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Molecular Scanning for Mutations in the Insulin Receptor Substrate-1 (*Irs-1*) Gene in Turkish with Type 2 Diabetes Mellitus

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Abstract. Insulin receptor substrate-1 (IRS-1) is an endogenous substrate for the insulin receptor tyrosine kinase, which plays a key role in insulin signaling. Recent studies have identified several polymorphisms in the human IRS-1 gene (Irs-1) that are increased in prevalence among type 2 diabetic patients. To determine whether variation in the Irs-1 contributes to genetic susceptibility to type 2 diabetes in Turkish people, PCR-RFLP and DNA sequencing method were utilized to analyze the coding region of Irs- 1 in 70 subject and 116 control patients. Three missense mutations were detected (Gly972Arg, Ala512Pro, Ser892Gly). There was no significant association found with any of these variants and diabetes. The Gly972Arg mutation, however, was relatively more common in with 10/70 diabetic patients and 15/116 non-diabetic controls being heterozygous and 1/70 being and 0/116 non-diabetic controls being homozygous for this variant. As a conclusion, Ala512Pro, Ser892Gly mutations were rare and Met613Val, Ser1043Tyr and Cys1095Tyr mutations were not found in the populations studied. Gly972Arg is more common than other known mutations in our population but may not be a major determinant in genetic susceptibility to type 2 diabetes.

Key words: Genetics, Insulin resistance, Type 2 diabetes mellitus, Restriction fragment length polymorphism
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TYPE 2 diabetes (non insulin dependent diabetes mellitus; NIDDM) is characterized by a combination of peripheral insulin resistance and impaired insulin secretory capacity of pancreatic β cell [1]. Insulin receptor substrate-1 (IRS-1) is a docking protein between the insulin receptor and a complex network of intracellular signaling molecules containing Src homology 2 (SH2) domains in the insulin-signaling cascade. In light of their role in insulin signaling the insulin receptor substrate gene (Irs-1) have been considered candidate genes for human metabolic disorders such as type 2 diabetes and obesity [2]. Mice rendered deficient in IRS-1 by targeted disruption display insulin resistance and impaired glucose tolerance, and mice heterozygous for defects in the genes for IRS-1 and the insulin receptor develop overt diabetes [3]. In several populations amino acid variants in IRS-1 have been identified [4–10]. These include Ala512Pro, Gly972Arg, Pro170Arg, Ser809Phe Met209Thr, Ser892Gly, Gly819Arg and Arg1221Cys. A common sequence polymorphism in Irs-1, which causes the replacement of glycine by arginine at codon 972, has been shown to be more frequent in patients with NIDDM than in control subjects and proposed to contribute to insulin resistance [5, 8].

Since these findings suggest the existence of an interaction between Irs-1 and type 2 diabetes, we analyzed the coding region of the IRS-1 gene for mutations in Turkish patients with type 2 diabetes, and compared the prevalence of variants that were found with diabetic and non diabetic-subject in our population.

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Materials and Methods

Samples

Seventy type 2 diabetic patients (30 female and 40 male) and 116 non-diabetic controls (55 female and 62 male) (BMI<25) were enrolled in the study. The subjects were recruited from the outpatient clinic of the Department of Endocrinology and Metabolism of Gulhane Military School of Medicine in Turkey and informed consent was obtained from the subjects. Inclusion criteria were based on the presence of two or more components of type 2 diabetes according to the guidelines defined by classification system of the World Health Organization (WHO, 1999). Patients who were treated with insulin or hyperglycemic drugs and/or showed hyperlipidemia were excluded. Control subjects underwent routine physical and laboratory evaluations to ensure that none had obesity, diabetes mellitus, hyperlipidemia, psychiatric, metabolic, hepatic or renal disease. None of the control subjects had a family history of hyperlipidemia or diabetes. Characteristics of the study population are given in Table 1.

Polymerase chain reaction (PCR)

Blood samples were obtained from each subject and genomic DNA was prepared from peripheral blood leukocytes (Puregene DNA isolation kit, Gentra, Minneapolis, MN, USA). In order to determine mutations in Irs-1, appropriate primer sets (Table 2) were prepared according to published cDNA and genomic DNA sequence of Irs-1 [13]. The 50 µl PCR mixture contains about 0, 5 µg of DNA, 0.2 mM dNTP mix, 1 µM each primer, 10× PCR buffer and 1.25 U of Taq polymerase (Perkin Elmer, USA). PCR was performed in a thermal cycler (MJ Research, USA) under standard conditions; for initial denaturation, 94°C for 5 min, 1 cycle; denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 45 sec, 35 cycles, final extension at 72°C for 7 min, 1 cycle.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis (Detection of Irs-1 missense mutations)

The restriction endonuclease enzymes used for Irs-1 mutation analyses and nucleotide substitutions are given in Table 3.

For detection of Ala512Pro, 10 µl of PCR product

Table 1. The clinical characteristics of the subjects studied

	patients	controls	t	*P**
Sex (F/M)	(30/40)	(55/61)	—	—
Age (years)	37.5 ± 12.2	35 ± 11.04	—	—
Body mass index (kg/m^2)	22.14 ± 3.98	23.86 ± 1.96	2.84	0.006
Fasting plasma glucose (mmol/l)	5.53 ± 0.76	4.08 ± 0.40	12.22	0.001
Fasting plasma insulin (pmol/l)	95.96 ± 34.45	54.70 ± 11.37	8.45	0.001
Cholesterol (mg/dl)	155.0 ± 28.1	174.4 ± 37.4	2.97	0.004
Triglycerides (mg/dl)	87.5 ± 36.3	141.7 ± 59.7	5.52	0.001
HOMA	3.34 ± 1.38	1.27 ± 0.30	11.05	0.001

Values are mean ± SD, F, Female, M, Male; **P*<0.05 patients vs controls.

Table 2. Primer sequences

A-	IRS1 5'-GC GG T GAG GAG GAG CTA AGC AAC-3'
	IRS4 5'-C TCC AAT GTC AGG AGA GCA GCC-3'
B-	IRS2 5'-GGA GGT GGC AGT GGA GGC CGA CTG CC-3'
	IRS5 5'-C CTC AGG GCC GTA GTA GCA GTC-3'
C-	IRS6 5'-CTG GAG CCC AGC CTT CCA CAT C-3'
	IRS8 5'-C CCT GGG CAG GCT CAC CTC CTC-3'
D-	IRS7 5'-GCT GAC ATG CGA ACA GGC ATT G-3'
	IRS9 5'-G CTA CTG ACG GTC CTC TGG CTG-3'

Table 3. Nucleotide substitutions (missense/nonsense)

Codon	nucleotide	aminoacid	enzyme
512	aGCC-CCC	Ala-Pro	Bst7 II (Bbv I)
612	cATG-GTG	Met-Val	Hsp92 II (Nla III)
892	gAGC-GGC	Ser-Gly	Apa I
972	cGGG-AGG	Gly-Arg	Xma I
1043	TCC-TAC	Ser-Tyr	DNA sequencing
1095	TGC-TAC	Cys-Tyr	Kpn I

generated with primer pair A (Table 2) was digested with *Bst7II* at 37°C for 1 h, followed by agarose gel electrophoresis, ethidium bromide staining, and ultraviolet transillumination. The predicted digestion product sizes are 490 bp and 168 bp for Ala512 homozygotes; 658 bp for Pro512 homozygotes; and 658 bp, 490 bp and 168 bp for Ala512Pro heterozygotes.

For detection of Met613Val, 10 µl of PCR product generated with primer pair B (Table 2) was digested with *Hsp92 II* at 37°C for 1 h and visualized as above. The predicted digestion product sizes are 435 bp, 84 bp, 60 bp, 36 bp, for Met613 homozygotes; 471 bp, 84 bp, and 60 bp for Val613 homozygotes; and 441 bp, 435 bp, 84 bp, 60 bp and 36 bp for Met613Val heterozygotes.

For detection of Ser892Gly, 10 µl of PCR product generated with primer pair B (Table 2) was digested with *Apa I* at 37°C for 1 h and visualized as above. The predicted digestion product sizes are 279 bp, 188 bp and 147 bp for Ser892 homozygotes, 222 bp, 188 bp, 147 bp, and 57 bp for Gly892 homozygotes, and 279 bp, 222 bp, 188 bp, 147 bp, and 57 bp for Ser892Gly heterozygotes.

For detection of Gly972Arg, 5 µl of PCR product generated with primer pair C (Table 2) was digested with *Xma I* at 37°C for 1 h and visualized as above. The predicted digestion product sizes are 435 bp, 174 bp for Gly972 homozygotes; 600 bp for Arg972 homozygotes; and 600 bp, 435 bp, 174 bp for Gly972*Arg* heterozygotes.

For detection of Cys1095Tyr, 5 µl of PCR product generated with primer pair D (Table 2) was digested with *Kpn I* at 37°C for 1 h and visualized as above. The predicted digestion product sizes are 699 bp for Cys1095 homozygotes; 450 bp and 249 bp for Tyr1095 homozygotes; and 699 bp, 450 bp, 249 bp for Cys1095Tyr heterozygotes.

DNA sequencing

For detection of Ser1043Tyr Irs-1, The PCR products generated with primer pair D were purified for sequencing (Quantum Prep PCR Kleen Spin Columns, Bio-Rad,USA). Purified PCR products were directly sequenced with Big Dye Terminator 3.1 kits (Perkin Elmer, USA). The same primers used for determination of the sequence as the initial PCR. The conditions were as follows: 25 cycles of denaturation at 96°C for 5 sec, annealing at 50°C for 5 sec, and extension at 60°C for 30 sec final extension at 72°C for 7 min, 1 cycle. The completed reaction mixture was then electrophoresed and resolved on an ABI Prism 310 Genetic Analyzer (ABI 310 automatic sequencer, Applied Biosystems).

Biochemical analysis

Cholesterol, triglyceride and, glucose concentrations in whole plasma were measured with a Technicon RA-1000 Autoanalyzer. Plasma insulin concentrations were measured in frozen samples by radioimmunoassay (Biodata Insulin Kit). The interassay coefficient of variation was 7.5%.

Statistical analyses

The statistical software SPSS version 11.0 was used. Data are given as mean SD. Comparison of parameters was performed using Student's *t* test. A p value of <0.05 was considered to be statistically significant.

Results

The mutational screening of the coding region of Irs-1 in 70 subjects and 116 controls revealed three missense mutations (Table 4). The Gly972Arg variation was more common in with 10/70 diabetic patients and 15/116 non-diabetic controls being heterozygous and 1/70 patients and 0/116 non-diabetic controls being homozygous for this change. In this Turkish population, the Ala512Pro variation was present in 0/70 diabetic patients and 1/116 non-diabetic controls. Ser892Gly

Table 4. IRS-1 mutation distributions in control and type 2 diabetes mellitus subjects

	Control group		type 2 diabetes mellitus group	
	n	%	n	%
$Gly^{972}Gly^{972}$	101	87.1	59	84.2
$Gly^{972}Arg^{972}$	15	12.9	10	14.2
$Arg^{972}Arg^{972}$	0	0	1	1.6
$Ala^{512}Ala^{512}$	115	0	70	100
Ala^{512} Pro^{512}	1	0	0	0
Pro^{512} Pro^{512}	0	0	0	0
$Ser^{892}Ser^{892}$	116	100	69	98.6
$Ser^{892}Gly^{892}$	0	0	1	1.4
$Gly^{892}Gly^{892}$	0	0	0	0

Table 5. The effect of Irs-1 972 polymorphism on clinical results.

		Gly/Gly	X/Arg	*P**	z
type 2 diabetes mellitus subjects	*n*	59	11		
	Body mass index (kg/m^2)	22.02 ± 4.24	22.81 ± 2.27	0.25	1.160
	Fasting plasma glucose (mmol/l)	5.50 ± 0.80	5.50 ± 0.54	0.85	0.186
	Fasting plasma insulin (pmol/l)	107.70 ± 38.45	93.71 ± 33.61	0.24	1.183
	Cholesterol (mg/dl)	154.6 ± 26.1	157.3 ± 38.6	0.96	0.045
	Triglycerides (mg/dl)	88.7 ± 36.6	81.2 ± 36.2	0.58	0.547
	HOMA	3.28 ± 1.26	3.64 ± 1.36	0.44	0.766
Non-diabetic subjects	*n*	100	16		
	Body mass index (kg/m^2)	23.96 ± 1.95	23.23 ± 2.02	0.49	0.685
	Fasting plasma glucose (mmol/l)	4.07 ± 0.37	4.12 ± 0.56	0.80	0.243
	Fasting plasma insulin (pmol/l)	56.17 ± 4.83	54.46 ± 12.15	0.37	0.901
	Cholesterol (mg/dl)	170.2 ± 37.3	199.6 ± 28.1	0.02	2.315
	Triglycerides (mg/dl)	135.6 ± 68.5	142.7 ± 58.9	0.83	0.214
	HOMA	1.37 ± 0.31	1.43 ± 0.20	0.30	1.043

Values are mean ± SD, *P<0.05 type2 diabetic subjects vs controls.

was found in 1/70 diabetic patient. Due to only one Ala512Pro and one Ser892Gly heterozygous subject were found, we failed to detect any difference between control and type 2 diabetic subjects for genotypic effect. The Met613Val, Ser1043Tyr and Cys1095Tyr variations were not found in our study population.

In our population, the Arg972 polymorphism was observed in 15.8% of type 2 diabetic patients and 12.9% of controls. The effects of the Irs-1 972 polymorphism on the individuals were analyzed for some clinical parameters and the results are presented in Table 5. Mean levels of insulin, glucose and body mass index (BMI) were found to be statistically different in both group, but were not associated with the presence of any of Irs-1 variant (Tables 1 and 5). In Irs-1 X/Arg carriers mean levels of insulin were found to be slightly lower than control levels but were not statistically significant (Table 5). In controls, there were also no observed difference in insulin and fasting glucose levels in carriers of the X/Arg genotype (Table 5). The HOMA index, as calculated from insulin and fasting glucose levels, was also not significantly different between carriers and non-carriers. Carriers of the combination of three mutations (NDDM n = 12; non-diabetic n = 16) did not have altered insulin level or HOMA index compared with the rest of the study group (data not shown). We found no significant differences in lipid profiles between carriers and non-carriers (Tables 1 and 5).

Discussion

Several studies have reported a higher prevalence of the Arg972 polymorphism in type 2 diabetic patients, while others have reported a weak or absent association between this variant and type 2 diabetes [4–12]. The Arg972 polymorphism is absent in Pima Indians [9]. In both diabetic and control Japanese populations, the prevalence of the Arg972 polymorphism appears to be lower than that observed in the corresponding Caucasian populations [5]. These results may account for the ethnic differences in the genetic background for type 2 diabetes. In the current study, the Arg972 polymorphism was observed in 15.8% of type 2 diabetic patients and 12.9% of controls in a Turkish population. Comparison of the allele frequencies at this site failed to show any statistically significant difference between control and subjects for the genotypic effect (Table 4). The Ala512Pro substitution has been found in Danish, and French Caucasians, and is absent or rare in Finns, Japanese and South Indians similar to in our study population [8, 14]. We found Ser892Gly variants in 1/70 diabetic patients, similar to the frequency reported in Finns and Mexican Americans [8, 15]. Met613Val, Ser1043Tyr and Cys1095Tyr variations were not found in our study population, although this might be due to the sample size. These mutations were found to be rare in other population studies [8, 15]. It has been proposed that certain genetic variations in the insulin receptor substrate-1 gene are associated with the development of insulin resistance and type 2 dia-

betes in some populations.

Various data suggest that the single molecular defect in insulin signaling involving the defective interaction between phosphatidylinositol-3-kinase and IRS-1, might result in both peripheral insulin resistance and impaired insulin secretion. In this study, we have shown that three gene variants in the Irs-1 are not associated with a detectable difference in type 2 diabetic patients phenotypes. Mean levels of insulin, glucose and body mass index (BMI) were found to be statistically different between patients and controls, but were not associated with the presence of any of these variants. The Irs-1 codon 972 variant did not confer insulin resistance but patients with this defect had slightly lower levels of fasting insulin and higher values for serum cholesterol ratio. Our results are in contrast with a German population of normal glucose-tolerant subjects in which a reduced insulin secretion in Irs-1 X/Arg carriers was reported and similar to that reported in Dutch [14]. Insulin resistance at the level of the [beta]-cell may result from reduced insulin secretion or may result from common polymorphism in the other components of the insulin- insulin receptor- insulin receptor substrate-1 functional complex. Mice with disruptions of the IRS genes also have abnormal lipid profiles [3]. In our study we found no significant differences in lipid profiles between carriers and non-carriers (Tables 1 and 5).

In conclusion, we suggest that Ala512Pro, Ser892Gly, mutations are rare and Gly972Arg is more common in our population but may not be a major determinant to genetic susceptibility to type 2 diabetes.

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