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ORIGINAL ARTICLE

Basic Study

Linked *PNPLA3* polymorphisms confer susceptibility to nonalcoholic steatohepatitis and decreased viral load in chronic hepatitis B

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Abstract

AIM: To investigate the association of *PNPLA3* polymorphisms with concurrent chronic hepatitis B (CHB) and nonalcoholic fatty liver disease (NAFLD).

METHODS: A cohort of Han patients with biopsy-proven CHB, with or without NAFLD (CHB group, n = 51; CHB + NAFLD group, n = 57), and normal controls (normal group, n = 47) were recruited from Northern (Tianjin), Central (Shanghai), and Southern (Zhangzhou) China. Their *PNPLA3* polymorphisms were genotyped by gene sequencing. The association between *PNPLA3* polymorphisms and susceptibility to NAFLD, and clinical characteristics of NAFLD were evaluated on the basis of physical indices, liver function tests, glycolipid metabolism, and histopathologic scoring. The association of *PNPLA3* polymorphisms and hepatitis B virus (HBV) load was determined by the serum level of HBV DNA.

RESULTS: After adjusting for age, sex, and body mass index, we found that four linked single nucleotide polymorphisms (SNPs) of *PNPLA3*, including the rs738409 G allele (CHB + NAFLD group *vs* CHB group: odds ratio [OR] = 2.77, 95% confidence interval [CI]: 1.18-6.54; P = 0.02), rs3747206 T allele (CHB + NAFLD group *vs* CHB group: OR = 2.77, 95%CI: 1.18-6.54; P = 0.02), rs4823173 A allele (CHB + NAFLD group *vs* CHB group: OR = 2.73, 95%CI: 1.16-6.44; P = 0.02), and rs2072906 G allele (CHB + NAFLD group *vs* CHB group: OR = 3.05, 95%CI: 1.28-7.26; P = 0.01), conferred high risk to NAFLD in CHB patients. In patients with both CHB and NAFLD, these genotypes of *PNPLA3* polymorphisms were associated with increased susceptibility to nonalcoholic steatohepatitis (NASH) (NAFLD activity score $\geqslant$ 3; P = 0.01-0.03) and liver fibrosis (> 1 Metavir grading; P = 0.01-0.04). As compared to those with C/C and C/G at rs738409, C/C and C/T at rs3747206, G/G and G/A at rs4823173, and A/A and A/G at rs2072906, patients in the CHB + NAFLD group with G/G at rs738409, T/T at rs3747206, A/A at rs4823173, and G/G at rs2072906 showed significantly lower serum levels of HBV DNA (P < 0.01-0.05).

CONCLUSION: Four linked SNPs of *PNPLA3* (rs738409, rs3747206, rs4823173, and rs2072906) are correlated with susceptibility to NAFLD, NASH, liver fibrosis, and HBV dynamics in CHB patients.

Key words: Chronic hepatitis B; Hepatitis B virus; Nonalcoholic fatty liver disease; *PNPLA3*; Single-nucleotide polymorphism

Core tip: The association of *PNPLA3* polymorphisms with concurrent chronic hepatitis B and nonalcoholic fatty liver disease was determined in Chinese Han patients. The effect of *PNPLA3* genotypes on hepatitis B virus load was also evaluated. Four linked single-nucleotide polymorphisms of *PNPLA3* (rs738409 G allele, rs3747206 T allele, rs4823173 A allele, and rs2072906 G allele) conferred susceptibility to nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, and liver fibrosis in chronic hepatitis B patients. Patients carrying these single-nucleotide polymorphisms showed a significantly lower serum level of hepatitis B virus DNA.

Pan Q, Zhang RN, Wang YQ, Zheng RD, Mi YQ, Liu WB, Shen F, Chen GY, Lu JF, Zhu CY, Zhang SY, Chen YM, Sun WL, Fan JG. Linked *PNPLA3* polymorphisms confer susceptibility to nonalcoholic steatohepatitis and decreased viral load in chronic hepatitis B. *World J Gastroenterol* 2015; 21(28): 8605-8614 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i28/8605.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i28.8605

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is an acquired, metabolic, stress-induced liver disease associated with insulin resistance and genetic susceptibility[1]. A "two-hit hypothesis" has been proposed to describe the pathologic progression of NAFLD, in which a high-fat diet causes hepatic fat accumulation as the first hit, and oxidative stress, mitochondrial dysfunction, cytokines, adipokines, bacterial endotoxins, and endoplasmic reticulum stress cause the second hit[2]. The clinical spectrum of NAFLD ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), but can progress to liver cirrhosis and finally hepatocellular carcinoma[3].

Adiponutrin, encoded by *PNPLA3* on chromosome 22q13, has both triacylglycerol hydrolase and acylglycerol transacetylase activity in hepatocytes. A single-nucleotide polymorphism (SNP) in *PNPLA3* (rs738409 C>G), which results in an adiponutrin variant with I148M (isoleucine-to-methionine substitution at residue 148), has been identified as a predisposing factor for liver steatosis. The adiponutrin variant is linked to reduced triglyceride hydrolysis in hepatocytes[4,5]. Moreover, the *PNPLA3* rs738409 G allele is closely associated with NASH[6], hepatic fibrosis/cirrhosis[4,7] and hepatocellular carcinoma[8], regardless of the degree of obesity[9]. Except for rs738409, *PNPLA3* rs2281135 was recently reported to be in tight linkage disequilibrium (LD) in four human ethnic groups (African, Caucasian, East Asian, and Mexican Americans) with NAFLD[10]. *PNPLA3* rs139051 TT is also associated with increased risk of NAFLD in the Chinese Han population[11]. Similar results were reported in a previous study, which showed that *PNPLA3* rs2294918 was associated with NASH in

European and American populations[12]. Some other *PNPLA3* polymorphisms, including rs2281135 and rs6006460, influence serum alanine aminotransferase activity[12] and hepatic fat content[13], respectively. Polymorphism of *PNPLA3*, therefore, seems to serve as the genetic basis for histologic progression of NAFLD.

With the increasing prevalence of obesity and metabolic syndrome, NAFLD has now become a commonly occurring liver disease and a public health burden in most areas of China[3]. In Western countries, high-fat diet-induced NAFLD occurs in the absence of hepatitis-virus-induced liver disease. However, the high prevalence of hepatitis B virus (HBV) infection in the Chinese population causes concurrent NAFLD and chronic hepatitis B (CHB) in China[14,15]. Of the studies published to date, hepatic steatosis occurs at rates of 60% (213 cases), 51.2% (86 cases), 13.5% (91 cases), and 14% (1915 cases) in patients from Greece[16], South Korea[17], Hong Kong (China)[18], and Hang Zhou (China)[19], respectively.

Recent experimental evidence indicates that *PNPLA3* polymorphisms may play a role in the concurrent development of NAFLD and chronic viral hepatitis. In patients infected with genotype 2 hepatitis C virus (HCV), the *PNPLA3* I148M variant correlates with significantly increased insulin resistance. In *PNPLA3* I148M carriers, the viral load is significantly lower at baseline and after 3-7 d of antiviral treatment[20]. However, the role of the *PNPLA3* polymorphism in patients with NAFLD and CHB has not yet been established.

Therefore, we characterized SNPs of *PNPLA3* in Han patients with biopsy-proven CHB with or without NAFLD from Northern (Tianjin), Central (Shanghai), and Southern (Zhangzhou) China. The association of *PNPLA3* polymorphisms with clinical, laboratory, and pathologic characteristics of NAFLD was evaluated. The correlation between *PNPLA3* polymorphism and HBV dynamics was also assessed.

MATERIALS AND METHODS

Study populations

Forty-seven normal controls (normal group), 57 patients with biopsy-proven CHB and NAFLD (CHB + NAFLD group), and 51 patients with only biopsy-proven CHB (CHB group) were recruited between January 2012 and June 2013. Subjects were enrolled from Tianjin Hospital of Infectious Diseases (Northern China, n = 54), Xinhua Hospital (Shanghai, Central China, n = 72), and Zhengxing Hospital (Zhangzhou, Southern China, n = 29). Subjects with the following were excluded: high alcohol intake (> 20 g/d for men and > 10 g/d for women), HCV infection, autoimmune hepatitis, Wilson's disease, hereditary hemochromatosis, and current or previous treatment that is known to cause steatosis. The study was reviewed and approved by the Ethics Committee of Xinhua Hospital. Informed consent was obtained from all participants. Clinical investigations were conducted in accordance with the principles of the Helsinki Declaration.

Biochemical and anthropometric analysis

Anthropometric parameters of height, weight, and body mass index (BMI) were characterized for the study population. A fasting blood sample was collected from each patient and control subject. The serum was separated and stored at -20 ℃ until required for further analysis. Biochemical tests were performed for measuring the enzyme activities of alanine aminotransferase, alkaline phosphatase, and γ-glutamyltransferase, as well as the level of total bilirubin by standard enzyme methodology using a multichannel automatic analyzer (Advia 1650; Bayer, Moss, Norway). Fasting blood glucose, total cholesterol, triglyceride, high-density lipoprotein, and low-density lipoprotein concentrations were analyzed using Wako Bioproducts (Wako Pure Chemical Industries, Richmond, VA, United States). The HBV-DNA titer was quantified by real-time PCR (Daangene, Guangzhou, China) on a Light Cycler (Roche Diagnostics GmbH, Mannheim, Germany).

Hepatic histopathologic assessment

Liver sections of CHB patients with and without NAFLD were obtained by needle biopsy after obtaining informed consent. Liver samples were fixed in 10% buffered formalin, embedded in paraffin, and sliced for further evaluation. Hematoxylin-eosin and Masson's trichrome staining was successively performed. Histologic changes were graded and staged according to the Kleiner classification, and the NAFLD activity score (NAS) was calculated using the grade of steatosis, lobular inflammation, and hepatocyte ballooning[21]. NASH was defined by NAS $\geqslant$ 5, including a ballooning degeneration score of $\geqslant$ 1.

Genotyping of SNPs

All blood samples were centrifuged at 1500 rpm for 10 min immediately after sample collection. The buffy-coat layer obtained from each blood sample was separated and transferred into 1.5-mL centrifuge tubes. Genomic DNA was extracted from the concentrated lymphocytes of the buffy coat using QiAamp DNA Mini Kit (Qiagen, Benlo, Limburg, Netherlands). The custom Ion AmpliSeq panel (Life Technologies of Thermo Fisher Scientific, Waltham, MA, United States) of *PNPLA3* was designed, with the overall coverage rate of 89.91%. The emulation PCR of the template was performed using the Ion OneTouch 2 System (Life Technologies) according to the manufacturer's instructions. *PNPLA3* variants were genotyped by DNA sequencing using the Ion 318 Chip (Life Technologies) following the Ion PGM 200 Sequencing kit protocol. In addition, the LD of *PNPLA3* SNPs was investigated by LD and haplotype block analysis using Haploview software (Broad Institute of MIT and Harvard, United States).

Table 1 Demographic and clinical data of all patients

Variable	Normal group (n = 47)	CHB group (n = 51)	CHB + NAFLD group (n = 57)	P value
Age (yr)	46.74 ± 6.97	36.76 ± 12.60	38.98 ± 13.55	< 0.001
Sex, n (%)	M: 29 (61.70)	M: 34 (66.67)	M: 42 (73.68)	0.420
	F: 18 (38.30)	F: 17 (33.33)	F: 15 (26.32)	
BMI (kg/m^2)	23.43 ± 2.59	22.84 ± 2.97	27.40 ± 3.24	< 0.001
TC (mmol/L)	4.40 ± 1.22	4.39 ± 0.96	4.89 ± 0.84	0.023
TG (mmol/L)	0.98 ± 0.31	1.12 ± 0.38	1.84 ± 1.35	< 0.001
HDL (mmol/L)	1.51 ± 1.22	1.50 ± 0.44	1.20 ± 0.28	0.142
LDL (mmol/L)	2.03 ± 0.85	2.22 ± 0.56	2.94 ± 1.02	< 0.001
FBG (mmol/L)	3.39 ± 1.30	4.11 ± 0.63	5.71 ± 1.92	< 0.001
ALT (U/L)	13. 72 ± 4.16	76.14 ± 53.70	68.51 ± 47.20	< 0.001
TBIL (μmol/L)	2.25 ± 0.51	23.78 ± 21.44	5.80 ± 3.55	< 0.001
GGT (U/L)	16.42 ± 7.31	94.33 ± 94.13	60.18 ± 36.81	< 0.001
ALP (U/L)	16.08 ± 5.48	95.23 ± 40.35	101.16 ± 88.46	< 0.001

CHB: Chronic hepatitis B; NAFLD: Nonalcoholic fatty liver disease; BMI: Body mass index; TC: Total cholesterol; TG: Triglyceride; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; FBG: Fasting blood glucose; ALT: Alanine aminotransferase; TBIL: Total bilirubin; GGT: γ-glutamyltransferase; ALP: Alkaline phosphatase.

Statistical analysis

The data are expressed as the mean ± SD. Odds ratios (ORs) adjusted for age, sex, and BMI were calculated using multivariant logistic regression with genotypes, age, sex, and BMI as the independent variables. The χ^2 test was used to assess whether the genotypes were in Hardy-Weinberg equilibrium and to test differences in genotype distribution. Prior to analyzing the data, all the variables were tested for normality, with non-normally distributed variables log-transformed to be better approximated by normality. Differences among the groups of genotypes were tested by analysis of variance using SPSS version 16.0 (SPSS Inc., Chicago, IL, United States). Differences were considered statistically significant at $P < 0.05$. The statistical methods of this study were reviewed by Guang-Yu Chen from Clinical Epidemiology Center, Shanghai Jiaotong University.

RESULTS

Demographic and clinical data

Patients with both NAFLD and CHB demonstrated a signficantly higher BMI than that of the normal group and CHB patients ($P < 0.001$, Table 1). Patents in the CHB + NAFLD had higher total cholesterol, trigcyleride, low-density lipoprotein and fasting blood glucose than the CHB group (all $P < 0.05$), and a trend for decreased high-density lipoprotein, indicated impaired glycolipid metabolism in CHB + NAFLD patients independent of CHB (Table 1). The coexistence of CHB, which reflects chronic hepatic inflammation, may provide an explanation for the insignificant difference in alanine aminotransferase activity between the CHB + NAFLD and the CHB groups.

Association of PNPLA3 polymorphisms and NAFLD in CHB patients

After genotyping for PNPLA3 polymorphisms, the G allele at rs738409, T allele at rs3747206, A allele at rs4823173, and G allele at rs2072906 were associated with NAFLD in CHB patients when compared with CHB only patients (Table 2). Furthermore, a significant association was found between NAFLD and *PNPLA3* SNPs rs738409, rs3747206, rs4823173, and rs2072906 after adjusting for sex and age, or sex, age, and BMI (all $P < 0.05$).

Surprisingly, haplotype block LD mapping showed that SNPs of rs738409, rs3747206, rs4823173, and rs2072906 were in tight LD in an 8-kb sequence (Figure 1). In contrast, the percentage of G allele at rs738409, T allele at rs3747206, A allele at rs4823173, and G allele at rs2072906 was similar between the normal and CHB groups, regardless of age, sex and BMI adjustment.

Quantitative phenotypes with PNPLA3 polymorphisms

The normal, CHB, and CHB + NAFLD groups were stratified by *PNPLA3* genotypes and subjected to demographic and clinical comparisons. In the normal group, subjects bearing G/G allele at rs738409, T/T allele at rs3747206, A/A allele at rs4823173, and G/G allele at rs2072906 demonstrated low-density lipoprotein (P = 0.01-0.06) and fasting blood glucose (P = 0.01-0.02) levels higher than those with C/C or C/G alleles at rs738409, C/C or C/T alleles at rs3747206, G/G or G/A alleles at rs4823173, and A/A or A/G alleles at rs2072906 (Table S1). These subjects were characterized by an increasing trend in total cholesteral levels and decreasing trend in high-density lipoprotein levels (Table S1).

Despite the lack of statistical significance, CHB patients with or without NAFLD carrying the G allele at rs738409, T allele at rs3747206, A allele at rs4823173, and G allele at rs2072906 also exhibited higher levels of triglycerides, low-density lipoprotein, and fasting blood glucose than those with the C allele at rs738409, C allele at rs3747206, G allele at rs4823173, and A allele at rs2072906 (Table S1). Thus, rs738409 (G allele), rs3747206 (T allele), rs4823173 (A allele),

Table 2 Association tests of *PNPLA3* single nucleotide polymorphisms

Groups	SNPs	Group (%)			OR (95%CI)			*P* value		
		Normal	CHB	CHB + NAFLD	Unadjusted	Adjusted for age, sex	Adjusted for age, sex, and BMI	Unadjusted	Adjusted for age and sex	Adjusted for age, sex, and BMI
CHB+NAFLD *vs* CHB	738409		C: 57.84 G: 42.16	C: 44.74 G: 55.26	1.695 (0.989-2.906)	2.334 (1.169-4.657)	2.774 (1.176-6.543)	0.054	0.016	0.020
	3747206		C: 57.84 T: 42.16	C: 44.74 T: 55.26	1.695 (0.989-2.906)	2.334 (1.169-4.657)	2.774 (1.176-6.543)	0.054	0.016	0.020
	4823173		G: 58.82 A: 41.18	G: 45.61 A: 54.39	1.703 (0.993-2.922)	2.134 (1.089-4.180)	2.734 (1.161-6.437)	0.052	0.027	0.021
	2072906		A: 58.82 G: 41.18	C: 44.74 G: 55.26	1.765 (1.028-3.029)	2.314 (1.174-4.559)	3.045 (1.277-7.262)	0.039	0.015	0.012
CHB+NAFLD *vs* normal	738409	C: 63.83 G: 36.17		C: 44.74 G: 55.26	2.180 (1.246-3.815)	2.529 (1.335-4.792)	3.018 (1.318-6.914)	0.00	0.004	0.009
	3747206	C: 63.83 G: 36.17		C: 44.74 T: 55.26	2.180 (1.246-3.815)	2.529 (1.335-4.792)	3.018 (1.318-6.914)	0.006	0.004	0.009
	4823173	C: 63.83 G: 36.17		G: 45.61 A: 54.39	2.104 (1.203-3.681)	2.536 (1.329-4.840)	3.212 (1.344-7.679)	0.009	0.005	0.009
	2072906	C: 63.83 G: 36.17		C: 44.74 G: 55.26	2.180 (1.246-3.815)	2.684 (1.403-5.134)	3.499 (1.452-8.430)	0.006	0.003	0.005
CHB *vs* normal	738409	C: 63.83 G: 36.17	C: 57.84 G: 42.16		1.286 (0.723-2.287)	1.356 (0.695-2.643)	1.315 (0.674-2.567)	0.391	0.372	0.422
	3747206	C: 63.83 G: 36.17	C: 57.84 T: 42.16		1.286 (0.723-2.287)	1.356 (0.695-2.643)	1.315 (0.674-2.567)	0.391	0.372	0.422
	4823173	C: 63.83 G: 36.17	G: 58.82 A: 41.18		1.235 (0.694-2.199)	1.439 (0.717-2.889)	1.325 (0.652-2.694)	0.472	0.306	0.437
	2072906	C: 63.83 G: 36.17	G: 58.82 A: 41.18		1.235 (0.694-2.199)	1.439 (0.717-2.889)	1.325 (0.652-2.694)	0.472	0.306	0.437

BMI: Body mass index; CHB: Chronic hepatitis B; CI: Confidence interval; NAFLD: Nonalcoholic fatty liver disease; OR: Odds ratio; SNPs: Single nucleotide polymorphisms.

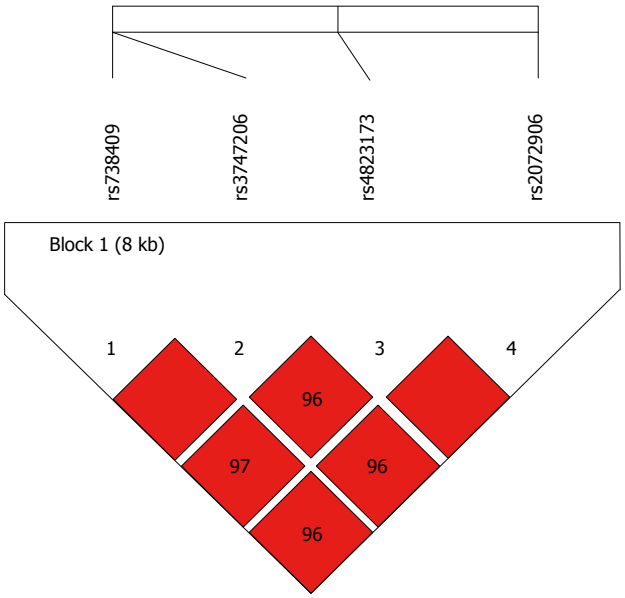

Figure 1 Block for liver disease of *PNPLA3* single-nucleotide polymorphisms.

and rs2072906 (G allele) may be involved in glycolipid metabolism.

Correlation between PNPLA3 polymorphisms and pathologic characteristics

Association of *PNPLA3* SNPs with the histologic features of NAFLD, including hepatocyte steatosis, lobular inflammation, hepatocellular ballooning, and liver fibrosis, were analyzed in the CHB + NAFLD group. As compared to those with C/C or C/G alleles at rs738409, C/C or C/T alleles at rs3747206, G/G or G/A alleles at rs4823173, and A/A or A/G alleles at rs2072906, CHB patients harboring the G/G allele at rs738409, T/T allele at rs3747206, A/A allele at rs4823173, and G/G allele at rs2072906 showed increased risk of hepatocellular ballooning (ballooning > 1; P = 0.01-0.03) and NASH (NAS $\geqslant$ 3; P = 0.01-0.03), but not with histologic features of steatosis and inflammation (Table 3). Another striking observation was the significant association between rs738409, rs3747206, rs4823173 and rs2072906 of *PNPLA3* and fibrogenesis. CHB + NAFLD patients with the G/G allele at rs738409, T/T allele at rs3747206, A/A allele at rs4823173, and G/G allele at rs2072906 were more susceptible to liver fibrosis (> 1 Metavir grading; P = 0.01-0.04) than those with heterozygote or homozygote C alleles at rs738409, C alleles at rs3747206, G alleles at rs4823173, and A alleles at rs2072906 (Table 3).

Effect of PNPLA3 polymorphisms on HBV dynamics

As defined by the serum level of HBV DNA, patients in the CHB + NAFLD and CHB groups showed active viral replication. However, there was a decreasing trend in HBV-DNA levels in the CHB + NAFLD patients (Figure 2A). In the CHB + NAFLD group, patients bearing the G/G allele at rs7384096, T/T allele at rs3747206,

Table 3 Association tests of *PNPLA3* single nucleotide polymorphisms with histological features in chronic hepatitis B + nonalcoholic fatty liver disease patients

Condition	*PNPLA3* genotype			*P* value
	CC (n = 12)	CG (n = 27)	GG (n = 18)	
Steatosis	rs738409: CC M: ≤ 1: 5 (41.67%); F: > 1: 7 (58.33%) rs3747206: CC M: ≤ 1: 5 (41.67%); F: > 1: 7 (58.33%) rs4823173: GG M: ≤ 1: 5 (38.46%); F: > 1: 8 (61.54%) rs2072906: AA M: ≤ 1: 5 (38.46%); F: >1: 8 (61.54%)	rs738409: CG M: ≤ 1: 11 (40.74%); F: > 1: 16 (59.26%) rs3747206: CT M: ≤ 1: 11 (40.74%); F: > 1: 16 (59.26%) rs4823173: GA M: ≤ 1: 11 (42.31%); F: > 1: 15 (57.69%) rs2072906: AG M: ≤ 1: 11 (44.00%); F: > 1: 14 (56.00%)	rs738409: GG M: ≤ 1: 4 (22.22%); F: > 1: 14 (77.73%) rs3747206: TT M: ≤ 1: 4 (22.22%); F: > 1: 14 (77.73%) rs4823173: AA M: ≤ 1: 4 (22.22%); F: > 1: 14 (77.73%) rs2072906: GG M: ≤ 1: 4 (21.05%); F: > 1: 15 (78.95%)	rs738409: 0.384; rs3747206: 0.384; rs4823173: 0.374; rs2072906: 0.275
Lobular inflammation	rs738409: CC M: ≤ 1: 5 (41.67%); F: > 1: 7 (58.33%) rs3747206: CC M: ≤ 1: 5 (41.67%); F: > 1: 7 (58.33%) rs4823173: GG M: ≤ 1: 6 (46.15%); F: > 1: 7 (53.85%) rs2072906: AA M: ≤ 1: 6 (46.15%); F: > 1: 7 (53.85%)	rs738409: CG M: ≤ 1: 10 (37.04%); F: > 1: 17 (62.96%) rs3747206: CT M: ≤ 1: 10 (37.04%); F: > 1: 17 (62.96%) rs4823173: GA M: ≤ 1: 9 (34.62%); F: > 1: 17 (65.38%) rs2072906: AG M: ≤ 1: 9 (36.00%); F: > 1: 16 (64.00%)	rs738409: GG M: ≤ 1: 5 (27.78%); F: > 1: 13 (72.22%) rs3747206: TT M: ≤ 1: 5 (27.78%); F: > 1: 13 (72.22%) rs4823173: AA M: ≤ 1: 5 (27.78%); F: > 1: 13 (72.22%) rs2072906: GG M: ≤ 1: 5 (26.32%); F: > 1: 14 (73.68%)	rs738409: 0.706; rs3747206: 0.706; rs4823173: 0.570; rs2072906: 0.509
Ballooning	rs738409: CC M: ≤ 1: 6 (50.0%); F: > 1: 6 (50.0%) rs3747206: CC M: ≤ 1: 6 (50.00%); F: > 1: 6 (50.00%) rs4823173: GG M: ≤ 1: 7 (53.85%); F: > 1: 6 (46.15%) rs2072906: AA M: ≤ 1: 7 (53.85%); F: > 1: 6 (46.15%)	rs738409: CG M: ≤ 1: 9 (33.33%); F: > 1: 18 (66.67%) rs3747206: CT M: ≤ 1: 9 (33.33%); F: > 1: 18 (66.67%) rs4823173: GA M: ≤ 1: 8 (30.77%); F: > 1: 18 (69.23%) rs2072906: AG M: ≤ 1: 8 (32.0%); F: > 1: 17 (68.0%)	rs738409: GG M: ≤ 1: 1 (5.56%); F: > 1: 17 (94.44%) rs3747206: TT M: ≤ 1: 1 (5.56%); F: > 1: 17 (94.44%) rs4823173: AA M: ≤ 1: 1 (5.56%); F: > 1: 17 (94.44%) rs2072906: GG M: ≤ 1: 1 (5.26%); F: > 1: 18 (94.74%)	rs738409: 0.021 (CC *vs* GG: P = 0.005; CG *vs* GG: P = 0.028); rs3747206: 0.021 (CC *vs* TT: P = 0.005; CT *vs* TT: P = 0.028); rs4823173: 0.012 (GG *vs* AA: P = 0.002; GA *vs* AA: P = 0.041); rs2072906: 0.009 (AA *vs* GG: P = 0.002; AG *vs* GG: P = 0.029)
NAS	rs738409: CC M: < 3: 10 (83.33%); F: ≥ 3: 2 (16.67%) rs3747206: CC M: < 3: 10 (83.33%); F: ≥ 3: 2 (16.67%) rs4823173: GG M: < 3: 11 (84.62%); F: ≥ 3: 2 (15.38%) rs2072906: AA M: < 3: 11 (84.62%); F: ≥ 3: 2 (15.38%)	rs738409: CG M: < 3: 11 (40.74%); F: ≥ 3: 16 (59.26%) rs3747206: CT M: < 3: 11 (40.74%); F: ≥ 3: 16 (59.26%) rs4823173: GA M: < 3: 10 (38.46%); F: ≥ 3: 16 (61.54%) rs2072906: AG M: < 3: 10 (40.0%); F: ≥ 3: 15 (60.0%)	rs738409: GG M: < 3: 8 (44.44%); F: ≥ 3: 10 (55.56%) rs3747206: TT M: < 3: 8 (44.44%); F: ≥ 3: 10 (55.56%) rs4823173: AA M: < 3: 8 (44.44%); F: ≥ 3: 10 (55.56%) rs2072906: GG M: < 3: 8 (42.11%); F: ≥3: 11 (57.89%)	rs738409: 0.039 (CC *vs* CG: P = 0.014; CC *vs* GG: P = 0.033); rs3747206: 0.039 (CC *vs* CG: P = 0.014; CC *vs* GG: P = 0.033); rs4823173: 0.020 (GG *vs* GA: P = 0.006; GG *vs* AA: P = 0.023); rs2072906: 0.021 (AA *vs* AG: P = 0.009; AA *vs* GG: P = 0.016)
Fibrosis grading	rs738409: CC M: ≤ 1: 11 (91.67%); F: > 1: 1 (8.33%) rs3747206: CC M: ≤ 1: 11 (91.67%); F: > 1: 1 (8.33%) rs4823173: GG M: ≤ 1: 12 (92.31%); F: > 1: 1 (7.69%) rs2072906: AA M: ≤ 1: 12 (92.31%); F: > 1: 1 (7.69%)	rs738409: CG M: ≤ 1: 17 (62.96%); F: > 1: 10 (37.04%) rs3747206: CT M: ≤ 1: 17 (62.96%); F: > 1: 10 (37.04%) rs4823173: GA M: ≤ 1: 16 (61.54%); F: > 1: 10 (38.46%) rs2072906: AG M: ≤ 1: 16 (64.0%); F: > 1: 9 (36.0%)	rs738409: GG M: ≤ 1: 8 (44.44%); F: > 1: 10 (55.56%) rs3747206: TT M: ≤ 1: 8 (44.44%); F: > 1: 10 (55.56%) rs4823173: AA M: ≤ 1: 8 (44.44%); F: > 1: 10 (55.56%) rs2072906: GG M: ≤ 1: 8 (42.11%); F: > 1: 11 (57.89%)	rs738409: 0.032 (CC *vs* GG: P = 0.009); rs3747206: 0.032 (CC *vs* TT: P= 0.009); rs4823173: 0.024 (GG *vs* GA: P = 0.044; GG *vs* AA: P = 0.006); rs2072906: 0.015 (AA *vs* GG: P = 0.004)

NAS: Nonalcoholic fatty liver disease activity score.

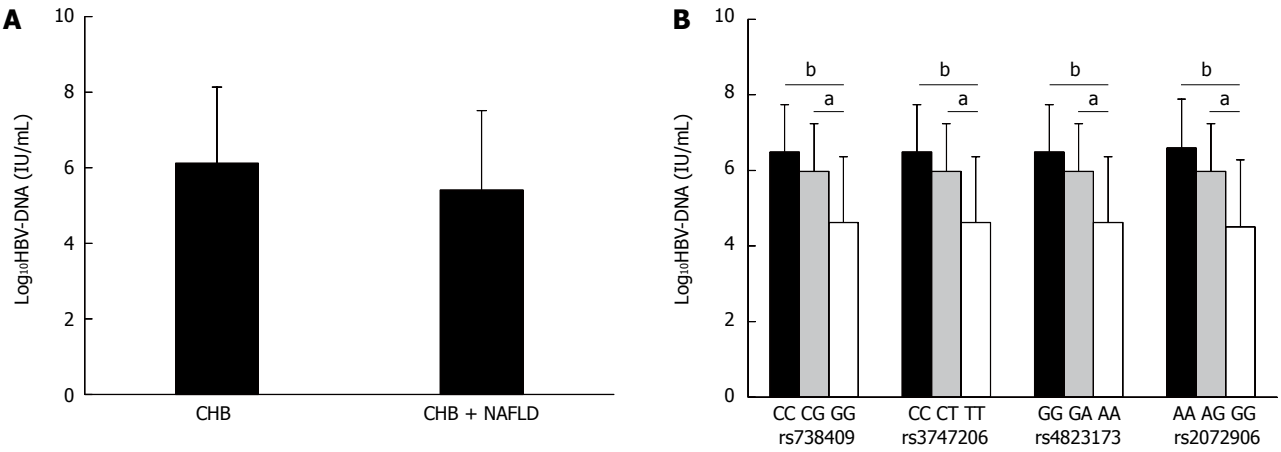

Figure 2 Association of *PNPLA3* SNPs and serum HBV-DNA level. A: Comparison of serum HBV-DNA level between CHB + NAFLD and CHB patients; B: Association of *PNPLA3* SNPs and serum level of HBV-DNA in CHB + NAFLD patients. CHB: Chronic hepatitis B; HBV: Hepatitis B virus; NAFLD: Nonalcoholic fatty liver disease; SNP: Single-nucleotide polymorphism. $^{a}P < 0.05$; $^{b}P < 0.01$.

A/A allele at rs4823173, and G/G allele at rs2072906 were characterized by an HBV-DNA level lower than the patients with C/C or C/G alleles at rs738409, C/C or C/T alleles at rs3747206, G/G or G/A alleles at rs4823173, and A/A or A/G alleles at rs2072906 (Figure 2B).

DISCUSSION

PNPLA3 is a hepatocyte-located adiponutrin, and has recently been shown to influence genetic predisposition to NAFLD[22-24]. A commonly prevalent *PNPLA3* SNP, the rs738409 G allele, contributes to the increased liver fat content in various populations[4-6,11,25], regardless of sex[5], age[26,27], or underlying diseases[6,28]. Moreover, association studies have confirmed that patients carrying the rs738409 SNP of *PNPLA3* are susceptible to NASH[7,25,29,30] and liver fibrosis[25,31-33]. There are similar findings for multiple *PNPLA3* SNPs, including rs2281135, rs139051, rs2294918, and rs6006460[10-13]. Therefore, polymorphism of *PNPLA3* plays an important role in the development of a full spectrum of NAFLDs, which includes simple steatosis, steatohepatitis, and liver fibrosis. Mechanistically, the effect of *PNPLA3* polymorphism on NAFLD has been attributed to abnormalities in plasma alanine and aspartate transaminase and ferritin levels[9,10,22,31], and abdominal fat (waist circumference-to-height ratio above 0.62)[9]. However, the *PNPLA3*-mediated liver enzyme change is independent of obesity, insulin resistance, and metabolism syndrome[29]. The rs738409 G allele of *PNPLA3* is even associated with decreased triglyceride levels in NAFLD patients of Malaysia[34].

In the present study, *PNPLA3* polymorphisms (rs738409, rs3747206, rs4823173, and rs2072906) were strongly associated with susceptibility to NAFLD in CHB patients from Northern, Central, and Southern China. This effect was independent of age, sex and BMI. Strikingly, these four SNPs were present in an 8-kb sequence of *PNPLA3* and showed strong LD with each other. When evaluated by NAS score ($\geqslant 3$), CHB patients with the rs738409 G allele, rs3747206 T allele, rs4823173 A allele, and rs2072906 G allele had greater susceptibility to suspected NASH or NASH than those with the rs738409 C allele, rs3747206 C allele, rs4823173 G allele, and rs2072906 A allele. These findings demonstrate that the role of *PNPLA3* polymorphisms in CHB patients is consistent with that in general population[11,24,32,35]. Thus, in contrast to the HCV-induced hepatocyte steatosis in chronic hepatitis C patients, host metabolism, rather than viral infection, is responsible for the development of fatty liver disease, especially NASH, in CHB patients.

rs738409, rs3747206, rs4823173, and rs2072906 of *PNPLA3* were not significantly associated with histologic parameters of NAFLD (steatosis and lobular inflammation), with the exception of ballooning. With regard to hepatosteatosis, there are conflicting reports concerning the correlation between hepatocyte steatosis and *PNPLA3* polymorphisms. The rs738409 G allele of *PNPLA3* was previously associated with steatosis grade in NAFLD patients in Italy and Japan[25,28], and with hepatic fat content in NAFLD patients in different ethnic subgroups (Caucasians, African Americans, and Hispanics) and different ages (children, adolescents and adults) in the United States[26,27]. However, the correlation between steatosis and rs738409 was not confirmed in four other groups of NAFLD patients from Malaysia, Northern Europe, and Japan[20,29,31,34]. Ethnic subgroups of Chinese, Indian, Malay, Japanese, Danish, Norwegian, Finnish, and Swedish populations were included in these studies. Similarly, *PNPLA3* polymorphism does not affect the lobular inflammation in Chinese, Indian, and Malay individuals with NAFLD[29]. On the basis of the slightly higher prevalence of hepatic steatosis and lobular inflammation, together with the prominent liver ballooning in subjects with the G allele at rs738409, T allele at rs3747206, A allele at rs4823173, and G allele

at rs2072906, we speculate that the accumulated effect of *PNPLA3* polymorphisms on pathologic characteristics may influence the occurrence of NAFLD in CHB patients.

Another noticeable result lies in the effect of *PNPLA3* polymorphisms on liver fibrosis. Liver fibrosis is one of the clinical outcomes of NAFLD[7], thus, most patients from the CHB + NAFLD group showed liver fibrosis, regardless of *PNPLA3* genotype. Perisinusoidal and portal fibrosis characterized their pathologic disorders on the basis of hepatosteatosis, ballooning, and lobular inflammation. Progressive liver fibrosis (> 1 Metavir grading), however, prevailed in CHB + NAFLD patients carrying G/G allele at rs738409 (C/C *vs* G/G: $P < 0.01$), T/T allele at rs3747206 (C/C *vs* T/T: $P < 0.01$), A/A allele at rs4823173 (G/G *vs* A/A: $P < 0.01$; G/G *vs* G/A: $P < 0.05$), and G/G allele at rs2072906 (A/A *vs* G/G: $P < 0.05$). These results suggest that *PNPLA3* polymorphism is a critical factor in the progression of NAFLD-related fibrosis in CHB patients, which is in accordance with previous reports in general populations[29,31]. NASH is the key step from simple steatosis to liver fibrosis. Therefore, the association between *PNPLA3* polymorphisms and liver fibrosis may be attributed to severe NASH correlating with rs738409, rs3747206, rs4823173, and rs2072906.

In addition to the interaction between *PNPLA3* polymorphisms and hepatic pathologic parameters, four linked SNPs of *PNPLA3* exhibited a striking association with viral dynamics in our study. When compared to that of CHB patients, the serum level of HBV-DNA showed a decreasing trend in patients with NAFLD and CHB. Furthermore, there was a significant reduction of log10 HBV-DNA in CHB + NAFLD patients with the G/G allele at rs738409, T/T allele at rs3747206, A/A allele at rs4823173, and G/G allele at rs2072906. *PNPLA3* polymorphisms seem to serve as the negative regulator of HBV replication.

NAFLD and CHB have been shown to share a common mechanism, namely, abnormality in the Toll-like receptor (TLR)-mediated innate immune response[35-38]. Suppression of the innate immune response in parenchymal and nonparenchymal liver cells underlies chronic infection with HBV[35]. In contrast, TLR4, one of the members of pattern recognition receptor family, is activated by lipopolysaccharide in hepatocytes and Kupffer cells during high-fat diet-induced NAFLD[35-37]. Activation of TLR4 initiates the innate immune response via MyD88-dependent and MyD88-independent signaling pathways. In patients with both NAFLD and CHB, inhibition of the TLR4-dependent innate immune system could be reactivated. Upregulation of TLR4/MyD88 signaling promotes the expression of various inflammatory cytokines, including interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor-α[39-42]. Replication of HBV, evaluated by HBV covalently closed circular DNA, is then repressed by tumor necrosis factor-α, IL-1β, and IL-6, partially via oxidative stress and inhibition of regulatory T cells[39-43]. In agreement with our findings, NAFLD-based downregulation of viral replication has been confirmed in a clinical trial of chronic hepatitis C patients[20] and a transgenic mouse model of HBV[44].

In summary, linked SNPs of *PNPLA3* (rs738409, rs3747206, rs4823173, rs2072906) are correlated with susceptibility to NAFLD, NASH, and liver fibrosis in Chinese CHB patients. In addition to fatty liver disease, these *PNPLA3* polymorphisms may exert an inhibitory effect on HBV-DNA level.

COMMENTS

Background

With the increasing prevalence of obesity and metabolic syndrome, nonalcoholic fatty liver disease (NAFLD) has become a commonly occurring liver disease and a public health burden in most areas of China. The high prevalence of hepatitis B virus (HBV) infection in the Chinese population causes concurrent NAFLD and chronic hepatitis B (CHB). Previous studies have indicated that *PNPLA3* polymorphisms play an important role in the development of NAFLD. However, the role of the *PNPLA3* polymorphism in patients with both NAFLD and CHB has not yet been established.

Research frontiers

PNPLA3 is a hepatocyte-located adiponutrin, and has recently been shown to influence genetic predisposition to NAFLD. In patients with concurrent CHB and NAFLD, the research hotspot lies in uncovering the association of *PNPLA3* polymorphisms with clinical, laboratory, and pathologic characteristics of NAFLD, and also with HBV dynamics.

Innovations and breakthroughs

To shed light on the effect of *PNPLA3*, a cohort of Han patients with biopsy-proven CHB, with or without NAFLD, and normal controls were enrolled from Northern (Tianjin), Central (Shanghai), and Southern (Zhangzhou) China. Their *PNPLA3* polymorphisms were genotyped by gene sequencing. The association between *PNPLA3* polymorphisms and susceptibility to NAFLD, and clinical characteristics of NAFLD were assessed on the basis of physical indices, liver function tests, glycolipid metabolism, and histopathologic scoring. Association of *PNPLA3* polymorphisms and HBV load was determined by serum level of HBV-DNA. This study found that four linked single nucleotide polymorphisms (SNPs) of *PNPLA3* (rs738409 G allele, rs3747206 T allele, rs4823173 A allele, and rs2072906 G allele) conferred susceptibility to NAFLD, nonalcoholic steatohepatitis (NASH), and liver fibrosis in CHB patients. Patients carrying these SNPs showed significantly lower serum levels of HBV-DNA.

Applications

The results suggest that four linked SNPs of *PNPLA3* (rs738409, rs3747206, rs4823173, and rs2072906) are correlated with susceptibility to NAFLD, NASH, and liver fibrosis in CHB patients. These *PNPLA3* polymorphisms may play a regulatory role in HBV dynamics.

Terminology

PNPLA3 is a gene on chromosome 22q13, which encodes adiponutrin, a protein with triacylglycerol hydrolase and acylglycerol transacetylase activity in hepatocytes. SNPs in *PNPLA3*, such as rs738409 C>G, result in an adiponutrin variant with I148M (isoleucine-to-methionine substitution at residue 148), which has been identified as a predisposing factor for liver steatosis.

Peer-review

This is an interesting work on the effect of *PNPLA3* polymorphisms and their association with NAFLD/NASH and HBV viral load in patients with CHB. The work is well designed, performed, and analyzed, and the findings are clear and

well presented in the Results section and Figures.

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