Meta Gene 2 (2014) 579–585

Contents lists available at ScienceDirect

Meta Gene

IRS1 gene polymorphisms Gly972Arg and Ala513Pro are not associated with insulin resistance and type 2 diabetes risk in non-obese Turkish population

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ARTICLE INFO

Article history:
Received 26 June 2014
Received in revised form 23 July 2014
Accepted 30 July 2014
Available online 24 August 2014

Keywords:
IRS1 gene
İnsulin resistance
Type 2 diabetes
Polymorphisms

ABSTRACT

Insulin receptor substrate 1 (IRS1), plays a critical role in insulin signaling and its control has an important place in the development of insulin resistance. The tyrosine phosphorylation of IRS1 serves as docking molecules for downstream effectors such as Phosphatidylinositol 3-kinase and phosphotyrosine phosphatase-2. We focused on the Gly972Arg and Ala513Pro variants of the *IRS1* gene, since these specific allelic variants are located near the Tyr-Met-X-Met (YMXM) motifs around Tyr987 and Tyr612. Thus, we aimed to investigate the effects of Gly972Arg/Ala513Pro polymorphisms in *IRS1* gene on development of insulin resistance and the risk of type 2 diabetes in a non-obese Turkish population. This work included 306 individuals comprising 178 subjects with type 2 diabetes and 128 healthy subjects matched for body mass index. Gly972Arg/Ala513Pro polymorphisms had no effect on type 2 diabetes risk and its phenotypes ($P > 0.05$). Although *IRS1* gene and its variants are associated with type 2 diabetes and insulin resistance in several studies worldwide, our data showed that there is no association between Gly972Arg and Ala513Pro variants in *IRS1* and disease in Turkish population.

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[1] Present study was carried out while Dr. Melda Aksoy Hepdogru was on duty at Selcuk University, Faculty of Medicine, Department of Medical Biology. At the moment Dr. Melda Aksoy Hepdogru continues her studies in Canada as an independent researcher.

http://dx.doi.org/10.1016/j.mgene.2014.07.008

Introduction

Insulin is an important determinant of early growth and plays a prominent role in maintaining glucose homeostasis. Insulin, secreted from pancreatic beta (β) cells, exhibits its effects by binding to insulin receptor (IR) on target tissues; leading to the activation of multiple signaling molecules and involving their pathways. Insulin-receptor interaction primarily induces autophosphorylation of tyrosine kinase domain on cytoplasmic surface of the receptor, than phosphorylation of a trio of tyrosine residues of cytosolic insulin receptor substrates (IRS). Subsequently, other signaling molecules such as Phosphatidylinositol 3-kinase (PI3- kinase) and Growth factor receptor-bound protein 2 (GRB2) are recruited leads to the activation of the Akt signaling pathway (mediates many metabolic effects that include glucose and lipid metabolism) and the mitogen-activated protein (MAP) kinase pathway (mediates cell proliferation), respectively (Wing, 2008).

IRS proteins have a similar structure consisting of an NH2-terminal pleckstrin homology (PH) domain adjacent to a phosphotyrosine binding (PTB) domain followed by a variable-length COOH-terminal tail. PH domain is essential for effective IR–IRS1 interactions. On the other hand, the PTB domain interacts directly with the juxtamembrane (JM) domain of the insulin receptors (Voliovitch et al., 1995). The COOH terminus of the IRS proteins contains a series of tyrosine (Tyr) phosphorylation sites (approximately 20 sites) that serve as on/off switches to recruit downstream effector molecules. Tyr residues that bind Src homology 2 (SH2) domains of their effector proteins, located in consensus motifs (YMXM or YXXM). Serine/Threonine (Ser/Thr) phosphorylation sites (about 50 sites) adjacent to these Tyr phosphorylation sites prevent binding of the SH2 domains of these effectors, thus inhibit insulin signaling (Myers and White, 1996). And also, it is suggested that this Ser/Thr phosphorylation may be responsible for tumor necrosis factor-α (TNFα)-mediated development of insulin resistance (Hotamisligil et al., 1996; Reis et al., 2012; Tanti et al., 1994).

Insulin resistance is a condition which the response of the target cells to normal levels of insulin is reduced, and insulin resistance plays a central role in the development of type 2 diabetes, an emerging epidemic of the 21st century.

So far, many polymorphisms described in *IRS1* gene, located in 2q36-37, especially Gly972Arg substitution, are shown to be associated with insulin resistance in studies with different several populations and meta-analyses about type 2 diabetes (Aileen et al., 2005; Burguete-Garcia et al., 2010; Celi et al., 2000; Jellema et al., 2003; Martínez-Gómez et al., 2011; Zaman Huri et al., 2012). The single nucleotide polymorphisms (SNPs) Gly972Arg, Pro170Arg and Met209Thr in *IRS1* gene have been reported to be associated with a reduction in phosphatidylinositol 3 kinase (PI3K) activity (Yoshimura et al., 1997) and subsequently development of insulin resistance (Armstrong et al., 1996; Garcia et al., 1993; Yoshimura et al., 1997). Additionally, the polymorphisms Gly972Arg and Ala513Pro located near the Tyr-Met-X-Met (YMXM) motifs around Tyr987 and Tyr612 have been noted to influence insulin resistance, hyperinsulinemia and fatty-acid composition of muscles (Garcia et al., 1993).

We investigated that whether these two important variants, Gly972Arg (rs1801278) and Ala513Pro (rs1801276) in *IRS1* gene are effective on insulin resistance and/or type 2 diabetes in Turkish in central region of Anatolia.

Materials and methods

Study subjects and assessment

In this study, 306 individuals comprised 178 subjects with type 2 diabetes and 128 healthy subjects matched for age and body mass index (BMI) were enrolled. Type 2 diabetic patients were diagnosed according to the American Diabetes Association diagnostic criteria, were over 35 years old and not using insulin, and having a body mass index (BMI) less than 30. In healthy nondiabetic group, subjects had no history of diabetes in first or second degree relatives. Informed written consent was obtained from each individual before participating into the study. The study was approved by the Ethical Committee of the Faculty of Medicine of Selcuk University.

Fasting plasma glucose, fasting insulin, HbA1C and c-peptide values were measured in both groups. In control group, oral glucose tolerance test (OGTT) was performed. For detecting insulin resistance, the homeostasis model assessment of insulin resistance (HOMA-IR) was calculated formulating as fasting

plasma glucose (mmol/l) multiplied by fasting serum insulin (pmol/l) and divided by 22.5. An individual with a HOMA-IR value higher than 2.5 was considered to be resistant to insulin.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the standard SDS and proteinase K procedure. SNPs Gly972Arg and Ala513Pro were genotyped using the PCR-RFLP analyses. The PCR analyses were performed using specific primers for scanning the SNPs Gly972Arg and Ala513Pro. The forward and reverse primers were: 5′AGTCTGGCTACTTGTCTGGC3′ and 5′ATGAGTTGTCCCCGTCAGA3′ for SNP Gly972Arg and 5′CGGTGAGGAGGAGCTAAGCA3′ and 5′GTGGGTAGGCAGGCATCATC3′ for SNP Ala513Pro, respectively. PCR amplification was performed in a 30 μl volume containing 50 ng genomic DNA, 1 × PCR buffer, 0.4 mM of each primer, 0.6 mM deoxyribonucleoside triphosphates, 0.1 units Taq polymerase. PCR reactions were carried out in a thermocycler (Bio-Rad, California, USA) as follows: an initial denaturation at 94 °C for 5 min was followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 57.2 and 58.4 °C (for Gly972Arg and Ala513Pro, respectively) for 30 s, elongation at 72 °C for 30 s, and final extension at 72 °C for 2 min.

RFLP analyses of the target SNPs Gly972Arg and Ala513Pro were achieved using the restriction enzymes *Alu*I (MBI Fermentas, Vilnius, Lithuania) and *Dra*III (MBI Fermentas, Vilnius, Lithuania) respectively according to the manufacturers' instructions. The resultant restriction fragments were ascertained on a 3% agarose gel and visualized.

Statistical analysis

Descriptive statistics were obtained for clinical and biochemical characteristics. Initial comparison was performed between patient and control groups using *t* test. The differences between the case and control groups in terms of genotype distribution or allele frequency were analyzed using Pearson Chi-square test. Also in order to evaluate Hardy–Weinberg equilibrium (HWE) in patient and control groups Chi-square goodness of fit test was carried out. Analyses were performed using dominant, additive, and recessive models. Dominance was defined in terms of allele 2 effects. In dominant allele 2 models, homozygous individuals for allele 1 were compared with carriers of allele 2. In recessive allele 2 models, homozygous individuals for allele 2 were compared with carriers of allele 1. Association between genotypes and type 2 diabetes was tested by conducting a case–control study. Allele frequencies of SNPs in patient and control groups were evaluated using odds ratio (OR). We performed logistic regression analysis by considering not only dominant, additive and recessive models of SNP genotypes but also sex and age.

We also checked if there is any association between genotypes and biochemical characteristics exclusive of type 2 diabetes. Therefore we carried out tests for normality of the biochemical characteristics. Fasting plasma insulin and HOMA-IR variables were skewed and were normalized by square root and log transformations respectively, before analysis. SNP genotypes were coded as 11, 12, and 22, respectively. Then single point (single SNP) regression analysis model was fitted for transformed fasting plasma insulin and HOMA-IR. For the other characteristics such as BMI, c-peptide and HbA1C, Kruskal–Wallis test was performed.

Individuals were classified according to their fasting glucose levels as <100, 100–125, 126–200, and >200. Chi-square test was then used to evaluate fasting glucose level-SNP genotype relationship.

All analysis was carried out using SPSS 18.0. In all analyses, a P value < 0.05 was considered statistically significant.

Results

Characteristics of study subjects

Clinical and biochemical characteristics of the individuals are shown in Table 1. Compared to the control subjects, type 2 diabetics had significantly different levels of fasting glucose, fasting insulin, HbA1C, and HOMA-IR ($P < 0.05$). When OGTT results were evaluated, individuals with fasting plasma glucose levels >200 at the 2nd hour were considered diabetic, and those with levels in the 140–200 range were considered to have impaired glucose tolerance. These individuals were not included in control group.

Table 1
Clinical characteristics of study subjects.

Variable	Type 2 diabetics	Healthy controls	P value
Sex (M/F)	178 (110/68)	128 (71/57)	0.27[a]
Age (years)[b]	56.13 ± 9.25	41.82 ± 9.55	0.00
BMI (kg/m^2)[c]	26.67 (18.60–35.30)	26 (17.6–34.9)	0.08
Fasting glucose (mg/dL)[c]	137 (77–526)	91 (58–129)	0.00
Fasting insulin (μU/mL)[d]	8.33 (7.61–9.08)	7.16 (6.58–7.76)	0.01
HbA1c (%)[c]	6.8 (6.67–7.5)	5.4 (4.0–5.60)	0.00
c-peptide (ng/mL)[c]	2.0 (0.50–7.20)	2.13 (0.4–15.7)	0.00
HOMA-IR[d]	2.82 (2.57–3.10)	1.52 (1.40–1.66)	0.00

BMI: Body mass index.
HbA1c: Hemoglobin A1c.
HOMA-IR: Homeostasis model assessment of insulin resistance.
[a] X^2 test.
[b] Values are expressed as means ± SD.
[c] Median (Minimum–Maximum).
[d] Back transformed mean and 95% CI.

Association study

According to the results of genotyping while Gly972Arg polymorphism was identified as heterozygous genotype in 9 patients (5.06%) and 8 healthy subjects (6.25%), Ala513Pro polymorphism was observed heterozygous genotype in only 2 patients (1.12%) (Table 2). None of these polymorphisms was associated with insulin resistance and type 2 diabetes ($P > 0.05$). Additionally, both of the polymorphisms had no effect on other phenotypes which are fasting glucose, fasting insulin, c-peptide and HbA1c ($P > 0.05$). Patient and control groups were in Hardy–Weinberg equilibrium ($P > 0.05$).

Discussion

Until recently, several genes were identified as type 2 diabetes risk genes due to known or potential biological functions in insulin secretion, insulin signaling, and adipocyte signaling, etc. by candidate gene approach. One of them was *IRS1* which is a central molecule in insulin signaling.

After binding insulin to its receptor, the tyrosine phosphorylation of IRS1 serves as docking molecules for downstream effectors such as PI3K and phosphotyrosine phosphatase 2 (SHP-2). Prolonged insulin stimulation and other stimuli triggered by inducers of insulin resistance activate IRS kinases that phosphorylate IRS1, on Ser/Thr residues (Boura-Halfon and Zick, 2009).

On the other hand, the binding specificity and affinity of the tyrosine phosphorylation sites are important for the response of IRS1 to tyrosine protein kinases, therefore polymorphisms within the tyrosine phosphorylation sites of the protein can alter those interactions (Garcia et al., 1993). Thus, the reduction of tyrosine phosphorylation of IRS1 inhibits downstream signaling of IRS1 (Aguirre et al., 2002).

Table 2
Association study and genotype distribution of SNPs in study groups.

SNP	Genotype	Type 2 diabetic n (%)	Control n (%)	Homozygote/heterozygote P value
Gly972Arg	GG	169 (94.94)	120 (93.75)	>0.05
	GA	9 (5.06)	8 (6.25)	
	AA	–	–	
Ala513Pro	GG	176 (98.88)	128 (100)	>0.05
	GC	2 (1.12)	–	
	CC	–	–	

Because no individual has homozygous genotype of rare allele for both SNPs, homozygous genotype of common allele was compared with heterozygous genotype.

Gly972Arg, Pro170Arg and Met209Thr polymorphisms which are around the tyrosine phosphorylation sites (Garcia et al., 1993; Ogihara et al., 1997), cause defects in tyrosine phosphorylation and have revealed different levels of reduction in PI3K activity (Yoshimura et al., 1997). And also, the Ser892Gly and Thr608Arg polymorphisms were reported in association with decrease in insulin-induced phosphorylation and PI3K activity in type 2 diabetic patients (Laakso et al., 1994).

We investigated the Gly972Arg and Ala513Pro variants of the *IRS1* gene, since these specific allelic variants are located near the YMXM motifs around Tyr987 and Tyr612. These variants have been reported to influence insulin resistance, hyperinsulinemia and fatty-acid composition of muscles with a non-sporadic prevalence (Borkman et al., 1993; Garcia et al., 1993). Gly972Arg polymorphism was found to be associated with type 2 diabetes in different populations (Aileen et al., 2005; Burguete-Garcia et al., 2010; Celi et al., 2000; Jellema et al., 2003; Martínez-Gómez et al., 2011; Zaman Huri et al., 2012), whereas several studies reported no relationship between Gly972Arg polymorphism and disease status (Florez et al., 2004; Zeggini et al., 2004). In a study conducted in the Turkish population, common polymorphism Gly972Arg and other rare polymorphisms were reported not to be associated with type 2 diabetes and related phenotypes (Orkunoglu Suer et al., 2005). Our data replicated that there is no association between Gly972Arg and Ala513Pro variants in *IRS1* and type 2 diabetes in Turkish population. Only a difference in allele frequency was observed, probably due to larger sample size of our study compared to previous study. Also, we determined Ala513Pro polymorphism in heterozygote form in only two patients while Orkunoglu Suer et al. (2005) detected in only one healthy subject. So, this polymorphism is rare in Turkish population as previously reported in other populations (Celi et al., 2000; Hart et al., 2002).

In our study, although fasting insulin, fasting glucose, HbA1c, c-peptide and HOMA-IR levels were found to be different between patient and healthy groups, both polymorphisms were not associated statistically with these phenotypes.

In recent years, with the contribution of Genome wide association (GWA) studies in type 2 diabetes pathogenesis, it has reached a great number of risk gene/allele, but the molecular mechanisms are mostly unknown. However, only a few risk genes related with type 2 diabetes development which include *IRS1* were confirmed by GWA studies (Rung et al., 2009; Voight et al., 2010). Contrary to expectations, the SNP identified in *IRS1* gene by GWA study was not Gly972Arg, the associated new SNP (rs2943641) has been located about 500 kb upstream of the *IRS1* gene (Rung et al., 2009). The rs2943641 in *IRS1* was found to be associated with type 2 diabetes, insulin resistance and hyperinsulinemia in 3 European populations comprised of over 14,000 individuals (Rung et al., 2009). *IRS1* gene, G972R and nearby markers detected by candidate gene approach and GWA studies are not in linkage disequilibrium (Rung et al., 2009). In recent years, although it has not reached genome-wide significance, in a large-scale meta-analyses an intronic SNP rs4675095 in *IRS1* which is not in LD neither with the SNP G972R (rs1801278) nor with the SNP rs2943641 was also associated with HOMA-IR ($P = 4.6 \times 10^{-3}$) (Dupuis et al., 2010). Furthermore in another meta-analyses performed using a two-stage genome-wide association, including up to 76,150 individuals, Kilpeläinen et al. (2011) identified three loci convincingly associated with body fat percentage. One of them was rs2943650 variant ($r^2 = 1.00$ with rs2943641) located 500 kb upstream of *IRS1*, an important mediator of insulin and insulin-like growth factor-1 (IGF-1) signaling (Kilpeläinen et al., 2011).

From the perspective of functional analyses, the risk allele was associated with reduced levels of IRS1 protein expression and decreased PI3K activity in human skeletal muscle biopsies (Rung et al., 2009). Null *IRS1* (Araki et al., 1994) and tissue-specific knockout (Nandi et al., 2004) experiments in mice have showed that IRS1 is a fundamental factor of insulin action both in peripheral insulin-sensitive tissues and in insulin-secreting pancreatic beta cells.

When the association data of different polymorphisms in *IRS1* gene and throughout or near this locus with diabetes evaluated with functional study data; it is clear that this locus is important in terms of affecting *IRS1* gene expression, further insulin signaling and type 2 diabetes development. On the other hand, we did not found any association in our population similar to some other populations (Florez et al., 2004; Zeggini et al., 2004). Lack of association in our study is possibly due to multiple different genes and variants thought to be effective in development of insulin resistance and type 2 diabetes.

In conclusion, our results replicated that the *IRS1* gene/variants have no contribution on genetic architecture of type 2 diabetes in the Turkish population. Even larger sample sizes will be required to characterize *IRS1* gene in Turkish population.

Acknowledgment

We would like to thank to Dr. Ahmet Arslan and Dr. Mustafa Sait Gonen for their assistance in this study. This study was supported by the Selcuk University Research Foundation (2003/010).

Financial disclosure

The authors declared no financial disclosure.

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