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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Association of glucokinase regulatory gene polymorphisms with risk and severity of non-alcoholic fatty liver disease: an interaction study with adiponutrin gene

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Abstract

Background Recent genome-wide association studies demonstrated an association between single nucleotide polymorphisms (SNPs) on the glucokinase regulatory gene (*GCKR*) with hepatic steatosis. This study attempted to investigate the association of *GCKR* rs780094 and rs1260326 with susceptibility to non-alcoholic fatty liver disease (NAFLD) and its severity.

Methods The genotypes were assessed on 144 histologically confirmed NAFLD patients and 198 controls using a Sequenom MassARRAY platform.

Results The *GCKR* rs1260326 and rs780094 allele T were associated with susceptibility to NAFLD (OR 1.49, 95 % CI 1.09–2.05, $p = 0.012$; and OR 1.51, 95 % CI 1.09–2.09, $p = 0.013$, respectively), non-alcoholic steatohepatitis (NASH) (OR 1.55, 95 % CI 1.10–2.17, $p = 0.013$; and OR 1.56, 95 % CI 1.10–2.20, $p = 0.012$, respectively) and NASH with significant fibrosis (OR 1.50, 95 % CI 1.01–2.21, $p = 0.044$; and OR 1.52, 95 % CI 1.03–2.26, $p = 0.038$, respectively). Following stratification by ethnicity, significant association was seen in Indian patients between the two SNPs and susceptibility to NAFLD (OR 2.64, 95 % CI 1.28–5.43, $p = 0.009$; and OR 4.35, 95 % CI 1.93–9.81, $p < 0.0001$, respectively). The joint effect of *GCKR* with adiponutrin rs738409 indicated greatly increased the risk of NAFLD (OR 4.14, 95 % CI 1.41–12.18, $p = 0.010$). Histological data showed significant association of *GCKR* rs1260326 with high steatosis grade (OR 1.76, 95 % CI 1.08–2.85, $p = 0.04$).

Conclusion This study suggests that risk allele T of the *GCKR* rs780094 and rs1260326 is associated with predisposition to NAFLD and NASH with significant fibrosis. The *GCKR* and *PNPLA3* genes interact to result in increased susceptibility to NAFLD.

H.-L. Tan and S. M. Zain contributed equally to this project and should be considered co-first authors.

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Keywords *GCKR* · NAFLD · NASH · Steatosis · Polymorphism

Abbreviations

ALT	Alanine aminotranferase
ANOVA	Analysis of variance
AST	Aspartate aminotranferase
BMI	Body mass index
CI	Confidence interval
GCKR	Glucokinase regulatory
GGT	Gamma-glutamyl transpeptidase
GMDR	Generalized Multifactor Dimensionality Reduction
GWAS	Genome-wide association study
HDL	High density lipoprotein
HWE	Hardy–Weinberg equilibrium
LDL	Low density lipoprotein
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
OR	Odds ratio
PNPLA3	Patatin-like phospholipase domain-containing protein 3
SD	Standard deviation
SNP	Single nucleotide polymorphism
UMMC	University of Malaya Medical Center
US	Ultrasonography

Introduction

Non-alcoholic fatty liver disease (NAFLD) was first characterized in 1980 by fatty infiltration of the liver among non-alcoholic patients [1]. This public health problem is an important cause of liver-related morbidity and mortality [2, 3]. NAFLD encompasses a wide clinicopathologic spectrum ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) [4]. Simple steatosis is relatively benign and characterized by hepatic steatosis in the absence of substantial inflammation or fibrosis. The progression of NASH is characterized by cellular necrosis, hepatocyte ballooning, and inflammatory infiltration which may eventually lead to complications such as cirrhosis, liver failure and hepatocellular carcinoma [5, 6].

The global prevalence of NAFLD is estimated to be 30–40 % in adult, 40 % of whom may progress to develop NASH [7]. Since NAFLD is known to be the hepatic manifestation of metabolic syndrome, the present global epidemic of diabetes and obesity increases the importance of NAFLD research.

Familial clustering of NAFLD led to the investigation of genetic variants as possible etiological factors [8]. Recent genome-wide association studies (GWAS) have provided insights into possible molecular pathway linking the genes to NAFLD [9–11].

Single nucleotide polymorphisms (SNPs) on genes encoding proteins in the lipogenesis pathways have been associated with NAFLD. Our previous candidate gene study had indicated a strong association between a common missense variant (rs738409) in the patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) gene with susceptibility to NAFLD and NASH [12]. We then extended our study to include two SNPs in the glucokinase regulatory protein (*GCKR*) gene, rs1260326 and rs780094. Glucokinase (GCK) is a phosphorylating enzyme which regulates hepatic glucose metabolism and activates hepatic lipogenesis [13]. The glucokinase regulatory protein (GCKRP), a product of *GCKR* gene, binds to GCK allosterically and thereby regulates its activity.

The rs1260326, a non-synonymous *GCKR* variant, is characterized by a C to T substitution encoding a proline-to-leucine substitution at amino acid position 446 (P446L). GWAS and several cohort studies had reported the association of this variant with triglyceride and fasting plasma glucose levels [14–16]. GWAS analyses performed in the Finnish, Swedish [14] and Danish populations [17] reported that the nonfunctional *GCKR* rs780094 variant is in strong linkage disequilibrium with rs1260326 (HapMap CEU $r^2 = 0.93$, CHB $r^2 = 0.82$). Furthermore, *GCKR* rs780094 in these studies was associated with triglyceride levels [11, 18, 19]. As NAFLD is characterized by triglyceride accumulation in the hepatocytes, we hypothesized that *GCKR* variants are associated with NAFLD.

The present study aims to explore the association between *GCKR* variants and NAFLD. Additionally, we explored whether race modified the association between *GCKR* variants and NAFLD. Given the potential role of the studied SNPs in computed tomography-measured hepatic steatosis, we aimed to determine whether these SNPs are associated with the histological features of NAFLD as assessed by liver biopsy. Furthermore, we investigated the contribution of *GCKR* together with *PNPLA3* gene variants on susceptibility to the disease.

Methods

Subject recruitment

This case-control study enrolled 144 patients with histologically confirmed NAFLD and 198 control subjects without NAFLD. Ethnicities of the subjects were validated by affirmations of no mixed marriages for at least three generations. All subjects were recruited from the University Malaya Medical Center (UMMC).

Blood samples were taken and the following data were obtained: age, sex, weight, height, HbA1c, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, total cholesterol, and serum triglyceride level. The biochemical tests were measured according to standard hospital clinical laboratory techniques. Body mass index (BMI) was defined as weight/height^2 (kg/m^2). Additional clinical parameters such as waist circumference, systolic and diastolic blood pressure and pulse rate were measured as standard protocol.

Controls were genetically unrelated healthy subjects with BMI (<25 kg/m^2), fasting plasma glucose (<110 mg/dL), normal lipid profile and normal liver enzymes. A liver ultrasonography (US) was performed on all controls, and they were excluded if they had any of the following: (i) slight diffuse increase in bright homogeneous echoes in the liver parenchyma with normal visualization of the diaphragm and portal and hepatic vein borders, and normal hepatorenal echogenicity contrast; (ii) diffuse increase in bright echoes in the liver parenchyma with slightly impaired visualization of the peripheral portal and hepatic vein borders; (iii) marked increase in bright echoes at a shallow depth with deep attenuation, impaired visualization of the diaphragm, and marked vascular blurring [20].

All NAFLD patients underwent a liver biopsy following a finding of increased echogenicity (compared to renal cortex) on ultrasound. All cases were confirmed by liver histology, and NASH was diagnosed based on recommendations by the NASH Clinical Research Network [21, 22]. All the biopsy specimens were 1.5 cm long, on average, and contained at least six portal tracts. Subjects were excluded from this study for the following reasons: presence of fatty accumulation around the liver, alcohol consumption of >10 g daily [23], viral hepatitis B and hepatitis C infection, primary biliary cirrhosis, use of drugs known to precipitate steatosis, autoimmune liver disease, Wilson's disease and α-1-antitrypsin deficiency.

Written informed consent was obtained from each participant prior to their participation in the study. The protocol was approved by the Medical Ethics Committee of UMMC.

Genotyping assay

Genomic DNA was extracted from peripheral white blood cells using the DNA Blood Mini Kit (Qiagen, German) according to the manufacturer's protocol. *GCKR* rs1260326 and rs780094 were genotyped using the Sequenom MassARRAY technology platform with the iPLEX GOLD chemistry (Sequenom, San Diego, CA). Designation of the specific assays with proximal SNPs filtering was performed using the MassARRAY Assay Design software package (v4.0).

A total of 1 μL of genomic DNA at a concentration of 10–20 ng/μL were employed in each amplification reaction. Reaction products were desalted with SpectroClean resin (Sequenom, San Diego, CA) after single-base extension. A volume of 10 nL of reaction products was spotted onto the SpectroCHIP using the MassARRAY Nanodispenser and the mass was determined using a MassARRAY Analyzer Compact MALDI-TOF mass spectrometer. The MassARRAY® Typer 4.0 software was used for data acquisition and analysis. Genotypes were assigned after cluster analysis using the default setting of Gaussian mixture model. Clusters were inspected to yield a clear cluster separation with good signal to noise cut-off. Manual review was performed to further clarify uncertain genotype calls.

A blank and five duplicates were introduced for quality control. The SpectroChip failed quality control of any of the following occurred: (i) Assay with <80 % call rate within the same SpectroChip; (ii) >25 % call rate in the blank control; (iii) <99.5 % concordance in duplicate checks; (iv) >10 % call rate in blank checks. These assays were repeated.

Statistical analysis

The genotype distribution was assessed for Hardy–Weinberg equilibrium (HWE) using χ^2-test. A p value of more than 0.05 indicates an agreement with HWE. Power analysis was carried out using Quanto by assuming a gene-only effect. A total of 116 NAFLD cases and 160 controls provide 80 % power at α of 0.05 according to the following assumptions: the allele frequency ranges from 0.30 to 0.40, baseline risk for the Malaysian population is 0.17 and the minimum detectable odds ratio was 2.0.

The NAFLD cases ($n = 144$) could be further classified, based on biopsy, to simple steatosis ($n = 33$), NASH ($n = 111$), NASH with no significant fibrosis (fibrosis score <2, $n = 37$), and NASH with significant fibrosis (fibrosis score ≥2, $n = 74$). Continuous data were tested using independent t test for normally distributed variables and Mann–Whitney U test for non-normally distributed variables.

Genotype was coded with 0, 1, or 2 corresponding to the number of minor alleles carried by each individual. The association between genotype and spectrum of NAFLD was evaluated using an additive model of inheritance. Regression analysis demonstrated a significant association with ethnicity but not with age and gender. Multiple logistic regression adjusted for potential confounding by ethnicity on susceptibility to NAFLD.

Association of histological ordinal variables and genotypes were determined using univariate analysis via the Jonckheere–Terpstra test. Multivariate analysis of histological ordinal variables was performed using ordinal

regression. Statistical analyses were performed using SPSS 16.0 (Chicago IL) with a two-sided $p < 0.05$ considered as statistically significant.

The association between the clinical parameters and genotypes were compared using the Kruskal–Wallis test for skewed variables and Analysis of Variance (ANOVA) for normally distributed variables. Data are expressed as mean and standard deviation (SD) unless otherwise stated.

The Generalized Multifactor Dimensionality Reduction (GMDR) method was used to determine the influence of *GCKR* and *PNPLA3* gene–gene interaction on NAFLD. Possible interactions were assessed using ten-fold cross validation with an exhaustive search considering ethnicity as covariate. The parameters outcome of GMDR analysis includes the cross validation consistency, testing balanced accuracy, and empirical *p* values [24]. The finding was then confirmed by performing an additive logistic regression model that takes into account all risk alleles of the SNPs.

Results

Table 1 outlines the demographic and clinical data of the subjects consisted of 144 NAFLD patients and 198 controls. The demographic and clinical data of the NAFLD patients who were stratified into simple steatosis and NASH are described in Table 2.

Table 1 Demographic and clinical data of the subjects

Characteristics	*n* (%) or Mean ± SD		*p* value
	Control (*n* = 198)	NAFLD (*n* = 144)	
Gender			
Males	85 (43)	77 (53)	0.054
Females	113 (57)	67 (47)	
Ethnicity			
Malays	80 (41)	59 (41)	0.044
Chinese	54 (27)	54 (38)	
Indians	64 (32)	31 (21)	
Age (years)	53.1 ± 11.5	51.1 ± 12.0	
BMI (kg/m^2)	22.7 ± 2.6	28.7 ± 4.4	<0.0001
HbA1c (%)	5.7 ± 0.8	6.6 ± 1.7	<0.0001
HDL cholesterol (mg/dl)	49.5 ± 12.9	48.5 ± 12.7	<0.0001
LDL cholesterol (mg/dl)	89.5 ± 22.4	117.1 ± 40.0	<0.0001
Total cholesterol (mg/dl)	176.4 ± 26.9	196.7 ± 44.0	0.001
Triglycerides (mg/dl)	118.4 ± 32.3	155.0 ± 62.7	<0.0001
AST (IU/L)	21.8 ± 9.5	42.9 ± 25.4	<0.0001
ALT (IU/L)	36.0 ± 16.6	83.0 ± 48.5	<0.0001
GGT (IU/L)	44.0 ± 25.4	111.6 ± 115.5	<0.0001

Data are expressed in mean ± SD for continuous data and percentage for categorical data

ALT Alanine transferase, *AST* aspartate aminotransferase, *BMI* body mass index, *GGT* gamma glutamyl transpeptidase, *HbA1c* haemoglobin A1c, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *NAFLD* non-alcoholic fatty liver disease

Genotypes and allele frequencies of GCKR polymorphisms

The genotype distribution of each SNP rs1260326 C>T and rs780094 C>T was observed in Hardy–Weinberg equilibrium for both NAFLD cases and controls, as well as after stratification by ethnicity. Overall, the frequency of risk allele T was higher in patients with NAFLD compared to controls (OR 1.49, 95 % CI 1.09–2.05, $p = 0.012$; and OR 1.51, 95 % CI 1.09–2.09, $p = 0.013$, for rs1260326 and rs780094, respectively). The association between the T allele and NAFLD was also significant among Indian patients (OR 2.64, 95 % CI 1.28–5.43, $p = 0.009$ and OR 4.35, 95 % CI 1.93–9.81, $p < 0.0001$, for rs1260326 and rs780094, respectively) but not in Malay and Chinese patients (Fig. 1, Supplementary Table 1).

Table 2 Demographic and clinical data of the NAFLD patients

Characteristics	*n* (%) or mean ± SD		
	Simple steatosis (*n* = 33)	NASH (*n* = 111)	*p* value
Gender, *n* (%)			0.461
Males	20 (61)	57 (51)	
Females	13 (39)	54 (49)	
Age (years)	50.7 ± 11.8	51.2 ± 12.1	0.82
BMI (kg/m^2)	26.7 ± 3.9	29.2 ± 4.4	0.003
HbA1c (%)*	6.1 ± 1.3	6.7 ± 1.7	0.021
Waist circumference (cm)	89.2 ± 11.2	95.2 ± 10.4	0.005
HDL cholesterol (mg/dl)	50.2 ± 15.1	48.0 ± 11.9	0.391
LDL cholesterol (mg/dl)	114.7 ± 42.8	117.8 ± 39.3	0.698
Total cholesterol (mg/dl)	191.6 ± 44.9	198.2 ± 43.8	0.448
Triglycerides (mg/dl)	124.6 ± 42.0	164.1 ± 65.1	0.001
AST (IU/L)*	37.6 ± 21.3	44.5 ± 26.3	0.139
ALT (IU/L)	71.9 ± 50.0	86.4 ± 47.8	0.134
GGT (IU/L)*	99.4 ± 106.6	115.3 ± 118.2	0.132
Systolic blood pressure (mmHg)	125.2 ± 13.0	134.2 ± 14.1	0.001
Diastolic blood pressure (mmHg)*	78.2 ± 9.1	83.8 ± 9.7	0.003

ALT Alanine transferase, *AST* aspartate aminotransferase, *BMI* body mass index, *GGT* gamma glutamyl transpeptidase, *HbA1c* haemoglobin A1c, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *NAFLD* non-alcoholic fatty liver disease, *NASH* non-alcoholic steatohepatitis

* *p* values obtained using Mann–Whitney *U* test, all other comparisons used independent *t* test

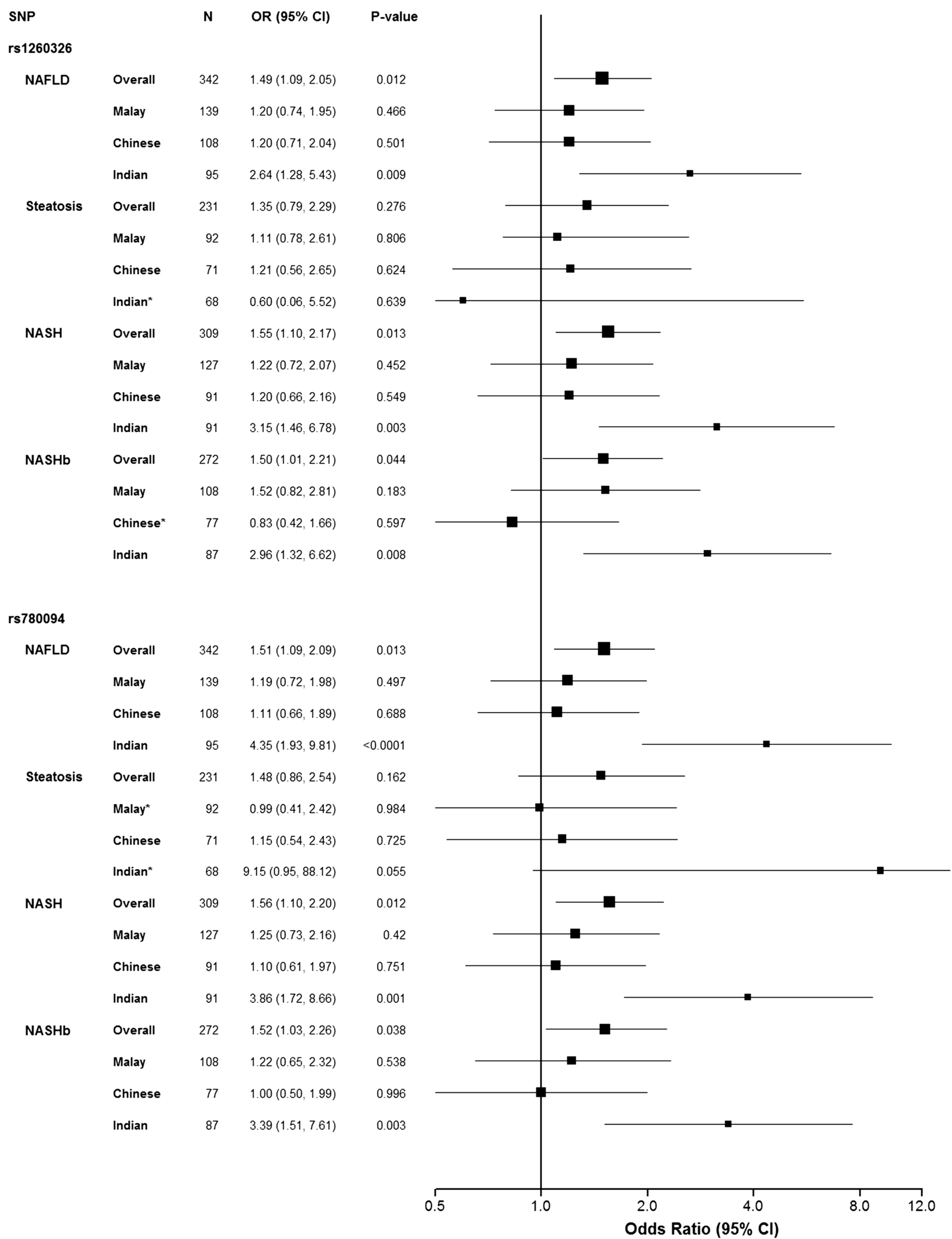

Fig. 1 The association between *GCKR* polymorphisms NAFLD spectrum. *The *horizontal line* representing the 95 % CI of the point estimate has been truncated due to space limitation. *NASHb* Non-alcoholic steatohepatitis with fibrosis

Significant association was replicated in the NASH (OR 1.55, 95 % CI 1.10–2.17, $p = 0.013$ and OR 1.56, 95 % CI 1.10–2.20, $p = 0.012$, for rs1260326 and rs780094, respectively) and NASH with significant fibrosis (OR 1.50, 95 % CI 1.01–2.21, $p = 0.044$ and OR 1.52, 95 % CI 1.03–2.26, $p = 0.038$, for rs1260326 and rs780094, respectively). These associations was also significant in Indian patients (OR 3.15, 95 % CI 1.46–6.78, $p = 0.003$ and OR 3.86, 95 % CI 1.72–8.66, $p = 0.001$, for rs1260326 and rs780094, respectively) but not in Malay and Chinese patients.

We observed no significant finding between both SNPs with simple steatosis and NASH with no significant fibrosis (Supplementary Table 1). Since *GCKR* is involved in the pathway of glucose metabolism [18], we expanded the analysis by categorizing the NAFLD patients into diabetic NAFLD ($n = 54$) and non-diabetic NAFLD ($n = 90$) categories. However, no association was seen between these SNPs and diabetes among these NAFLD patients.

GCKR polymorphisms and liver histology

There was an association between hepatic steatosis grade and T allele (homozygous TT compared to homozygous CC, had a mean score of 2.18 vs 1.63, $p = 0.008$). Following multivariate adjustment, carriers of allele T had exhibited 76 % higher odds of developing hepatic steatosis of higher grade compared to non-carriers (OR 1.76; 95 % CI 1.08–2.85, $p = 0.004$). This association remained significant even after adjustment for other histological features ($p = 0.04$) (Table 3). There was no association between T allele and lobular inflammation, hepatocellular ballooning and fibrosis.

Combined effect of *GCKR* and *PNPLA3* gene with risk of NAFLD

Variants of both the *GCKR* and *PNPLA3* genes [12] have been postulated to confer risk of NAFLD in our study population. Hence, we investigated the interaction between the two risk genes on occurrence of NAFLD. We derived two best models for the interaction. In order to obtain a perfect cross-validation consistency, we suggest that the three-locus model (*GCKR* rs1260326, *GCKR* rs780094, *PNPLA3* rs738409) would be the best model (empirical $p = 0.003$). The combined effect of the three SNPs (OR 4.14, 95 % CI 1.41–12.18, $p = 0.010$) confers a greater risk for NAFLD than either SNP alone: 1.49 risk for *GCKR* rs1260326, 1.51 risk for *GCKR* rs780094 and 2.23 risk for *PNPLA3* rs738409. The combined effect of *GCKR* and *PNPLA3* showed no significant association with histological parameters ($p > 0.05$).

Statistical power

All significant findings were checked for study power, whereby estimated powers of 75, 82, 77, 94 and 76 % were obtained for association between rs1260326 with NAFLD, rs780094 with NAFLD, rs1260326 with NAFLD in Indian patients, rs780094 with NAFLD in Indian patients, and rs1260326 with NASH, respectively.

Discussion

We identified a positive association between a common intronic SNP (rs780094) and a loss of function SNP (rs1260326) on *GCKR* with NAFLD. This association was modified by race with a strong and significant association seen in the Indians and a weak and nonsignificant association seen in the Malays and Chinese. We also report an association between rs1260326 T allele with increased hepatic steatosis severity, and an interaction between the *GCKR* and *PNPLA3* genes.

Association studies have previously reported the positive association between *GCKR* SNPs with NAFLD. We add to this evidence base by presenting the association between *GCKR* rs1260326 and rs780094 T allele and

Table 3 Association of T allele of rs1260326 with histological features in NAFLD patients

Histology	Univariate p value[a]	Multivariate p value[b] (FDR q value[g])	OR (95 % CI)
Steatosis >33 vs. <33 %	0. 004	0.040 (0.016)	1.76 (1.08–2.85)[c]
Lobular inflammation ≥2 foci vs. <2 foci	0. 750	0.639 (0.750)	1.18 (0.73–1.91)[d]
Hepatocellular ballooning ≥1 vs. <1	0. 739	0.281 (0.985)	1.05 (0.47–2.33)[e]
Fibrosis ≥2 vs. <2	0. 414	0.859 (0.828)	0.81 (0.51–1.29)[f]

OR odds ratio, *CI* confidence interval

[a] Jonckheere–Terpstra test

[b] Ordinal regression

[c, d, e, f] Multivariate logistic regression

[g] False discovery rate, $q < 0.05$ is significant

NASH. Subsequently, we observed an association between the risk allele with NASH with significant fibrosis. These findings are similar to the meta-analysis of 7,176 subjects reported by the Genetics of Obesity-related Liver Disease (GOLD) consortium [11]. However, no NASH specific results were available in the GWAS report. The associations with NASH are clinically important because of its progressive nature [25].

Another interesting finding from our study is that *GCKR* rs1260326 T allele was significantly associated with a higher grade of hepatic steatosis. This is consistent with a study among obese children and adolescents in the United States among Caucasians, African Americans, and Hispanics [26]. The role of the *GCKR* gene in encoding GCKRP, a regulatory enzyme in the modulation of hepatic GCK activity [27, 28], can explain our findings. The *GCKR* rs1260326 functional variant P446L increases GCK activity by downward regulation of fructose 6-phosphate [29]. Increased GCK activity is associated with increased hepatic glycolytic flux, de novo lipogenesis and hepatic triglyceride level [13, 30]. Malonyl coenzyme A (CoA), a substrate for de novo lipogenesis, increases fat deposition in the liver and blocks fatty acid oxidation [29, 31].

The finding of gene interaction between the *GCKR* and *PNPLA3* genes on susceptibility to NAFLD may be related to the role of the two genes in the development of hepatic fat content [9, 26]. A synergistic multiplicative interaction was seen, whereby the effect size contributed by the combined genes resulted in a greater risk of NAFLD than either gene alone. Intriguingly, genes that share a similar pathway or outcome as the *PNPLA3* gene have been shown to interact with each other [32, 33]. The etiology of NAFLD is complex and more studies are required to clarify the genetic underpinnings of NAFLD.

The study population was multiracial and comprised of Malays, Chinese and Indians. The Malaysian Chinese and Indians migrated from Southern China [34] and Southern India [35] in the late eighteenth century and early nineteenth century, respectively. The association between the *GKCR* variants and NAFLD and NASH was modified by race, being stronger and more significant in Indians compared to Malays and Chinese. Compared to the Malays and Chinese, there is genetic admixture of the Indian subpopulation with West Eurasian ancestry, resulting in their genetic affinity towards both Asian and European [36, 37]. The higher prevalence of NAFLD in those with European ancestry compared to Asian ancestry [38] can explain our study results.

Though our race-stratified analysis was similar, we failed to replicate Yang's results in our Chinese subjects [19]. This may be attributed to the difference in genetic variability of Southeast Asian Chinese compared to Chinese from Shanghai and Beijing Han Chinese (CHB) [39]. The minor allele frequency in our Chinese control subjects (44 %) are different than Chinese from Shanghai (53 %) [19] and the CHB (57 %). There were also methodological differences in case definitions, with no liver biopsy carried out in this other study [19].

The strength of our study is the availability of a histological profile of the biopsy-proven NAFLD patients that provides a definite diagnosis of the disease. This allows a complete investigation of the *GCKR* variants and NAFLD spectrum: simple steatosis, NASH with no significant fibrosis and NASH with significant fibrosis [40]. It is very important to profile NAFLD according to the severity of the condition because unlike simple steatosis, which has a benign long-term prognosis, NASH and hepatic fibrosis signify greater risk of progression to cirrhosis and end-stage liver disease [41–43]. Another strength of this study was the specific ethnic comparisons of the association between *GCKR* polymorphisms and NAFLD among three major Asian ethnic groups, Malays, Chinese and Indians. This allows the investigation of how ethnicity modifies the association between *GCKR* variants and NAFLD spectrum.

A limitation of this study is that liver biopsy was not carried out in controls due to ethical concerns. This might result in misclassification of the controls. However, controls had to meet strict selection criteria that included normal BMI, fasting plasma glucose, lipid profile and liver enzymes. This criteria minimized the possibility of misclassification in this study.

To our knowledge, this is the first report of a genetic interaction between *GCKR* rs1260326 and rs780094 together with *PNPLA3* rs738409 on the increased susceptibility to NAFLD. However, further evidence is needed to confirm this interaction.

In conclusions, we found a positive significant association between G*CKR* rs1260326 and rs780094 with NAFLD and NASH. Furthermore, the two SNPs may also increase the risk of NASH with significant fibrosis, and rs1260326 variant was associated with increased steatosis grade.

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Conflict of interest The authors declare no conflict of interest.

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