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Insulin Receptor Gene Mutations in Iranian Patients with Type II Diabetes Mellitus

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ABSTRACT

Background: Patients with diabetes mellitus type II suffer from hyperglycemia because they are not able to use the insulin that they produce, often due to inadequate function of insulin receptors. There are some evidences that this deficiency is inherited in a dominant autosomal manner and leads to the malfunction of the pancreatic beta cells resulting in insulin excretion disorders. In this study, we sought to identify mutations in the insulin receptor (INSR) gene, which can cause insulin resistance in type II diabetic patients. **Methods:** DNA was extracted from peripheral blood cells of the patients (n = 128) diagnosed with type II diabetes. All 22 exons of the INSR gene of the patients were analyzed for mutations running PCR, conformation-sensitive gel electrophoresis and DNA sequencing, consecutively. **Results:** Approximately 26% of the patients had genetic mutations; however, most of them were not reported. These mutations include exon 2 (His171Asn, Ile172Ser, Cys196Ser and Ser210Arg), exon 3 (Gly227Asp and Gly232Ser), exon 8 (Thr543Ser), exon 9 (a heterozygote was observed with no change in phenylalanine at position 669), exon 13 (two heterozygotes: Arg890Pro with Asn865 remaining unchanged), exon 14 (Ala906Gly and Pro918Trp with Arg902 unchanged), exon 17 (Val1086Glu) and exon 19 (His1157Gln with Thr1172 unchanged). **Conclusion:** The lack of similar mutation records in literature and genetic data banks may suggest a geographic pattern for these INSR gene variants in our population. *Iran. Biomed. J. 13 (3): 161-168, 2009*

Keywords: Diabetes type II, Insulin resistance, PCR, Conformation-sensitive gel electrophoresis (CSGE), Iran

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INTRODUCTION

Patients with diabetes mellitus type II, also known as non-insulin-dependent diabetes mellitus (NIDDM) [1] suffer from hyperglycemia because they are not able to use the insulin produced at their body, often due to inadequate function of insulin receptors. This affliction has many health effects such as blindness [2]. Symptoms generally start after the age of 40, thus it is also called adult diabetes. NIDDM is more often a problem in overweighting people after the age of 35 with high blood insulin concentrations. About 85-90% of diabetics over the age of 40, are diagnosed as type II [2]. Racial and geographical differences in the distribution of diabetes type II indicate the heterogeneity of the disease [3]. Although it appears to be familial, the inheritance pattern of NIDDM is already unknown and suspected as dominant autosomal [4]. Such resistance leads to the malfunctioning of the pancreatic beta cells resulting in insulin excretion disorders [5].

In 1988, Kadowaki *et al.* [6] brought up the question of whether mutations in the insulin receptor (INSR) gene, located on chromosome 19 (p13.3-p13.2), account for the insulin resistance in patients with NIDDM. Taira *et al.* [7] reported cases of NIDDM carrying mutations at INSR. These mutations end in receptors with slightly decreased kinase activity or affinity for insulin. With such cases, environmental factors including obesity may trigger the onset of diabetes. A reduction in the number of insulin receptors in obese people is also

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now known to cause resistance to insulin [7]. Proper diet and normal blood sugar levels may in turn improve the insulin excretion capacity by improving the function of the receptors [7].

The aim of this research was to identify any mutations in each of the 22 exons of the INSR gene in Iranian patients diagnosed with NIDDM.

MATERIALS AND METHODS

Patients and sampling. We selected subjects diagnosed with NIDDM based on the following inclusion criteria**:** 40 years old or older at the onset of disease, no history of diabetic ketoacidosis (DKA), overweight (BMI $\geq$ 20 kg/m^2) and NIDDM cases in at least one of the first relatives. Subjects with any of the following exclusion criteria were not entered in the study: personal history of DKA, BMI $\leq$ 20 kg/m^2 or significant idiopathic weight loss during illness (without hyperthyroidism, digestion syndrome, malignancy, and so forth), any pancreatic illness (chronic, trauma, neoplasm, hemo-chromatosis), drug dependency (thyroid hormones, interferon, glucocorticoid and nicotine), infectious disease (such as congenital rubella or cytomegalovirus virus), genetic syndromes (such as Duane, Klinefelter or Turner), history of gestational diabetes. For analysis of exon 8, non-diabetic subjects were tested as controls. Patients (n = 128) visited by an endocrinologist and diagnosed with type II diabetes were selected based on the above mentioned inclusion criteria and referred to the Cellular and Molecular Biology Research Center of Shahid Beheshti University (Tehran, Iran). All the patients signed consent and then peripheral blood (5 cc, EDTA) was collected. The samples were kept frozen until use.

DNA extraction. DNA was extracted from peripheral blood by the sodium perchlorate method. Briefly, blood (500 µl) was suspended in 1 ml of lysis buffer (10 mM Tris-HCl, pH 8, 0.1 mM EDTA, 0.15 M NaCl, 0.5% Triton X-100) and centrifuged at 8000 ×g at 4°C for 5 min. Then, sodium perchlorate (100 µl, 4 M), SDS (10 µl, 10%) and TE buffer (400 µl; 10 mM Tris, 1 mM EDTA) and NaCl (100 µl, 5N) were added to the pellet. This solution was mixed and centrifuged at 12,000 ×g for 10 min. The supernatant was transferred to a new tube and the DNA was precipitated by alcohol and dissolved in 100 µl dH_2O.

Primer design. For each exon, we designed a pair of primers (Table 1) that anneal to the respective exon and 50 to 100 nucleotides of flanking introns. PCR products were subject to conformation-sensitive gel electrophoresis (CSGE) for mutation detection. It must be mentioned here that the mutation detection in the first and last bases of PCR product is not reliable.

PCR amplification. A total of 50 µl reaction volume in each tube contained 200 ng genomic DNA, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 40 pmol each of forward and reverse primers, 1× PCR buffer, 1.25 unit of *Taq* DNA polymerase (CinnaGen, Iran). Termal cycle conditions consisted of initial denaturation at 94°C for 5 min, followed by 30 cycles including denaturation at 94°C for 30 s, annealing at exon primer specific temperature for 60 s and extension at 72°C for 30 s. The final incubation was also included at 72°C for 5 min [8].

PCR product electrophoresis. The PCR products were electrophoresed in 1.5% agarose gel. The gel was stained with ethidium bromide, and the bands were visualized under UV transillumination and photographed [9].

Scanning of PCR products by CSGE. All the PCR products were analyzed by CSGE for any possible mutation [10]. A 10% polyacrylamide gel containing 99:1 acrylamide (Sigma-Aldrich, St. Louis, MO, USA) was used as the base of electerpphoresis to Bis(acryloyl)piperazine (BAP); Fluka, Switzerland), 10% ethylene glycol (Sigma, Germany), 15% formamide (Gibco, Carlsbad, CA, USA), 0.1% ammonium per sulfate (Sigma-Aldrich, Germany), 0.07% N,N,NV,NV-tetra methyl ethylene diamine (Sigma-Aldrich, Germany) and 0.5× TTE buffer (44 mM Tris, 14.5 mM taurine, 0.1 mM EDTA buffer, pH 9.0). The gel was loaded onto a vertical cast preparation of standard gel apparatus (Bio-Rad Laboratories, Hercules, CA, USA) with 18-cm glass plates. A pre-run for 30 min at 30 mA with 0.5× TTE electrophoresis buffer did set the gel construct at its optimal status. PCR products were first heated up to 98^{o}C for 5 min to be denatured completely and then were cooled down to 65^{o}C for 30 min to let the strands to re-anneal again. PCR products were mixed with 5 µl loading buffer (30% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol FF) and then loaded onto the gel, and electro-phoresed at room temperature at 40 mA for 4 h.

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Table 1. Primers for amplification of INSR gene.

Exon number	Primer sequence	Size of PCR Product (bp)
1	INSR1 F 5` - GAG AGC CGA GAG ACA GTC CCG G– 3` INSR1 R 5` - ATT TTG GCT TGG GTG GGG TCC TCT – 3	500
2	INSR2 F 5` - TGT GTC CCG GCA TGG ATA TC – 3` INSR2 R 5` - CCC CTA CCT AAT GAC CAT TT – 3`	653
3	INSR3 F 5`- TTT CCC TCT CTC TCT CTC TC – 3` INSR3 R 5` - AGA CCT CAC TCA TAG CCA AT – 3`	472
4	INSR4 F 5` - CCC CTT TCT CTT TCT CTC TC – 3` INSR4 R 5` - CGA CCA TCC TAA AAG TGC TG – 3	310
5	INR5 F 5`- ATG AGA AGA TTG AAA TAT GT-3` INR5 R 5`- CTA ATA CAC GAA CTT CCT AG-3`	259
6	INR6 F 5`- TCT TGG AGT TGT AGA AGA CC -3` INR6 R 5`- ACC ATC TTC CAC TAA ACC GG -3`	329
7	INR7 F 5`- TGG TCT GAA ACT ACA CTG AA -3` INR7 R 5`- AAG CAC AGA GCC AGC CAG CC -3`	239
8	INR8 F 5`- TCA GTG TGA CGG TCT TGT AA -3` INR8 R 5`- GAA TTC ACA TTC CCA AGA CA -3`	330
9	INR9 F 5`- AGC TTT CTT TGC ACA CTG TT -3` INR9 R 5`- TGC ATC AGA CAC ACG TGT GC -3`	279
10	INR10 F 5`- TGT ATG TGT GTT CAG CCG CA -3` INR10 R 5`- CAA CAC CAA GCC AAT TGG CA -3	309
11	INR11 F 5`- CTG TCT AAT GAA GTT CCC TC -3` INR11 R 5`- CAG AGA AAC CCC TGG GTT CT -3`	179
12	INR12 F 5`- TAT TCT CCA GTG TCA CTT TT -3` INR12 R 5`- AAG TCA GCC TTG ATG TCC CA -3`	402
13	INR13 F 5`- TGG GAT CTC ATC CAA GAG TT -3` INR13 R 5`- ACT CTG AAG GGG CAT GCT GA -3	249
14	TK14F 5`- CTC CTT CTC CTC CTC TCT TC –3` TK14R 5`- CTG AGG CTG CCA TGG AGA C –3`	210
15	TK15F 5`- TTC TAT TTC AGT AGA CGT CCC- 3` TK15R 5` -GCA CAC CAC TGA ACT ACT TG –3`	140
16	TK16F 5`- CCA TGA GAA TCT CAA GCT AAC G- 3` TK16R 5`- GGA TGG TAC TCA CCA TCA CTG G -3`	132
17	TK17F 5`- GCA TGG GTC CTG GAT CAC AG –3` TK17R 5`- TAG GAG GAT ACA CCC TGT GTC –3`	480
18	INR18 F 5`- CCT GGT GAG TCG AAT CAC GG-3` INR18 R 5`- GAG GAG GCC AGG AGC GGG TG-3`	227
19	INR 19 F` 5`- GAT CCC AGT GCT GCT GAA AC-3` INR 19 R 5`- ACC TGG CCT GGG TCG TTA TG-3`	250
20	INR 20 F 5`- GGT GCT AGG ACC AAG GCT GA-3` INR 20 R 5`- GAA TTC AAG CCC AGC GTC CA -3`	228
21	INR 21 F 5`- GTG TGT GTC TAA ATG GCT TC-3` INR 21 R 5`- TAT GCA AAC ACA AAC ACA CC-3`	330
22	INR 22 F 5`- CTG CAG GGA CAA GAG TGG GG-3` INR 22 R 5`- TTT GGT TTT TTC TTT CGA AAT TTT G-3`	873

The gel was stained with ethidium bromide (1 mg/ml) for 10 min and destained in dH_2O for 10 min.

Sequencing the suspected mutations. PCR products of the samples with a suspected mutation, detected by CSGE method, were purified using a DNA extraction kit (Fermentas, Lithuania Cat. # K0513) and subjected to sequencing by the dideoxy chain-termination method [11].

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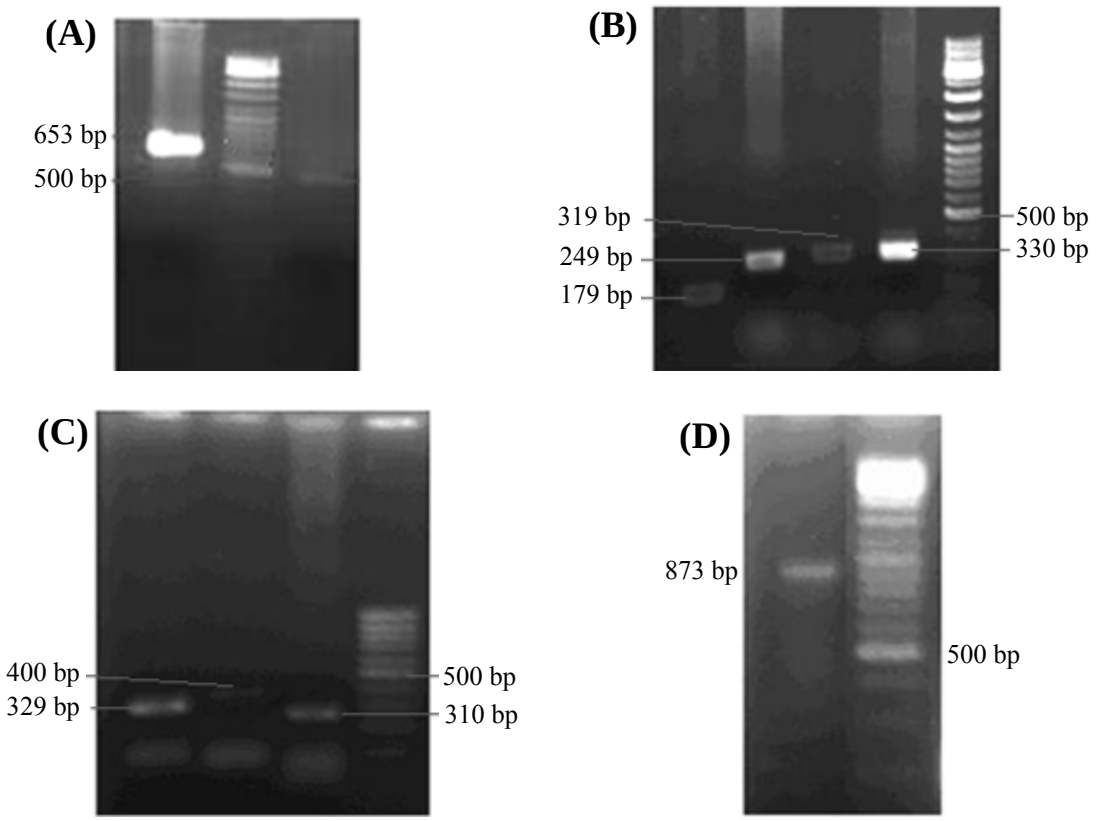

Fig. 1. Two percent of agarose gel electrophoresis of PCR product. **(A)** Lane 1, PCR product of exon 2 of insulin receptor gene; lane 2, 100 bp DNA ladder and lane 3, PCR product of exon 3 of insulin receptor gene. **(B)** Lane 1, PCR product of exon 11 of insulin receptor gene; lane 2, PCR product of exon 8 of insulin receptor gene gene; lane 3, PCR product of exon 10 of insulin receptor gene; lane 4, PCR product of exon 13 of insulin receptor gene and lane 5, 100 bp DNA ladder. **(C)** Lane 1, PCR product of exon 6 of insulin receptor gene; lane 2, PCR product of exon 12 of insulin receptor gene; lane 3, PCR product of exon 4 of insulin receptor gene and lane 4, 100 bp DNA ladder. **(D)** Lane 1, PCR product of exon 22 of insulin receptor gene insulin receptor gene and lane 2, 100 bp DNA ladder.

RESULTS

The PCR products relevant to a few exons of insulin receptor gene are shown in Figure 1 along side with DNA ladder marker. The mutations of exons 14, 15, 16 and 17 (tyrosine kinase domain) were determined by direct sequencing of the PCR product, while the rest was first screened through CSGE and then the suspected samples were submitted for sequencing. Any double-bond sample observed on the illuminated gel under UV light was suspected for mutation (Fig. 2). Because of the specific design of primers, we were able to discriminate the mutation existing on exon sequence or donor/acceptor or branch sites of the introns.

Screening of mutations by CSGE method. We detected mutations on the INSR gene sequence of 33 (25.78%) of the 128 Iranian type II diabetes patients (Table 2). On exon 2 of four patients, we detected four mutations: at position 511 C→A, at position 514 T→G, at positions 586 and 628 T→A resulted in amino acid changes His 171 Asp, Ile 172 Ser, Cys 196 Ser, and Trp 628 Arg, respectively. On exon 3 of four patients, at position 694 G→C resulting in a Gly 232 Ser change. On exon 3 of four patients, at position 680 G→A resulting in a Gly 227 Asn change, establishing a recognition site for the PvuII restriction enzyme (CAGCTG), which was confirmed by restriction digestion with PvuII enzyme (data not shown). This enzyme does not

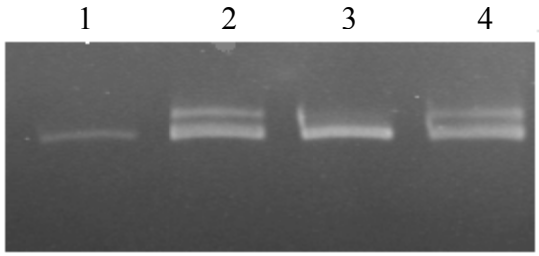

Fig. 2. CSGE electrophoresis of PCR product. Lane 1, PCR product of normal sample and lanes 2-4, PCR product of suspected ones.

Table 2. Mutations detected in the INSR gene of Iranian NIDDM patients, based on GenBank accession number M10051.

GenBank accession number	Exon number/ nucleotide position	Amino acid position	Nucleotide variation	Codon variation	Amino acid variation	Mutations Frequency	No. of patients
EF207606	2/511	171	C→A	CAC→AAC	His→Asn	4	4
	2/515	172	T→ G	ATC→AGC	Ile→Ser	4	
EF207605	2/586	196	T → A	TGC→AGC	Cys→Ser	4	
EF207605	2/628	210	T →A	TGG→AGG	Trp→Arg	4	
EU331144	3/679	227	G → A	GGC→AGC	Gly→Ser	4	4
EU331144	3/694	232	G→C	GGC→AGC	Gly→Ser	4	
DQ333190	8/1627	543	A → T	ACG→TCG	Thr→Ser	5	5
EF207612	9/2007	669	C→ C/T	TTC →TTT	Phe→Phe	3	3
EF207609	13/2595	865	C → C/T	AAC→AAT	Asp→Asp	3	3
EF207608	13/2669	890	G → G/C	CGA→CCA	Arg →Pro	3	
DQ068255	14/2706	902	C → G	CGC→CGG	Arg→Arg	8	8
DQ068255	14/2717	906	C → G	GCT→GGT	Ala→Gly	8	
EF025510	14/2752 and 2753	918	CC → TG	CCG→TGG	Pro→Trp	8	
DQ311689	17/3257	1086	T → A	GTG→gAG	Val→Gln	4	4
EF207610	19/3471	1157	T → T/A	CAT→CAA	His →Gln	2	2
EF207610	19/3516	1172	T → T/G	ACT→ACG	Thr→Thr	2	

normally have a recognition site on exon 3. On exon 8 of five patients, at position 1627 an A→T mutation resulted in a Thr 543 Ser change. In intron 9, mutations were detected in nucleotides 18 and 19 (AT → TG). On exon 9 of three patients, we observed a heterozygote C→C/T at position 2007, leaving Phe 669 unchanged. We also observed two heterozygote mutations on exon 13 of three patients. At position 2595 and 2669, nucleotides C and G were observed as C/T and C/G, respectively (Asp 865 Asp and Arg 890 Pro). On exon 14 of eight patients, C→G at positions 2706 and 2717 resulted in Arg 902 Arg and Ala 906 Gly, respectively. A missense mutation was found in exon 14: C at positions 2752 and 2753 (CCG → TGG) was replaced with T and G, respectively, resulting in Pro 918 Trp replacement (Fig. 3). Two heterozygotes were observed on exon

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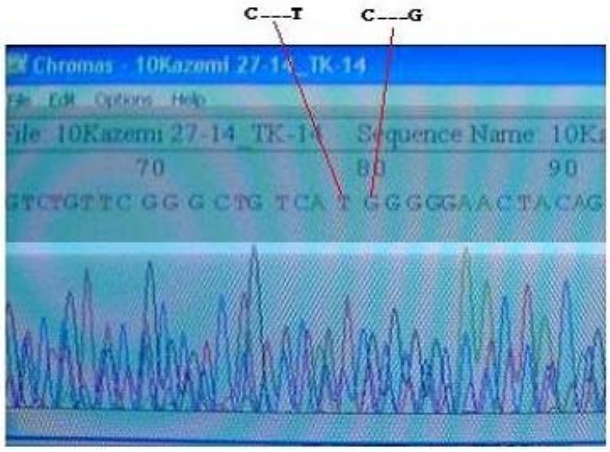

Fig. 3. Chromatogram shows C at position 2752 and 2753 (CCC→TGG) were replaced with T and G on exon 14 of insulin receptor gene, replaced of proline 918 with tryptophan.

19 of two patients. Nucleotide T at positions 3471 and 3516 was detected as T/A and T/G, respectively (His 1157 Gln and Thr 1172 Thr). No changes were observed on other exon sequences.

Controls. We identified no modifications on the INSR gene in exon 8 in non-diabetic control subjects.

Sequencing. The sequences of the PCR products, suspected to have mutations by their kinetic patterns on the CSGE gel, were submitted to GenBank with the accession numbers as shown in Table 2. DQ333190, DQ311689, DQ068255, EF207605, EF207606, EF207608, EF207609, EF207610, EF207612, EU331144

DISCUSSION

It is estimated that up to 90% of the people with type II diabetes suffer from insulin resistance [12]. Both insulin secretion and insulin receptor action are under genetic control; therefore, mutation in either set of genes (for insulin secretion or insulin receptor action) could theoretically be the primary event in diabetes. Several INSR mutation studies have been carried out in order to improve new therapy for diabetic patients [13].

This study represents the first effort in Iran to perform a complete molecular analysis of all 22 exon sequences of the INSR gene in type II diabetic patients to identify mutations and compare the results to the mutations reported in other populations.

The genetic analysis of the insulin receptor started in 1983, but the first clinical report of a patient with defective INSR was recognized in 1975. In recent years, INSR mutations were found to cause resistance to insulin. In case of INSR gene mutations that change the function of the receptor, although normal insulin secretion continues, the receptor does not react to the existing insulin, indicating that the insulin signal pathway within the cell is either inactive or defective.

A number of researchers studied different mutations in the INSR gene and reported that such mutations do not result in type II diabetes. For example, Moller *et al.* [14] studied a 1611-bp segment of the glucose transporter II promoter gene using single-strand conformation-polymorphism analysis and direct sequencing in patients affected by type II diabetes in Denmark. They detected four mutations (Gly 471 Ala, Asp 149 Ala, Thr 122 Asp and Gly 447 Ala), though none of them contributed to the type II diabetes [14]. Hansen *et al.* [15] reported Met 326 Ile and Gly 1020 Ala in alpha subunit of P85 phosphatidyl inositol 3 kinase in NIDDM, with no function in diabetes. They followed the Met 326 Ile mutation in the p85 alpha regulatory subunit of the phosphoinositide 3-kinase in 1190 Caucasian men for 20 years. Hansen *et al.* [15] also studied the relevant protein, and reported that this mutation had no relationship with type II diabetes and had no effect on the role of insulin [16]. On the other hand, there are some reports relating NIDDM to genomic mutations.

Cocozza *et al.* [17] studied tyrosine kinase domain of insulin receptor gene of 103 patients and detected Arg 1152 Gln mutation. Nozaki *et al.* [18] detected Gly 1008 Val in tyrosine kinase domain of insulin receptor gene which related to insulin resistance. Iwanishi *et al.* [19] detected Leu 1193 Trp mutation, causing defective tyrosine kinase activity. Cama *et al.* [20] reported Ileu 1153Met mutation in tyrosine kinase domain of insulin receptor gene. Kan *et al.* [21] reported Thr 831 Aal and Try 1334 Cys mutations and Elbein *et al.* [22] reported Met 958 Val mutation. Imano and Kawamori [23] detected homozygote mutations in tyrosine kinase domain of insulin receptor gene, and proposed the more severity of the disease compared to those harboring heterozygote mutations in tyrosine kinase domain of insulin receptor gene. Kusari *et al.* [24] reported missense (Arg 981 Gln) and nonsense mutations in tyrosine kinase domain of insulin receptor gene and

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Hojlund *et al.* [25] reported missense mutation (Arg 1174 Gln) in tyrosine kinase domain of insulin receptor gene related to NIDDM. Accili *et al.* [26] detected a homozygote mutation (Val 382 Phe) in a family resistant to insulin; however, Roach *et al.* [27] reported Ser 323 Leu mutation in an insulin resistance patient.

Tyrosine kinase receptors are a family of receptors with a similar structure. They each have a tyrosine kinase domain (which phosphorylates proteins on tyrosine residues), a hormone binding domain, and a carboxyl terminal segment with multiple tyrosines for autophosphorylation. When hormone binds to the extracellular domain, the receptors aggregate. When the receptors aggregate, the tyrosine kinase domains phosphorylated the C terminal tyrosine residues [28]. The tyrosine kinase is attached to insulin hormone and its mutations are very important in patients.

We searched in the literature for each of the mutations found in the present study; however, no similar mutations have been reported in type II diabetes patients. Therefore, it is possible that these mutations could be specific for the Iranian population. More investigations are needed to determine the incidence of such mutations in Iranian patients, and more importantly, whether these mutations correlate with clinical signs and symptoms. We observed many novel mutations in our analysis of the insulin receptor gene of Iranian type II diabetes patients. The fact that these mutations have not been reported in studies performed in other countries suggests the possibility of a geographic pattern for these variants in the INSR gene.

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