A 3'-untranslated region variant (rs2289046) of insulin receptor substrate 2 gene is associated with susceptibility to nonalcoholic fatty liver disease

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Abstract

Purpose **: Nonalcoholic fatty liver disease (NAFLD) is an increasing global health concern defined by excessive hepatic fat content in the absence of excessive alcohol consumption. Regarding the key role of insulin and insulin resistance in NAFLD, we investigated whether insulin receptor substrate 1 (IRS1) and insulin receptor substrate 2 (IRS2) gene variants were associated with NAFLD risk.**

Methods **: In this case-control study, 305 subjects including 151 cases with biopsy-proven NAFLD and 154 controls were enrolled. All the subjects were genotyped for IRS1 (rs1801278) and IRS2 (rs2289046) gene variants using PCR-RFLP method.**

Results **: Our findings showed that the IRS2 rs2289046 "GG+AG" genotype compared with "AA" genotype to be a marker of decreased NAFLD susceptibility and the difference remained significant even after adjustment for confounding factors including age, BMI, sex, smoking status, systolic blood pressure, and diastolic blood pressure (P=0.014 ; OR=0.50, 95%CI= 0.29-0.87). Furthermore, the IRS2 "G" allele was significantly underrepresented in the cases with NAFLD than controls (P=0.026 ; OR=0.62, 95%CI=0.41-0.94). However, no significant difference was found for IRS1 rs1801278 gene variant.**

Conclusions **: This study suggests, for the first time, that the IRS2 gene rs2289046 variant may play a role in NAFLD susceptibility. Nevertheless, this observation warrants further investigations in other populations.** (Acta gastroenterol. belg., **2020**, 83, **271-276**).

Keywords : Insulin, *IRS1* gene, *IRS2* gene, NAFLD, variant.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease and its prevalence is rapidly increasing worldwide. NAFLD is considered the hepatic expression of metabolic syndrome and defined by excessive hepatic fat content in the absence of excessive alcohol consumption. The spectrum of NAFLD ranges from simple steatosis to non-alcoholic steatohepatitis (NASH) with potential for progression to cirrhosis and hepatocellular carcinoma. Given its very high global prevalence, twenty-five percent, it is of paramount importance that the pathogenesis of NAFLD to be completely understood (1).

Despite considerable efforts, the exact underlying mechanisms of NAFLD development and progression have not yet been fully elucidated. Nonetheless, previous epidemiological studies have revealed that NAFLD patients are predisposed to metabolic disorders such as abnormal glucose tolerance (2), insulin resistance (IR) (3, 4), type 2 diabetes (T2D) (5), and obesity (6). Additionally, circulating levels of insulin in patients with NAFLD are higher than controls (7). IR is the root cause of NAFLD and the severity of histological progression of NAFLD is strongly associated with insulin sensitivity independent of body mass index (BMI) (8). Interestingly, IR is more severe in patients with NASH than those with simple fatty liver (9). Moreover, NAFLD patients with IR compared with those without IR have much higher rates of elevated liver enzymes of aspartate aminotransferase and alanine aminotransferase (10).

The binding of insulin to its receptor, insulin receptor (INSR), phosphorylates it and triggers insulin signaling. INSR uses docking proteins such as insulin receptor substrates (IRSs) to mediate insulin action. Insulin receptor substrate 1 (product of the *IRS1* gene) and insulin receptor substrate 2 (product of the *IRS2* gene) are cytoplasmic proteins that are expressed in almost all cells and play a crucial role in insulin signaling and glucose metabolisim and their impaired responsiveness to insulin leads to IR (11). Previous studies have also demonstrated that *IRS1* and *IRS2* gene variants are significantly associated with insulin secretion (12), circulating insulin levels (13,14), IR (13-16), T2D (16-18), and obesity (19-21). Despite the biological plausibility, however, the possible associations between *IRS1* and *IRS2* gene variants and NAFLD risk have not yet been investigated. Consequently, this study was designed to explore whether *IRS1* (rs1801278) and *IRS2* (rs2289046) gene variants were associated with NAFLD. Our criteria for selecting these two SNPs were based on their position in the gene

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(exon or regulatory regions), commonly use in previous studies, and functional importance.

2. Materials and Methods

2.1. Participants

The study population consisted of 151 cases with biopsy-proven NAFLD (age range, 32-88 years) and 154 controls (age range, 33-80 years). In this hospital based case-control study all the participants were Iranian and genetically unrelated. They were informed about the aims of the study and their demographic, anthropometric, and clinical information was collected by self-administered questionnaires. NAFLD diagnosis was established in accordance with the following criteria : (1) ultrasonographic evidence of fatty liver and high serum levels of liver enzymes (ALT, AST, GGT) (2) alcohol consumption < 20 g/day in men and < 10 g/day in women (3) excluding patients with other causes of liver disease including viral hepatitis, Wilson's disease, alpha-1 antitrypsin deficiency, and use of drugs known to induce steatosis (4) performing liver biopsy and histological confirmation of NAFLD as the gold standard method of NAFLD diagnosis by an experienced pathologist who was unaware of the patients' clinical and biochemical data and scored biopsies using the Brunt's criteria. Steatosis and necroinflammation were graded from 0 to 3 and fibrosis was staged from 0 to 4 (22).

Various systems for scoring liver biopsies have been developed and there are pros and cons to each scoring system. We used Brunt criteria mainly because it has been widely utilized in previous studies. More importantly, even the most sophisticated systems will not replace an analytical report done by an experienced pathologist. In this study, the controls had neither liver steatosis (examined by abdominal ultrasonography), elevated liver enzymes, nor viral hepatitis infection (examined by blood test). None of them was also alcoholic or drank regularly and none was on regular medications. The controls subjects were recruited from the institute staff and medical students. The Ethical Committee of the Institute reviewed and approved this study which was conducted according to the principles of the Helsinki Declaration. BMI of each subject was calculated by the standard formula : weight (kg) / height squared (m^2).

2.2. Genotype analysis

Five milliliters of peripheral blood samples from each of the 305 subjects were collected in tubes containing ethylene diaminetetraacetic acid (EDTA) as anticoagulant and stored at 4ºC. In this study, genomic DNA was purified from peripheral blood leucocytes using standard methods. *IRS1* rs1801278 and *IRS2* rs2289046 gene variants were genotyped using PCR-RFLP method by laboratory personnel who were blinded to case or control status. Table 1 indicates the details of the PCR and RFLP conditions. The PCR products were digested with the appropriate restriction enzymes (Fermentas, Leon-Rot, Germany) and the digested products were run on 2.5 to 3.5% agarose gels and then stained with ethidium bromide for visualization under UV light. Genotyping of the subjects were denoted on the basis of the digestion patterns and the presence or absence of the respective restriction enzymes sites. The concordance of genotyping was validated by duplicate analysis of approximately 20% of the randomly selected samples.

2.3. Statistical methods

Chi-square (χ^2) test or t-test were used to compare differences in demographic, anthropometric or clinical parameters between the cases with NAFLD and controls. We also calculated the differences in allele frequencies of the polymorphisms between different groups using χ^2 test. To examine the distribution of the genotype frequencies logistic regression analysis was used. Logistic regression was applied to adjust confounding factors such as age and BMI too. For all the alleles and genotypes, the odds ratios (OR) which present the measure of associations were given with the respective 95% confidence intervals (95% CI). Statistical analyses were performed with SPSS software for Windows, version 25.0 (SPSS Inc. Chicago, IL, USA). In all statistical tests, a $P<0.05$ was considered to indicate a statistically significant difference.

3. Results

Table 2 presents demographic, anthropometric, clinical, and biochemical characteristics of the cases with NAFLD and the controls. The cases were older ($P<0.001$) and more likely to be overweight / obese ($P<0.001$), male ($P<0.001$), and smoker ($P=0.014$) than the controls. Moreover, systolic blood pressure (SBP), diastolic blood pressure (DBP), as well as circulating levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma glutamyl transferase (GGT) were higher in the cases with NAFLD compared with the controls ($P<0.001$).

Table 1. — **Information for the studied SNPs in insulin receptor substrate 1 (IRS1) and insulin receptor substrate 2 (IRS2) genes**

Gene (SNP ID)	Location (Base change)	Forward Primer Reverse Primer	PCR program (35 cycles)	PCR fragment size (bp)	Restriction enzyme, Incubation temperature	Alleles: RFLP fragments size (bp)
IRS1 (rs1801278)	Exon 1 (G/A)	5'-CTTCTGTCAGGTGTCCATCC-3' 5'-TGGCGAGGTGTCCACGTAGC-3'	93 ºC 45s, 64 ºC 30s, 72 ºC 45s	263	MvaI, 37 ºC	Allele G: 263 Allele A: 156+107
IRS2 (rs2289046)	3'-UTR (A/G)	5'-TTGGACTTTGAAGACGGATTAC-3' 5'-TTCCATCAATAACATAGGGGCT-3'	94 ºC 45s, 61 ºC 40s, 72 ºC 45s	471	PvuII, 37 ºC	Allele A: 471 Allele G: 399+72

Table 2. — **Characteristics of the cases with nonalcoholic fatty liver disease (NAFLD) and the controls** [a]

Variables	Controls (n=154)	Cases (n=151)	P-value
Age (years)	29.2(7.5)	38.8(9.4)	<0.001
BMI(kg/m^2)	23.8(3.1)	29.3(5.4)	<0.001
Gender			
Men	80(51.9)	111(73.5)	
Women	74(48.1)	40(26.5)	<0.001
Smoking status			
Never smoker	141(91.6)	113(74.8)	
Former smoker	9(5.8)	19(12.6)	
Current smoker	4(2.6)	19(12.6)	0.014
SBP (mmHg)	114.4(13.3)	123.5(15.1)	<0.001
DBP (mmHg)	69.6(8.5)	74.8(9.3)	<0.001
AST (IU/L)	19.6(7.2)	39.0(17.1)	<0.001
ALT (IU/L)	19.5(10.3)	71.6(40.3)	<0.001
GGT (IU/L)	18.5(8.4)	58.1(31.1)	<0.001
Steatosis			
Grade 0		-	
Grade 1		39(25.8)	
Grade 2		81(53.7)	
Grade 3		31(20.5)	
Necroinflammation			
Grade 0		47(31.1)	
Grade 1		58(38.4)	
Grade 2		44(29.2)	
Grade 3		2(1.3)	
Fibrosis			
Stage 0		88(58.3)	
Stage 1		56(37.1)	
Stage 2		7(4.6)	
Stage 3		-	
Stage 4		-	

The distribution of genotypes and alleles of *IRS1* rs1801278 and *IRS2* rs2289046 gene variants in the cases with NAFLD and controls are provided in Table 3. The carriers of the *IRS2* rs2289046 "GG+AG" genotype compared with the carriers of the "AA" genotype were associated with a decreased risk for NAFLD, and the difference remained significant even after adjustment for confounding factors including age, BMI, sex, smoking status, SBP, and DBP (P=0.014 ; OR=0.50, 95%CI= 0.29-0.87). Furthermore, the *IRS2* rs2289046 "AG" genotype compared with "AA" genotype seems to be a marker of decreased NAFLD susceptibility (P=0.021 ; OR=0.49, 95%CI=0.27-0.90). Moreover, the *IRS2* "G" allele was significantly underrepresented in the cases with NAFLD than the controls (P=0.026 ; OR=0.62, 95%CI=0.41-0.94). Nevertheless, as shown in Table 3, no statistically significant difference in genotype or allele frequencies between the two groups of cases and controls was found for *IRS1* rs1801278 gene variant either before or after adjustment for confounding factors.

4. Discussion

This case-control study was conducted to investigate whether *IRS1* and *IRS2* gene variants were associated with NAFLD. The *IRS2* rs2289046 "G" allele as well as the "GG+AG" and "AG" genotypes had a protective effect for NAFLD susceptibility. However, we found no significant association between *IRS1* gene rs1801278 variant and NAFLD.

Increasing evidence suggests that NAFLD results from environmental factors acting on a susceptible

Table 3. — **Association between genotypes and alleles of insulin receptor substrate 1 (IRS1) and insulin receptor substrate 2 (IRS2) genes and risk of nonalcoholic fatty liver disease (NAFLD)**[a]

Gene (Variant)	Controls (n=154)	Cases with NAFLD (n=151)	OR (95% CI) P-value [b]
IRS1 (rs1801278)			
Genotype-wise comparison			
GG	147(95.5)	147(97.4)	1.0(reference)
GA	7(4.5)	4(2.6)	0.53(0.05-6.29)0.615
AA	0(0.0)	0(0.0)	-
Allele-wise comparison			
G	301(97.7)	298(98.7)	1.0(reference)
A	7(2.3)	4(1.3)	0.52(0.10-2.59)0.414
IRS2 (rs2289046)			
Genotype-wise comparison			
AA	59(38.3)	84(55.6)	1.0(reference)
AG	76(49.4)	54(35.8)	0.49 (0.27-0.90) 0.021
GG	19(12.3)	13(8.6)	0.51(0.19-1.29) 0.153
AG and GG	95(61.7)	67(44.4)	0.50(0.29-0.87)0.014
GG versus others	19(12.3)	13(8.6)	0.69(0.28-1.72)0.432
Allele-wise comparison			
A	194(63.0)	222(73.5)	1.0(reference)
G	114(37.0)	80(26.5)	0.62(0.41-0.94)0.026

[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.

polygenic background, and significant associations have been found between genetic polymorphisms and the presence and severity of NAFLD (23). However, the underlying mechanisms of NAFLD pathogenesis still remain unclear. Previous epidemiological studies have shown that IR plays a pivotal role in the development and progression of NAFLD. IR expedites the release of free fatty acid from adipose tissue and its influx into liver (3,4,24). The risk of NAFLD progression is increased in the patients with IR. HOMA-IR index is associated with the severity of fibrosis. It is an independent predictor of advanced liver fibrosis too (25,26). It appears that insulin secretion in NAFLD patients is increased to compensate for reduced insulin sensitivity to maintain glucose homeostasis in these patients. As insulin signaling pathway genes play a crucial role in glucose homeostasis and IR, it is not surprising that they are potential candidate genes for metabolic disorders such as NAFLD and their dysregulation may lead to NAFLD (18). Despite the biological plausibility, however, no studies to date have evaluated the association between *IRS1* and *IRS2* gene variants and NAFLD risk. IRS1 and IRS2 mediate insulin signals to the downstream molecules. While IRS1 controls peripheral insulin action and body growth, IRS2 regulates glucose homeostasis and body weight control.

In this study, we found a significant association between *IRS2* gene rs2289046 polymorphism and NAFLD susceptibility. The *IRS2* gene contains 2 exons and is located on the long arm of chromosome 13 and encodes IRS2 protein. *IRS2* gene is a polymorphic gene and more than one thousand SNPs have been found in it. IRS2 has a vital role in glucose metabolisim and in pancreatic beta-cell mass development and survival. In actual fact, IRS2 / phosphatidylinositol 3-kinase (PI3K) / protein kinase B (AKT) signaling pathway plays an essential role in the regulation of hepatocyte insulin sensitivity and hepatic IR (11,27,28). Therefore, any defects in *IRS2* gene and in the function of IRS2 protein may impair the biological response to insulin and lead to IR and obesity that are involved in the etiology of NAFLD (3, 6). Previous studies have also demonstrated that *IRS2* knockout mice suffer from reduced beta-cell mass and insulin secretion, an increased peripheral IR and impaired glucose metabolism (29). Furthermore, IRS2 has a key role in differentiation of preadipocytes into adipocytes (29). As stated, a significant association between the rs2289046 variant — located in the 3'UTR of *IRS2* gene — and NAFLD susceptibility was found in the present study. The *IRS2* rs2289046 "GG+AG" genotype and "AG" genotype compared with "AA" genotype, as well as "G" allele copmpared with "A" allele had a protective effect for NAFLD susceptibility. Accumulating evidence suggests that *IRS2* gene polymorphisms are associated with metabolic disorders including circulating insulin levels (14), IR (14), 2h OGTT levels (30), T2D (17), BMI (19, 20), and hepatocellular carcinoma (31). It has also been shown that mutations in 3'UTR region are linked to some diseases. The 3'UTR region plays a central role in regulating gene expression and its alterations may directly affect the function of protein. Alternatively, these variations per se might not be functional, instead they can be associated with epigenetic modifications that have functional effects on gene expression (32,33). Nonetheless, the molecular mechanism by which the rs2289046 variant may affect the function of *IRS2* gene and NAFLD susceptibility is not clear. A possible hypothesis is that the rs2289046 "A" allele gives rise to a defect in the function of IRS2 protein, which in turn impairs the biological response to insulin and leads to insulin resistance and finally NAFLD. Such a mechanism is speculative at the present but biologically plausible, and in accordance with it, the *IRS2* rs2289046 "G" allele has a protective effect for obesity, as well as the carriers of the "G" allele (GG+AG) have a lower BMI (34, 35). Additionally, the metabolic syndrome subjects carrying the "G" allele (GG+AG) show lower circulating insulin levels and HOMA-IR compared with AA subjects (35). These findings are in concordance with ours. The other possible hypothesis linking the rs2289046 variant with NAFLD risk is through linkage disequilibrium. Rs2289046 may not be a functional polymorphism ; instead it might be in complete or near-complete linkage disequilibrium with a yet unknown functional polymorphism of *IRS2* gene.

The other gene studied here, *IRS1*, contains 1 exon and is located on the long arm of chromosome 2 and encodes IRS1 protein. Considering the role of IRS1/PI3K/Akt signaling pathway in the pathogenesis of NAFLD, restoration its activity would be beneficial for the improvement of insulin sensitivity and the amelioration of NAFLD (36). In this study, we found no significant association between the *IRS1* gene rs1801278 variant — located in Exon 1 — and NAFLD susceptibility. The rs1801278 (Gly972Arg or G972R) polymorphism is a glycine-to-arginine substitution at codon 972 in *IRS1* gene. The Gly972Arg variant may cause a change in the tertiary structure of IRS1 protein and decrease IRS1 activity and inhibit INSR autophosphorylation and activity (37). Transgenic mice expressing a mutated human 972Arg allele indicate increased IR and decreased pancreatic beta-cell function leading to hyperglycaemia (38). Previous studies have also reported that Gly972Arg variant is associated with circulating insulin levels (13), IR (13,15,16), T2D (16, 18), obesity (21) and hepatic fibrosis severity (18). The IRS-1 Gly972Arg polymorphism is associated with increased diabetes risk in the patients with NAFLD. This gene variant affects insulin receptor activity and reduces hepatic insulin signaling, which in turn can give rise to the progression of liver damage in the patients with NAFLD (18). Notwithstanding the biological plausibility, we suggest that *IRS1* is not a predisposing gene for NAFLD. In complex diseases, such as NAFLD, the effect of a majority of genes may be difficult to identify due to their modest individual effects and complex interactions (39). Therefore, to conclude that *IRS1* gene does not have a role in the development

and progression of NAFLD, the Gly972Arg and other *IRS1* gene variants should be investigated in other larger populations.

The present case-control study was well designed and we conducted multicenter collaborative research. We also used liver biopsy as the gold standard method for confirming the diagnosis of NAFLD. Nevertheless, when interpreting our findings, some potential limitations should be considered. One limitation was the modest sample size that precluded us from doing detailed stratified analyses. If we had had a larger study population, we have been able to perform multiple sub-analyses too, for example the evaluation of the association between the *IRS2* and *IRS2* gene variants and the risk of simple fatty liver and NASH separately. Given the small sample size of each subgroup and low statistical power, it was impossible to detect the possible small effects of the gene variants in the sub-analyses. And if we did these sub-analyses, the obtained results were not valid and reliable to be discussed. Another limitation was lack of information on serum levels of insulin, glucose, as well as HOMA-IR index. Unfortunately, due to budget limitations, we were unable to assess some biochemical parameters related to glucose homeostasis and insulin resistance. For this reason, the possible association between the *IRS2* and *IRS2* gene variants and serum insulin level and IR was not investigated. We were also not able to explore whether the association between these genes and NAFLD is influenced by circulating insulin level and IR. The other limitation was that by testing only one variant in each gene, the coverage of the genes was incomplete. And finally, there was a potential information bias from the case-control study design. Taken together, however, despite all the limitations that were due largely to the limited budget, this study deserves attention considering its novelty and interesting findings.

In conclusion, to the best of our knowledge, this study suggests, for the first time, that the *IRS2* gene rs2289046 variant may play a role in NAFLD susceptibility. This observation is relevant from a theoretical standpoint ; nonetheless, our findings warrants further investigations in other populations.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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