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Original Article

Association of Adiponectin gene polymorphisms and nonalcoholic fatty liver disease

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Abstract: Objective: To investigate the correlation between Adiponectin gene polymorphisms and the genetic susceptibility of nonalcoholic fatty liver disease (NAFLD). Methods: 357 NAFLD patients from January 2005 to December 2013 and 357 cases of healthy controls among the Han population were collected; polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used to detect three tagSNPs (Rs2241767, rsl501299 and rs3774261) of Adiponectin. Risk factors were analyzed by multivariate logistic regression and haplotype analysis was performed using SHEsis software. Results: Rs2241767, rsl501299 and rs3774261 polymorphisms were associated with the risk of NAFLD. Haplotype analysis showed that, A-T-A haplotype was a protective factor of NAFLD (OR: 0.154, 95% CI: 0.011-0.576, P = 0.004) and G-G-A (OR: 4.012, 95% CI: 2.118-10.324, P < 0.001) and G-T-G (OR: 5.219, 95% CI: 2.751-12.651, P < 0.001) haplotype was risk factors of NAFLD. Conclusion: There was an association between Adiponectin gene polymorphisms and the genetic susceptibility of NAFLD.

Keywords: Adiponectin, gene polymorphism, NAFLD

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a kind of metabolic stress liver damage closely related to genetic susceptibility and metabolic syndrome [1, 2]. Its spectrum of disease includes nonalcoholic simple fatty liver disease, nonalcoholic steatohepatitis and the related liver cirrhosis and hepatocellular carcinoma [3-5]. Adiponectin is expressed and secreted by adipose tissues, playing an important role in anti-atherosclerosis, promoting fatty acid oxidation and the regulation of insulin sensitivity, which is an important factor for the regulation of lipid metabolism and glucose metabolism and closely related with the incidence and disease progression of NAFLD [6-8]. Previous studies have shown that some Adiponectin polymorphisms were correlated with the incidence and disease severity of NAFLD [9-12], but these findings are inconsistent. These inconsistent findings may be attributed to the differences in race and research design. In this study we used HapMap database selecting tagSNPs to explore the relevance of Adiponectin gene polymorphisms with NAFLD in Chinese Han population.

Materials and methods

Subjects

This study was approved by the Ethics Committee of Affiliated Hospital of Luzhou Medical College, and all subjects were informed consent.

All selected subjects were the people receiving physical examination in the Affiliated Hospital of Luzhou Medical College from January 2005 to December 2013. 357 cases were respectively included in NAFLD and healthy control groups.

Diagnostic criteria of NAFLD

According to the diagnostic criteria proposed by the group of fatty liver and alcoholic liver disease, the branch of live disease of Chinese Medical Association in 2006, individuals with the items of I~5 and 6 or 7 can be diagnosed with NAFLD. 1) no history of drinking or drinking

Table 1. The primers' sequence

SNPs	Primers sequences (5'-3')	Annealing temperature (°C)	Endonuclease
rsl501299 (G > T)	Sense: CCCGGAGTCCTGCTTCCCCTGCCC	55	Bsm I
	Antisense: TGTCTAGGCCTTAGTTAATAATGAA		
rs2241767 (A > G)	Sense: GGTGAGAAGGGTGAGAAAGGAG	60	Bsu36 I
	Antisense: TACTGGGAATAGGGATGAGGG		
rs3774261 (A > G)	Sense: TGGCATTCAACCACATTTAC	58	Rsa I
	Antisense: AAGCCTTCATTCTTCATCAG		

Table 2. Characteristics of included subjects

Indices	Control group (n = 357)	NAFLD group (n = 357)	*P* value
Gender (M/F)	115/242	118/239	0.887
Age (Years)	55.12 ± 12.11	56.10 ± 11.34	0.134
BMI (Kg/m^2)	21.44 ± 2.19	26.14 ± 2.77	< 0.01
Waistline (cm)	75.24 ± 6.44	90.13 ± 5.41	< 0.01
Hips (cm)	90.76 ± 6.09	99.11 ± 7.31	< 0.01
WHR	0.82 ± 0.07	0.92 ± 0.09	< 0.01
SBP (mmHg)	122.4 ± 13.5	136.1 ± 15.2	< 0.01
DBP (mmHg)	77.5 ± 10.3	85.45 ± 11.5	< 0.01
FPG (mmol/L)	5.6 ± 1.3	6.6 ± 1.5	< 0.01
AST (u/L)	25.6 ± 9.3	34.1 ± 9.7	< 0.01
ALT (u/L)	18.9 ± 8.8	33.2 ± 10.3	< 0.01
TG (mmol/L)	l.24 ± 0.80	2.20 ± 1.12	< 0.01
TC (mmol/L)	5.33 ± 1.22	5.76 ± 1.24	< 0.01
HDL-C (mmol/L)	1.55 ± 0.56	1.23 ± 0.40	< 0.01
LDL-C (mmol/L)	2.64 ± 0.88	2.88 ± 0.97	< 0.01
Fasting insulin (mIU/L)	6.12 ± 2.35	11.44 ± 7.21	< 0.01
High-sensitivity CRP (mg/L)	2.44 ± 1.01	3.89 ± 1.32	< 0.01

but the converted alcohol content < l40 g/week for men, < 70 g/week for women; 2) except for viral hepatitis, specific diseases, such as drug-induced liver disease, total parenteral nutrition and hepatolenticular degeneration, can lead to fatty liver; 3) in addition to the clinical manifestations of the primary disease, there were other nonspecific signs, such as fatigue, indigestion, liver pain, and liver and spleen enlargement ; 4) there may be overweight/visceral obesity, fasting hyperglycemia, blood lipids disorders, hypertension and other components of metabolic syndrome; 5) levels of plasma transaminase and r-glutamyltransferase may have mild or moderate increase, less than five times of the normal upper limit, particularly for ALT; 6) liver imaging findings were consistent with the radiologic diagnostic criteria of diffuse fatty liver; 7) histological changes of liver biopsy were consistent with the pathological diagnostic criteria of fatty liver disease.

In this study, the diagnosis was mainly based on B ultrasound: l) the near-field diffuseness of liver was enhanced (stronger than the kidney and spleen), and the far-field echo gradually attenuated; 2) intrahepatic duct structure was not clear; 3) mild or moderate liver enlargement, with blunt edge angle; 4) color doppler flow imaging (CDFI) prompted that the intrahepatic color flow signal was reduced or difficult to display, but the trend of liver blood vessels was normal; 5) the echo of right lobe capsule and diaphragm displayed unclear or incomplete; patients with mild fatty liver should meet the first item and one of those 2 to 4; patients with moderate fatty liver should meet the first item and two of 2 to 4; severe fatty liver should meet the first item two of 2 to 4 as well as the fifth item.

Abnormal standard of clinical indices

Standard of the metabolic syndrome and its components adopted the consensus of Asia-Pacific region in 2007; 1) obesity: waist circumference of men > 90 cm, that of women > 80 cm, and/or body mass index > 25 kg/m^2, male or female; 2) hypertriglyceridemia (TG): TG > l.7 mmol/L or accepted special treatment of TG abnormalities; 3) hypo high-density lipoprotein cholesterol (HDL-C) cholesterolemia: HDL-C < l.03 mmol/L for male and HDL-C < l.29 mmol/L for female. 4) hypertension: systolic blood pressure (SBP) > l30 mmHg and/or diastolic blood pressure (DBP) > 85 mmHg, or had already been diagnosed with hypertension and received treatment; 5) high fasting plasma glucose

Table 3. Distributions of genotypes and alleles

SNPs	Alleles (1/2)	Groups	Genotypes (n, %)			*P* value	MAF	*P* value	*HWE*
			1/1	1/2	2/2				
rs2241767	G/A	Case	30 (0.084)	138 (0.387)	189 (0.529)	0.002	0.246	< 0.001	P > 0.05
		Control	9 (0.025)	158 (0.443)	190 (0.532)		0.277		P > 0.05
rsl501299	T/G	Case	80 (0.224)	164 (0.459)	113 (0.317)	< 0.001	0.454	< 0.001	P > 0.05
		Control	31 (0.087)	165 (0.462)	161 (0.451)		0.318		P > 0.05
rs3774261	A/G	Case	48 (0.134)	179 (0.501)	130 (0.364)	< 0.001	0.385	< 0.001	P > 0.05
		Control	131 (0.367)	155 (0.434)	71 (0.199)		0.584		P > 0.05

(FPG): FPG > 5.6 mmol/L or have been diagnosed with type 2 diabetes; 6) patients in line with no less than three of above items were diagnosed with metabolic syndrome.

Other indicators: total cholesterol (TC) increase: TC > 5.72 mmol/L; low-density lipoprotein cholesterol (LDL-C) increase: LDL-C > 3.64 mmol/L; ALT increase: ALT > 40 IU/L for male; ALT > 3l IU/L for female; aspartate aminotransferase (AST) increase: Male AST > 37 IU/L; female AST > 3l IU/L.

TagSNPs selection

We downloaded the Adiponectin gene information of Chinese Han population from HapMap database of International HapMap Project; Haploview 4.2 software was used to select three tagSNPs (rs2241767, rsl501299 and rs3774261).

Adiponectin genotyping

DNA was extracted using the extract kit (Bioteck, Beijing, China); genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). PrimerPremier 5.0 software was used to design primers (**Table 1**).

The reaction system was as follows: the total system was 25 uL, containing genomic DNA 2 uL, PCR Mi × 12.5 uL, upstream and downstream primers each l uL, Nuclease-Free Water 8.5 uL. Reaction conditions: pre-denaturation at 94°C for 5 min, 35 cycles; denaturation at 94°C, annealing for 30 s (annealing temperature was shown in **Table 2**), extending at 72°C for 30 s, and finally extending at 72°C for 5 min to terminate the reaction. Target amplified fragments were detected by gel electrophoresis; the restriction analysis of target fragments was performed with the restriction enzyme, and then genotypes were analyzed by 2-3% agarose gel electrophoresis.

Statistical analyses

SPSS l7.0 statistical software (Chicago, IL, USA) was used for statistical analysis. Group representativeness of samples was tested by Hardy-Weinberg equilibrium. Measurement data were tested by normality test and homogeneity of variance analysis. The data in line with normal or approximate normal distribution and the transformed data in line with normal distribution were presented as mean ± standard and analyzed by *t* test; count data were analyzed by χ^2 test; multivariate analysis was performed using Logistic regression analysis. Haplotype analysis was conducted using SHEsis software (http://analysis.bio-x.cn/SHEsisMain.htm).

Results

Characteristics of included subjects

BMI, SBP, DBP, FPG, ALT, AST, TG, TC and LDL-C in NAFLD group were significantly higher than those in control group (P < 0.05), while HDL was significantly lower than that in control group (P < 0.05) (**Table 2**).

Distribution of genotype and allele frequencies

As shown in **Table 3**, genotype frequencies of rs2241767 GG, rs1501299 TT and rs3774261 GG were significantly higher in the NAFLD group (All *P* values < 0.05). The allele frequencies also were differences between NAFLD group and control subjects (All *P* values < 0.05).

Logistic regression analysis of risk factors for NAFLD

Firstly, age, gender and three tagSNPs (rs2241767, rs1501299, and rs3774261) were

Table 4. Logistic regression results

Factors	β	SE	Wald χ^2	*P* value	OR	95% CI
Constant	-22.033	3.130	68.332	< 0.001	0.000	-
Rs2241767	2.110	0.449	19.323	< 0.001	3.232	1.210-7.332
Rs1501299	1.871	0.454	10.211	< 0.001	2.913	1.301-6.310
Rs3774261	1.776	0.409	8.991	0.004	2.121	1.103-4.332
Gender	1.761	0.665	18.321	< 0.001	3.198	1.391-6.323
BMI	0.655	0.093	23.21	< 0.001	2.021	1.440-5.231
TG	1.236	0.411	9.911	0.008	1.443	1.011-4.102
HDL-C	-1.636	0.454	10.542	0.001	0.328	0.099-0.877

Table 5. Haplotype analysis

Haplotype	Frequency of haplotype		χ^2	P	OR	95% CI
	Case	Control				
A-G-A	0.265	0.244	0.508	0.121	1.132	0.786-1.567
A-T-A	0.010	0.088	8.766	0.004	0.154	0.011-0.576
A-T-G	0.401	0.452	1.601	0.216	0.843	0.654-1.231
G-G-A	0.056	0.016	11.980	< 0.001	4.012	2.118-10.324
G-T-A	0.225	0.239	0.012	0.876	0.961	0.754-1.201
G-T-G	0.062	0.010	13.870	< 0.001	5.219	2.751-12.651

used as independent variables to perform dichotomous unconditional logistic regression analysis, stepwise selecting independent variables. It showed that there were statistical significances in rs2241767, rs1501299 and rs3774261 between patients and control subjects. After further adjustment of the risk factors, such as BMI, SBP, DBP, FPG, TG, TC, HDL-C, LDL-C, ALT and AST, the differences remain statistically significant (**Table 4**).

Haplotype analysis

Haplotype analysis of the three tagSNPs was conducted using SHEsis software. It showed that there were a total of six common haplotypes, and there were significant differences in the distribution of three between the two groups. A-T-A haplotype was a protective factor for NAFLD, while G-G-A and G-T-G haplotypes were the risk factors for NAFLD (**Table 5**).

Discussion

Adiponectin is an adipose cell cytokine, and its plasma Adiponectin level correlates with insulin resistance and metabolic syndrome [3]. It also has confrontation on NAFLD and insulin resistance [4, 5]. Our results showed that there were significant differences between the two groups from clinical data. Obesity, hypertension, high cholesterol and other indicators of metabolic syndrome were significantly increased in NAFLD group, which was consistent with the characteristics of the metabolic syndrome, indicating that NAFLD was closely related to the metabolic syndrome.

Previous studies on Adiponectin gene polymorphism and NAFLD were limited in functionality selecting individual locus [9-12]. NAFLD was a complex disease and closely related to metabolic syndrome, which may be the results of multiple loci commonly affected mutations [16, 17]. The study and analysis of individual loci will lead to deviation. We chose tagSNP to investigate 3 tagSNPs of Adiponectin gene in Han population in this study. It comprehensively covered all the common Adiponectin gene polymorphisms, and we did haplotype analysis on related loci in order to get more accurate assessment about the influence of entire Adiponectin gene polymorphisms on NAFLD. Previous studies have shown that Adiponectin gene rs2241766 and rsl501299 were associated with NAFLD [18]. However, due to the lower frequency rs2241766 locus mutation, it failed to enter the HapMap database, therefore it was not included in our study. Hashemi et al.'s study found that Adiponectin rs1501299 polymorphism was closely related to the incidence of NAFLD, and they were correlated with liver inflammation and fibrosis, which were consistent with our findings. We found that TT genotype of rsl501299 locus was a risk factor of NAFLD. In addition, we also found GG genotype of rs2241767 locus was a risk factor of NAFLD. For now the related research between rs2241767 polymorphism and NAFLD had not been found internationally. DU et al. [20] found that in rs2241767 locus AG/GG genotype Han population groups, compared with the AA genotype, plasma Adiponectin level was lower significantly, and increased the risk of type 2 diabetes, indicating that rs2241767 polymorphism was associated with

the metabolic syndrome and NAFLD. While in rs3774261, our study showed that the GG genotype was a risk factor of NAFLD. No report about the correlation between this SNP and NAFLD was reported. LING et al. [21] found that in Turkey and southern European populations, compared with AA genotype, Adiponectin level of GG/GA genotype was significantly increased, indicating that rs3774261 polymorphisms affect blood glucose, lipid metabolism, and associated with NAFLD.

Haplotype analysis showed the risk of NAFLD was significantly reduced in people with A-T-A haplotype. The risk of NAFLD was increased significantly in people with G-G-A and G-T-G, respectively. We chose tagSNPs instead of selecting SNP loci from biological functions. Our research showed that tagSNP rs3774261 and rs2241767 polymorphisms were associated with NAFLD. Future studies need to be taken to analysis its association and function between these two tagSNP and associated SNP in order to further elucidate the specific mechanisms of NAFLD.

Limitation of our study

The limitations of our study included the followings: First, we do not detect the concentration of plasma Adiponectin, therefore we were unable to clarify whether these polymorphic loci could influence the NAFLD by affecting the concentration of plasma Adiponectin. Second, we diagnose NAFLD and its severity only by B-ultrasound without a more accurate clinical diagnosis by liver biopsy. Third, our sample size is small, future studies need larger sample size to confirm our results.

Conclusion

In conclusion, our study indicated that there was an association between Adiponectin gene polymorphisms and the genetic susceptibility of NAFLD in Chinese Han population.

Disclosure of conflict of interest

None.

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