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ORIGINAL RESEARCH

Genetic Variations of *AKT1* are Associated with Risk Screening for Non-Alcoholic Fatty Liver Disease

Yajie Ding[1,*], Zongzhe Tang[1,*], Ru Zhang[1], Mengting Zhang[1], Qing Guan[1], Liuxin Zhang[2], Hongliang Wang[3], Yue Chen[1], Wei Zhang[4], Jie Wang[1]

[1]Department of Fundamental and Community Nursing, School of Nursing, Nanjing Medical University, Nanjing, Jiangsu, People's Republic of China; [2]The Nethersole School of Nursing, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong (SAR), People's Republic of China; [3]Department of General Practice, Ninghai Road Community Health Service Center, Nanjing, Jiangsu, People's Republic of China; [4]Department of Epidemiology, Shanghai Cancer Institute, Shanghai, People's Republic of China

*These authors contributed equally to this work

Correspondence: Jie Wang, Department of Fundamental and Community Nursing, School of Nursing, Nanjing Medical University, 101 Longmian Avenue, Nanjing, Jiangsu, 211166, People's Republic of China, Tel +86-25-86869557, Fax +86-25-86868501, Email wangjienjmu@126.com

Purpose: Protein kinase B (PKB/AKT) has shown a high profile in the research of metabolic diseases. This research sought to determine whether the *AKT1* gene's single nucleotide polymorphisms (SNPs) and the risk of developing non-alcoholic fatty liver disease (NAFLD) were related.

Patients and Methods: Recruited in this case–control study were 2693 subjects, including 815 with NAFLD and 1878 without NAFLD. Three SNPs of *AKT1* (rs2494732, rs2494752 and rs1130233) were genotyped. To examine the correlation between SNPs and NAFLD susceptibility, logistic regression was performed.

Results: After adjusting for sex, age, triglyceride and glucose, *AKT1* rs2494732-C (all $P < 0.05$ in co-dominant model, dominant model and additive model) and rs2494752-G ($P < 0.05$ in co-dominant model) were linked to a lower risk of NAFLD. The combined effect of both SNPs on NAFLD risk was statistically significant, showing a dose dependence ($P_{trend} = 0.010$). Sex, body mass index, hypertension, hyperglycemia, hypertriglyceridemia, high-density lipoprotein-cholesterol, alanine aminotransferase, and beneficial alleles were all significant predictors of NAFLD risk (all $P < 0.05$). The prediction model achieved good discrimination, with an area under the receiver operating characteristic curve of 0.779. The Hosmer–Lemeshow test suggested an inadequate calibration of the model ($\chi^2 = 21.073$, $P = 0.007$).

Conclusion: *AKT1* rs2494732 and rs2494752 may be related to Chinese NAFLD susceptibility. The prediction model combining both SNPs with clinical factors displays a strong ability to discriminate NAFLD patients. Both SNPs may be exploited to design new models for early screening of NAFLD high-risk population.

Keywords: NAFLD, susceptibility, *AKT1* gene, polymorphism, risk screening

Introduction

The prevalence of nonalcoholic fatty liver disease (NAFLD) keeps rising globally, especially in adolescents and young adults.[1,2] Characterized by intemperate amassing of triglycerides and cholesterol in hepatocytes, NAFLD is regarded as a hepatic sign of metabolic disorder.[3] It causes nonalcoholic steatohepatitis, liver fibrosis, cirrhosis, and liver cancer,[4] and also involves an increased risk of extrahepatic diseases, such as type 2 diabetes mellitus (T2DM),[5] cardiovascular disease,[6] and chronic kidney disease,[7] impairing the health-related quality of life of patients.[8] Therefore, early screening and prevention should be enhanced through tools designed with factors associated with its susceptibility.

The pathogenesis of NAFLD remains unsolved but may involve multiple factors, including insulin resistance, adipose tissue dysfunction, mitochondrial dysfunction, endoplasmic reticulum stress, inflammatory activation, intestinal microbiota, as well as genetic and epigenetics.[9,10] Metabolic associated fatty liver disease (MAFLD), a more appropriate term, has been proposed to redefine NAFLD in an international consensus, emphasizing the association with metabolic comorbidities.[11]

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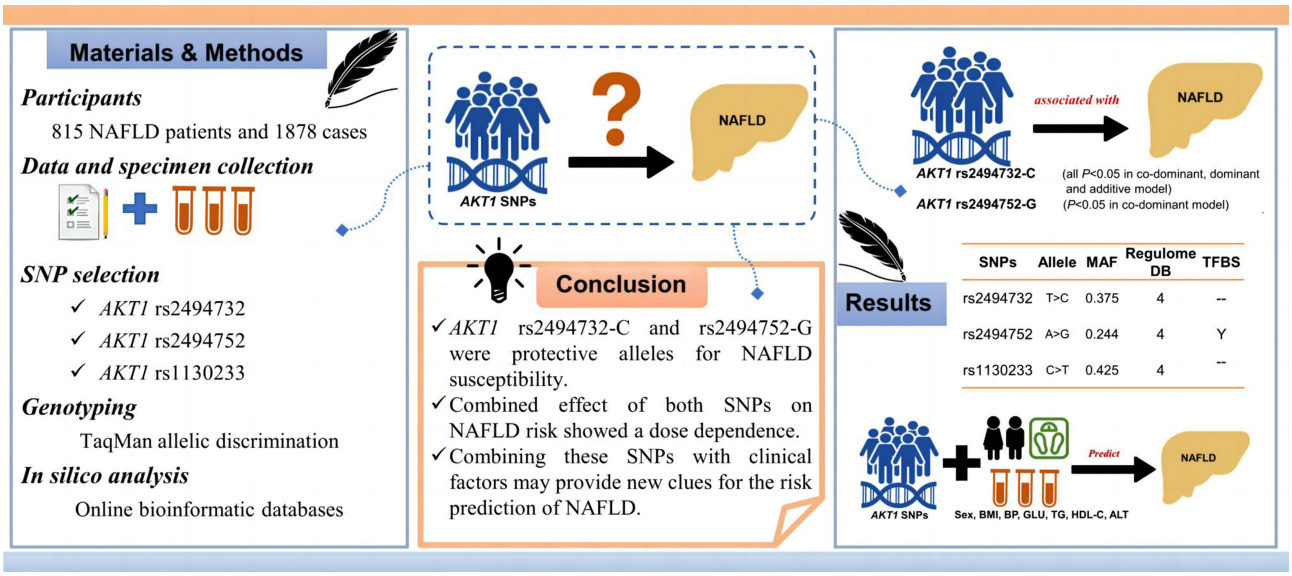
Graphical Abstract



During the progression of NAFLD, protein kinase B (PKB, also known as AKT) is overactivated to promote the maturation of proteins associated with lipid accumulation (a type of metabolic disorder).[12] AKT, as a serine and threonine kinase, is activated by phosphoinositide 3-kinase (PI3K) through phosphorylation, thereby regulating a range of cellular processes, such as metabolism, cell survival, motility, lipid synthesis, protein synthesis and degradation.[13] Implications of AKT in tumors, diabetes, obesity, neurological disorders, inflammation, and other diseases have been reported.[14–16] *AKT* contains three isoforms (*AKT1, AKT2*, and *AKT3*), each with distinctive but overlapping functions.[17] *AKT1* is mainly involved in growth control, *AKT2* in metabolic regulation, and *AKT3* in brain development.[18] In recent years, the role of *AKT1* in metabolism has attracted mounting attention. *AKT1* deletion protects mice from insulin resistance and obesity induced by diet, indicating a role of *AKT1* in energy metabolism.[17] In addition, studies have identified associations between *AKT1* variants and metabolic-related diseases, such as T2DM,[19] obesity,[20] and metabolic syndrome.[21] Considering that NAFLD is a metabolic stress-related disease, we speculate that *AKT1* may regulate the susceptibility to NAFLD.

As a genetic marker, single nucleotide polymorphisms (SNPs) have shown their value in etiological studies, due to their high stability across phenotypes of diseases.[16] Therefore, this present work attempted to investigate the associations between functional SNPs of *AKT1* gene and NAFLD susceptibility, and evaluate the ability of *AKT1* polymorphisms combined with clinical variables in predicting NAFLD risk.

Materials and Methods

Study Participants and Conception

This case–control study involved 2693 individuals, including 815 in the NAFLD case group and 1878 in the control group, all recruited between April and October 2020 from a community in Nanjing, Jiangsu, China. Ethical approval was obtained from the Institutional Ethics Review Committee of Nanjing Medical University, with a project approval number of (2019) 740. Prior to the trial, written informed permission was obtained from each participant.

Included were individuals with Han nationality, ages over 18 years, post-fasting blood samples, sufficient comprehending and communicating skills to complete the questionnaire. Excluded were those with infection, autoimmune diseases, malignant tumors, other liver diseases (such as viral hepatitis, alcoholic liver disease, and drug-induced fatty hepatitis), excessive alcohol consumption (≥20 g/day in females and ≥30 g/day in males), willingness to undergo liver transplantation within one year, or advanced liver diseases with complications such as variceal bleeding or ascites.

The cases diagnosed with NAFLD were classified into the case group in accordance with the 2018 modification of the Chinese NAFLD guideline,[22] while the subjects in the same community without NAFLD were assigned to the control group. Detailed diagnostic criteria for NAFLD include (1) no history of abusing alcohol excessively (<20 g/day in females and <30 g/day in males); (2) exclusion of conditions such drug-related liver disease, viral hepatitis, complete parenteral feeding, hepatomegaly, and autoimmune liver disease (all of which are causes of fatty liver); and (3) histological changes on liver biopsy. Given the difficulty in obtaining a histologic diagnosis of the liver, the working definition of NAFLD, provided that (1) and (2) were met, was (i) liver imaging findings that meet the diagnostic criteria for diffuse fatty liver with no other explanation; and/or (ii) metabolic syndrome-related components presenting with unexplained persistent elevations of serum alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) and γ-glutamyl transpeptidase (γ-GT) for more than 6 months.

Age (±3 years) was frequency-matched between the two groups. Sample size was estimated based on preliminary study and PASS 11.0 software, with two-sided $\alpha = 0.05$, power = 80, odds ratio (OR) = 1.5, frequency of gene variation in the general population = 20%. The sample sizes in two groups met the requirements (at least 534 in each group).

Data and Specimen Collection

Demographic data were gathered from self-administered questionnaire surveys. Clinical data about medical history, anthropometric, biochemical and imaging information were obtained from electronic medical records.

Participants who were fasting had their venous blood drawn (at least 5 mL) and kept in tubes containing ethylene diamine tetraacetic acid (EDTA), an anticoagulant. Plasma and blood cells were separated from each blood sample within 2 hours and frozen at −80°C before genotyping assays.

SNP Selection Criteria

The following methods were used to select SNPs: (1) The genotype information of *AKT1* (upstream and downstream extended by 2000bp each) in Han Chinese in Beijing (CHB) was downloaded from the 1000 Genomes Project database (http://www.1000genomes.org/) and imported into Haploview 4.2 software (Broad Institute, Cambridge, MA, USA). (2) Tagging SNPs (tagSNPs) of *AKT1* gene were selected according to the correlation coefficient $r^2 \geq 0.8$, the minor allele frequency (MAF) > 0.05 and Hardy–Weinberg *P*-value cutoff = 0.05 in the software. At this point, 62 tagSNPs were generated. (3) The MAFs of tagSNPs in the Chinese population were available from NCBI dbSNP (http://www.ncbi.nlm.nih.gov/); the SNPs with MAF ≤ 0.05 were excluded, leaving 18 tagSNPs with high frequency. (4) Finally, the SNPs associated with other diseases (especially metabolic syndrome, type 2 diabetes, hypertension, hyperlipidemia, etc.) were selected through literature review. Based on the above four processes, three polymorphisms (rs2494732, rs2494752, and rs1130233) in the *AKT1* gene were selected for further genotyping.

DNA Extraction and Genotyping

By using a magnetic bead technique based on blood genomic extraction kit (Pangu Genome Nanotechnology Co., Ltd.; Nanjing, China), genomic DNA was isolated from blood samples, and its concentration was set to 50 ng/mL.

Genotyping was performed in a 384-well plate on a Light Cycler 480 II Real-Time PCR System (Roche, Switzerland) using the TaqMan allelic discrimination method. Primer and probe information are detailed in Supplementary Table S1. A random selection of 10% of the samples was used in subsequent tests to confirm the quality control of the experimental data, and the concordance was 100%. The entire genotyping was performed in a blinded manner (all technicians were unaware of the participants' information) and followed the manufacturer's instructions. The success rate of genotyping was more than 96% for all SNPs.

In silico Analysis

Potential biological function of *AKT1* gene polymorphisms in NAFLD susceptibility was explored using several online bioinformatic databases. (1) Genetic variation sites on chromosomes were determined through NCBI dbSNP (https://www.ncbi.nlm.nih.gov/snp). (2) The RegulomeDB scores of SNPs were obtained using the RegulomeDB database (http://www.regulomedb.org/), with a lower score implying a higher profile in transcriptional regulation. (3) The functions of SNPs were

predicted in the SNP Function Prediction database (https://snpinfo.niehs.nih.gov/). (4) Whether genetic variation sites were associated with histone modifications was checked using the UCSC Genome Browser database (http://genome.ucsc.edu/). (5) The RNAfold WebServer (http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi) projected the influence of genetic variation of positive SNPs on *AKT1* mRNA secondary structure.

Statistical Analysis

Statistical analyses were conducted using SPSS 23.0, R software v3.4.3, and MedCalc 19.1. The Kolmogorov–Smirnov test and histogram were used to evaluate data normality. Descriptive analysis was conducted according to data type, including constituent ratios, mean ± standard deviation, and median (interquartile range). Baseline information for both groups was compared using Chi-square (χ^2) test (for categorical variables), Student's *t*-test (for normal continuous variables), and Mann–Whitney *U*-test (for non-normal continuous variables). A goodness-of-fit χ^2-test was used to determine Hardy–Weinberg equilibrium. Logistic regression analysis based on four genetic models (co-dominant model, dominant model, recessive model and additive model) was performed to construct odds ratio (OR) and 95% confidence interval (CI) to investigate the correlations of SNPs with susceptibility to NAFLD, with age, sex, triglyceride, and glucose adjusted for potential confounding effects. The rules for implementing the four genetic models are detailed in Table 1. To account for the impact of multiple comparisons, the false discovery rate (FDR) is adjusted. Cochran–Armitage trend was tested to analyze the combined effects of statistically significant SNPs. We further performed subgroup analysis to explore the influence of confounding factors, and heterogeneity between subgroups was assessed by *Q* test. To identify NAFLD predictors and proceed to construct a predictive model, multivariate stepwise logistic regression and receiver-operating characteristic curve (ROC) were used. The discrimination and calibration of the prediction model were assessed by the area under the receiver operating characteristic curve (AUROC) and the Hosmer–Lemeshow test, respectively. In all analyses, a P-value < 0.05 in the two-tailed test was considered statistically significant.

Results

Basic Characteristics of Study Subjects

Table 2 summarizes the demographic and clinical features of the NAFLD cases and the controls. Between the two groups, there was no discernible age difference ($P > 0.05$). Sex, body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transpeptidase (γ-GT), total cholesterol (TC), triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), and glucose (GLU) were the variables where there were significant differences (all $P < 0.05$). Besides, all SNPs in the control group's genotype frequencies were in Hardy–Weinberg equilibrium (all $P > 0.05$) (Supplementary Table S1), indicating that the selected samples were representative of the population.

Associations Between *AKT1* SNPs and NAFLD Susceptibility

The genotype information for the three SNPs in both groups is displayed in Table 1. After adjusting for sex, age, TG, and GLU, logistic regression analyses revealed that *AKT1* variants rs2494732-C (TC genotype vs TT genotype: adjusted OR = 0.784, 95% CI = 0.648–0.947, $P = 0.012$; dominant model: adjusted OR = 0.775, 95% CI = 0.647–0.928, $P = 0.006$; additive model: adjusted OR = 0.823, 95% CI = 0.714–0.949, $P = 0.007$) and rs2494752-G (AG genotype vs AA genotype: adjusted OR = 0.826, 95% CI = 0.684–0.999, $P = 0.048$) were significantly correlated with low NAFLD risk in different models. After false discovery rate correction for multiple comparisons (Supplementary Table S2), their associations were still significant with all $P_{FDR} \leq 0.25$.[23] *AKT1* 1130233 and NAFLD susceptibility did not, however, appear to be significantly correlated with any of the models (all $P > 0.05$).

As shown in Table 3, beneficial alleles (rs2494732-C and rs2494752-G) were counted to evaluate the combined effects of both SNPs on NAFLD susceptibility. A lower incidence of NAFLD was observed in the subjects with more beneficial alleles. Those with beneficial alleles ("1–2" or "3–4") showed a significantly lower risk of NAFLD, compared to those carrying none of these beneficial alleles (adjusted OR = 0.820, 95% CI = 0.677–0.993, $P = 0.042$; adjusted OR = 0.683,

Table 1 Genotype Distributions of Three *AKT1* SNPs and Their Associations with NAFLD Risk

SNP	Controls n (%)	NAFLD Cases n (%)	OR (95% CI)[a]	*P*[a]
rs2494732				
TT	892 (49.5)	436 (56.0)	1.00 (ref)	
TC	754 (41.9)	283 (36.4)	**0.784 (0.648–0.947)**	**0.012**
CC	155 (8.6)	59 (7.6)	0.734 (0.520–1.037)	0.080
Dominant model			**0.775 (0.647–0.928)**	**0.006**
Recessive model			0.815 (0.582–1.140)	0.232
Additive model			**0.823 (0.714–0.949)**	**0.007**
rs2494752				
AA	884 (48.9)	407 (51.8)	1.00 (ref)	
AG	766 (42.4)	305 (38.9)	**0.826 (0.684–0.999)**	**0.048**
GG	158 (8.7)	73 (9.3)	1.030 (0.750–1.416)	0.855
Dominant model			0.861 (0.720–1.029)	0.100
Recessive model			1.122 (0.826–1.525)	0.462
Additive model			0.936 (0.815–1.074)	0.347
rs1130233				
CC	478 (26.6)	221 (28.3)	1.00 (ref)	
CT	888 (49.5)	378 (48.5)	0.916 (0.740–1.133)	0.417
TT	429 (23.9)	181 (23.2)	0.933 (0.725–1.200)	0.589
Dominant model			0.921 (0.754–1.125)	0.421
Recessive model			0.987 (0.798–1.220)	0.904
Additive model			0.964 (0.849–1.093)	0.565

Notes: [a]Logistic regression model, adjusted for sex, age, triglycerides and glucose. Bold type indicates statistically significant results. Four genetic models: co-dominant model (heterozygote vs wild homozygote, mutant homozygote vs wild homozygote), dominant model (heterozygote + mutant homozygote vs wild homozygote), recessive model (mutant homozygote vs wild homozygote + heterozygote), and additive model (mutant homozygote vs heterozygote vs wild homozygote). Take rs2494732 as an example: codominant model (TC vs TT; CC vs TT), dominant model (TC + CC vs TT), recessive model (CC vs TC + TT), and additive model (CC vs TC vs TT).

Abbreviations: SNPs, single nucleotide polymorphisms; NAFLD, nonalcoholic fatty liver disease; OR, odds ratio; 95% CI, 95% confidence interval.

95% CI = 0.488–0.957, $P = 0.027$, respectively). It appears that the combination allele affects NAFLD susceptibility in a dose-dependent way ($P_{trend} = 0.010$) as more beneficial alleles were linked to a reduced incidence of NAFLD.

The combined effect of rs2494732-C and rs2494752-G was further evaluated in subgroups stratified according to sex, age, BMI, BP, GLU, TG, HDL-C, ALT, AST, and γ-GT using additive models (Supplementary Table S3). The stratification was based on the health industry standard of the People's Republic of China (WS/T 404.1–2012), clinical guidelines and the population characteristics in this study.[22,24–26] We found that the combined protective effect of two SNPs was more pronounced in the subgroups of males, age <40 years, BMI <24 kg/m^2, SBP <140 and DBP <90 mmHg, GLU ≥5.6 mmol/L, TG <1.7 mmol/L, HDL-C >1.04 mmol/L, ALT ≥40 U/L, AST <40 U/L, and γ-GT <50 U/L (all adjusted $P < 0.05$). Except for age ($P = 0.022$), the effects in the remaining subgroups showed no significant heterogeneity (all $P > 0.05$).

Table 2 Distributions of Demographic and Clinical Characteristics in NAFLD Case and Control Groups

Variables	Controls (N=1878)	NAFLD Cases (N=815)	χ^2/t/Z	P
Sex, n (%)			175.956	**<0.001**[a]
Male	1217 (64.8)	731 (89.7)		
Female	661 (35.2)	84 (10.3)		
Age, year	42.74±8.06	43.40±9.19	−1.772	0.077[b]
BMI, kg/m^2	22.92±2.77	25.47±2.44	−23.432	**<0.001**[b]
SBP, mmHg	124.37±15.52	130.32±14.33	−9.458	**<0.001**[b]
DBP, mmHg	77.93±10.71	83.10±10.06	−11.757	**<0.001**[b]
ALT, U/L	18.00 (13.00, 26.00)	28.50 (20.00, 40.00)	−17.771	**<0.001**[c]
AST, U/L	20.00 (17.00, 24.00)	22.00 (19.00, 27.38)	−10.243	**<0.001**[c]
γ-GT, U/L	22.00 (16.00, 32.00)	32.00 (24.00, 48.75)	−16.954	**<0.001**[c]
TC, mmol/L	4.67 (4.19, 5.27)	4.91 (4.37, 5.44)	−5.834	**<0.001**[c]
TG, mmol/L	1.10 (0.78, 1.56)	1.63 (1.24, 2.36)	−18.142	**<0.001**[c]
HDL-C, mmol/L	1.39 (1.19, 1.65)	1.17 (1.02, 1.33)	−18.395	**<0.001**[c]
LDL-C, mmol/L	2.70 (2.28, 3.18)	3.04 (2.61, 3.49)	−11.510	**<0.001**[c]
GLU, mmol/L	5.44±0.84	5.71±0.99	−6.652	**<0.001**[b]

Notes: [a]χ^2 test; [b]Student's *t*-test; [c]Mann–Whitney *U*-test. Bold type indicates statistically significant results.
Abbreviations: NAFLD, nonalcoholic fatty liver disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GT, γ-glutamyl transpeptidase; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; GLU, glucose.

Table 3 Combined Effect of *AKT1* rs2494732-C and rs2494752-G on NAFLD Risk

Variables	Controls n (%)	NAFLD Cases n (%)	NAFLD Prevalence (%)	OR (95% CI)	P
0	569 (30.3)	280 (34.4)	33.0	1.00 (Ref)	
1–2	1122 (59.7)	471 (57.8)	29.6	**0.820 (0.677–0.993)**	**0.042**[a]
3–4	187 (10.0)	64 (7.9)	25.5	**0.683 (0.488–0.957)**	**0.027**[a]
Trend				**0.824 (0.711–0.955)**	**0.010**[b]

Notes: [a]*P*-value of the logistic regression model, adjusted for sex, age, triglycerides and glucose. [b]*P*-value of the Cochran–Armitage trend test. Bold type indicates statistically significant results.
Abbreviations: NAFLD, nonalcoholic fatty liver disease; OR, odds ratio; 95% CI, 95% confidence interval.

Functional Prediction of Positive SNPs

The Regulome DB score for both *AKT1*-rs2494732 and rs2494752 was 4. The SNP function prediction database predicted rs2494752 as a transcription factor binding site. UCSC database predictions showed that both rs2494732 and rs2494752 were enriched close to the H3K4Me1 marker (Figure 1). The impact of rs2494732-C and rs2494752-G on *AKT1* mRNA secondary structure was predicted by RNAfold WebServer (Figure 2). The arrows indicated the position of the variation. The T and C alleles of rs2494732 were calculated to have minimum free energy (MFE) values of −38.6 and −39.1 kcal/mol, respectively. The MFE value for rs2494752 was calculated to be −24.1 kcal/mol for both the A and G alleles. The results of the linkage disequilibrium (LD) test with Haploview software showed no strong LD among the three candidate SNPs (Supplementary Figure S1), implying that the haplotype analysis was not necessary.

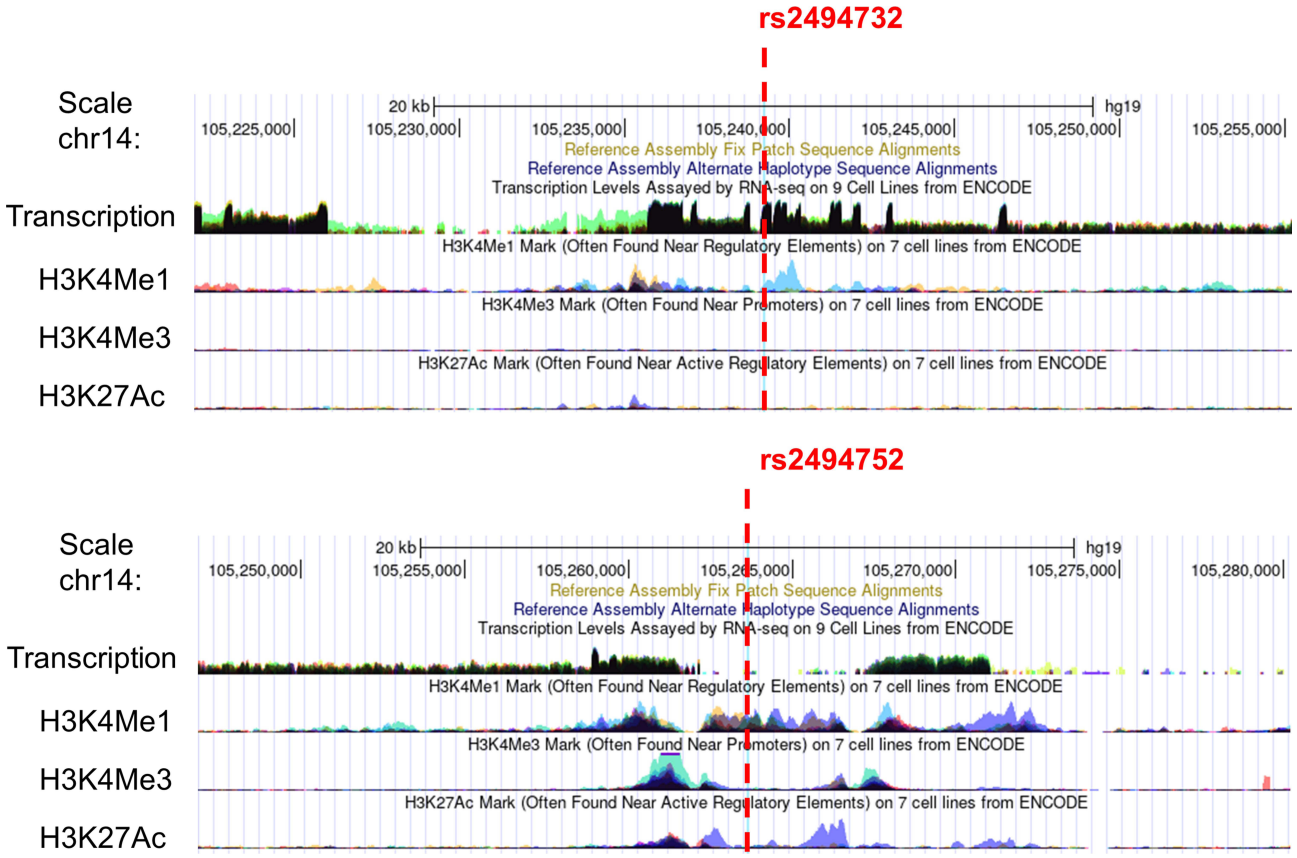


Figure 1 Functional prediction of positive single nucleotide polymorphisms in *AKT1*. The red dotted line indicates the position of *AKT1* rs2494732 and rs2494752 (available at http://genome.ucsc.edu/).

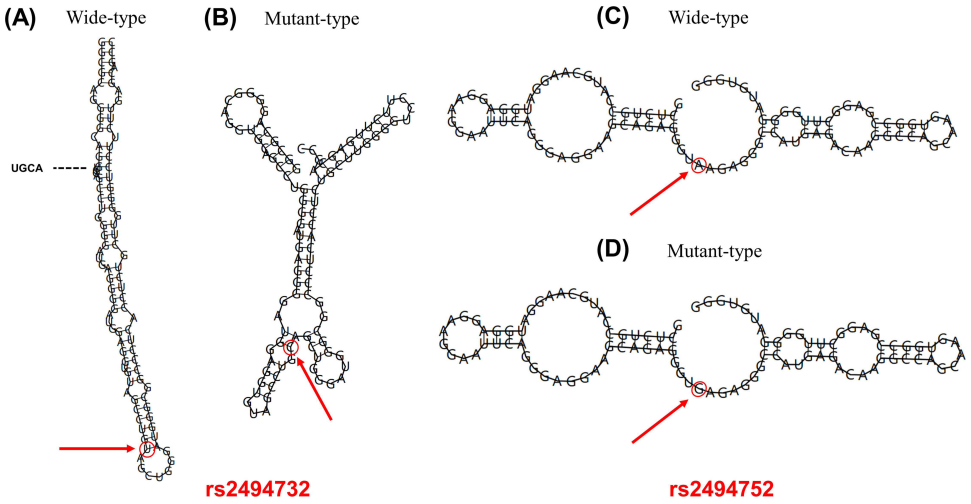


Figure 2 Effects of rs2494732 and rs2494752 variants on *AKT1* mRNA secondary structure. The red arrows represent the position of the variation (50 bases upstream and 50 bases downstream from the variation). (**A**) The minimum free energy (MFE) value for the rs2494732-T was −38.6 kcal/mol. (**B**) The MFE value for rs2494732-C was −39.1 kcal/mol. (**C**) The MFE value for rs2494752-A was −24.1 kcal/mol. (**D**) The MFE value for rs2494752-G was −24.1 kcal/mol.

Independent Predictors of NAFLD

A stepwise regression model was conducted incorporating sex, age, BMI, hypertension, hyperglycemia, hypertriglyceridemia, HDL-C, ALT, AST, γ-GT, and combined beneficial alleles (rs2494732-C and rs2494752-G). These variables showed no evident multi-collinearity (Supplementary Table S4). As outlined in Table 4, sex (OR = 0.413, 95% CI = 0.314–0.542, $P < 0.001$), BMI

Table 4 Multivariate Stepwise Logistic Regression Analysis for Independent Factors Influencing NAFLD Risk

Variables	b	SE	Wald χ^2	OR (95% CI)	P
Sex (male[a] vs female)	−0.885	0.140	40.243	0.413 (0.314–0.542)	<0.001
BMI (<24[a] vs ≥24 kg/m^2)	1.165	0.101	132.490	3.207 (2.630–3.911)	<0.001
Hypertension (no[a] vs yes)	0.390	0.107	13.337	1.477 (1.198–1.821)	<0.001
Hyperglycemia (no[a] vs yes)	0.216	0.100	4.709	1.242 (1.021–1.510)	0.030
Hypertriglyceridemia (no[a] vs yes)	0.659	0.107	37.913	1.932 (1.567–2.383)	<0.001
Low HDL-C (no[a] vs yes)	0.356	0.124	8.221	1.428 (1.119–1.822)	0.004
ALT (<40[a] vs ≥40 U/L)	0.567	0.127	19.943	1.762 (1.374–2.260)	<0.001
Beneficial alleles (0 vs 1–2 vs 3–4)	−0.167	0.081	4.308	0.846 (0.723–0.991)	0.038
Constant	−1.714	0.116	216.626		

Notes: Hypertension:[26] systolic blood pressure ≥140 mmHg or/and diastolic blood pressure ≥90 mmHg; Hyperglycemia:[22] glucose ≥5.6 mmol/L; Hypertriglyceridemia:[25] triglycerides ≥1.7 mmol/L; Low HDL-C:[25] HDL-C ≤1.04 mmol/L. [a]Reference in analyses of categorical variables.
Abbreviations: NAFLD, nonalcoholic fatty liver disease; OR, odds ratio; 95% CI, 95% confidence interval; BMI, body mass index; HDL-C, high-density lipoprotein-cholesterol; ALT, alanine aminotransferase.

(OR = 3.207, 95% CI = 2.630–3.911, $P < 0.001$), hypertension (OR = 1.477, 95% CI = 1.198–1.821, $P < 0.001$), hyperglycemia (OR = 1.242, 95% CI = 1.021–1.510, P=0.030), hypertriglyceridemia (OR = 1.932, 95% CI = 1.567–2.383, $P < 0.001$), low HDL-C (OR = 1.428, 95% CI = 1.119–1.822, $P = 0.004$), ALT (OR = 1.762, 95% CI = 1.374–2.260, $P < 0.001$), and beneficial alleles (OR = 0.846, 95% CI = 0.723–0.991, $P = 0.038$) were independent predictors of NAFLD.

By combining these 8 variables, a NAFLD risk prediction model was constructed. As shown in Figure 3, the AUROC of the prediction model was 0.779 (95% CI = 0.763–0.795). At a cutoff value of −1.098, the sensitivity and specificity of this model were 79.7% (76.7–82.5%) and 63.7% (61.4–65.9%), respectively. The calculated positive and negative predictive values were 48.8% and 87.9%, respectively. The NAFLD risks estimated by the model and those observed in the real setting differed statistically significantly, according to the Hosmer–Lemeshow test ($\chi^2 = 21.073$, $P = 0.007$).

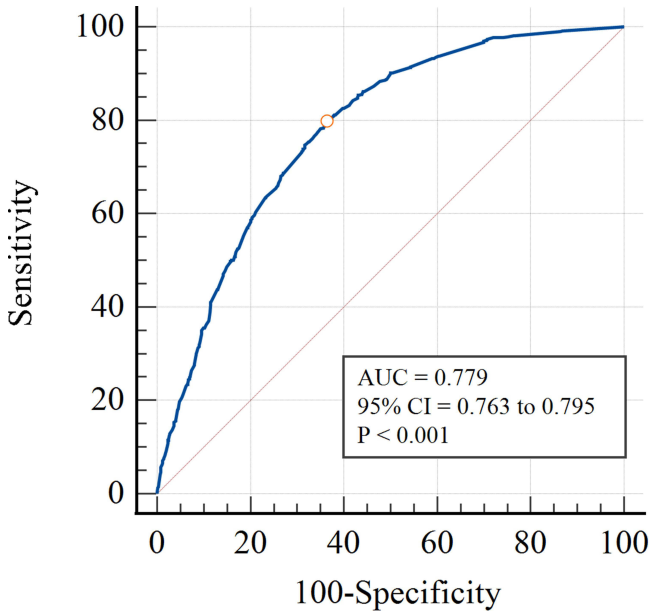


Figure 3 The receiver-operating characteristic curve for the risk prediction model of nonalcoholic fatty liver disease.
Abbreviations: AUC, area under the curve; 95% CI, 95% confidence interval.

Discussion

This research investigated the links between *AKT1* SNPs and the risk of NAFLD in a Chinese Han cohort. Our findings demonstrated that *AKT1* rs2494732-C and rs2494752-G were beneficial alleles related to low NAFLD risk, but no association of rs1130233 with NAFLD risk was found. Furthermore, the combination of beneficial alleles and clinical factors showed an ideal predictive ability for the risk of NAFLD.

The *AKT* gene is located in the chromosome 14q32.33 region. As a downstream effector of the PI3K pathway, it can be activated to regulate the function of multiple substrates in various physiological processes, such as metabolism, cell survival, motility, transcription and cell cycle.[27] Genetic variants of *AKT* are associated with the development of cancer, neurological disorders, obesity, diabetes and other metabolic diseases.[15] Among the three isoforms of *AKT, AKT1* is the most widely expressed and associated with growth and adipogenesis.[28,29] McKenzie et al[20] found that *AKT1* variants might influence obesity-associated metabolic phenotypes in elderly Caucasians. The results of genotyping experiments by Eshaghi et al[21] also suggested an association between *AKT1* polymorphisms and the components of metabolic syndrome. All these findings verify the implication of *AKT1* gene in metabolism disorders.

NAFLD is a metabolic condition that is intimately linked to metabolic syndrome,[11] T2DM,[5] and obesity,[30] and its association with *AKT1* variants has not been explored. This study is the first to show a connection between *AKT1* polymorphisms and the likelihood of developing NAFLD. Additionally, we discovered that people with both rs2494732-C and rs2494752-G had a dose-dependently decreased risk of developing NAFLD. These findings offer fresh arguments for decriminalizing NAFLD.

rs2494732 belongs to an intron polymorphic locus of *AKT1* gene, and studies have focused on its association with schizophrenia,[31] psychosis in cannabis users,[32] and cancers, such as head and neck squamous cell carcinoma[33] and non-small cell lung cancer.[34] For the first time, we discovered in the current investigation, those who carried the rs2494732-C allele had a decreased chance of developing NAFLD. Lieburg et al explored the association of *AKT1* with BMI in cannabis users, finding that the rs2494732 polymorphism was associated with BMI, glycosylated hemoglobin (HBA1c) level and total metabolic risk.[35] BMI, a known risk predictor for NAFLD, has been incorporated into several NAFLD risk assessment models, such as the ZJU index,[36] fatty liver index (FLI),[37] and hepatic steatosis index (HIS).[38] In addition, HBA1c, an index of insulin resistance and type 2 diabetes, is usually increased during the development of NAFLD.[39] More importantly, we noticed that the rs2494732-C variant might affect the expression level of *AKT1* by changing its RNA secondary structure (Figure 2). All these evidences provide clues to explain an association between rs2494732 and NAFLD was found in this study.

Similarly, we also identified rs2494752-G of *AKT1* as a protective genotype against NAFLD. In the previous studies, rs2494752 was found to be involved in various cancers, including liver cancer,[40] esophageal squamous cell carcinoma,[41] gastric cancer,[42] breast cancer,[43] and non-small cell lung cancer.[44] No studies have been conducted to analyze the role of rs2494752 polymorphism in NAFLD, but our data from the UCSC database indicated that the locus was located in the promoter region of *AKT1* gene and close to the peak of H3k4me1 level. It also served as a transcription factor binding site, as shown by the database predicting SNP functions. These findings imply that rs2494752 variant may influence cis-regulatory modules in transcription and translation to regulate the protein level and biological effects of AKT, thus reducing the risk of NAFLD. Further genetic and epigenetic studies are needed to determine the role of this polymorphism in metabolism. No association of rs1130233 polymorphism with NAFLD susceptibility was found in this study, although Eshaghi et al[21] revealed that rs1130233 was connected to key metabolic syndrome markers as hs-CRP and BMI. Genotyping of Iranians showed that *AKT1* rs1130233 was not linked to an increased risk of cardiovascular disease or the metabolic syndrome,[45] which is similar to our findings. Therefore, whether rs1130233 is associated with susceptibility to NAFLD remains to be explored.

Stratified analysis revealed that in the majority of subgroups, the combined effects of rs2494732 and rs2494752 were statistically significant, meanwhile exhibiting no heterogeneity between subgroups (except for that in the age subgroup), suggesting that these variables did not alter this effect. Although age may confound the results, we have adjusted it as a covariate in our analysis. Further multivariate stepwise logistic regression results showed that female and beneficial alleles were independent protective factors for NAFLD. A meta-analysis of the burden of NAFLD in China from 2008 to 2018 suggested that the prevalence of NAFLD was higher in males.[46] This may be related to the greater levels of serum

TG, glucose, and body fat, as well as higher prevalence of hypertension and liver enzyme abnormalities in males.[47] Our results also showed that BMI, hypertension, hyperglycemia, hypertriglyceridemia, low HDL-C, and high ALT were independent risk factors for NAFLD. Variables other than ALT are risk factors proposed by the diagnostic criteria for metabolic syndrome.[48] ALT elevates mildly in NAFLD, making it an indicator of the biochemistry in the liver.[49] What's more, the importance of these variables in screening NAFLD has also been written into the NAFLD guideline.[22] The AUROC of the prediction model implied a good discriminative power. Disappointingly, the results of Hosmer–Lemeshow test showed poor calibration of the predictive model. More studies are needed to establish NAFLD-predicting models with strong abilities of discrimination and calibration. Our predictive models combining genetic and clinical factors may be used in early screening for high-risk NAFLD populations. However, their costs and benefits should be analyzed in future studies.

Some limitations deserve our attention. First, this study was a single-center study and the sample size might not be large enough. We performed frequency matching based on age. Considering the sample size, we did not match sex in two groups. Consequently, we adjusted for sex as a covariate, and performed multivariate and stratified analyses to control the effect of confounding factors. Second, only three SNPs of *AKT1* were selected. There is a need to investigate the combined effect of multiple genes on NAFLD susceptibility. In addition, bioinformatic analysis has limitations in predicting the biological functions of SNPs. Further functional studies are needed. Finally, we did not include behavioral factors (such as diet and exercise) in our predictive models. In future, the combined effects of multiple genetic and behavioral factors on NAFLD susceptibility need to be explored in a prospective context and in large multicenter samples of different ethnicities.

Conclusion

In conclusion, this study revealed for the first time that *AKT1* (rs2494732 and rs2494752) variants are associated with NAFLD susceptibility in the Chinese Han population. These beneficial SNPs and clinical variables work well together to predict NAFLD susceptibility. Our findings offer hints for further functional research as well as markers for screening high-risk NAFLD populations.

Data Sharing Statement

The original contributions presented in the study were included in the manuscript/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics Statement

The studies involving human participants were reviewed and approved by the Institutional Ethics Review Committee of Nanjing Medical University [NO. (2019) 740], complying with the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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