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Resistin gene polymorphism and nonalcoholic fatty liver disease risk

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ABSTRACT – **Background** – Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disease and one of the main global health issues in which liver fat surpasses 5% of hepatocytes without the secondary causes of lipid accumulation or excessive alcohol consumption. Owing to the link between NAFLD and insulin resistance (IR) and obesity and the role of resistin in theses metabolic disorders, we explored the possible association between resistin gene (*RETN*) variant and NAFLD. **Methods** – A total of 308 unrelated subjects, including 152 patients with biopsy-proven NAFLD and 156 controls were enrolled and genotyped for the *RETN* gene rs3745367 variant using PCR-RFLP method. **Results** – NAFLD patients had higher liver enzymes, systolic blood pressure (SBP), and diastolic blood pressure (DBP) than the controls (P<0.001). However, we observed no significant difference in genotype and allele frequencies between the cases with NAFLD and the controls for the *RETN* rs3745367 polymorphism either before or after adjustment for confounding factors including age, BMI, sex, smoking status, SBP, and DBP. **Conclusion** – To our knowledge, this study is the first one that investigated the association between *RETN* gene rs3745367 variant and biopsy-proven NAFLD. Our findings do not support a role for this gene polymorphism in NAFLD risk in Iranian population; nonetheless, they need to be further investigated in other populations.

Keywords – Gene; NAFLD; resistin; RETN; variant.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disease and one of the most important global health issues in which liver fat surpasses 5% of hepatocytes in the absence of the secondary causes of lipid accumulation or excessive alcohol consumption. Characterized by the hepatic fat accumulation, NAFLD comprises a wide spectrum of disorders ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) which may progress into cirrhosis. There is an increasing interest in NAFLD due largely to its growing burden – it affects approximately 23% to 25% of adults worldwide[1]. Notwithstanding the fact that the etiology of NAFLD has not yet been fully elucidated, insulin resistance (IR) and obesity are known to be implicated in NAFLD pathogenesis. NAFLD is positively connected with abnormal glucose tolerance[2], circulating insulin levels[3], and type 2 diabetes (T2D)[4]. In addition, IR[5, 6] and obesity[7] are risk factors for NAFLD. The severity of histological progression of NAFLD and IR are linked, and IR is less severe in the patients with simple steatosis than those with NASH[8, 9]. The increased levels of liver enzymes are also much lower in the NAFLD patients without IR than those with IR[10]. Previous reports have also shown that in addition to insulin (INS), insulin receptor (INSR), insulin receptor substrates (IRSs), insulin-like growth factors (IGFs) and insulin-like growth factor binding proteins (IGFBPs) are all involved in IR and insulin signaling pathway[11]; and it is interesting that significant associations have been found between NAFLD risk and *INSR*, *IRS2*, *IGF1*, and *GHRL* gene polymorphisms[12-17].

Human resistin, the product of the *RETN* gene, is a cysteine-rich protein contains 108 amino acids. Resistin is largely produced by adipose tissue and inflammatory cells, such as macrophages and monocytes. Previous reports suggest that resistin plays a key role in energy homeostasis, IR, and inflammation and participates in the pathogenesis of NAFLD. By desensitizing fat cells, skeletal muscle cells, and liver cells to insulin, resistin induces hepatic IR[18, 19]. Furthermore, serum resistin level is positively associated with body mass index (BMI)[20], waist circumference[21], IR[22], and NAFLD[21,23]. Resistin also increases the expression of pro-inflammatory cytokines such as TNF-α, IL6, and IL12[24,25]. Finally, significant associations between *RETN* gene variants and the expression of *RETN* gene[26], serum resistin levels[27], obesity[28], T2D[18], and hypertension[18] have been found. Accordingly, the present study investigated the possible association of the rs3745367 polymorphism of *RETN* gene with NAFLD risk. This single nucleotide polymorphism (SNP) was selected based on its high degree of heterozygosity and commonly use in the previous genetic association studies.

METHODS

Study population

This was a retrospective case-control study where 308 Iranian and genetically unrelated individuals [cases with biopsy-proven NAFLD (n=152, age range, 32–87 years) and controls (n=156, age range, 31–82 years)] were enrolled after informed consent. The

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present study had the approval of the Institute's Ethics Committee, following the principles of the Declaration of Helsinki. For the NAFLD group, participants were enrolled after a diagnosis of fatty liver defined by (I) ultrasonographic evidence of fatty liver (II) high serum levels of liver enzymes including aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma glutamyl transferase (GGT) (III) excluding patients with other causes of liver disease such as that caused by alcohol abuse (alcohol consumption >70 g/wk. in women or >140 g/wk. in men), viral hepatitis, Wilson's disease, alpha-1 antitrypsin deficiency, or use of drugs likely to cause NAFLD (IV) the confirmation of liver biopsy consistent with NAFLD by a seasoned pathologist who was blinded to clinical and laboratory data of the patients and analyzed the liver biopsy samples using the Brunt's criteria. Grading of steatosis and necroinflammation was from 0 to 3 and staging of fibrosis was from 0 to 4[29]. For the control group, subjects who were free of elevated liver enzymes and viral hepatitis infection (examined by blood test), and had no liver steatosis (examined by abdominal ultrasonography), and were not alcoholic or on regular medications were enrolled. Controls were recruited from medical students and the research staff of the Institute. All the participants were asked to complete self-administered questionnaires in which they listed their demographic, anthropometric, and clinical characteristics. The formula for calculation of BMI was weight in kilograms (kg) divided by height in meters squared (m^2)[30].

Genotyping

The genomic DNA was purified from 5 mL EDTA-anticoagulated whole blood using phenol chloroform extraction and ethanol precipitation protocol and was stored at -20ºC for further analysis[31]. The genotypes of *RETN* rs3745367 were determined using PCR-RFLP method. Genotyping was performed without the knowledge of case or control status of the participants by different laboratory personnel. The *RETN* gene rs3745367 polymorphism was evaluated using 5'-AGAGACCTCACTGATCCCTG-3' as the forward primer and 5'-TGCTGCTTATTGCCCTAAATAC-3' as the reverse primer. PCR was performed with an initial denaturation at 95ºC for 10 min, followed by 36 cycles of denaturation at 95ºC for 42 s, annealing at 61ºC for 35 s, and extension at 72ºC for 45 s. The final extension was at 72ºC for 10min. PCR products were separated by 3.0 % agarose gels after digestion with the restriction enzyme of BtgI (Fermentas, Leon-Rot, Germany) in a water bath at 37ºC overnight[32]. They were then stained with ethidium bromide (0.5 µg/mL) and visualized with a UV transilluminator. The digestion patterns and the presence ("G" allele) or absence ("A" allele) of the BtgI determined the genotypes of the RETN gene rs3745367 (A/G) polymorphism for each subject. BtgI digestion reveals genotypes denoted AA (358 bp), AG (358, 220, and 138 bp), or GG (220 and 138 bp). Around 20 % of all the samples were genotyped twice to validate the genotyping results with a reproducibility of 100 %.

Statistical analyses

SPSS statistics software for Windows, version 25.0 (SPSS Inc. Chicago, IL, USA) was used for statistical analyses. Continuous variables were expressed as mean (standard deviation) and compared using *t*-test. Categorical clinical variables were presented as number (percent) and compared using chi-square (χ^2) test. To compare the allele frequencies between NAFLD and control groups, we also used χ^2 test. Logistic regression analysis was performed to evaluate the association between the genotype frequencies and NAFLD risk, as well as, to adjust confounding factors[33]. The measure of associations was assessed by the odds ratio (OR) and the corresponding 95% confidence interval (95%CI). Significant statistical level was chosen as $P<0.05$.

RESULTS

Demographic, anthropometric, clinical, and biochemical characteristics of the study population are depicted in TABLE 1. Age and BMI of the NAFLD patients were significantly higher than the controls ($P<0.001$). The cases with NAFLD were also more likely to be male ($P<0.001$) and smoker ($P=0.019$) than the controls. Moreover, systolic blood pressure (SBP), diastolic blood pressure (DBP), and circulating levels of AST, ALT, and GGT were significantly different between the case and control groups, being higher in the case group ($P<0.001$).

TABLE 1. Clinical, demographic and biochemical characteristics of the study subjects[a].

Characteristics	Controls (n=156)	Cases with nonalcoholic fatty liver (n=152)	*P*-value
Age (years)	29.1 (7.5)	38.2 (9.4)	<0.001
BMI (kg/m^2)	23.1 (3.3)	29.5 (5.0)	<0.001
Gender			
Male	81 (51.9)	111 (73.0)	
Female	75 (48.1)	41 (27.0)	<0.001
Smoking status			
Never smoker	142 (91.0)	112 (73.6)	
Former smoker	9 (5.8)	20 (13.2)	
Current smoker	5 (3.2)	20 (13.2)	0.019
SBP (mmHg)	114.4 (13.6)	123.5 (15.1)	<0.001
DBP (mmHg)	69.5 (8.2)	74.1 (9.5)	<0.001
AST (IU/L)	19.6 (7.3)	39.3 (17.0)	<0.001
ALT (IU/L)	19.4 (10.3)	71.8 (40.3)	<0.001
GGT (IU/L)	18.8 (8.7)	58.2 (31.6)	<0.001
Steatosis			
Grade 0			
Grade 1		39 (25.7)	
Grade 2		82 (53.9)	
Grade 3		31 (20.4)	
Necroinflammation			
Grade 0		46 (30.3)	
Grade 1		58 (38.1)	
Grade 2		46 (30.3)	
Grade 3		2 (1.3)	
Fibrosis			
Stage 0		89 (58.6)	
Stage 1		56 (36.8)	
Stage 2		7 (4.6)	
Stage 3			
Stage 4			

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma glutamyl transferase.
[a]Variables presented as mean (SD) or number (%).

The distribution of genotypes and alleles of the *RETN* gene rs3745367 variant in the patients with NAFLD and the controls is provided in TABLE 2. No significant difference was observed for the *RETN* rs3745367 in either genotype or allele frequencies between the patients and the controls. This difference remained insignificant even after adjustment for confounding factors including age, BMI, sex, smoking status, SBP, and DBP.

TABLE 2. The genotype and allele frequencies of resistin gene (*RETN*) rs3745367 variant in the cases with nonalcoholic fatty liver and in the controls[a].

Gene (SNP)	Controls (n=156)	Cases (n=152)	OR (95%CI) *P*-value[b]
RETN (rs3745367)			
Genotype-wise comparison			
GG	47 (30.1)	64 (42.1)	1.0 (reference)
GA	86 (55.1)	66 (43.4)	0.38 (0.13–1.10) 0.076
AA	23 (14.8)	22 (14.5)	0.67 (0.14–3.12) 0.609
GA and AA	109 (69.9)	88 (57.9)	0.43 (0.16–1.17) 0.099
AA versus others	23 (14.8)	22 (14.5)	1.12 (0.27–4.64) 0.875
Allele-wise comparison			
A	180 (57.7)	194 (63.8)	1.0 (reference)
G	132 (42.3)	110 (36.2)	0.77 (0.49–1.18) 0.230

[a]Variables presented as number (%); [b]Adjusted for age, body mass index (BMI), sex, smoking status, systolic blood pressure (SBP), and diastolic blood pressure (DBP) in genotype-wise comparisons.

DISCUSSION

NAFLD which is becoming a worldwide epidemic is a complex metabolic condition in which like other complex diseases the interactions between different genetic and environmental factors determine the presence and severity of the disease. Owing to the fairly small individual effects and complex interactions of these genes, their discovery is not easy. A common approach to identifying novel susceptibility genes is to study the SNPs in candidate genes; although it is difficult to establish whether a SNP is pathogenic or not. Ethnic variation in NAFLD prevalence and familial clustering show that NAFLD has a genetic component. Genes involved in IR, fatty acid metabolism, oxidative stress, immune regulation, and fibrosis development are among the candidate genes for NAFLD[34-37]. Considering the role that resistin plays in IR, obesity, and inflammation, and the key role of theses metabolic disorders in NAFLD pathogenesis, it appears sensible that *RETN* gene might be involved in the development and progression of NAFLD. IR can increase the release of free fatty acids from adipose tissue and their influx into liver[5, 6]. Resistin level is directly associated with IR[22], BMI[20], waist circumference[21], oxidative stress[38], hepatic fat content[39], and fibrosis severity[40]. The expression of resistin mRNA is increased in the liver of NASH patients[41]. Resistin desensitizes the cells of fat, muscle, and liver tissues to insulin and induces hepatic insulin resistance and increases glucose production[42]. On the other hand, high glucose concentration up regulates resistin production in leukocytes[38]. Therefore, maybe resistin is involved in the signaling pathways underlying liver damage and the progression of simple steatosis to steatohepatitis[23]. Alternatively, resistin can cause NAFLD through stimulating inflammation which is a major factor in the pathogenesis of NAFLD. Resistin is a pro-inflammation adipokine and a physiological modulator of inflammation. The expression of resistin is positively associated with the severity of inflammation and liver fibrosis[38]. Resistin participates in liver fibrogenesis by its proinflammatory action[18]. C-reactive protein (CRP), an inflammatory biomarker, is directly linked to circulating level of resistin[43]. Resistin also stimulates the expression level of pro-inflammatory cytokines such as TNF-α, IL6, and IL12 through a nuclear factor-kappa B-dependent pathway. The circulating levels of TNF-α and IL6 which are higher in NAFLD patients can be improved by having a healthy lifestyle and results in improvement of liver damage. Resistin can also up-regulate the TNF-α and IL-1β expression via MEK and ERK pathways by inhibiting some microRNAs (miRNAs). MiRNAs are small non-coding RNA molecules with about 22-25 nucleotides in length which act in post-transcriptional regulation of gene expression[24, 44, 45].

In the present investigation, we conducted a case-control study to explore the possible association between the *RETN* gene rs3745367 variant and NAFLD risk. No statistically significant difference was found for this gene variant in either genotype or allele frequencies between the cases and controls. The *RETN* gene is located on chromosome 19p13.2 and contains four exons and three introns. This gene which has highly conserved intron sequence boundaries encodes 108 amino acids. The rs3745367 polymorphism is located in the intron 2 of *RETN* gene; alterations in intronic sequences can influence RNA splicing and the expression of protein[46]. To the best of our knowledge, no previous studies have investigated the association between *RETN* gene rs3745367 variant and NAFLD risk. Only one report by Zhang et al.[18] studied and found a relationship between this SNP and NAFLD risk in patients with type 2 diabetes mellitus. Previous studies have also shown significant associations between the rs3745367 polymorphism and resistin levels[47] and hypertension[48]. There have been associations between other *RETN* polymorphisms and the expression of *RETN* gene[26], serum resistin levels[27] obesity[28], and NAFLD[36, 49]. Consistently, the findings for the relationship between circulating resistin level and susceptibility to NAFLD are also conflicting. Some researchers have reported that serum levels of resistin in patients with NAFLD compared to healthy controls were higher[23, 25], lower[50], or of no significant difference[51-53]. Inconsistent findings are not rare in genetic association studies and there are several explanations for that such as racial differences in genetic background, genotyped markers, statistical methods, variations in the lifestyle or dietary factors, or even differences in disease definition and the diagnosis methods used for NAFLD[54]. Alternatively, the rs3745367 and other *RETN* variants including rs1862513 may be in linkage disequilibrium with other unknown functional variants of *RETN* gene that explains the discrepancies observed.

This case-control study had the following limitations: (I) Because of the modest sample size it was not sensible to perform sub-analyses. (II) Resistin serum level was not measured due to budget limitations. (III) Owing to the fact that only one polymorphism in the *RETN* gene was genotyped, the coverage of the gene for this genetic association study was not complete. Notwithstanding the aforementioned limitations, the design of this study was good and liver biopsy which is generally considered as the gold standard method to confirm NAFLD diagnosis was used. This study is the first one that investigated the association between *RETN* gene rs3745367 variant and biopsy-proven NAFLD.

CONCLUSION

Our findings revealed that *the RETN* gene rs3745367 variant does not play a role in NAFLD susceptibility; however, this observation need to be investigated in other populations with more participants.

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Authors' contribution

Tabaeian SP: acquisition of data; performing the experiments; survey execution; performing statistical analyses; drafting of manuscript. Mahmoudi T: performing the experiments; study conception and design; analysis and interpretation of data; drafting of manuscript. Rezamand G: acquisition of data; performing the experiments; survey execution; drafting of manuscript. Nobakht H, Dabiri R and Farahani H: acquisition of data; performing the experiments; drafting of manuscript. Asadi A i: performing the experiments; analysis and interpretation of data; coordination responsibility; supervising the project; acquisition of the financial support; drafting of manuscript. Zali MR: acquisition of data; drafting of manuscript.

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Tabaeian SP, Mahmoudi T, Rezamand G, Nobakht H, Dabiri R, Farahani H, Asadi A, Zali MR. Risco de polimorfismo genético resistina e doença hepática gordurosa não alcoólica. Arq Gastroenterol. 2022;59(4):483-7.

RESUMO – Contexto – A doença hepática gordurosa não alcoólica (DHGNA) é uma doença hepática crônica e um dos principais problemas de saúde global em que a gordura hepática ultrapassa 5% dos hepatócitos sem as causas secundárias de acúmulo lipídico ou consumo excessivo de álcool. Devido à ligação entre a DHGNA e resistência à insulina (IR) e obesidade e o papel da resistina em distúrbios metabólicos, exploramos a possível associação entre a variante do gene resistina (RETN) e a DHGNA. **Metodos** – Foram selecionados 308 indivíduos não relacionados, incluindo 152 pacientes com DHGNA comprovada por biópsia e 156 controles para a variante do gene *RETN* rs3745367 usando o método PCR-RFLP. **Resultados** – Pacientes com DHGNA apresentaram enzimas hepáticas mais elevadas, assim como pressão arterial sistólica e pressão arterial diastólica maiores do que os controles (P<0,001). No entanto, não se observou diferença significativa nas frequências genótipo e alelo entre os casos com DHGNA e os controles para o polimorfismo *RETN* rs3745367 antes ou depois do ajuste para fatores de confusão, incluindo idade, índice de massa corporal, sexo, estado de tabagismo, pressão arterial sistólica e pressão arterial diastólica. **Conclusão** – Para nosso conhecimento, este estudo foi o primeiro que investigou a associação entre a variante do gene *RETN* rs3745367 e a DHGNA comprovada em biópsia. Nossas descobertas não suportam um papel para este polimorfismo genético no risco DHGNA na população iraniana; no entanto, eles precisam ser mais investigados em outras populações.

Palavras-chave – Gene; DHGNA; resistina; RETN; variante.

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