Invited original article

Title:

Associations of hydroxysteroid 17-beta dehydrogenase 13 variants with liver histology in Chinese patients with MAFLD

Short title:

HSD17B13 variants and MAFLD

Authors' name:

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Abbreviations:

MAFLD = metabolic associated fatty liver disease; MAFL = metabolic associated fatty liver; HCC = hepatocellular carcinoma ; BMI = body mass index; HOMA = homoeostasis model assessment; IR = Insulin resistance; GWAS = genome-wide association studies; SNP = single nucleotide polymorphism; MAF = minor allele frequency; *HSD17B13* = hydroxysteroid 17-beta dehydrogenase 13; *PNPLA3* = patatin-like phospholipase domain containing protein 3; TLR3 = toll-like receptor 3; IFNL3 = interferon lambda-3 ; TLL1 = tolloid like 1; MICA = MHC class I polypeptide-related chain A

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Informed consent: Written informed consent was obtained from all participants included in the study.

Abstract

Background and objective:

In Europeans, variants in the hydroxysteroid 17-beta dehydrogenase 13 (*HSD17B13*) gene impact the liver histology of metabolic associated fatty liver disease (MAFLD). The impact of these variants in ethnic Chinese is unknown. The aim of this study was to investigate the potential associations in Chinese patients.

Methods:

427 Han Chinese with biopsy-confirmed MAFLD were enrolled. Two single nucleotide polymorphisms in *HSD17B13* were genotyped - rs72613567 and rs6531975. Logistic regression was used to test the association between the SNPs and liver histology.

Results:

In our cohort, the minor allele TA of the rs72613567 variant was related to an increased risk of fibrosis [OR = 2.93 (1.20-7.17), P = 0.019 for the additive model; OR = 3.32 (1.39-7.91), P = 0.007 for the recessive model], an inverse association as compared to the results from European cohorts. In contrast, we observed a protective effect on fibrosis for the minor A allele carriers of the *HSD17B13* rs6531975 variant [OR = 0.48 (0.24-0.98), P = 0.043 for the additive model; OR = 0.62 (0.40-0.94), P = 0.025 for the dominant model]. *HSD17B13* variants were only associated with fibrosis but not other histological features. Furthermore, *HSD17B13* rs6531975 modulated the effect of *PNPLA3* rs738409 on hepatic steatosis.

Conclusions:

The *HSD17B13* rs72613567 variant is a risk variant for fibrosis in a Han Chinese MAFLD population but with a different direction for allelic association to that seen in Europeans. This data exemplifies the need for studying diverse populations in genetic studies in order to fine map GWAS signals.

Keywords:

Metabolic associated fatty liver disease; Non-alcoholic fatty liver disease; Hydroxysteroid 17-beta dehydrogenase 13; Single nucleotide polymorphism

Introduction

Metabolic associated fatty liver disease (MAFLD) is recognized as a leading cause of liver-related morbidity and mortality.[1,2] In China, MAFLD burden is increasing, with prevalence rising from 18% to 29% in the last decade.[3] MAFLD comprises a spectrum of disease ranging from simple steatosis or MAFL to the presence of steatohepatitis with varying degrees of fibrosis and cirrhosis.[4] MAFLD arises from "multiple hits", with genes acting as an important modifier of the clinical phenotype.[5] Though our understanding of the underpinnings of MAFLD has been enhanced by numerous genetic association studies, all identified polymorphisms to date explain only 10–20% of disease heritability.[6,7]

It is broadly acknowledged that there is over-representation of subjects of European ancestry in human genetics research with ~79% of all GWAS participants of European descent. This overrepresentation hinders a complete understanding of the human genetic architecture. Moreover, it can also have a negative impact including prediction accuracies between 1.6-4.9 fold lower in other ethnicities compared to Europeans.[8] Hence, increasing the representation of diverse populations and studying other ethnicities has become a research priority.

Several variants in the hydroxysteroid 17-beta dehydrogenase 13 (*HSD17B13*) gene encoding a hepatic lipid droplet protein have been identified to impact the histological features of MAFLD. However, the impact of *HSD17B13* gene variants on MAFLD histology among those of Chinese ancestry is unknown. Notably, allele frequencies, haplotype patterns and the effect size of polymorphisms vary considerably across populations and ethnicities.[6] As *HSD17B13* has been proposed as a therapeutic target for MAFLD, it is pivotal to explore whether the effect of this variant observed in Caucasian populations extends to other populations, as also the effect size.

It is known that the genetic association of variants in *HSD17B13* with the histological features of MAFLD is complex, with different potentially causative SNPs and various SNPs associated with different phenotypic patterns. For example, alleles of rs6834314 and rs72613567 associate with decreased injury and with increased hepatic fat.[9] However, there are other studies that show no association of rs72613567 with steatosis.[10,11] Noncoding SNPs (rs6531975) not in linkage disequilibrium with rs72613567 have also

been associated with decreased hepatic fat.[9] Adding to this complexity, a recent study of 487 patients suggested that those harboring the 'protective' *TA*-allele of rs72613567 have a numerically increased risk for mortality, liver-related death and hepatic decompensation.[12] Likewise, while some reports suggested that there is a potential interaction between *HSD17B13* and variants in patatin-like phospholipase domain containing protein 3 (*PNPLA3*) in MAFLD, subsequent reports have failed to discern an association.[13,14] Given these controversies, the aims of this study were 1) to explore the role of variants in the *HSD17B13* gene in a cohort of Han Chinese with biopsy-confirmed MAFLD, 2) to clarify the role of the variants on the various morphological features of MAFLD and 3) to discern if there is any interaction between the variants and variants in *PNPLA3*.

Materials and Methods

Study population

We recruited 427 consecutive Han Chinese with biopsy-confirmed MAFLD from the PERSONS cohort (2017.01-2019.05). The definition of MAFLD was based on the criteria proposed by an international expert panel.[15] The study cohort included patients from a previously published study as well as additional subjects.[16] To ascertain the effects of the *HSD17B13* variant on liver disease solely due to MAFLD, patients with other causes of liver disease including alcohol use disorder or viral hepatitis were excluded. Briefly, all consecutive patients aged ≥ 18 with biopsy-proven MAFLD without alternative causes of liver disease were recruited to the study. The study protocol was approved by the ethics committee of the First Affiliated Hospital of Wenzhou Medical University (2016-246, 1 December 2016) and registered in the Chinese Clinical Trial Registry (ChiCTR-EOC-17013562). Written informed consent was obtained from each subject before their participation in the study. Patients identifiers were anonymized and replaced by the health examination number.

Clinical and biochemical data

Clinical and biochemical data were collected from all patients within 24 hours of liver biopsy. Body mass index (BMI) was calculated as weight (kg) divided by the square of height (m). Insulin resistance (IR) was estimated according by the homoeostasis model assessment (HOMA-IR).[17] Diagnosis of diabetes was based on criteria of the American Diabetes Association.[18]

Assessment of liver histology

Liver biopsies were performed using a 16-gauge needle under ultrasound guidance. The histology was reviewed by a single liver pathologist (X.D. Wang) who was blinded to the clinical and biochemical data. Histologic scoring was based on the Activity Score.[19] Steatohepatitis was diagnosed as a score ≥ 4 and a score of at least one for each of steatosis, ballooning and lobular inflammation. Severe steatosis, severe ballooning and severe lobular inflammation were defined if their scores were more than or equal to 2.

Genetic analysis

Genotyping for the HSD17B13 (rs72613567 and rs6531975) and PNPLA3 (rs738409) variants were performed using the MassARRAY or TaqMan assays platform according to the manufacturer's protocol. For the purpose of genotyping, each sample used approximately 20 ng of genomic DNA. Locus-specific PCR and detection primers were designed using Assay Design Suite v3.1.

Statistical analysis

Statistical analyses were performed using R software (version 3.5.2, R Foundation for Statistical Computing, Vienna, Austria) and SPSS 19.0 (SPSS Inc. Chicago, IL, USA). Continuous variables are expressed as mean ± SD and compared using the One-way ANOVA analysis. Categorical variables are expressed as frequency (%) and compared using the chi-square test. Multivariable logistic regression models were undertaken to test the association between the aforementioned SNPs and liver histology features. $P < 0.05$ was considered to be statistically significant.

Results

Patient characteristics

The study comprised 427 consecutive biopsy-confirmed MAFLD patients; their clinical, biochemical, and histological features are depicted in **Supplementary Table 1**. The average age was 41 years with 73.8% being male. About 287 (67.2%) had fibrosis (≥ F1), 226 (52.9%) had severe steatosis (S2-S3), 157 (36.8%) had severe ballooning (B2) and 84 (19.7%) had severe inflammation (A2-A3).

Genotype distribution, HWE calculations

Two single nucleotide polymorphisms in *HSD17B13* were genotyped - rs72613567 and rs6531975. Genotype distribution of rs72613567 and rs6531975 in *HSD17B13* was in Hardy–Weinberg equilibrium (All $P > 0.05$). The minor allele frequency (MAF) for rs72613567 and rs6531975 were 0.32 and 0.30 in our cohort, respectively. It is close to the MAF in general East Asian population in the 1000 genomes project.[20] The overall genotype distribution of rs72613567 T/T, T/TA and TA/TA was 47.3%, 42.0%, and 10.7%, while the distribution of rs6531975 G/G, G/A and A/A was 49.8%, 40.5%, and 9.8%, respectively.

Clinical and laboratory characteristics stratified by *HSD17B13* variants

The baseline characteristics of study participants according to rs72613567 genotypes is presented in **Table 1**. There were significant differences in levels of fasting glucose, triglycerides and HDL-cholesterol among rs72613567 genotypes (All $P < 0.05$). **Table 2** shows the baseline characteristics of study participants according to rs6531975 genotypes. No significant differences were observed among rs6531975 genotypes.

HSD17B13 variants and hepatic steatosis

The proportion of severe steatosis in rs72613567 T/T, T/TA and TA/TA was 103 (52.0%), 91 (51.7%) and 27 (60.0%), while the proportion of severe steatosis in rs6531975 G/G, G/A and A/A was 113 (54.1%), 84 (49.4%) and 24 (58.5%) (**Table 3**). No association between *HSD17B13* variants and severe steatosis was observed in multivariable analysis (**Table 4**).

HSD17B13 variants and hepatocyte ballooning and lobular inflammation

The proportion of severe ballooning in rs72613567 T/T, T/TA and TA/TA was 73 (36.9%), 58 (33.0%) and 21(46.7%), while the proportion of severe ballooning in rs6531975 G/G, G/A and A/A was 79 (37.8%), 63 (37.1%) and 11 (26.8%). The proportion of severe inflammation in rs72613567 T/T, T/TA and TA/TA was 35 (17.7%), 35 (19.9%) and 12 (26.7%), while the proportion of severe inflammation in rs6531975 G/G, G/A and A/A was 40 (19.1%), 35 (20.6%) and 8 (19.5%) (**Table 3**). Both severe ballooning and inflammation were unrelated to *HSD17B13* variants in multivariable analysis (**Table 4**).

HSD17B13 variants and fibrosis

The prevalence of having fibrosis in rs72613567 T/T, T/TA and TA/TA was 135 (68.2%), 111 (63.1%) and 38 (84.4%). A higher prevalence of fibrosis was observed in patients with the TA/TA genotype in rs72613567 ($P < 0.05$) (**Table 3**). In rs6531975 genotypes, the prevalence of having fibrosis in G/G, G/A and A/A was 150 (71.8%), 109 (64.1%) and 23 (56.1%). The A allele carriers of rs6531975 showed a non-significant trend for a reduced prevalence of having fibrosis ($P = 0.082$) (**Table 3**).

To further understand the association between *HSD17B13* variants and liver histology in Chinese patients with MAFLD, multivariable logistic regression models were undertaken. As shown in **Table 4**, rs72613567 TA/TA increased the risk of fibrosis with an odds ratio (OR) of 2.93 (TA/TA vs. T/T, 95% CI: 1.20-7.17, $P = 0.019$) for the additive model and an OR of 3.32 (TA/TA vs. T/T+T/TA, 95% CI: 1.39-7.91, $P = 0.007$) for the recessive model after adjusting for age, sex, BMI, presence of diabetes,

fasting glucose, triglycerides and HDL-cholesterol. In contrast, the rs6531975 A allele appeared to have a protective impact on fibrosis with an OR of 0.48 (A/A vs. G/G, 95% CI: 0.24-0.98, P = 0.043) for the additive model and an OR of 0.62 (G/A + A/A vs. G/G, 95% CI: 0.40-0.94, P = 0.025) for the dominant model after adjusting for age, sex, BMI and presence of diabetes.

Interaction of *PNPLA3* and *HSD17B13* variants

Next, we conducted interaction analysis for *HSD17B13* (rs72613567 and rs6531975) and *PNPLA3* (rs738409) variants for their impact on liver histology. For fibrosis, no interaction effects were observed between the two genes. In contrast, there was an interaction between rs6531975 and rs738409 with regard to hepatic steatosis (**Figure 1**). For the rs738409 risk allele carriers (CG + GG), the proportion of severe steatosis was lower in patients with the rs6531975 A allele (G/A + A/A), compared to that with rs6531975 G/G **(Figure 1A**). Using the latter as reference, patients with the rs6531975 A allele (G/A + A/A) attenuated the risk effect of the rs738409 G allele (C/G + G/G) on steatosis with an OR of 0.57 (95% CI: 0.34-0.96, P = 0.034) after adjusting for age, sex, BMI and presence of diabetes (**Figure 1B**). The interaction between rs72613567 and rs738409 on liver steatosis was also performed (**Figure 2**), however no effect was observed.

Discussion

We characterized the impact of *HSD17B13* gene variants on histological features in a cohort of Han Chinese with MAFLD. This study has three key findings. First, we confirmed the *HSD17B13* region as a susceptibility locus for MAFLD related fibrosis but extend these findings to identify an inverse allelic direction of association as compared to that reported in Europeans. Second, the *HSD17B13* variants are only associated with fibrosis and not any other histological feature. Third, the *HSD17B13* variants modulates the effect of *PNPLA3* rs738409 on hepatic steatosis, but not other histological features.

The association between *HSD17B13* variants and liver histological features seems to be complex, with multiple suggested functional variants. Notably, in our cohort the minor allele TA of the rs72613567 variant was related to an increased risk of fibrosis, an inverse association as compared to the results in European cohorts. Hence, if there is a shared causal variant across European and Chinese populations, it is unlikely to be rs72613567. In this regard, we observed a protective effect for the minor A allele carriers of the *HSD17B13* rs6531975 variant, but this is not in strong linkage disequilibrium with rs72613567. Thus, further fine-mapping studies in Han Chinese populations and comparison to other populations would be helpful to identify shared causal variants across different ethnicities.

The differential effect size and allele direction of variants discovered by GWAS between ethnicities is not uncommon. In one Chinese MAFLD cohort, researchers found that the neurocan (*NCAN*) rs2228603 T variant associated with a higher level of HDL,[21] while it is positively related to liver steatosis in the US population.[22] Similarly, toll-like receptor 3 (*TLR3*) rs3775290[23,24] and interferon lambda-3 (*IFNL3*) rs12979860[25,26] variants in Chinese hepatocellular carcinoma (HCC) populations show opposite effects to those in non-Asian populations. Inconsistent results have also been observed in other Asian populations such as among Japanese. For example, tolloid like 1 (*TLL1*) rs17047200[27] and MHC class I polypeptide-related chain A (*MICA*) rs2596542[28] variants were suggested to have protective impacts on fibrosis and HCC in Caucasians. The association was inverse to those of a Japanese cohort[29,30]. Besides, there are several MAFLD-related SNPs in Europeans for which there has been no association in Chinese populations[31-33]. Along the same line, lower genetic prediction accuracies between 1.6-4.9 fold lower was observed in other ethnicities compared to Europeans.[8] Hence, increasing the representation of diverse populations and studying other ethnicities has recently become a research priority to enhance understanding of the human genetic architecture and its translational implications.

The ethnic differences in the characteristics of patients with MAFLD might also contribute to the observed differences in the genetic findings. There is growing evidence for example that MAFLD disease course in Asian populations is different to that in Caucasians. As an example, for the same BMI, there is a higher prevalence of MAFLD in Asians. Published reports also indicate that lean MAFLD accounts for

36.9% of cases in China,[3] but only 17.3 % of the total disease burden in the United States.[34] Differences in metabolic adaptation has been reported between lean and non-lean MAFLD patients suggesting that lean fatty liver disease likely has a distinct pathophysiology.[35]

Another intriguing aspect of this study is the lack of association between *HSD17B13* variants and other histological features. To date the nature of the association between the rs72613567 allelic variant and histological features of MAFLD, particularly steatosis is unclear. Abul-Husn and colleagues suggested a lack of association between the rs72613567 TA variant and steatosis in human liver,[10] consistent with the study of Pirola et al.[11] However, a study by Ma et al. found a significant association with hepatic steatosis.[9] Similarly, in animal and *in-vitro* studies, inconsistent results have been reported for an effect of *HSD17B13* on hepatic lipid accumulation. Abul-Husn et al showed no differences in lipid accumulation according *HSD17B13* isoforms.[10] Similarly Ma et al. reported that *HSD17B13* over-expression or knockout in HepG2 cells did not affect lipid content.[9] On the other hand, Marion et al. noted hepatic steatosis in *HSD17B13* knockout mice,[36] whilst Su et al. observed steatosis in mice that over-expressed *HSD17B13*.[37] Collectively, these results imply that *HSD17B13* variants could have a direct impact on fibrosis rather than effects on steatosis. These findings may be associated with retinol metabolism since retinol plays a crucial role in the activation and transformation of hepatic stellate cells to matrix secreting myofibroblasts and the development of hepatic fibrosis.[38] Since *HSD17B13* participates in the rate limiting step of retinol metabolism,[9] the mutant in *HSD17B13* might conceivably influence the process of fibrosis.

The interaction between *HSD17B13* and *PNPLA3* variants in MAFLD is also a subject of controversy.[14,39] In this work, we noted an interaction between these variants with regard to steatosis, but not with other histological features. Furthermore, rs72613567 TA variant in *HSD17B13* was found to be associated with obesity in Europeans.[11] Potential interaction effects may exist between fat distribution and *HSD17B13* variants. We performed a logistic regression model to explore the interaction effects, however, nothing was found in adiposity measures and SNP genotypes on liver phenotypes (data was not shown in Result section). As *HSD17B13* has been suggested as a potential therapeutic target for MAFLD and the growing

concerns about the failure of phase 2 and 3 clinical trials in this disease[40,41] that is at least partially attributed to clinical heterogeneity, our study highlights the importance of first understanding the functional basis of the various proposed genomic and other targets before therapeutic development.[40,42] Collectively, our data suggest supports such an approach. The data from *HSD17B13*-knockout mice in fact suggests that HSD17B13 triggers steatosis and inflammation[36] which is opposite to what has been reported in humans.

The present study has limitations. Firstly, the sample size is modest. In case the opposite finding is due to the sample size, we have performed a post-hoc power analysis. The power calculated for the model was 72%. It is close to but less than 0.80. Considering the low proportion of the rs72613567 TA variant in the general population, we think it is acceptable. In addition, lack of a validation cohort from populations in other parts of China or those of Chinese ancestry living outside mainland China is another limitation.

In conclusion, the *HSD17B13* rs72613567 variant is a risk variant for hepatic fibrosis in a Han Chinese MAFLD population with a different direction for allelic association to that seen in Europeans.

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Competing interests

The authors declare that there are no conflicts of interest associated with this manuscript.

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Table Legends

Table 1. Baseline characteristics of biopsy-confirmed MAFLD patients according to rs72613567 genotypes.

Table 2. Baseline characteristics of biopsy-confirmed MAFLD patients according to rs6531975 genotypes.

Table 3. Liver histology features of biopsy-confirmed MAFLD patients according to *HSD17B13* genotypes.

Table 4. Association between *HSD17B13* variants and liver histology features in Chinese MAFLD patients.

Supplemental Table 1. Baseline characteristics of biopsy-confirmed Chinese MAFLD patients.

Figure Legends

Figure 1. The interaction of *HSD17B13* rs6531975 and *PNPLA3* rs738409 on liver steatosis.

Panel A shows the prevalence of mild steatosis and severe steatosis according to rs6531975 and rs738409 genotypes. Panel B shows the interaction effect of rs6531975 and rs738409 on steatosis after adjusting for age, sex, BMI and presence of diabetes. Patients with the rs6531975 A allele (G/A + A/A) attenuated the risk effect of the rs738409 G allele (C/G + G/G) on steatosis with an OR of 0.57 (95% CI: 0.34-0.96, P = 0.034).

Figure 2. The interaction of HSD17B13 rs72613567 and PNPLA3 rs738409 on liver steatosis.

Panel A shows the prevalence of mild steatosis and severe steatosis according to rs72613567 and rs738409 genotypes. Panel B shows the interaction effect of rs72613567 and rs738409 on steatosis after adjusting for age, sex, BMI and presence of diabetes. No interaction effect was observed in rs72613567 and rs738409.