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Original Article

Interactions of central obesity with rs3918242 on risk of non-alcoholic fat liver disease: a preliminary case-control study

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Abstract: NAFLD is a complex disease characterized by inflammation and insulin resistance which is determined by an interaction of genetics and environmental factors. *MMP* gene has been implicated in relation to inflammation and insulin resistance. The preliminary case–control study aimed to investigate the association between Matrix metalloproteinase (MMP)-9-1562C/T (rs3918242), MMP-2-1306C/T (rs243865) and risk of NAFLD and to further evaluate the interactions of central obesity with rs3918242 and rs243865. Two variants, rs3918242 and rs243865, were genotyped by polymerase chain reaction -restriction fragment length polymorphism. Gene-environment interactions on risk of NAFLD was preliminarily investigated by generalized multifactor dimensionality reduction (GMDR) and further confirmed by unconditional logistic regression methods. After adjusting for covariates, increased risk of NAFLD were observed in subjects carrying TT/CT genotypes in rs3918242 ($_{Adjust}$OR=1.64, 95% CI: 1.24, 2.11, *P*=0.006). However, decreased risk of non-alcoholic fat liver disease was found when *MMP-2* rs243865 (TT/CT) genotype carriers compared with CC carrier ($_{Adjust}$OR=0.65, 95% CI: 0.47, 0.72, *P*=0.000).Interactions of central obesity with rs3918242 was preliminarily found by GMDR, with a maximum prediction accuracy (67.61%) and a maximum Cross-validation Consistency (10/10).The unconditional logistic regression method indicated central obesity-positive subject with genotype TT/CT had 4.54 times risk of NAFLD compared to central obesity-negative subjects with genotype CC (OR_{add}^{a}=4.54, 95% CI: 2.81, 7.21, P_{add}^{a}=0.000), which further confirmed the interactions. The results indicate that both rs3918242 and rs243865 is associated with risk of NAFLD. Furthermore, rs3918242 and central obesity have synergistic effects on risk of NAFLD.

Keywords: Interactions, central obesity, matrix metalloproteinase, polymorphisms, non-alcoholic fat liver disease

Introduction

Non-alcoholic fat liver disease (NAFLD) is a common liver disease characterized by a spectrum of histological features ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), NASH-related cirrhosis or even hepatocellular carcinoma (HCC) [1]. In the past decade, due to alterations of lifestyle and prevalence of obesity, the morbidity of NAFLD has increased significantly in china [2]. It is well established that fundamental risk factors for NAFLD are adipose expansion, inflammation and insulin resistance [3]. Nevertheless, the pathogenesis of NAFLD cannot be fully elucidated by these classic risk factors.

Matrix metalloproteinase (MMPs), a family of zinc-dependent proteinases responsible for extracellular matrix remodeling, has a wide range of biological actions on adipose tissue expansion [4], inflammation [5] and insulin resistance [6]. Finding of previous of studies had indicated that circulating MMP-2, MMP-9 altered in obesity, diabetes mellitus, dyslipidemia and even metabolic syndrome [7], suggesting that MMP-2,-9 can be used as an independent predictor of NAFLD. However, to date, no genetic study is available concerning the association between variants in *MMP-2, MMP-9* and risk of NAFLD. Therefore, we examined whether functional polymorphism in *MMP* gene have any bearing on risk of NAFLD. Considering that mounting epidemiological evidence points to an association between obesity and NAFLD [3] and that rs3918242 and rs243865 increased risk of obesity [8, 9], the present study

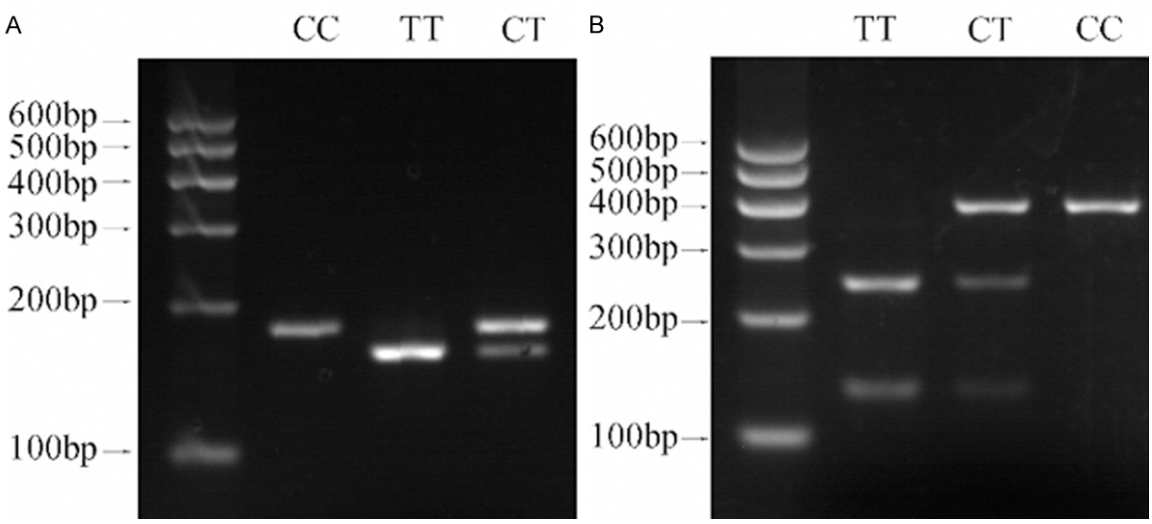

Figure 1. Electrophoretograms of amplified and digested PCR products. A: rs243865, B: rs3918242.

Table 1. Characteristics of patients with NAFLD and controls subjects

Variable	Case (545)	Control (636)	*P*
Gender (M/F)	301/244	324/312	0.141
Age (year)	45.6±10.3	46.9±13.3	0.064
BMI (Kg/m^2)	23.6±4.3	22.9±3.5	0.002
WC (Cm)	86.9±12.3	85.3±12.2	0.020
TC (mmol/l)	3.6±1.3	3.5±1.9	0.225
TG (mmol/l)	1.8±0.9	1.7±0.9	0.057
LDL-C (mmol/l)	2.5±0.6	2.4±0.8	0.017
HDL-C (mmol/l)	1.7±0.7	2.0±0.9	0.000
FBG (mmol/l)	4.8 (3.3, 8.7)	3.6 (3.5, 6.9)	0.039
HOMA-IR	3.1±0.6	2.4±0.8	0.000
Central obesity (%)	46.05	36.79	0.001

also aimed to investigate the interactions of central obesity with rs3918242 and rs243865.

Methods

Study population

636 controls subjects and 545 patients with NAFLD were recruited from hepatology outpatient unit between January 2012 and September 2014. The diagnosis of NAFLD was made according to criteria proposed by the fatty liver and alcoholic liver disease study group of the Chinese Liver Disease Association [10] and the details were previously described [11]. Briefly, all NAFLD patients accorded with sonographic feature findings and had no history of specific diseases that could result in fatty liver and habit of drinking (alcohol consumption: male >140 g/week, female >70 g/week). Healthy controls were recruited according to normal hepatic sonographic feature. Central obesity was diagnosed by criteria made in the guidelines for prevention and treatment of hypertension in China (waist circumference: female >85 cm, male >90 cm). This study was approved by the Ethics Committee in Rennin Hospital of Wuhan University. Written informed consent, as well as assent, was given for each subject.

Anthropometric and biochemical measurements

Height (m) and weight (kg) were obtained to calculate the body mass index (BMI) as weight (kg)/height (m^2). Waist circumference (WC) (narrowest diameter between xiphoid process and iliac crest) were measured. Fasting plasma lipid profiles, such as total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) were examined by enzymatically (Hitachi Ltd., Tokyo, Japan). Fasting plasma glucose was measured by the glucose oxidase method, Serum fasting insulin was determined by a radioimmunoassay technique (Leinco, Sh anghai, China). Insulin resistance was often estimated by the homeostasis model assessment of insulin resistance (HOMA-IR). (HOMA-IR=fasting serum insulin (mIU/l) × fasting plasma glucose (mmol/l)/22.5).

MMP-2,-9 polymorphism genotyping

Genomic DNA was obtained from peripheral blood leukocytes by classic phenol-chloroform

Table 2. Association analysis of genetic polymorphisms with risk of NAFLD

Genotypes	distributions		Without adjustment		With adjustment	
	Controls	Cases	*OR* (95% CI)	*P*	OR_a (95% CI)	*P*
CC	417	290	1			
CT	189	220	1.67 (1.31,2.14)	0.000	1.63 (1.21,2.01)	0.009
TT	30	35	1.68 (1.01,2.79)	0.045	1.64 (1.00,2.51)	0.049
CT/TT	219	255	1.67 (1.32,2.12)	0.000	1.64 (1.24,2.11)	0.006
CC	361	360	1			
CT	234	161	0.69 (0.54,0.88)	0.003	0.57 (0.51,0.81)	0.001
TT	41	24	0.59 (0.35,0.99)	0.045	0.56 (0.31,0.92)	0.024
CT/TT	275	184	0.68 (0.53,0.86)	0.001	0.65 (0.47,0.72)	0.000

[a]Adjusting for WC, FBG, HDL-C, HOMA-IR and Central obesity rate as covariates.

extraction protocols. Rs3918242 and rs-243865 were genotyped using the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method. The PCR primers for rs3918242 and rs243865 were as following: 5'-GCCT GGCACATAGTAGGCCC -3' (primer forward) and 5'-CTTCCTAGCCAGC-CGGCATC-3' (primer reverse); and 5'-CTTCCTAG-GCTGGTCCTTACTGA-3' (primer forward) and 5'-CTGAGACCTGAAGACCTAAAGAGCT-3' (primer reverse), respectively. PCR was performed as following: initial denaturation at 94°C for 5 min; 30 cycles of 95°C for 30s, 58°C (rs243865), 65°C (rs3918242) for 45 s; and final extension at 72°C for 10 min. The PCR products were digested with XspI (rs243865), PaeI (rs-3918242) restriction enzymes and were separated by 2% agarose gel electrophoresis with ethidium bromide staining. Digestion of MMP-2-1306C/T: 188-bp and 5-bp fragments for CC genotype; 188-bp, 162-bp, 5-bp, and 26-bp fragments for CT genotype; and 162-bp, 26-bp, and 5-bp fragments for TT genotype; Digestion of MMP-9-1562C/T: 435-bp fragment for CC genotype; 435-bp, 247-bp and 188-bp fragments for CT genotype; and 247-bp and 188-bp fragments for TT genotype (**Figure 1**).

Statistical analysis

Statistical analysis was performed using SPSS 19.0 (SPSS Inc., Chicago, Illinois, USA). Kolmogorov Smirnov test was performed to determine the distribution characteristics of variables. Continuous variables are described as means±standard deviation or median (min-max) according to presence or absence of normal distribution. The distribution of demographic characteristics and Hardy-Weinberg equilibrium (*HWE*) were examined using Chi-square. Unconditional logistic regression (ULR) method was utilized for confounder effect adjustment and association of the two variations with risk of NAFLD. To evaluate the gene-environment interactions, generalized multifactor dimensionality reduction (GMDR) method, previously described in detail elsewhere [12], was conducted with three output parameters including cross-validation consistency, the testing balanced accuracy, and empirical *P* values after adjusting potential confounders as covariates [12]. In the present study, one-to three factor models were performed and the model with the highest prediction accuracy and maximum cross-validation consistency score was defined as the "best model" [12]. Empirical *P* values of prediction accuracy obtained from permutation testing as a benchmark based on 1000 shuffles [12]. Finally, multiplicative interaction and additive interaction was conducted to confirm the results from GMDR analyses. Additive interaction was evaluated by three parameters including relative excess risk due to interaction (RERI), attributable proportion due to interaction (AP) and synergy index (S) [13]. The 95% CIs of RERI and API without 0 and the 95% CI of the S index without 1 can be considered that an additive interaction exists. Multiplicative interaction was assessed by unconditional logistic regression after adjusting for potential confounders [13]. All reported $P<0.05$ was considered as statistically significant.

Results

Characterization of study population

A total of 1181 with no sibship Chinese subjects were recruited in this study. The average age of the 545 cases was (45.6±10.3) years, and the average age of 636 controls was (46.9±13.3) years, respectively. 55.23%, 50.94% of participants were male in cases and controls, respectively. The distributions of demographic, and clinical characteristics of the

Table 3. GMDR models of gene-environmental interactions on risk of NAFLD

Model[a]	PA	CVC	*P*
central obesity	56.36%	8/10	0.001
rs3918242, central obesity	67.61%	10/10	0.000
rs3918242, rs243865, central obesity	61.37%	9/10	0.001

[a]Adjusting for WC, BMI, LDL-C, HDL-C, FBG HOMA-IR as covariates.

subjects are shown **Table 1**. There were no significant differences between the case group and the control group in terms of age, gender, TC, TG. While terms of WC, BMI, LDL-C, HDL-C, FBG, HOMA-IR and central obesity-positive rate were significantly different between NAFLD patients and controls subjects.

Association analysis of rs3918242 and rs243865 with NAFLD

Rs3918242 and rs243865 were studied in our association analysis. The genotype distributions in the control and case group were in *HWE* (rs3918242: x^2=2.00, *P*=0.156, x^2=0.62, *P*=0.433; rs243865: x^2=0.14, *P*=0.710, x^2=1.20, *P*=0.273). The distributions of two variants between NAFLD patients and controls and their association with risk of NAFLD are shown in **Table 2**. Taking the subjects carrying the TT genotype in rs3918242 as a reference, the subjects carrying genotype CT, CC, CT/CC had an increased risk of NAFLD (OR=1.67, 95% CI: 1.31, 2.14, *P*=0.000; OR=1.68, 95% CI: 1.01, 2.79, *P*=0.045; OR=1.67, 95% CI: 1.32, 2.12, *P*=0.000), and after adjusting for WC, BMI, LDL-C, HDL-C, FBG HOMA-IR, central obesity-positive rate, the increased risk was still found (OR_a=1.63, 95% CI: 1.21, 2.01, P_a=0.009; OR_a=1.64, 95% CI: 1.00, 2.51, P_a=0.049; OR_a=1.64, 95% CI: 1.24, 2.11, P_a=0.006). However, subjects carrying genotype CT, CC, CT/CC had an decreased risk of NAFLD (OR=0.69, 95% CI: 0.54, 0.88, *P*=0.003; OR=0.59, 95% CI: 0.35, 0.99, *P*=0.045; OR=0.68, 95% CI: 0.53, 0.86, *P*=0.001), and after adjusting confounding factors including WC, BMI, LDL-C, HDL-C, FBG HOMA-IR and central obesity rate, the decreased risk was found (OR_a=0.57, 95% CI: 0.51, 0.81, P_a=0.001; OR_a=0.56, 95% CI: 0.31, 0.92, P_a=0.024; OR_a=0.65, 95% CI: 0.47, 0.72, P_a=0.000).

Gene-environment interaction

Firstly, GMDR was performed to preliminarily investigate the gene-environment interactions among rs3918242, rs243865 and central obesity. The results obtained from GMDR analysis for one-factor to three factor models are listed in **Table 3**. Among all models, the two-factor interaction model of rs3918242 and central obesity, with highest prediction accuracy (PA) (67.61%) and maximum cross-validation consistency (CVC) (10/10), was defined as is the best model after adjusting for WC, BMI, LDL-C, HDL-C, FBG HOMA-IR as covariates, suggesting that there was a potential gene-environment interaction between rs3918242 and central obesity affecting risk of NAFLD.

Secondly, to further confirm the potential gene-environment interaction mentioned above, multiplicative interaction and additive interaction analysis was conducted. As described in **Table 4**. After adjusting for WC, BMI, LDL-C, HDL-C, FBG HOMA-IR as covariates, central obesity-negative subjects with the genotype CT/CC had 1.59 times risk of NAFLD compared to central obesity-negative sujects carrying the genotype TT ($OR_{add}{}^a$=1.59, 95% CI: 1.15, 2.12; central obesity-positive subjects with genotype TT had 1.43 times risk of NAFLD that of central obesity-negative subjects carrying genotype TT ($OR_{add}{}^a$=1.43, 95% CI: 1.05, 1.94, additive gene-environment interactions were found between rs3918242 CT/CC and central obesity affecting risk of NAFLD (OR_{add}=4.54, 95% CI: 2.81, 7.21. 0 was not included in 95% CIs of RERI and API, and 1 was not included in 95% CI of S (RERI=2.52, 95% CI: 1.32, 3.72; API=0.56, 95% CI: 0.24, 0.88; and S=3.47, 95% CI: 1.63, 6.84, respectively). At the same time, gene-environment multiplicative interactions were also found ($OR_{multi}{}^a$=1.99, 95% CI: 1.19, 3.43, P_{multia}=0.004). Therefore, it can be considered that a significant gene-environment interaction between rs3918242 and central obesity.

Discussion

In this preliminary case-control study, we selected two variants in *MMP* to investigate the association with NAFLD. Our results indicated that rs3918242 and rs243865 were associated with risk of NAFLD after adjusting for WC, BMI, LDL-C, HDL-C, FBG, HOMA-IR. To our best knowledge, this is the first study concerning the association between rs3918242, rs243865 and NAFLD. There are other reports concerning

Table 4. Gene-environmental interactions analysis between rs833061 central obesity on risk of NAFLD

Genotype	Obesity	Distributions		OR_{add}[a] (95% CI)	P_{add}[a]	OR_{multi}[a] (95% CI)	P_{multi}[a]
		NAFLD	Controls				
TT	NO	122	215	1		1.99 (1.19,3.43)	0.004
CT/CC		172	187	1.59 (1.15,2.12)	0.006		
TT	Yes	168	202	1.43 (1.05,1.94)	0.027		
CT/CC		83	32	4.54 (2.81,7.21)	0.000		
RERI				2.52 (1.32,3.72)			
API				0.56 (0.24,0.88)			
S				3.47 (1.63,6.84)			

[a]Adjusting for WC, BMI, LDL-C,HDL-C, FBG HOMA-IR as covariates. P_{add}: *P*-value from additive gene-environmental interactions analysis. P_{multi}: *P*-value from multiplicative gene-environmental interactions analysis.

the association between the *MMP* polymorphism and other forms of metabolism disease, most notably obesity whose genetic and molecular pathogenesis was partly identical to NAFLD [3]. Indeed, there is extensive evidence that rs3918242 and rs243865 might be associated with obesity [8, 9]. Andrade et al reported that rs3918242 may contribute to pathogenetic mechanisms involved in the development of obesity in women [9]. More recently, rs243865 was also found to be associated with NAFLD obesity [8]. Unsurprisingly, the rs3918242 variant increased the type 2 diabetes risk by 1.82-fold [14]. Taken together, our findings and those from previously published reports demonstrate that the *MMP* polymorphism increases the risk of metabolism disease. Until now, the pathogenesis of rs3918242 and rs243865 affecting susceptibility of NAFLD is still unclear. It may be elucidated by that the genetic variants significantly affects serum MMP level [8, 9], which influences insulin sensitivity and inflammatory factors [5, 6], eventually driving the development of NAFLD.

Obesity, a major public health problem with devastating consequences, is commonly accompanied by inflammation, adipokine dysregulation and subsequent insulin resistance [3, 14] which contributes development of NAFLD. It is now more acceptable that it is the distribution of body fat especially visceral fat (not total fat) that is associated with NAFLD [15]. In general practice, the BMI is often used as a surrogate marker of obesity defined as a BMI>30 kg/m^2 [16]. However, BMI neither distinguishes between the accumulation due to muscle or fat nor takes the distribution of fat into account [16]. Therefore, we recommends WC, a sensitive marker of visceral fat accumulation [16], as a universal criterion of central obesity and non-central obesity in our study.

Given that NAFLD is a complex disease characterized by an intricate interplay of both genetic and environmental factors and that rs3918242 and rs243865 increases risk of obesity [8, 9], the pre-sent study also aimed to investigate the in-teractions of central obesity with rs3918242 and rs243865. Statistical methods such as ULR and GMDR method were applied [17]. The results obtained from GMDR indicated that gene-environment interactions were likely to exist between rs3918242 and central obesity affecting the risk of NAFLD, with synergistic effects on risk of NAFLD. However, interactions between rs243865 and central obesity were not found. To further confirm the synergistic effects, ULR method was conducted. Synergistic effects was further confirmed by the existence of potential additive and multiplicative gene-environment interactions analysis, and central obesity-positive subjects with genotype CT/TT in rs833061 had 4.54 times risk of NAFLD compared to central obesity-negative subjects with genotype CC. The underlying mechanism might be that rs833061 not only increases the risk of NAFLD, but also enhances susceptibility of obesity [9].

Some inevitable limitations warrant consideration. First, the main one of this study was that NAFLD was diagnosed by hepatic ultrasound whose sensitivity and specificity was lower compared with hepatic biopsy [18]. Second, only a limited number of genetic variants and environmental factors related to NAFLD were investigated. Last but not the least, sample size was relatively small and consequently limited the generalizability of our conclusions.

In conclusion, our study has investigated the association between rs3918242, rs243865 and NAFLD and even explored the interactions of central obesity with rs3918242, rs243865. Our findings support that rs3918242 increases risk of NAFLD and rs243865 decreases the risk. Furthermore, rs3918242 is likely to have an interaction with central obesity, and they have synergistic effects on risk of NAFLD. Further well-designed researches are needed to confirm our results.

Disclosure of conflict of interest

None.

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