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Original Article

USF-1 genetic polymorphisms confer a high risk of nonalcoholic fatty liver disease in Chinese population

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Abstract: Genetic polymorphisms in upstream transcription factor 1 (*USF1*) were investigated for their links to increased risk of nonalcoholic fatty liver disease (NAFLD) in Chinese population. Between January 2013 and April 2014, 174 patients with NAFLD in the First Affiliated Hospital of China Medical University were selected for this study. A group of 100 healthy subjects were identified as the control group. The MALDI-TOF-MS, a mass spectrometry based technique, was used to detect *USF-1* genetic polymorphisms using PCR amplified DNA products. Furthermore, Automatic Chemistry Analyzer (ACA) was used to determine the clinical indicators. Genotypes, allele frequencies and clinical indicators were measured to assess NAFLD risk in relation to the SNPs. *USF-1* rs6427573 genetic polymorphisms were associated with an increased risk of NAFLD (AA vs. GG: OR = 3.16, 95% CI = 1.56-6.43, *P* = 0.001; GA + AA vs. GG: OR = 1.87, 95% CI = 1.13-3.09, *P* = 0.015; GG + AA vs. AA: OR = 2.96, 95% CI = 1.49-5.88, *P* = 0.001; G vs. A: OR = 2.10, 95% CI = 1.43-3.09, *P* < 0.001). Similarly, rs2516839 polymorphisms also conferred a risk for NAFLD (AA vs. GG: OR = 2.49, 95% CI = 1.43-4.34, *P* = 0.001; GA + AA vs. GG: OR = 1.69, 95% CI = 1.02-2.78, *P* = 0.041). On the other hand, rs3737787 and rs2774279 showed no statistical significances in the NAFLD group and control group (*P* > 0.05). Two *USF-1* genetic polymorphisms, rs6427573 and rs2516839, may present an increased risk of NAFLD.

Keywords: Upstream transcription factor 1, nonalcoholic fatty liver disease, polymorphism, susceptibility, rs3737787, rs2774279, MALDI-TOF-MS, increased risk

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease, which has become a major public health crisis all over the world [1, 2]. NAFLD is classified into two main types, the non-alcoholic steatohepatitis and non-alcoholic fatty liver [3]. The incident rate in the world is high with as many as 15 to 30 adults out of 100 are diagnosed with NAFLD, and 13-18 per 100 are affected by NAFLD [4]. NAFLD ranges from benign steatosis to nonalcoholic steatohepatitis (NASH), and approximately 2-3% of NASH will progress into liver cirrhosis and/or develop hepatocellular carcinoma [5]. Alarmingly, the incidence rate of NAFLD appears to increase with advancing age, and NAFLD is common among the older age group [6]. Insulin resistance and metabolic derangements related to hypertension are the major risk factors that contribute to the development and progression of NAFLD [7]. In addition, some environmental factors including air pollution, industrial chemicals and water pollutants may have an important influence in NAFLD development [8]. A growing body of evidence supports the idea that specific genetic factors, such as upstream-stimulatory factor-1 (USF-1), may play a determining role in the onset and progression of the NAFLD [9, 10].

Upstream stimulating factor 1 (USF1), a 43-kDa protein that belongs to helix-loop-helix-leucine zipper family, regulates gene transcription via its ability to bind strongly to E-box regulatory elements [11]. USF1 is ubiquitously expressed and plays an important role by activating transcription of genes involved in cellular proliferation (*cyclin B1* gene), cell division controlling (*cdc2* gene), inflammation (*Cox2)* and bone remodeling (*Osteopontin* genes) [12]. The transcriptional activity mediated by USF1 can be

altered by mutations/variations in the E-box DNA binding motif or critical changes in the phosphorylation sites targeted by kinases (e.g., p38 stress-activated kinase and extracellular signal-regulated kinase ½) [13, 14]. USF1 plays an essential role in NO synthase transciption in mesangial cells, to control the effects of excessive NO in the mesangium and glomerulus, which is important in the homeostatic regulation of glomerular and vascular function, as well as NO dependent cellular functions, including DNA replication, transcription, and apoptosis [15]. USF1 mediated hepatic lipid overexpression, observed in HepG2 cells induced by glucose, suggest the existence of a glucose-dependent pathway leading to NAFLD [16]. We are interested in genetic polymorphisms within the USF1 gene, since USF1 has multiple roles in normal cell function which might involve in the process of NAFLD.*USF1* gene is 6.73 kb in length, located on chromosome 1q23, and contains eleven exons [17]. *USF1* polymorphisms are associated with angiotensinogen expression in human fat biopsies and the knockdown of *USF1* leads to decreased expression and reduces the circulating pool of angiotensinogen, thereby regulating arterial pressure [17]. Importantly, *USF1* SNPs and haplotypes also associate with neuro-pathological lesions caused by disorders of lipid metabolism and elevated cholesterol, which also impacts synaptic plasticity in the central nervous system [18, 19]. *USF1* variants (rs3737787 and rs2073658) are associated with atherosclerosis, linked to coronary heart disease and sudden cardiac death [20]. We selected 4 gene polymorphisms of *USF1* gene, namely, rs6427573, rs2774279, rs3737787 and rs2516839, by using a combination of NCBI-dbSNP, HapMap databases and Haploview 4.2 software, to analyze the relationship of *USF1* gene polymorphism and the risk of NAFLD in Chinese population. Our aim, ultimately, is to understand the role of USF1 in lipid regulation.

Material and methods

Objects of study

Between January 2013 and April 2014, 174 patients with nonalcoholic fatty liver disease (NAFLD) were examined at First Affiliated Hospital of China Medical University and selected for this study. The patient group included 151 males and 23 females and the average age of 44.22 ± 11.41 years. A total of 100 healthy subjects were selected, and, included 42 males and 58 females and the average age was 32.70 ± 10.80 years. All subjects provided written informed consent to participate in the study. Based on the guidelines for the diagnosis and management of NAFLD issued by the Fatty Liver and Alcoholic Liver Disease Study Group of the Chinese Liver Disease Association, clinical diagnostic criteria are as follows: (i) no history of alcohol drinking or less than 140 g/week ethanol intake in male (less than 70 g/week in female); (ii) specific diseases lead to steatosis, such as viral hepatitis, drug-induced liver disease, Wilson's disease, total parenteral nutrition and autoimmune liver disease must be excluded; (iii) the histological findings of liver biopsy are in accord with the pathological diagnostic criteria of fatty liver disease [21].

Clinical indicators

The height and weight of all objects were measured and the body mass index (BMI) was calculated. 10 ml venous blood of all objects with an empty stomach was collected in the morning. Hitachi 717S analyzer automatically recorded the triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), glutamic-pyruvic transaminase (ALT/GPT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total protein (TP), albumin (ALB), total bile acid (TBA), total bilirubin (T.BIL), urea nitrogen (BUN), creatinine (Crea), fasting blood-glucose (FBG), uric acid (UA).

DNA extraction

Genomic DNA was extracted from blood clots according to the manufacturer's instructions using a commercially available blood DNA purification kit (CWBIO, Beijing). Blood clots (300 µl) were transferred to 2 ml centrifugal tube (Eppendorf), 550 µl GIT lysis buffer was added and the mix were homogenized by the mini-homogenizer (VWR, US). Proteinase K (30 µl) was added and was tumbled for 10 minutes to mix samples. The centrifugal tubes were incubated in an incubator at 56°C until all the white granules on the tube wall completely dissolved. Buffer PP (220 µl) was added to centrifugal tubes and vortexed for 20 seconds, incubated on ice for 5-7 minutes and recovered to room temperature. The sample was centrifuged for 3 minutes at 12,000 rpm and the supernatant

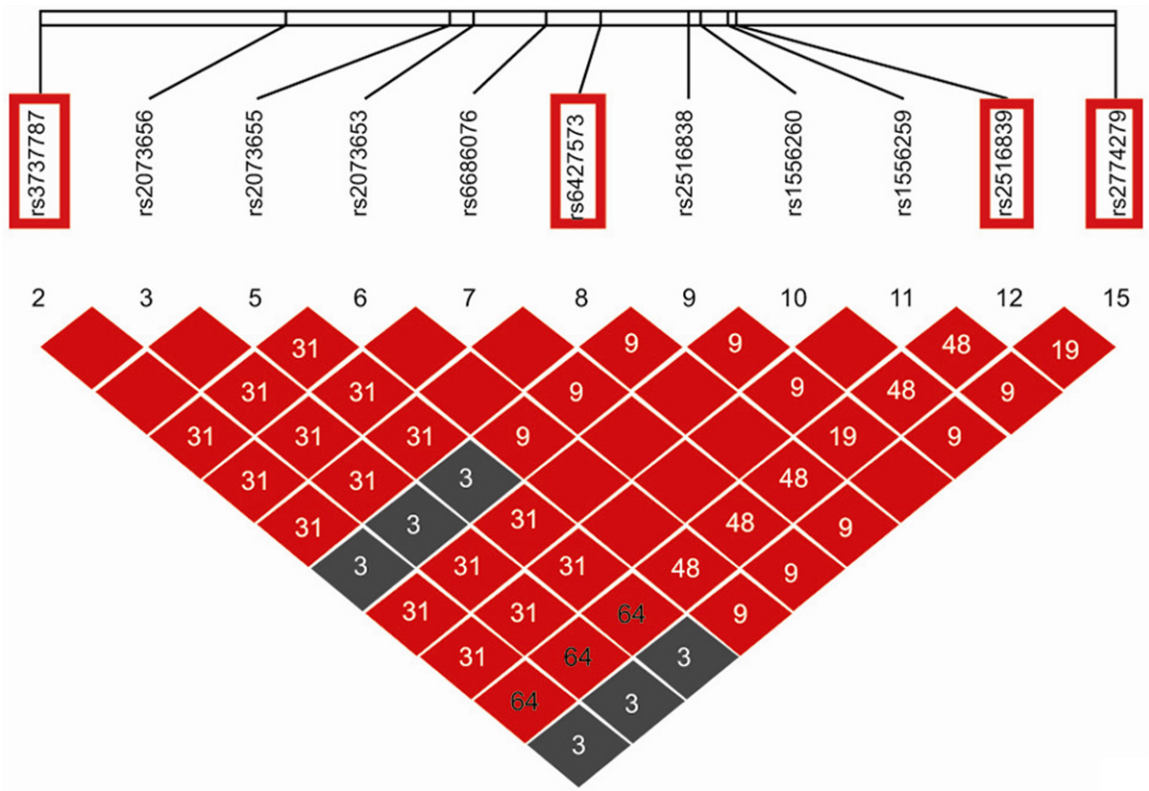

Figure 1. Linkage disequilibrium maps of four SNPs in HapMap CHB population.

was transferred to a new 2 ml centrifuge tube and absolute ethyl alcohol one-half of the volume of supernatant was added. The centrifuge tube was vortexed vigorously for 15 seconds and centrifuged briefly at 2000 rpm to collect the liquid. The liquid containing DNA was transferred to the spin column with a collection tube. The tubes were centrifuged for 1 minute at 12,000 rpm and the waste was discarded, and the spin column was placed back into the collection tube. Buffer GW1 (500 μl) containing absolute ethyl alcohol and Buffer GW2 (500 μl) was added to the spin column in that order and centrifuged again for 1 min at 12, 000 rpm. The spin column was placed at room temperature to air dry and placed in a fresh 1.5 ml centrifuge tube. Buffer GE (100-200 ml) was added to the center part of spin column and kept at room temperature for 2 to 5 minutes to elute bound DNA and centrifuged for 1 minute at 12,000 rpm. DNA solution was collected and stored at -20°C.

Detection of DNA content and purity

A Shimadzu UV 260 spectrophotometer (Shimadzu, Kyoto, Japan) was used to measure the absorbance at wavelengths between 260 nm and 280 nm. Beer-Lambert law was used to calculate sample concentration according to equation: c = A260/ (ε × b), where ε is molar absorption coefficient, b is optical path length, c is molar concentration. A260/A280 ratio was used to determine the sample purity. The DNA purity was between 1.6 and 1.9.

Selection of USF1 SNPs

NCBI-dbSNP (http: //www.ncbi.nlm.nih.gov/SNP/) and HapMap (httP: //SNP.cshl.org/cgi-Perl/gbrowse/haPmaP27_B36/) databases were used to retrieve the data packet of SNPs of human *USF1* gene on chromosome 1 (1q22-23). Haploview 4.2 program (http: //www.broad. mit.edu/mpg/haploview/) was used to select the tag SNPs of *USF1* and the parameters were set at CHB (Han Chinese in Beijing, China), MAF (minor allele frequency) > 10%, r^2 > 0.8, D' = 1. The nucleotide sequences of selected site were suitable for primer design for PCR amplification. Four tag SNPs, namely, rs6427573, rs2774279, rs3737787 and rs2516839, were chosen in this study which could stand for all the other sites in the *USF1* gene (**Figure 1**).

Table 1. Primers for PCR amplification of *USF-1* gene polymorphisms

SNP	Primers for PCR amplification (5'-3')	Length (nt)	Molecular Weigh t (g/M)
rs3737787	F: 5'-ACGTTGGATGAGAGGAGCACAAGGGCCCA-3'	29	9016
	R: 5'-ACGTTGGATGCAGTGGTGTGAAACACACAA-3'	30	9296
rs6427573	F: 5'-ACGTTGGATGAAGTGCTGGGATTACAGGTG-3'	30	9371
	R: 5'-ACGTTGGATGAGAGCAAGATTCTGTCTCAG-3'	30	9278
rs2774279	F: 5'-ACGTTGGATGTCACAGTCCTTCACCTTTCC-3'	30	9080
	R: 5'-ACGTTGGATGAGAACCCCAGGTTCCTGAC-3'	29	8896
rs2516839	F: 5'-ACGTTGGATGCTGGTCCTTTTTTGGAGGTC-3'	30	9257
	R: 5'-ACGTTGGATGTCTACCAGGACTTAGCACTC-3'	30	9176

SNP, single-nucleotide polymorphism; F, forward; R, reverse.

Table 2. Clinical indicators in the NAFLD group and the control group

Clinical indicators	NAFLD group (N = 174)	Control group (N = 100)	*P* value
Age (years)	44.22 ± 11.41	38.70 ± 10.80	0.041[a]
Sex ratio (Male/Female)	151/23	42/58	0.032[b]
BMI (kg/m^2)	27.27 ± 3.00	21.95 ± 2.89	< 0.001[a]
TG (mg/dl)	1.99 ± 1.77	0.85 ± 0.39	< 0.001[a]
TC (mg/dl)	4.95 ± 0.89	4.26 ± 0.57	0.003[a]
HDL-C (mg/dl)	1.13 ± 0.27	1.45 ± 0.30	0.807[a]
LDL-C (mg/dl)	3.13 ± 0.77	2.52 ± 0.57	0.008[a]
ALT (U/L)	33.74 ± 21.11	14.10 ± 5.36	< 0.001[a]
ALP (U/L)	70.46 ± 16.92	59.85 ± 21.46	< 0.001[a]
GGT (U/L)	59.08 ± 56.33	18.93 ± 6.97	< 0.001[a]
TP (g/L)	71.91 ± 3.68	71.22 ± 3.01	0.272[a]
ALB (g/L)	47.72 ± 2.70	44.25 ± 2.21	< 0.001[a]
TBA (μmol/L)	3.44 ± 2.65	2.81 ± 1.81	0.043[a]
TBIL (μmol/L)	12.83 ± 5.50	12.81 ± 4.89	0.781[a]
BUN (mmol/L)	5.14 ± 1.23	4.60 ± 1.13	0.020[a]
Crea (umol/L)	71.97 ± 11.28	61.87 ± 11.94	< 0.001[a]
FBG (mmol/L)	5.98 ± 1.55	5.12 ± 0.42	0.001[a]
UA (mmol/L)	375.2 ± 78.56	305.2 ± 67.33	< 0.001[a]

NAFLD, non-alcoholic fatty liver disease; SD, standard deviation; [a]*P* value of student's t test; [b]χ^2 test; BMI, body mass index; WHR, waist-hip ratio; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma glutamyl trans peptidase; TP, total Protein; ALB, albumin; TBA, total bile acid; TBIL, total bilirubin; BUN, blood urea nitrogen; FBG, fasting blood-glucose; UA, uric acid.

Primer design

Assay Designer 3.1 was applied to design primer and test the feasibility of the primer based on the following conditions: (i) Sequences of the primers and the templates should be closely complementary. (ii) Avoid stable dimers or hairpins between primers. (iii) Avoid DNA mismatch with the template at non-target sites. The design of upstream and downstream primers for PCR reaction should be strictly complied with the design principles of primers. Four primers were shown in **Table 1**.

Genotyping

MALDI-TOF-MS was used to determine the SNP genotype. Reaction conditions of PCR were as follows: 15 min initial denaturation at 94°C, 20 s denaturation at 94°C, 30 s annealing at 56°C, 1 min extension at 72°C, 45 cycles, followed by a final extension step of 72°C for 3 min. The product was stored at -80°C. PCR amplification products were run on agarose gel electrophoresis. Shrimp alkaline phosphatase (SAP) was used to remove excess nucleotides and purify PCR products. The purified reaction conditions

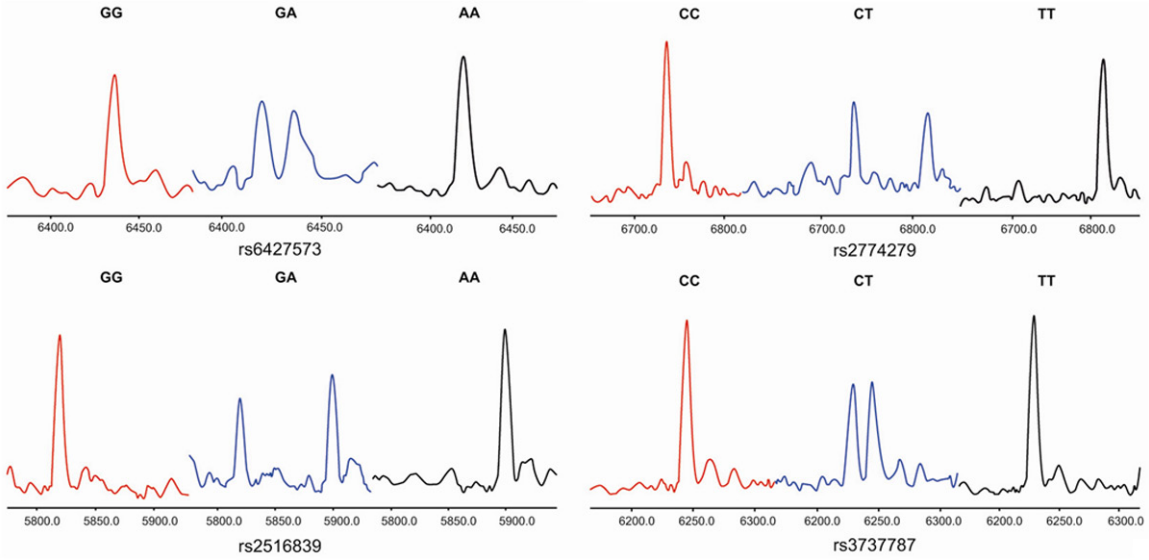

Figure 2. Genotyping profiles of *USF-1* gene polymorphisms.

were as follows: 37°C for 40 min, 85°C for 5 min and the products were stored at 4°C. Single base extension was applied to achieve the molecular weight of single base by MALDI-TOF-MS. Reaction system included × 10 iplex buffer, terminator mix, primer and enzyme. Reaction conditions of single base extension were as follows: 40 cycles of 94°C for 30 s, 94°C for 5 s, 52°C for 5 s; 5 cycles of 80°C for 5 s, 72°C for 3 min and the products were stored at 4°C. TYPER4.0 software was used for data analysis and SNP map.

Statistical analysis

Data was analyzed with SPSS 17.0. For each SNP, Hardy-Weinberg equilibrium was used to test and confirm the representation of the sample in the population. Data were expressed as mean ± standard deviation (mean ± SD). χ^2 test was used to compare differences between groups. Logistic regression analysis was used to calculate the odds ratios (OR) and 95% confidence intervals (CI) of each genotype, representing the relative risk. $P < 0.05$ was considered statistically significant.

Results

Clinical indicators in the NAFLD group and the control group

Except for Crea, the levels of BMI, TG, TC, LDL-C, ALT, ALP, GGT, ALB, TBA, BUN, FBG, and UA of the NAFLD group were significantly higher than those of the control group (all $P < 0.05$). However, the levels of HDL-C, TP and TBIL had no statistical significances between the NAFLD group and the control group (**Table 2**).

Genotyping profiles of USF-1 gene polymorphisms

MALDI-TOF-MS detected two alleles and three kinds of genotypes in each SNP. C allele and T allele were detected in rs3737787 and rs2774279, with C allele as wild-type allele, and T allele as the mutant allele. The locus showed CC genotype as homozygous wild type, CT genotype as heterozygous mutant type and TT genotype as homozygous mutant type. G allele as wild allele and A allele as mutant allele were detected in rs6427573 and rs2516839. In the two sites, GG genotype stands for homozygous wild type, GA genotype as heterozygous mutant type and AA genotype as homozygous mutant type (**Figure 2**).

Distribution of genotypes and allele frequencies

The χ^2 test of goodness of fit was used to analyze the genotype and allele frequencies distributions of rs3737787, rs6427573, rs2774279 and rs25168394 in the NAFLD group and the control group. It showed that the frequencies distributions of four SNPs in the two groups were in accordance with Hardy-Weinberg equi-

Table 3. Distribution of genotypes and allele frequencies

Genotype	NAFLD group (n = 174)	Control group (n = 100)	OR (95% CI)	χ^2	*P* value
rs3737787					
CC	109 (62.6%)	65 (65%)	Ref.		
CT	60 (34.5%)	32 (32%)	1.12 (0.66-1.90)	0.172	0.678
TT	5 (2.9%)	3 (3%)	0.99 (0.23-4.30)	< 0.001	0.993
CT + TT	65 (37.4%)	35 (35%)	1.11 (0.66-1.85)	0.152	0.697
CC + CT	169 (97.1%)	97 (97%)	Ref.		
TT	5 (2.9%)	3 (3%)	0.96 (0.22-4.09)	0.004	0.952
C allele	278 (79.9%)	162 (81%)	Ref.		
T allele	70 (20.1%)	38 (19%)	1.07 (0.69-1.67)	0.01	0.752
rs6427573					
GG	83 (47.7%)	63 (63%)	Ref.		
GA	41 (23.6%)	25 (25%)	1.25 (0.69-2.26)	0.52	0.471
AA	50 (28.7%)	12 (12%)	3.16 (1.56-6.43)	10.69	0.001
GA + AA	91 (52.3%)	37 (17%)	1.87 (1.13-3.09)	5.971	0.015
GG + GA	124 (71.3%)	88 (88%)	Ref.		
AA	50 (28.7%)	12 (12%)	2.96 (1.49-5.88)	10.16	0.001
G allele	207 (59.5%)	151 (75.5%)	Ref.		
A allele	141 (40.5%)	49 (24.5%)	2.10 (1.43-3.09)	14.39	< 0.001
rs2774279					
CC	125 (71.8%)	78 (78%)	Ref.		
CT	45 (25.9%)	19 (19%)	1.48 (0.81-2.71)	1.605	0.205
TT	4 (2.3%)	3 (3%)	0.83 (0.18-3.82)	0.056	0.813
CT + TT	49 (28.2%)	22 (22%)	0.82 (0.44-1.53)	0.379	0.538
CC + CT	170 (97.7%)	97 (97%)	Ref.		
TT	4 (2.3%)	3 (3%)	0.76 (0.17-3.47)	0.125	0.723
C allele	295 (84.8%)	175 (87.5%)	Ref.		
T allele	53 (15.2%)	25 (12.5%)	1.26 (0.75-2.10)	0.775	0.379
rs2516839					
GG	60 (34.5%)	47 (47%)	Ref.		
GA	91 (52.3%)	43 (43%)	2.49 (1.43-4.34)	10.51	0.001
AA	23 (13.2%)	10 (10%)	1.80 (0.78-4.15)	1.939	0.164
GA + AA	114 (65.5%)	53 (53%)	1.69 (1.02-2.78)	4.18	0.041
GG + GA	151 (88.3%)	90 (90%)	Ref.		
AA	23 (13.2%)	10 (10%)	1.37 (0.62-3.01)	0.621	0.431
G allele	211 (60.6%)	137 (68.5%)	Ref.		
A allele	137 (39.4%)	63 (31.5%)	1.41 (0.98-2.04)	3.392	0.066

NAFLD, non-alcoholic fatty liver disease; OR, odds ratio; CI, confidence interval.

librium ($P > 0.05$). Rs6427573 of *USF1* gene was associated with an increased risk of NAFLD (AA vs. GG: OR = 3.16, 95% CI = 1.56-6.43, $P = 0.001$; GA + AA vs. GG: OR = 1.87, 95% CI = 1.13-3.09, $P = 0.015$; GG + AA vs. AA: OR = 2.96, 95% CI = 1.49-5.88, $P = 0.001$; G vs. A: OR = 2.10, 95% CI = 1.43-3.09, $P < 0.001$). Similarly, rs2516839 sites polymorphisms may also carry the risk of NAFLD (AA vs.GG: OR = 2.49, 95% CI = 1.43-4.34, $P = 0.001$; GA + AA vs. GG: OR = 1.69, 95% CI = 1.02-2.78, $P = 0.041$). However, genotypes and allele frequency differences of rs3737787 and rs2774279 showed no statistical significances in both the NAFLD group and the control group ($P > 0.05$) (**Table 3**).

Discussion

NAFLD spectrum ranges from benign steatosis to nonalcoholic steatohepatitis, which might advance to cirrhosis and end-stage liver dis-

eases [7]. Insulin resistance, oxidative stress, inflammation, obesity related hypertension, atherogenic dyslipidemia and metabolic syndromes are major risk factors for the development of NAFLD [1]. Metabolic syndrome is present in 60% of women and 30% of men, and is defined by increased BMI, ALP, TG FBG and decreased level of HDL-C [22]. In line with those findings, our result showed significantly higher levels of BMI, TG, TC, LDL-C, ALT, ALP, GGT, ALB, TBA, BUN, FBG, and UA, but lower levels of Crea in the NAFLD group compared to the control group.

In recent years, gene polymorphisms and the development of NAFLD have generated considerable interest. Al-Serri et al. found that *SOD2* C47T polymorphism was related with advanced fibrosis in non-alcoholic steatohepatitis where oxidative stress could play an important role in hereditary hemochromatosis and liver injury induced by drug [23]. Similarly, microsomal triglyceride transfer protein (*MTTP*) rs3816873 polymorphism is a risk factor for NAFLD, by its influence on MTTP expression, resulting in high production of low-density lipoprotein and removal of lipids from hepatocytes [24]. Other factors, such as *CDKN1A* variants, encoding protein p21, are associated with the rapid progression of idiopathic pulmonary fibrosis, non-small cell lung cancer, and NAFLD [25]. Our results indicate significant relationships between two *USF1* SNPs (rs6427573 and rs2516839) and the risk of NAFLD in Chinese populations according to the logistic regression analyses. To the best of our knowledge, this is the first study to describe a relationship between *USF1* SNPs and the risk of NAFLD in Chinese population. In our study, we found that the polymorphisms of *USF1* rs6427573 (genotype AA) and rs2516839 (genotype AA) are associated with an increased risk of NAFLD. The presence of the homozygous wild type of rs6427573 and rs2516839 significantly enhanced the development of NAFLD. However, for rs3737787 and rs2774279, no statistical significance was observed between both genotypes and allele differences and the risk of NAFLD. Hoffstedt et al. found that the polymorphisms of rs3737787 and rs2073658 in the *USF1* gene were in complete linkage disequilibrium, and they were associated with increased lipolytic effect of noradrenaline, dobutamine, terbutaline, CGP12177 and forskolin in fat cells, localized at the postadrenoceptor level [26], indicating the role of USF1 in lipid metabolism. Laurila et al. also found evidences of genetic interaction between *USF1* (rs2516839) and *APOA5* (rs3135506), reiterating the strong correlation of the genetic variants with regulation of lipid levels [27]. Rs2516839, located in the 5' untranslated region of *USF1*, could affect USF-1 mRNA stability, and is associated with formation of large fibrotic lesion areas and higher triglycerides levels in Australian ADL families [27]. Zeggini et al. also found a link between TM2D and rs2516839 of the *USF1* gene in regulating the triglyceride levels in serum. Our study is the first case-control study based on Chinese population to analyze the relationship of *USF1* gene and the risk of NAFLD.

We must exercise caution based on the limitation of this study. The first limitation of our study is inherent to retrospective studies, which is limited sample size and lack of follow-up data. Second, only 4 SNPs of *USF1* gene were studied which may not fully represent the risk of NAFLD in relation to all available SNPs of the gene. Further study with large sample, multicenter study and multi-population approach should be established. Furthermore, systematic analysis covering the entire *USF1* gene locus and its links to the development of NAFLD should be explored.

In conclusion, we provide evidence that *USF-1* rs6427573 and rs2516839 genetic polymorphisms may increase the risk of NAFLD in the Chinese population. Thus *USF-1* gene could be a potent genetic marker for the diagnosis of NAFLD. Further studies are needed to replicate our findings and strengthen the confidence in data by using larger studies.

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Disclosure of conflict of interest

None.

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