that were studied. However, it was interesting to note that transheterozygosity of both the variants conferred a higher risk of the disease irrespective of ethnicity.

**Applications**

Individuals can be screened for these variants to assess their risk of developing NAFLD. Further, life style based modifications can be suggested to delay the onset/progression of the disease.

**Terminology**

NAFLD describes a range of liver conditions that begins with accumulation of fat in the liver, a condition referred to as steatosis.
of fat in the liver (fatty liver) and progresses to fat accumulation along with inflammation and scarring non-alcoholic steatohepatitis, hepatic cells replaced by scar tissue (cirrhosis) finally leading to hepatocellular carcinoma.

**Peer-review**
The present work deals with a human study in which genetic susceptibility to NAFLD in two Indian ethnicities is evaluated. This study constitutes an interesting work as the identification of population at risk is always desirable.

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