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Association of PNPLA3 Polymorphisms in Patients with Metabolic Dysfunction Associated Steatotic Liver Disease in North Coastal Andhra Pradesh: A Case-Control Study

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KEYWORDS

Polymorphisms, Metabolic Dysfunction Associated

ABSTRACT:

Background: Metabolic dysfunction-associated steatotic liver disease (MASLD) is a common cause of chronic liver diseases. It is indeed the leading cause of liver-related morbidity and mortality worldwide. The increasing prevalence of obesity, type 2 diabetes, and metabolic syndrome is leading to a rise in cases of MASLD globally. Genetic predisposition plays a significant role in MASLD development. PNPLA3 rs738409 polymorphism is associated with increased liver fat accumulation and can influence the progression of liver disease. Individuals carrying the variant may have a higher risk of developing conditions like MASLD and other liver complications.

Aim: This study aimed to investigate the relationship between MASLD susceptibility and PNPLA3 polymorphisms in a cohort from North Coastal Andhra Pradesh, India.

Methods:A total of 300 individuals, 150 MASLD cases (93 male, 57 female; mean age: 42.89±11.31 years), and 150 healthy controls (67 male, 83 female, mean age: 40.07±10.43 years) were included in the study. DNA was isolated from peripheral blood using the salting out method. The genotypes of PNPLA3 were determined by the PCR-RFLP (restriction fragment length polymorphism).

Results: The frequencies of rs738409 genotypes CC, CG, and GG were 40%, 36%, and 24% in MASLD cases, while in the control group, they were 23%, 70%, and 7%, respectively. The Chi square test statistic was 38.7871 with ap value of less than 0.0001 indicating a significant difference in genotype distribution between cases and controls. Individuals with the GG genotype have approximately 4.42 times higher odds of having the condition compared to those with the CC genotype.

Conclusion: This study concludes that PNPLA3 (rs738409) polymorphisms may play a key role in MASLD susceptibility in the cohort. Additionally, the higher prevalence of the CG genotype in controls, where G is the mutant allele, suggests that the CG genotype may confer a protective effect against MASLD. The GG is associated with increased risk. These findings indicate the genetic basis of MASLD and the importance of genotypic variation in disease risk assessment. particularly CG and GG, were observed to play a crucial role in determining the risk and
susceptibility to MASLD. Correlating disease stage with BMI and biochemical parameters like AST, ALT, along with comprehensive clinical follow-up, could enhance treatment outcomes.

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1. Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) is a perpetual cause of chronic liver disease. It is becoming a more significant concern for the global health. MASLD is the updated term of Nonalcoholic fatty liver disease associated with metabolic syndrome. MASLD is defined by the accumulation of lipids in hepatocytes exceeding 5% of liver weight. The disease starts with indolent course and in certain proportion of cases can lead to chronic liver disease and lead to Metabolic dysfunction-associated steatohepatitis (MASH), cirrhosis, and liver cancer (Hepatocellular carcinoma. Initially, the fat accumulates in the liver (steatosis), followed by inflammation (MASH), fibrosis, cirrhosis, and complete liver damage [1, 2]. It is a complex disease that arises from a combination of environmental factors and genetic variations.

Its prevalence differs across various regions. In Asia, it is usually between 30-32% and more common particularly in the Middle East [3]. In India, the prevalence is 9-32% in the general population [4]. MASLD is more prevalent in individuals with obesity, insulin-resistance, and metabolic syndrome [5]. Oxidative stress can also impact the development of MASLD [6]. Yamamoto and his team (2019) forecast that the number of overweight or obese individuals will exceed 2 billion by 2030. This implies that more people are likely to develop MASLD, due to increased obesity levels in the general population [7]. The prevalence of MASLD is high, and it can result in severe liver disease and other morbidities like diabetes, metabolic syndrome, and heart disease, making it a major health concern [8].

Genetic variations can play a role in determining the severity of MASLD. A review authored by Barbara and team delved into the essential genetic variances in MASLD from a constantly growing list [9]. The patatin-like phospolipase domain - containing protein 3 (PNPLA-3) gene plays a crucial role in MASLD and may be a key factor in its future treatment approaches [10].

PNPLA3, also known as adiponutrin, is a 481-unit protein. Hepatocytes show the highest levels of expression. It can also be located in adipocytes and dermal tissue, and it is regulated by our dietary intake. PNPLA3 plays an essential role in fat metabolism, indicating its ability to metabolize and transport fats within the body [11, 12].

This PNPLA3 gene is located on human chromosome 22 (chr22q13.31). In the PNPLA3 rs738409 C > G single nucleotide polymorphism (SNP), there is a genetic modification that results in the replacement of cytosine with guanosine. As a result of this change, the body synthesizes the incorrect amino acid, i.e., methionine at position 148 instead of isoleucine, Ile [ATC] to Met [ATG]. This SNP occurs within the third exon of the PNPLA3 gene. PNPLA3 levels are greatly influenced by fluctuations in energy consumption and dietary habits. The consumption of carbohydrates enhances the expression of PNPLA3 in human liver cells via a carbohydrate response element protein (chREBP) and sterol regulatory element-binding protein 1 c (SREBP-1c) [13-15].

PNPLA3 plays an essential role in fat metabolism. It is responsible for the degradation of fat molecules within the liver and prevents fats from moving into peripheral adipose tissue, thus leading to hepatic steatosis and associated disorders. The PNPLA3 gene is linked not just to fat in the liver, but also associated with inflammation in the liver, hepatic steatohepatitis, fibrosis, and cirrhosis, highlighting its crucial role in the progression of MASLD [16]. Patients carrying the PNPLA3 I148M variant experience more severe liver damage compared to those with the normal variant. MASLD is very common and might produce undesirable effects. Recognizing the elements that effect the emerging of this condition is key for suitable treatment methods whichenable in successful prevention and management [17].

2. Objectives

The objectives of the study were to estimatevarious biochemical parameters and analyze the PNPLA3 polymorphism in MASLD patients and healthy controls, and investigate the additive effect of PNPLA3 variant on the risk of MASLD patients in the North coastal Andhra Pradesh population.

3. Materials and methods

The present study, comprises 150 patients diagnosed with fatty liver and 150 healthy individuals aged

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between 18 to 70 years from the North Coastal Andhra Pradesh region, India. Patients in whom steatosis was diagnosed by ultrasonography (USG) at KIMS-ICON Hospital, Sheela Nagar, Visakhapatnam were included. Patients with alcohol consumption, the presence of hepatitis B or C virus infection or other liver diseases such as autoimmune hepatitis and Wilson's disease were excluded from the study.

The study was approved by the Institutional Ethics Committee, Andhra University, Visakhapatnam, India.

Methods

Anthropometric measurements, including height, weight were recorded, and 5 ml of peripheral blood was collected in EDTA tubes from all 300 subjects with their consent. All subjects were assessed fortheir lipid profile, random blood sugar (RBS), aspartate aminotransferase (AST), alanine aminotransferase (ALT) by standard laboratory methods [18]. Type 2 diabetes was diagnosed based on the examination of RBS levels along with the clinical symptoms.

Ultrasonographic examination

Diagnosis and grading of liver steatosis were performed by using ultrasonography (USG) by radiologists. Grading of diffuse hepatic steatosis on USG is based on the following criteria: Grade I: Fine diffuse increase in echogenecity of liver texture, Grade II: diffuse increase coarse echogenecity of liver texture with mild attenuation of ultrasound's sound beams, Grade III: diffuse increase coarse echogenecity of liver texture resulting in poor visibility of portal vein radical walls and right hemi diaphragm [19].

SNP genotyping

Genomic DNA was extracted from peripheral blood using the salting out method (Lahiri and Nurnberger) [20]. The PNPLA3 rs738409 SNP genotyping was carried out by PCR-RFLP. For PNPLA3, primers were PNPLA3-F(5'-AGTTCCCCGTTCTTTTGACCC-3') and PNPLA3-R(5'TCAGCGCTAGCAGAGAAAGC-3') [21]. The conditions for PCR reactions (10 µL) were, initial denaturation at 94° C for 5 minutes, subsequently 40 cycles of 94°C denaturation for 20 seconds, 65° C for annealing for 20 seconds, 72° C extension for 20 seconds, and a final extension for 10 minutes. The PCR product size was 247 base pairs (bp).

The RFLP assay for the PNPLA3 rs738409 genotype was performed by digesting the PCR product with 5 µL of BtsCI restriction endonuclease at 37° C for 12 hours. Both PCR amplification products and restriction digestion fragments were analyzed on agarose gel electrophoresis.

Statistical Analysis

Categorical variables were summarized as frequencies and percentages; continuous variables were expressed asmean ± standard deviation. Categorical variables were analyzed using the chi-square test. Odd ratio (OR) was performed to evaluate the risk association of individual genotypes among cases and controls. ANOVA was performed to evaluate the risk association among biochemical parameters and genotypes in patients with MASLD and control subjects.

4. Results

Of the 150 patients with fatty liver included in the study, 93 patients were male with a mean age of 42.89±11.31 years. The mean BMI of patients was 27.56 kg/m^2 .The mean BMI of patients was significantly higher than that in control subjects. Diabetes mellitus was present in 13% of patients while 18% were hypertensive. Metabolic syndrome (MS) was found to be present in 56.6% of the patients. The mean values of the biochemical parameters are presented in Table 1. AST, ALT, total cholesterol, high density lipoprotein cholesterol (HDL) and low density lipoprotein cholesterol(LDL) levels showed a significant association with the MASLD patients. Elevated AST and ALT levels are the markers for the liver inflammation or damage. Elevated cholesterol levels can be closely linked to metabolic dysfunction, it can lead to changes in lipid metabolism, resulting in higher levels of LDL while lowering the HDL.

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Table 1: Characteristics of the Patients with MASLD and Healthy Controls.

Characteristics	Total Cases(N=150)	Total Controls(N=150)	p value
Male	93(62%)	67(45%)	0.0028
Female	57(38%)	83(55%)	0.0028
Age	42.89 ± 11.31	40.07 ± 10.43	0.0255
BMI	27.56 ± 3.44	23.65 ± 1.92	< 0.0001
RBS	117.49 ± 45.14	115.91 ± 21.68	0.6995
AST	30.79 ± 14.25	26.43 ± 6.17	0.0007
ALT	33.23 ± 18.08	28.67 ± 5.74	0.0035
CHOL	158.01 ± 47.93	107.22 ± 18.74	< 0.0001
TGL	141.06 ± 59.62	132.89 ± 18.66	0.1103
HDL	41.17 ± 5.6	39.52 ± 3.74	0.0029
LDL	72.28 ± 48.14	41.59 ± 15.05	< 0.0001
VLDL	28.22 ± 11.92	26.65 ± 3.77	0.1251

Mean ±SD; MASLD: Metabolic dysfunction associated steatotic liver disease, BMI: Body mass index, RBS: random blood sugar, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, CHOL: Total cholesterol, TGL: triglycerides, HDL: high density lipoprotein, LDL: low density lipoprotein, VLDL: very low density lipoprotein.

Seventy five percent of the patients had Grade I Fatty Liver while 21% and 4% of the patients had Grade II Fatty Liver and Grade III Fatty Liver, respectively as shown in Table 2.

Table 2: List of Genotypes among the different Grades in MASLD Patients

Grading of fatty liver	CC	CG	GG	Total
GRADE I FATTY LIVER	56	37	19	**112**
GRADE II FATTY LIVER	4	16	12	**32**
GRADE III FATTY LIVER	0	1	5	**6**
Total	**60**	**54**	**36**	**150**

In the analysis of the rs738409 polymorphism the band patterns observed after the digestion of the PCR product with BtsCI were as follows: 173 and 74 bp bands correspond to the CC genotype, while 247 bp band represents the GG genotype while the presence of all three bands corresponds to CG genotype. The

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frequencies of rs738409 genotypes CC, CG, and GG were 40%, 36%, and 24%, respectively in MASLD cases, while in the control group, the frequencies were 23%, 70%, and 7%, respectively. The genotype and allele frequencies of the I148M polymorphism in cases and controls are shown in Table 3. Analysis of data revealed that the genotypes and alleles in MASLD cases and controls were in Hardy-Weinberg equilibrium.

The Chi square test statistic was 38.7871 with a p value < 0.0001, indicating significant genotype distribution differences between cases and controls. This suggests a significant relationship between the polymorphism and MASLD condition.Individuals with the GG genotype have about 4.42 times higher odds of having the condition compared to those with the CC genotype (Table 3).

Table 3: Risk Association of Individual Genotype and Allele Frequencies of rs738409 in Cases and Controls with Test for Heterogeneity

Marker	Genotype	Cases (%)	Controls (%)	Odds ratio (95% CI)	p value	Test for Heterogeneity
rs738409	CC	60 (40.00%)	34 (23%)	2.2745 (1.3757 to 3.7606)	0.0014	χ^2=38.7871; P= <0.0001
	CG	54 (36.00%)	106 (70%)	0.2335 (0.1438 to 0.3790)	< 0.0001	
	GG	36 (24.00%)	10 (7%)	4.4211 (2.1031 to 9.2938)	0.0001	
	C allele	174 (0.58)	174 (0.58)	1.0000 (0.7231 to 1.3830)	1	
	G allele	126 (0.42)	126 (0.42)			

Analysis of Various Parameters with the rs738409 Genotypes.

To investigate the association of various parameters and genotypes of rs738409, age, BMI, RBS, liver enzymes, and lipid profile levels were compared between MASLD patients and controls(Table 4).BMI, AST, ALT, Total cholesterol, TGL, LDL and VLDL levels were higher in patients than in controls. The G-allele of rs738409 was significantly associated with an increase in AST (p = 0.007) and ALT levels (p = 0.039) and also associated with BMI (p =0.005).

Table 4: Comparison of Various Parameters among the different Genotypes of rs7384089 in PNPLA3 in MASLD Patients and Controls.

Cases	CC	CG	GG	p value
Age	42.50 ± 10.76	43.70 ± 11.24	42.25 ± 10.54	0.777
BMI	26.47 ± 3.25	28.45 ± 3.17	28.05 ± 3.75	0.005
RBS	122.90 ± 49.37	115.98 ± 39.91	110.47 ± 44.79	0.362
AST	27.90 ± 14.15	33.02 ± 15.44	32.14 ± 11.62	0.007
ALT	30.06 ± 13.81	36.75 ± 23.89	32.92 ± 12.46	0.039
CHOL	153.00 ± 44.14	160.36 ± 53	161.72 ± 46.23	0.344
TGL	130.51 ± 35.62	152.07 ± 84.48	140.81 ± 40.46	0.129
HDL	40.95 ± 5.61	41.38 ± 5.82	41.11 ± 5.31	0.986
LDL	62.65 ± 27.91	80.49 ± 66.67	75.09 ± 37.97	0.095

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VLDL	26.14 ± 7.123	30.41 ± 16.9	28.16 ± 8.09	0.133

Controls	CC	CG	GG	p value
Age	40.02 ± 10.54	40.13 ± 10.52	39.90 ± 10.79	0.982
BMI	23.82 ± 2.08	23.06 ± 1.26	23.82 ± 2.08	0.664
RBS	114.38 ± 19.42	117.13 ± 22.14	108.20 ± 24.38	0.415
AST	26.64 ± 6.09	26.01 ± 6.12	30.00 ± 6.41	0.146
ALT	28.58 ± 5.60	28.35 ± 5.72	32.20 ± 5.65	0.128
CHOL	107.67 ± 18.92	108.03± 18.73	97.00 ± 16.79	0.203
TGL	133.67 ± 17.97	133.58 ± 18.59	122.80 ± 20.55	0.210
HDL	39.17 ± 3.74	39.66 ± 3.70	39.20 ± 4.44	0.778
LDL	41.61 ± 15.12	42.36 ± 15.27	33.20± 10.53	0.184
VLDL	27.08 ± 3.44	26.70 ± 3.81	24.60 ± 4.16	0.234

Mean ±SD; MASLD: Metabolic dysfunction associated steatotic liver disease, BMI: Body mass index, RBS: random blood sugar, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, CHOL: Total cholesterol, TGL: triglycerides, HDL: high density lipoprotein, LDL: low density lipoprotein, VLDL: very low density lipoprotein. The data of each parameter from MASLD and control subjects were compared for the different rs738409 genotypes. P values were analyzed using the Anova test in MASLD patients and control subjects.

5. Discussion

MASLD is a significant public health issue due to its association with a range of serious health conditions. Patients with MASLD exhibit a higher prevalence of metabolic disorders compared to healthy individuals. In our study of MASLD patients, we found significant differences in BMI and liver enzymes (AST and ALT) across genotypes. CG and GG genotypes associated with higher values, indicating a potential higher risk or more severe disease. In healthy controls, these parameters did not show significant differences across genotypes. MASLD is frequently linked with insulin resistance and obesity. In the present study, the two conditions often coexist, with MASLD potentially exacerbating the metabolic disturbances associated with diabetes (13%), hypertension (18%) and metabolic syndrome (56%) risk factors. In 2001,Marchesini et al., and few other also reported coexistence of insulin resistance and obesity in their study [22-24].

Table 5: Overview of the CC, CG and GG Genotypes based on the findings from present study and other studies.

S.No	Author (references)	Genotype		OR	Genotype		OR	Genotype		OR
		CC case	CC control		CG case	CG control		GG case	GG control	
1	Alam,S [35]	45	37	0.398	27	43	1.365	3	19	5.700
2	Bhatt, S. P[36]	149	112	0.299	16	35	2.704	8	15	2.105
3	Baclig, M. O [37]	26	14	0.299	8	12	2.100	2	6	3.923
4	Hudert, C. A [38]	175	45	0.498	71	31	1.444	11	19	6.401
5	Krishnaswamy L [28]	59	19	0.261	29	50	2.288	14	36	3.280
6	Karoli,R [39]	51	20	0.240	32	55	2.597	17	25	1.627
7	Oniki, Kentaro [40]	223	38	0.248	394	111	0.346	121	45	0.662
8	Niriella, M. A[41]	25	54	0.605	134	464	0.993	232	842	1.114
9	Valenti, L [42]	118	103	0.355	56	114	1.801	5	36	5.733

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USG is indeed a valuable diagnostic tool for assessing fatty liver disease, particularly in detecting moderate to severe cases [25]. The 5-point scoring system for liver fibrosis (F0-F4) based on FibroScan results provides a way to assess the severity of liver disease[26]. Scores above 6 kPa indicate some degree of liver disease, with higher scores correlating with more severe fibrosis. In the present study, 75% of the patients had Grade I fatty liver, 21% had Grade II fatty liver and the remaining 4% had Grade III fatty liver. All the patients had mild to moderate fibrosis (F0-F2) [26].

PNPLA3 is a 481-unit protein primarily expressed in adipose tissues but shows the highest levels of expression in hepatocytes. The PNPLA3 protein is responsible for the breakdown of triglycerides and aids in relocating certain healthy fats, such as polyunsaturated fatty acids [27].The I148M mutant is inefficient in fat breakdown. This process results in an accumulation of triglycerides and fat droplets that are high in polyunsaturated fatty acids, contributing to an excess of fat in the liver. Additionally, it appears to evade proteasomal degradation, resulting in its accumulation in liver cells. This dual effect of impaired lipid metabolism and increased stability of the protein can significantly influence liver health. In Chennai based south Indian population study Krishnasamy Narayanasamy et al., 2020 also reported that the rs738409 polymorphism increases the risk of MASLD with an elevated TGL levels [28]. In our study TGL levels were found to be elevated in CG+GG versus CC genotypes in cases than in controls.

The PNPLA3 has been evaluated in several studies around the world. Plenty of studies have found an association between the SNP and MASLD [29-34].The present study also found significant association between PNPLA3 rs738409 and MASLD. According to the findings from the present study, it is assumed that the PNPLA3 rs738409 plays a prominent role in the progression of the disease. The GG genotype was strongly associated with an increased risk of MASLD, with statistically significant difference between cases and controls. 24% of the cases had GG genotype and a noteworthy fact is that all the cases who exhibited Grade III fatty liver had atleast one or two G alleles. However other factors (e.g. environmental or lifestyle) may also contribute to the progression of the disease.

The Chi-square test statistic was 38.7871, with $p< 0.0001$ indicating significant genotype distribution differences between cases and controls. G is the mutant allele, The GG genotype was significantly associated with the risk of MASLD ($p < 0.0001$). In the present study, individuals with the GG genotype have about 4.42 times higher odds of having the condition compared to those with the CC genotype, and a higher prevalence of the CG genotype in controls than in MASLD patients was observed. The association of this genetic variation with the development of MASLD and damage to the liver has been proven in Argentinian and Caucasian populations [28, 29]. The results of the current study revealed that the G-allele of rs738409 is strongly associated with MASLD.The association of G-allele of rs738409 with the levels of AST and ALT is controversial. The G-allele of rs738409is significantly associated with high levels of AST and ALT with MASLD among Argentinians [28], Hispanics [29], and Italian adults [32]. However, in European American, African American [29], and German [30] populations, rs738409 is not associated with the levels of AST and ALT. In our study, it was observed that the G-allele of rs738409 is linked to the increased levels of AST and ALT.

Limitation of our study lies in the lack of comprehensive MASH diagnoses, as we did not utilize targeted tests like liver biopsies. Long-term follow-up are necessary to validate and extend our observations. Strength of our study is derived from employing biochemical and molecular analysis to determine the risk factors for this disease. This information could be helpful in medical practice, in screening methods, and treatment interventions for people at higher risk of MASLD.

Future implications: Ongoing research into genetic mutations associated with MASLD in South India, particularly in Andhra Pradesh, is essential for deepening our understanding of the disease's etiology.

Conclusion:

This study concludes that PNPLA3 rs738409 polymorphism may play a key role in MASLD susceptibility in the cohort. The GG genotype is

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associated with increased risk of MASLD. These findings indicate the genetic basis of MASLD and the importance of genotypic variation in disease risk assessment. Particularly, CG and GG were observed to play a crucial role in determining the risk and susceptibility to MASLD. Correlating disease stage with BMI and biochemical parameters like AST and ALT, along with comprehensive clinical follow-up, could enhance treatment outcomes. Our study suggests that PNPLA3 may be involved in the progression of MASLD in the North Coastal Andhra Pradesh population.

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