Insulin receptor substrate 2 gene Gly1057Asp polymorphism is a risk factor for nonalcoholic fatty liver disease

Shadi Nouri, MD[1], Touraj Mahmoudi, MS[2], Farzaneh Hojjati, MS[2], Zeinab Nourmohammadi Najafabadi, MD[3], Radmehr Shafiee, MD[4], Shiva Sayedsalehi, MD[2], Atefeh Dehghanitafti, MD[2], Abbas Ardalani, MS[2], Kiarash Kohansal, MD[5], Gholamreza Rezamand, MD[6,7], Asadollah Asadi, PhD[8], Hossein Nobakht, MD[9], Reza Dabiri, MD[9], Hamid Farahani, PhD[10], Seidamir Pasha Tabaeian, MD[6,7], Mohammad Reza Zali, MD[2]

[1]Department of Radiology, School of Medicine, Arak University of Medical Sciences, Arak, Iran, [2]Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [3]School of Dentistry, Isfahan University of Medical Sciences, Isfahan, Iran, [4]Department of Clinical Pathology, Faculty of Veterinary Medicine, Tehran University, Tehran, Iran, [5]Physiology Research Center, [6]Department of Internal Medicine, School of Medicine, and [7]Colorectal Research Center, Iran University of Medical Sciences, Tehran, Iran, [8]Department of Biology, Faculty of Science, University of Mohaghegh Ardabili, Ardabil, Iran, [9]Internal Medicine Department, Semnan University of Medical Sciences, Semnan, Iran, [10]Department of Physiology and Pharmacology, School of Medicine, Qom University of Medical Sciences, Qom, Iran. Corresponding author: Seidamir Pasha Tabaeian; noblautoumah@gmail.com

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Abbreviations: NAFLD, nonalcoholic fatty liver disease; IRS2, insulin receptor substrate 2; NASH, nonalcoholic steatohepatitis; IR, insulin resistance; T2D, type 2 diabetes; IGF, insulin-like growth factor; SNV, single nucleotide variant; BMI, body mass index; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; HWE, Hardy-Weinberg equilibrium; PI3K-Akt, phosphatidylinositol 3 kinase (PI3K)-protein kinase B (AKT)

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ABSTRACT

Objective: Nonalcoholic fatty liver disease (NAFLD), which is an emerging global chronic liver disease, has a close association with insulin resistance. We aimed to determine whether the Gly1057Asp (rs1805097) polymorphism of the insulin receptor substrate 2 (*IRS2*) gene is associated with NAFLD.

Methods: Using the polymerase chain reaction-restriction fragment length polymorphism method, 135 patients with biopsy-proven NAFLD and 135 controls underwent *IRS2* genotype analysis.

Results: Genotype and allele distributions of the *IRS2* gene Gly1057Asp variant conformed to the Hardy-Weinberg equilibrium in both the case and control groups ($P > .05$). The Asp/Asp genotype of *IRS2* gene Gly1057Asp polymorphism compared with Gly/Gly genotype was associated with a 2.1-fold increased risk for NAFLD after adjustment for confounding factors ($P = .029$; odds ratio = 2.10, 95% CI = 1.23-3.97).

Conclusion: Our findings revealed for the first time that the Gly1057Asp Asp/Asp genotype of the *IRS2* gene is a marker of increased NAFLD susceptibility; however, studies in other populations are required to confirm the results.

Introduction

Nonalcoholic fatty liver disease (NAFLD), which has recently become a major global health problem, is typified by ectopic lipid accumulation in the liver (more than 5% of hepatocytes) without excessive drinking. NAFLD ranges in severity from simple steatosis to nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis. In spite of the high prevalence of NAFLD worldwide (approximately 1 in 4 adults), its exact pathogenesis has still largely remained unsolved.[1] However, previous research has demonstrated that insulin resistance (IR)[2,3] and obesity[4] are the main contributors in the etiology of NAFLD. NAFLD is also linked with type 2 diabetes (T2D),[4] hyperinsulinemia,[5] impaired glucose tolerance,[6] high deposition of visceral adipose tissue,[2] dyslipidemia,[4] and high blood pressure.[4] Peripheral IR modifies lipid metabolism, and visceral and peripheral fat content facilitates the generation of IR in the hepatocytes and NAFLD development.[3] Consistently, the intensity of IR in NASH patients is higher vs subjects with simple steatosis,[7] and NAFLD patients with IR have higher circulating liver enzymes than patients without IR.[8]

During insulin signaling, insulin binds to its receptor, insulin receptor, leading to its phosphorylation, which in turn results in the

phosphorylation and activation of the substrates of the insulin receptor or insulin receptor substrates (IRSs), including IRS2. This initiates a cascade of second messengers, such as PI3K and Akt, which eventually leads to the regulation of glucose and lipid metabolism. IRS2 is a scaffolding protein that is heavily involved in the insulin signaling pathway and in pancreatic β-cell development and survival. It controls downstream signaling of insulin-like growth factor 1 (IGF1) and its receptor. At molecular levels, the impairment of the IRS2- phosphatidylinositol 3 kinase (PI3K)-protein kinase B (AKT) pathway has a key role in hepatic IR.[9–11] The *IRS2* gene appears to be implicated in the development of some metabolic disorders; significant associations have been detected between single nucleotide variants (SNVs) in the *IRS2* gene and IR,[12] T2D,[13–15] and obesity.[16,17] Finally, significant associations between NAFLD risk and some polymorphisms in the insulin pathway genes of *INSR* and *IGF1* have been found.[5,18] This study aimed to determine the possible effect of the *IRS2* gene Gly1057Asp (rs1805097) variant on the susceptibility to NAFLD. This SNV was chosen on the basis of its usage in prior genetic association studies, its position in the gene, and its high degree of heterozygosity.

Materials and Methods

Study Population

A total of 270 genetically unrelated participants including 135 patients with biopsy-proven NAFLD (age range, 31-79 years) and 135 controls (age range, 30-76 years) were enrolled in this case-control study. The study protocol was permitted by the Ethics Committee of the Research Institute for Gastroenterology and Liver Diseases of Shahid Beheshti University of Medical Sciences (Tehran, Iran), and all the subjects were informed about the study's aim and their consent was obtained before entering the study. The study adhered to the principles of the Declaration of Helsinki and subsequent amendments. Self-administered questionnaires were used to obtain general characteristics of the participants who had the same ethnic background. In this study, NAFLD patients were enrolled on the basis of the following criteria for the diagnosis of NAFLD: (1) detection of hepatic steatosis on ultrasonography; (2) considerable rise in the circulating liver enzymes levels; (3) absence of secondary causes of hepatic fat accumulation, including history of alcoholism (alcohol intake of more than 70 g/week for women or 140 g/week for men), hepatitis B or hepatitis C virus infection, Wilson's disease, autoimmune hepatitis, or medication-induced liver steatosis; and (4) liver biopsy evidence of NAFLD provided by an experienced pathologist whose analyses of the biopsy samples were in accordance with Brunt's criteria. The grades of steatosis and necroinflammation were from 0 to 3, and the stages of fibrosis were from 0 to 4. We recruited the control group from students of Shahid Beheshti University of Medical Sciences or the staff of the Research Institute for Gastroenterology and Liver Diseases. The controls consisted of individuals without abnormalities in abdominal ultrasound imaging. They all had normal serum liver enzymes levels, and none of them had a viral hepatitis infection, were addicted to alcohol, or were prescribed regular medications. Body mass index (BMI) was computed as weight divided by height squared.[19,20]

SNV Genotyping

The laboratory assistants conducted the experiments blinded to the participants' clinical data. Peripheral venous blood (5 mL) was collected in anticoagulative tubes containing EDTA and kept refrigerated at 4°C. We extracted the DNA of each individual from their blood samples using phenol chloroform extraction and ethanol precipitation protocol and stored it at −20°C until use. Genotyping of the exon 1 variant of the Gly1057Asp in the *IRS2* gene was done by the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method. In brief, genomic DNA was amplified by using the primers 5'-AGCTCCCCCAAGTCTCCTAA-3' and 5'-GGCCACACCAAAAGCCATCT-3' to discover the genotypes of the *IRS2* gene. The PCR conditions were as follows: (1) predenaturation at 95°C for 10 minutes; (2) 35 cycles for denaturation at 95°C for 45 seconds, annealing at 59°C for 35 seconds, and extension at 72°C for 40 seconds; and (3) a final extension at 72°C for 10 minutes. We used a negative control for each experiment. The PCR products (291 bp) were then analyzed by using RFLP: overnight digestion with the restriction enzyme of HhaI (Fermentas) at 37°C in a water bath. Electrophoresis was performed on 3.5% agarose gel stained with ethidium bromide and then the RFLP products (291 bp, 221 bp, and 70 bp) were visualized using ultraviolet light transillumination. The Gly allele of the *IRS2* Gly1057Asp SNV had bands of 221 bp and 70 bp, whereas its Asp allele had a band of 291 bp; thus an individual with band(s) at 221 bp and 70 bp, at 291 bp only, or at 291 bp, 221 bp, and 70 bp was defined as the Gly/Gly homozygotic genotype, Asp/Asp homozygotic genotype, and Gly/Asp heterozygotic genotype, respectively. Fifteen percent of the samples were retested randomly to check the reproducibility of the genotyping.

Statistical Analysis

Data were analyzed by SPSS software, version 25.0. For comparing continuous variables, such as age and BMI, which were expressed as mean (SD), the Student *t*-test was used. To compare categorical clinical variables of sex and smoking status, which were presented as number (%), the χ^2 test was applied. Identified genotype distributions of the *IRS2* gene Gly1057Asp variant in the case and control groups were also analyzed separately using the χ^2 test to see if they met Hardy-Weinberg equilibrium (HWE) requirements. This test was used to assess the potential differences in the allele frequencies between the 2 groups. Logistic regression analysis was performed to evaluate the possible associations between the genotype frequencies and susceptibility to NAFLD as well as for adjusting confounding factors, including age, BMI, sex, smoking history, systolic blood pressure, and diastolic blood pressure. The odds ratios (ORs) with their 95% CIs were calculated to assess the strength of the genetic associations. A significant difference was defined as $P < .05$.

Results

The characteristics of the study population are described in **TABLE 1**. The age ($P < .001$), BMI ($P < .001$), smoking rate ($P = .036$), sex ratio (male-female) ($P < .001$), systolic blood pressure ($P < .001$), and diastolic blood pressure ($P < .001$) in the NAFLD group were significantly higher than those in the control group. Compared with the control subjects, the NAFLD patients had significantly higher serum levels of aspartate aminotransferase ($P < .001$), alanine aminotransferase ($P < .001$), and γ glutamyl transferase ($P < .001$).

TABLE 2 depicts the genotype and allele frequencies of the *IRS2* gene Gly1057Asp variant in the study population. Genotype and allele

TABLE 1. General characteristics of the populations studied[a]

Characteristics	Controls (n = 135)	Patients with NAFLD (n = 135)	*P* value[b]
Age, y	31.2 (7.1)	37.5 (9.0)	<.001
Body mass index, kg/m^2	25.3 (3.4)	28.8 (5.2)	<.001
Sex			
Male	73 (54.1)	95 (70.4)	
Female	62 (45.9)	40 (29.6)	<.001
Smoking			
No	119 (88.1)	106 (78.5)	
Former	9 (6.7)	14 (10.4)	
Current	7 (5.2)	15 (11.1)	.036
Systolic blood pressure, mmHg	115.0 (12.9)	122.6 (14.2)	<.001
Diastolic blood pressure, mmHg	68.8 (8.1)	73.5 (8.9)	<.001
Aspartate aminotransferase, IU/L	20.8 (7.6)	38.5 (17.0)	<.001
Alanine aminotransferase, IU/L	19.7 (10.2)	70.8 (38.6)	<.001
Gamma glutamyl transferase, IU/L	17.8 (7.7)	57.4 (30.5)	<.001
Steatosis			
Grade 0		-	
Grade 1		31 (23.0)	
Grade 2		75 (55.5)	
Grade 3		29 (21.5)	
Necroinflammation			
Grade 0		44 (32.6)	
Grade 1		43 (31.9)	
Grade 2		45 (33.3)	
Grade 3		3 (2.2)	
Fibrosis			
Stage 0		70 (51.8)	
Stage 1		54 (40.0)	
Stage 2		9 (6.7)	
Stage 3		2 (1.5)	
Stage 4		-	

NAFLD, nonalcoholic fatty liver disease.
[a]*Variables are presented as mean (SD) or number (%).*
[b]*P value less than .05 was considered statistically significant.*

distributions of this SNV conformed to the HWE test both in the case and control groups ($P > .05$). This implies that, in this study, we used a representative sample population. Analysis of the *IRS2* gene Gly1057Asp polymorphism showed a significant difference between the patients and the controls. The Asp/Asp genotype of *IRS2* gene Gly1057Asp variant compared to the Gly/Gly genotype was significantly more frequent in the cases with NAFLD than the controls even after adjustment for confounding factors such as age and BMI ($P = .029$; OR = 2.10, 95% CI = 1.23-3.97).

Discussion

In this study, we found further evidence of the role of *IRS2* gene in NAFLD. Carrying the Gly1057Asp Asp/Asp genotype of the *IRS2* gene was associated with a 2.1-fold rise in NAFLD risk.

TABLE 2. Insulin receptor substrate 2 (*IRS2*) gene rs1805097 polymorphism in patients with nonalcoholic fatty liver disease (NAFLD) and controls and logistic regression analysis[a]

IRS2 (rs1805097)	Controls (n = 135)	NAFLD (n = 135)	OR (95% CI)	*P* value[b]
Genotype-wise comparison				
GG	57 (42.2)	46 (34.1)	1.0 (reference)	
GA	61 (45.2)	62 (45.9)	1.33 (0.75-2.90)	.617
AA	17 (12.6)	27 (20.0)	2.10 (1.23-3.97)	.029
GA and AA	78 (57.8)	89 (65.9)	1.48 (0.67-3.24)	.208
AA vs others	17 (12.6)	27 (20.0)	1.71 (0.64-3.11)	.305
Allele-wise comparison				
G	175 (64.8)	154 (57.0)	1.0 (reference)	
A	95 (35.2)	116 (43.0)	1.36 (0.70-2.47)	.175

[a]*Variables presented as number (%).*
[b]*Adjusted for age, body mass index, sex, smoking history, systolic blood pressure, and diastolic blood pressure in genotype-wise comparisons.* P *value less than .05 was considered statistically significant.*

A better comprehension of NAFLD pathogenesis will assist in the development of personalized treatments. Search for NAFLD genes has remained challenging due largely to the fact that the nature of NAFLD is supposed to be multifactorial and it derives from the interactions between multiple genes and environmental factors. Detecting the genes, however, is difficult due to, for example, the observed inconsistencies in association studies, which in turn stem from diversity in disease definition, diet, lifestyle, genetic background, or statistical analyses.[21–24] Familial clustering and ethnic diversity in NAFLD prevalence are due to genetic predisposition to the disease. Considering the fact that IR and obesity are very often among the main characteristics of NAFLD and IR is the key mechanism in the development and progression of NAFLD, the genes involved in IR are presumably candidate genes for NAFLD. The insulin signaling pathway regulates metabolism of lipids, and IR is closely linked to hepatic lipid accumulation and advanced fibrosis in NAFLD patients. IRS2 has a key role in the proximal insulin pathway, regulation of glucose homeostasis, and control of bodyweight.[25,26]

To date, there is only 1 study that investigated the role of the *IRS2* gene in susceptibility to NAFLD. Dabiri et al[27] showed that the rs2289046 3'-UTR polymorphism of the *IRS2* gene might contribute to the pathogenesis of NAFLD. In accordance with their finding, this study suggested that the Asp/Asp genotype of the *IRS2* gene Gly1057Asp variant was a risk factor for NAFLD when compared with the Gly/Gly genotype. The *IRS2* gene is located on chromosome 13q34 with numerous polymorphisms, including Gly1057Asp, which is a common nonsynonymous SNV with significant associations with various diseases. The Gly1057Asp variant causes an amino acid substitution of Gly to Asp at codon 1057 in the *IRS2* gene. Apparently, this amino acid substitution, which is in the proximity of 2 putative tyrosine phosphorylation sites at positions 1042 and 1072, may make a change to the tertiary structure and function of the IRS2 protein, which in turn leads to impaired signal transduction. Thus, this SNV, by introducing a charged amino acid (Asp) in place of a neutral one (Gly), may affect the insulin signaling pathway and alter the downstream signaling through IRS2.[14,28] Prior investigations have also indicated that subjects with the

Gly1057Asp polymorphism have a higher grade of obesity-independent IR[12] and reduced pancreatic β-cell function.[15] The Gly1057Asp variant interacts with obesity to affect pancreatic β-cell function and under conditions of obesity or high circulating levels of nonesterified fatty acids, has a detrimental effect on β-cells.[29] In comparison with the Gly/Gly+Gly/Asp genotype, the Asp/Asp genotype of the Gly1057Asp variant increases the susceptibility to T2D by interacting with obesity.[14,15] Individuals with the Asp/Asp genotype also have a higher serum C-peptide concentration, which is probably linked to lower insulin sensitivity.[13] Consistent with the above literature, we showed that the *IRS2* Gly1057Asp Asp/Asp genotype was a risk factor for NAFLD. More evidence corroborates the hypothesis that IRS2 might be involved in NAFLD pathogenesis. Insulin resistance with deficiency of IRS2-associated PI3K activity leads to a rise in intracellular fatty acid-derived metabolites. Metformin, by ameliorating the hepatic IRS2-PI3K-Akt pathway improves IR. Eugenol, which has hepatoprotective, anti-hyperglycemic, and anti-inflammatory properties, modulates insulin sensitivity and improves IR by upregulating IRS2 in rats with NAFLD. Previous reports have also demonstrated that the effects of miR-190b as a microRNA on IR is mediated via IRS2-PI3K-Akt signaling.[11,30] IRS2 also participates in preadipocyte differentiation through the upregulation of specific transcriptional factors like peroxisome proliferator-activated receptor γ.[17] Finally, the disruption of IRS2 in knockout mice results in metabolic defects in liver, hepatic IR, fatty acid accumulation in hepatic cells, steatosis, beta-cell failure, reduced β-cell mass and insulin secretion, and diabetes.[9,11,15,28,29] Consequently, there is accumulating evidence suggesting that the *IRS2* gene might contribute to NAFLD development and progression; however, the mechanism of action remains to be clarified by further research.

Some limitations of this study must be reported. A limitation is that circulating concentrations of insulin and glucose as well as sufficient genetic marker sites were not obtained; this was because of limited funding. The other limitation of this study was its modest sample size, also mostly due to budget limitations. The strengths of this report also warrant attention. The design was good and multicenter research was carried out. In addition, the gold standard method (liver biopsy) was applied and NAFLD diagnosis was not based primarily on ultrasonographic results. Finally, this study put forward some interesting and novel findings that were in accordance with prior publications.

In summary, the Gly1057Asp Asp/Asp genotype of the *IRS2* gene might predict susceptibility to NAFLD, but future larger studies are needed to confirm this finding.

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Conflict of Interest Disclosure

The authors have nothing to disclose.

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