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Association of Tumor Necrosis Factor-alpha Polymorphisms and Risk of Coronary Artery Disease in Patients With Non-alcoholic Fatty Liver Disease

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Background: Cardiovascular events account for the main cause of death in patients with non-alcoholic fatty liver disease (NAFLD), and are largely influenced by genetic factors. Although multiple studies showed that tumor necrosis factor-alpha (TNF-α) polymorphisms are risk factors in the progression of NAFLD, few papers on the association of the polymorphisms and the developing coronary artery disease (CAD) in NAFLD patients have been reported.
Objectives: The present study was designed to evaluate the association of TNF-α polymorphisms at residues -238 and -308, with the risk of developing CAD in Chinese patients with NAFLD.
Patients and Methods: The TNF-α polymorphisms at residues 238 and 308 were genotyped in B-type ultrasonography proven NAFLD patients with (n = 246), without (n = 247) CAD and healthy controls (n = 304), using polymerase chain reaction (PCR). Serum lipid profiles were determined using biochemical methods. Statistical analyses were performed using SPSS statistical software, version 20.0 for Mac.
Results: We found a significant association between TNF-α-238 guanine to alanine (GA) polymorphism and carriers of variant allele A between NAFLD patients with and without CAD ($P < 0.05$). Carriers of the A allele of TNF-α-238 had higher serum triglycerides (TG) and low density lipoprotein (LDL) levels in NAFLD patients with CAD ($P = 0.025$ and 0.001, respectively) and a higher TG level in NAFLD patients without CAD ($P = 0.017$), than their non-carrier counterparts.
Conclusions: In the Chinese Han population that we studied, NAFLD patients who carry the TNF-α-238 GA polymorphism have an increased risk of developing CAD. Mechanisms underlying this potentially important association require further investigation.

Keywords: Tumor Necrosis Factor-alpha; Polymorphism; Genetic; Non-alcoholic Fatty Liver Disease; Coronary Artery Disease

1. Background

Non-alcoholic fatty liver disease (NAFLD), the most common form of liver disease, is now recognized as a major public health problem across the world (1-4). As a hepatic manifestation of the metabolic syndrome, NAFLD may also promote atherosclerosis (5-9). As such, NAFLD patients are at high risk of death due to cardiovascular disease (CVD) (8). Tumor necrosis factor-alpha (TNF-α) is a proinflammatory cytokine that is produced and secreted mainly by inflammatory cells. The TNF-α promotes inflammatory responses to injury and regulates insulin resistance (IR) through insulin signal transduction pathways in the liver (10). Therefore, TNF-α may play an important role in the development and progression of metabolic disorders in humans (11-13). Expression of TNF-α is upregulated by inflammatory mediators, as well as genetic variations in the TNF-α promoter region, which alter the transcriptional activity and protein expression levels of TNF-α (14-18). Previous studies have shown a strong association between the presence of TNF-α polymorphisms and the risk of developing NAFLD, especially in patients of Chinese origin (19-21). A recent meta-analysis further confirmed these data (22). However, other studies examining the association between TNF-α polymorphisms and risk of coronary artery disease (CAD) have yielded discrepant results (23-31). While in Caucasians, TNF-α variants were indeed associated with CAD development, progression, and complications (26), such an association did not appear to exist in the Chinese population (31). Considering the high incidence of CAD-related morality in NAFLD patients, it is of particular importance to further identify the potential association between TNF-α polymorphisms and relative risk of developing CAD in NAFLD patients. Such studies may ultimately provide novel means to combat the development of CAD and to decrease the morality rate of NAFLD patients.

2. Objectives

In the present study, we selected and genotyped two of the most widely studied TNF-α polymorphisms, namely the substitution of guanine (G) by adenine (A) at residues 238 and 308 of the promoter region, in NAFLD patients,

with or without CAD, versus healthy controls. We aimed to assess the effect of these two TNF-α polymorphisms for their association with the risk of Chinese Han NAFLD patients for developing CAD.

3. Patients and Methods

3.1. Study Subjects

This study was approved by the Ethical Committee of Qingdao Municipal Hospital (Qingdao, China) and a written informed consent form was obtained from each subject before participation in the study. This study was performed in accordance with the principles of the Declaration of Helsinki (32).

To test our hypothesis, we selected a total of 493 unrelated adult Chinese NAFLD patients of both genders and 304 healthy controls matched for sex and age with the patients (152 males, 152 females, mean age 61.31 ± 9.40 years) between April 2010 and May 2014. These cases included 493 NAFLD patients diagnosed by B-type ultrasonography [246 patients with CAD (127 males, 118 females, mean age 61.54 ± 10.28 years) and 247 patients without CAD (126 males, 120 females, mean age 62.13 ± 9.74 years)]. These healthy controls were volunteers recruited from the Departments of Gastroenterology and Cardiology of Qingdao Municipal Hospital. All subjects were of Northern Han Chinese origin. The diagnosis of NAFLD was based on the standard clinical evaluation according to the Chinese Association of Medicine in 2010 (33). The CAD was diagnosed by a percutaneous coronary angiogram, analyzed by two experienced interventional cardiologists, and defined by presence of at least 50% stenosis in at least one of the coronary arteries. The control group consisted of individuals who were confirmed as being healthy by echocardiography, medical history, and general and laboratory examinations at the same hospital. Subjects were excluded if they had other liver diseases, cardiac disorders, diabetes mellitus, infectious disease, concurrent major renal and malignant disease, or a history of medication. Basic clinicopathological information (name, age, etc.) was obtained using a standard study questionnaire.

3.2. Biochemical Analyses

Blood samples were taken from each subject after a 12-hour overnight fast and collected into ethylene diamine tetraacetic acid-containing tubes. For biochemical analyses, blood samples were obtained and assessed for serum levels of total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) using routine enzymatic methods. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations were also measured, as described previously (34).

3.3. Genomic DNA Extraction and Genotyping

Genomic DNA was extracted from peripheral blood using the Genomic DNA Purification Kit (Beijing Bioteke Biotechnology, Beijing, China) following the manufacturer's instructions and stored at -20 °C until use. To genotype TNF-α single nucleotide polymorphisms (SNPs), we performed polymerase chain reaction (PCR) analysis using the following primers for TNF-α-238 polymorphism: 5'-AGAAGACCCCCCTCGGAACC-3' and 5'-ATCTGGAGGAAGCGGTAGTG-3'. The PCR amplification profile was achieved by 35 cycles of denaturation, annealing and extension at 94 °C, 59 °C and 70 °C for 1 min, respectively. The resulting PCR products were then separated in 2% gel with a 152-base pair product in size. The primers for the 308 polymorphism were 5'-AGGCAATAGGTTTTGAGGGCCAT-3'and 5'-CATCAAGGATACCCCTCACACTC-3'. The PCR conditions were 35 cycles of denaturation, annealing and extension for 1 min each at 94 °C, 60 °C and 72 °C to obtain a 134-base pair PCR product. The TNF-α genotypes were then detected by direct DNA sequencing using the ABI Prism Sequence Detection System ABI3730 (Foster City, CA, USA).

3.4. Statistical Analysis

Statistical analyses were performed using SPSS statistical software, version 20.0 for Mac (SPSS Inc., Chicago, IL, USA). Genotype and allele frequencies were estimated by counting the DNA sequencing data of each subject. The distributions between patients and controls were analyzed by Pearson's χ^2 test or Fisher's exact test, where appropriate. The Hardy-Weinberg equilibrium between expected and observed genotype distributions was assessed using the χ^2 test. Baseline characteristics are shown as mean ± standard deviation (SD). Differences in characteristics between different groups were examined using the Student's t test, paired samples t test or the χ^2 test. The strength of the association between the polymorphism and presence/absence of CAD was evaluated by logistic regression analysis and estimated by the odds ratio (OR) with 95% confidence interval (CI). A $P < 0.05$ was considered as statistically significant.

4. Results

4.1. Characteristics of the Study Population

The basic characteristics of this study population are shown in Table 1. Healthy controls were matched for sex and age with the NAFLD patient group, with or without CAD ($P > 0.05$). As expected, NAFLD patients had a high prevalence of traditional risk factors for fatty liver, including hypertension, increased body-mass index (BMI), serum TG, TC, and LDL levels and decreased HDL levels, compared to controls (all $P < 0.05$). Moreover, serum ALT and AST levels were also higher in patients compared to controls ($P < 0.05$).

Importantly, NAFLD patients with CAD had a higher prevalence of tobacco use and hypertension, increased BMI, ALT, AST, TG, TC, and LDL, and decreased HDL levels

compared to healthy controls (all P < 0.05). In NAFLD patients without CAD, we also found an increased prevalence of tobacco use, hypertension, TG, and LDL, and a lower HDL level compared to those with CAD (all P < 0.05).

4.2. TNF-α-238 and -308 Genotypes and Allele Distribution

The genotype distributions of the two TNF-α variants were in accordance with the Hardy-Weinberg equilibrium in both patients and controls (all P > 0.05). We found no evidence of the AA homozygote for these two TNF-α variants in our study population. To ensure the accuracy of our genotyping, we randomly repeated the DNA sequencing in 100 subjects for reverse sequencing. The success rate of duplicated genotyping was 100%. As shown in Table 2, at position TNF-α-238, there was significant difference in genotypic and allelic distributions between the NAFLD patients and their control counterparts (OR: 3.007, 95% CI: 1.819-4.971, P = 0.000; OR: 2.760, 95% CI: 1.701-4.478, P = 0.000; OR: 1.824, 95% CI: 1.068-3.116, P = 0.026; OR: 1.759, 95% CI: 1.047-2.957, P = 0.031, respectively). Moreover, a statistically significant difference was observed between NAFLD patients with and without CAD (OR: 1.648, 95% CI: 1.036-2.624, P = 0.034; OR: 1.568, 95% CI: 1.009-2.439, P = 0.044, respectively). In contrast, there was no statistical difference observed for TNF-α-308 SNPs between cases and controls (all P > 0.05).

Table 1. Basic Characteristics of the Study Population [a,b]

Characteristics	Groups			P Value		
	CAD + NAFLD (n = 246)	CAD - NAFLD (n = 247)	Control (n = 304)	P1	P2	P3
Age, y	61.54 ± 10.28	62.13 ± 9.74	61.31 ± 9.40	0.514	0.782	0.316
Gender, female/male	118/127	120/126	152/152	0.991	0.491	0.518
Smoker, No. (%)	115 (46.7)	90 (36.4)	100 (32.9)	0.02	0.001	0.384
BMI, Kg/m^2	25.83 ± 3.20	26.05 ± 3.36	23.13 ± 2.96	0.457	< 0.001	< 0.001
Hypertension, No. (%)	125 (50.8)	99 (40.1)	68 (22.4)	0.017	< 0.001	< 0.001
ALT, U/L	41.88 ± 23.80	42.09 ± 23.12	20.75 ± 9.63	0.92	< 0.001	< 0.001
AST, U/L	40.57 ± 22.01	41.49 ± 22.39	20.33 ± 8.13	0.647	< 0.001	< 0.001
TG, mmol/L	2.34 ± 0.99	1.88 ± 0.81	1.35 ± 0.58	< 0.001	< 0.001	< 0.001
TC, mmol/L	5.22 ± 0.96	5.05 ± 0.94	4.32 ± 0.93	0.057	< 0.001	< 0.001
HDL, mmol/L	1.21 ± 0.38	1.36 ± 0.41	1.56 ± 0.42	< 0.001	< 0.001	< 0.001
LDL, mmol/L	2.90 ± 0.86	2.70 ± 0.86	2.61 ± 0.75	0.011	< 0.001	0.208

[a] Abbreviations: ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; BMI, Body Mass Index; CAD + NAFLD, Non-Alcoholic Fatty Liver Disease Patients With Coronary Artery Disease; CAD – NAFLD, NAFLD Patients Without CAD; HDL, High-Density Lipoprotein; LDL, Low-Density Lipoprotein; P1, CAD + NAFLD vs. CAD – NAFLD; P2, CAD + NAFLD vs. Control; P3, CAD – NAFLD vs. Control; TC, Total Cholesterol; TG, Triglyceride.
[b] Data are Presented as Mean ± SD or No. (%).

Table 2. Distribution of TNF-α-238 and -308 Polymorphisms in These Study Groups [a,b]

Genotype	CAD + NAFLD	CAD - NAFLD	Controls	OR (95% CI)	P1	OR (95% CI)	P2	OR (95% CI)	P3
238									
GG	192 (78.0)	211 (85.4)	278 (91.4)						
GA	54 (22.0)	36 (14.6)	26 (8.6)	1.648 (1.036-2.624)	0.034	3.007 (1.819-4.971)	< 0.001	1.824 (1.068-3.116)	0.026
AA	0	0	0						
Allele G	438 (89.0)	458 (92.7)	582 (95.7)						
Allele A	54 (11.0)	36 (7.3)	26 (4.2)	1.568 (1.009-2.439)	0.044	2.760 (1.701-4.478)	< 0.001	1.759 (1.047-2.957)	0.031
308									
GG	221 (89.8)	224 (90.7)	283 (93.1)						
GA	25 (10.2)	23 (9.3)	21 (6.9)	1.102 (0.607-1.999)	0.750	1.384 (0.747-2.564)	0.302	0.656 (0.358-1.203)	0.173
AA	0	0	0						
Allele G	467 (94.9)	471 (95.3)	587 (96.5)						
Allele A	25 (5.1)	23 (4.7)	21 (3.5)	1.096 (0.613-1.959)	0.756	1.365 (0.746-2.497)	0.311	1.496 (0.827-2.707)	0.180

[a] Abbreviations: CAD + NAFLD, Non-Alcoholic Fatty Liver Disease Patients With Coronary Artery Disease; CAD – NAFLD, NAFLD Patients Without CAD; P1, CAD + NAFLD vs. CAD – NAFLD; P2, CAD + NAFLD vs. Control; P3, CAD – NAFLD vs. Control.
[b] Data are presented as No. (%).

4.3. TNF-α Polymorphism Association With Clinical Parameters in Non-alcoholic Fatty Liver Disease Patients

To assess whether these two TNF-α polymorphisms associate with clinical parameters, we compared TNF-α genotype with the clinical characteristics between NAFLD patients with or without CAD and the control group (Table 3). In NAFLD patients with CAD, serum TG and LDL levels were significantly higher in the GA genotype carriers than those with TNF-α GG genotype at position -238 (P = 0.025 and 0.001, respectively). The TNF-α-238 GA genotype of NAFLD patients with CAD showed higher TC levels (GA, 5.34 ± 1.15 vs. GG, 5.18 ± 0.90) and lower HDL levels (GA, 1.18 ± 0.41 vs. GG, 1.22 ± 0.37) than those with the GG genotype. However, such an association was not statistically significant (P > 0.05). Furthermore, CAD-free NAFLD patients with the TNF-α-238 GA genotype exhibited higher TG levels than those with the GG genotype (P = 0.017). The TNF-α-308 GA genotype of NAFLD patients with CAD was higher than in those of the GG genotype (P = 0.030). No statistically significant differences were observed between the controls and NAFLD patients without CAD, with regards to presence of TNF-α-308 (P > 0.05).

Table 3. Comparison of TNF-α-238 and -308 Genotypes With Clinicopathological Parameters [a,b]

	TNF-α-238 genotypes		TNF-α-308 genotypes	
	GG	GA	GG	GA
CAD + NAFLD	n = 192	n = 54	n = 221	n = 25
Age, y	61.51 ± 10.00	61.67 ± 11.32	61.67 ± 10.27	60.40 ± 10.46
Female/male	89/103	29/25	104/116	14/11
Smoker	89 (46.4)	26 (48.1)	107 (48.4)	8 (32)
BMI, kg/m^2	25.90 ± 3.16	25.61 ± 3.37	25.82 ± 3.06	25.96 ± 4.32
Hypertension	95 (49.5)	30 (55.6)	118 (53.4)	10 (40)
ALT, U/L	42.10 ± 22.52	41.07 ± 23.09	41.54 ± 22.46	42.88 ± 23.85
AST, U/L	41.00 ± 21.48	39.01 ± 23.95	39.94 ± 20.76	40.12 ± 23.00
TG, mmol/L	2.26 ± 0.92	2.60 ± 1.16[c]	2.29 ± 1.02	2.78 ± 0.99[d]
TC, mmol/L	5.18 ± 0.90	5.34 ± 1.15	5.25 ± 0.98	5.27 ± 0.98
HDL, mmol/L	1.22 ± 0.37	1.18 ± 0.41	1.21 ± 0.39	1.24 ± 0.37
LDL, mmol/L	2.81 ± 0.87	3.23 ± 0.83[e]	2.89 ± 0.88	2.96 ± 0.72
CAD – NAFLD	n = 201	n = 46	n = 224	n = 23
Age, y	62.17 ± 9.71	61.96 ± 9.97	62.4 ± 9.61	61.57 ± 10.86
Female/male	100/101	20/26	113/111	7/16
Smoker	73 (36.3)	17 (30.4)	81 (36.2)	9 (39.1)
BMI, kg/m^2	25.97 ± 3.32	26.40 ± 3.54	25.97 ± 3.29	26.91 ± 3.90
Hypertension	83 (41.3)	16 (34.8)	84 (37.5)	10 (43.5)
ALT, U/L	41.86 ± 22.83	43.10 ± 24.58	42.06 ± 22.69	42.38 ± 23.55
AST, U/L	41.34 ± 22.06	42.11 ± 23.99	41.51 ± 21.93	41.26 ± 22.03
TG, mmol/L	1.83 ± 0.81	2.12 ± 0.77[f]	1.84 ± 0.81	2.05 ± 0.78
TC, mmol/L	5.06 ± 0.91	5.02 ± 1.06	5.07 ± 0.93	4.90 ± 0.96
HDL, mmol/L	1.37 ± 0.41	1.32 ± 0.40	1.37 ± 0.41	1.26 ± 0.35
LDL, mmol/L	2.71 ± 0.86	2.67 ± 0.87	2.68 ± 0.86	2.87 ± 0.85
Controls	n = 268	n = 36	n = 283	n = 21
Age, y	61.21 ± 9.22	62.11 ± 10.75	61.09 ± 9.14	64.29 ± 10.22
Female/male	131/137	21/15	139/144	13/8
Smoker	93 (34.7)	12 (33.3)	100 (35.3)	8 (38.1)
BMI, kg/m^2	23.15 ± 3.00	22.98 ± 2.68	23.16 ± 2.99	22.79 ± 2.53
Hypertension	90 (33.6)	11 (30.6)	78 (27.6)	7 (33.3)
ALT, U/L	20.96 ± 9.94	19.19 ± 8.87	20.89 ± 9.80	18.81 ± 8.95
AST, U/L	20.36 ± 8.15	20.11 ± 8.02	20.43 ± 8.06	19.05 ± 8.12
TG, mmol/L	1.35 ± 0.60	1.38 ± 0.45	1.34 ± 0.59	1.45 ± 0.48
TC, mmol/L	4.29 ± 0.91	4.50 ± 1.02	4.33 ± 0.93	4.29 ± 0.96
HDL, mmol/L	1.57 ± 0.42	1.52 ± 0.44	1.57 ± 0.41	1.44 ± 0.46
LDL, mmol/L	2.61 ± 0.76	2.63 ± 0.64	2.63 ± 0.76	2.59 ± 0.54

[a] Abbreviations: ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; BMI, Body Mass Index; CAD + NAFLD, Non-Alcoholic Fatty Liver Disease Patients With Coronary Artery Disease; CAD – NAFLD, NAFLD Patients Without CAD; HDL, High-Density Lipoprotein; LDL, Low-Density Lipoprotein; TG, Triglyceride; TC, Total Cholesterol.
[b] Data are Presented as Mean ± SD or No. (%).
[c] P = 0.025.
[d] P = 0.017.
[e] P = 0.001.
[f] P = 0.030.

5. Discussion

The current study is the first report to associate TNF-α polymorphisms with risk of developing CAD in NAFLD patients. We found that in these patients, presence of the TNF-α-238 GA genotype and the A allele increased the risk of CAD compared with the GG homozygotes. Future studies are required to address whether and how this polymorphism modulates TNF-α expression, as well as the role of TNF-α in the pathogenesis of CAD in the setting of NAFLD.

The human TNF-α gene is localized at chromosome 6p21.3 and codes a protein that acts as a proinflammatory cytokine. It is mainly produced by monocytes and macrophages after Gram-negative bacteria infection or lipopolysaccharide (LPS) (35, 36). As a biomarker of systemic inflammation, TNF-α is centrally involved in the pathophysiology of cardiovascular and metabolic syndromes, including CAD and NAFLD (37). Overexpression of TNF-α may contribute to the development of NAFLD, atherosclerosis and CAD by affecting major lipid metabolism, reducing insulin receptor signaling, and blocking insulin actions (38-40). Valenti et al. (41) showed that TNF-α-238, and not -308, had higher prevalence in Italian patients with NAFLD compared to controls. And carriers of TNF-α-238 A allele had higher insulin resistance indices. Similar findings in Chinese populations were also reported (19-21). We also found that compared to the control subjects, the frequencies of TNF-α-238 A allele, instead of -308 A allele were higher in subjects with NAFLD. Moreover, TNF-α promoter polymorphism at position -238 A allele increased TG and LDL levels in patients with NAFLD, indicating that A allele of TNF-α-238 may contribute to the development of NAFLD. These results are in accordance with other Chinese data (19-21).

The CAD is a multifactorial disorder. Studies examining the association between TNF-α polymorphisms and the risk CAD or other cardiovascular events have generated controversial results. A previous study in non-diabetic Japanese males showed that presence of a TNF-α-863 variant was significantly related to CAD. Specifically, these investigators found that the TNF-α-863 A allele may protect against the progression of CAD (23). On the other hand, Vendrell et al. (26) reported that TNF-α-308 polymorphism may increase the risk of developing CAD in European women with type 2 diabetes mellitus. In agreement with previous studies, Korean researchers showed that TNF-α G to A polymorphism at position -238 was significantly associated with CAD and that carriers of the -238 A allele exhibited an increased risk of developing CAD. It was therefore argued that this allele could be used as a predictive marker for CAD in Koreans (25). However, because of differences in ethnicity, phenotype, and environment factors, discrepant results have been reported in other studies (27-30). In the present report, the frequency of TNF-α-238 GA heterozygote genotype and A allele in NAFLD patients with CAD was substantially higher than in those without CAD. Also, NAFLD patients with CAD who carried the TNF-α-238 A allele had an increase in serum TG and LDL levels than those who did not carry the A allele. There was no association observed between TNF-α-308 polymorphism and risk of developing NAFLD. However, we observed detrimental effects of TNF-α-308 A allele on lipid levels in NAFLD patients with CAD, suggesting that TNF-α A allele could contribute to insulin resistance in CAD patients, which in turn, may exacerbate the risk of cardiovascular complications. In a previous study, it was also shown that TNF-α induced phosphorylation of p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) in human aortic smooth muscle cells, and that these post-translational modifications were inhibited by Phaseolus *vulgaris* extract treatment (42). In chronic heart failure patients, high TNF-α levels have been associated with greater disease severity (43). Interestingly, anti-TNF-α therapy significantly controlled the aortic stiffness, carotid atherosclerosis, and calprotectin in patients with inflammatory arthropathy, indicating that long-term anti-TNF-α therapy reduced aortic stiffness and carotid intima media thickness progression in patients with inflammatory arthropathy (44). All of these studies demonstrated that TNF-α contributed to CAD and thus, further study is needed to associate TNF-α-238 SNP with expression of TNF-α protein.

Our current study is subject to several methodological limitations that are worth nothing (45). First, due to difficulty in obtaining liver biopsy in this epidemiological survey, we resorted to the use of ultrasonography for diagnosing NAFLD. Secondly, we did not associate TNF-α polymorphisms and the level of expression with insulin resistance or disease severity in NAFLD patients. Thirdly, future studies with larger sample sizes and multiple ethnic groups are required to confirm our current data.

In conclusion, this study provided preliminary evidence in favor of an association between presence of a TNF-α-238 polymorphism and the development of CAD in NAFLD patients of Chinese Han origin. TNF-α-238 GA genotype may increase the risk for CAD in NAFLD patients. In addition, the TNF-α-308 GA heterozygote genotype was positively associated with increased levels of TG in NAFLD patients with CAD, suggesting the potential role of TNF-α-308 GA genotype in the development of CAD in these patients. However, mechanisms underlying the association between TNF-α gene polymorphisms and the risk of CAD in NAFLD patients will require further investigation.

Authors' Contributions

Study concept and design: Yuting Cheng, Man Jiang. Acquisition of data: Baiquan An. Analysis and interpretation of data: Yuting Cheng. Drafting of the manuscript: Yuting Cheng. Critical revision of the manuscript for important intellectual content: Shiying Xuan. Statistical analysis: Yuting Cheng, Baiquan An. Administrative, technical, and material support: Yongning Xin. Study supervision: Shiying Xuan.

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