

Original Research

## Differential activation and expression of insulin receptor substrate 1 (IRS1) in mononuclear cells of type 2 diabetes patients after insulin stimulation

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**Abstract:** Insulin regulates the glucose homeostasis by inducing tyrosine phosphorylation of insulin receptor substrate (IRS) proteins. IRS1 is the best studied member of this family and insulin-induced Tyrosine phosphorylation of (YXXM) motifs provides docking site for SH2 domain-containing proteins. Recent studies have suggested that genetic and/or environmental factors may affect the expression and phosphorylation levels of IRS1, and these could be important for development of insulin resistance. To shed light to the molecular basis of type 2 diabetes we wanted to determine whether YXXM motifs are genetically modified in these patients. We have isolated mononuclear cells of eighteen type 2 diabetes patients and prepared genomic DNA and protein lysates from these cells. The genomic DNA was used to sequence IRS1 gene, and protein lysates were used to determine the expression and phosphotyrosine levels of IRS1 after insulin stimulation. Although, we did not detect any mutations at/or near the YXXM coding regions in patients' DNA, immunoprecipitation analysis of IRS1 indicated decreased levels of expression and tyrosine phosphorylation of IRS1 in patient's samples compared to that of healthy controls. Our results suggest that mononuclear cells of patients can be used to test the levels of insulin responsiveness before therapy.

**Key words:** Diabetes mellitus, Type 2, Insulin Receptor Substrate protein, insulin resistance, insulin responsiveness.

### Introduction

Type 2 diabetes is a chronic metabolic disorder affecting >5% of the population in Western countries (1,2). Insulin resistance is predisposed to developing type 2 diabetes, and a common pathological state in which target cells fail to respond to ordinary levels of circulating insulin (3,4).

Insulin is the primary hormone that regulate the glucose homeostasis via inducing complex signaling system and insulin receptor substrate (IRS) proteins are the key players in this system (5,6). IRS1 is widely expressed in human tissues and the best studied member of IRS protein family (7,8). IRS1 is an adaptor molecule that connect insulin receptor to downstream elements of insulin signaling such as phosphatidylinositol-3-kinase (PI3K) and mitogen activated protein kinases (MAPK's) (9,10). IRS1 interacts with tyrosine kinase receptors through its pleckstrin homology domain (PH) and a phosphotyrosine binding domain (PTB) (11). IRS1 has several YXXM motifs on C-terminal, and tyrosine phosphorylation of these motifs by insulin receptor (IR) trigger intracellular signaling cascades which activates PI3K and AKT (12-14). Activation of AKT regulates glucose uptake as well as glycogen and protein synthesis (12,15).

IRS1 function is regulated by post-translational modifications (16). Although tyrosine phosphorylation of IRS1 promotes insulin signaling, its Ser/Thr phosphorylation generally inhibits the insulin signaling by inducing degradation and dissociation of IRS1 from the insulin receptor and inhibition of its tyrosine phosphorylation (17). These regulations are highly

conserved under physiological conditions (18).

Recent studies have shown that genetic and/or environmental factors affect the expression levels as well as phosphorylation status of IRS1, and some of these Ser/Thr phosphorylations are important in developing insulin resistance (19,20). Most of the results claiming the importance of Ser/Thr phosphorylations in development of insulin resistance originates from studies done in cell lines or in animal models (21-23). Limited number of studies using human samples have shown that there is a strong correlation between reduced tyrosine phosphorylation, diminished expression, and increased Ser/Thr phosphorylations of IRS1 and type 2 diabetes (24-27). In addition to these, some missense mutations in IRS1 gene have also been identified. These changes include Ala512Pro, Gly972Arg, Pro170Arg, Ser809Phe, Met209Thr, Ser892Gly, Gly819Arg, Arg1221Cys (28-31). Among these, Gly972Arg is the most common variation which has been associated with development of Type 2 Diabetes (32-34).

In this study, we wanted to determine whether inactivation mutations on YXXM motifs are responsible for development of type 2 diabetes. Therefore, we applied simple and non-invasive method to evaluate IRS1 signaling in mononuclear cells of patients with

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## Type 2 Diabetes.

**Materials and Methods****Clinical parameters of patients included in this study**

Eighteen patients aged from 23 to 52, diagnosed with Type 2, had BMI (Body Mass Index)'s higher than 19 and did not take any treatment such as metformin were included in this study. Patients who were pregnant, had acute or chronic diseases, and taking any medications were excluded in this study.

**Tissue procurement**

Blood samples were taken from 18 patients and 15 non-diabetic controls. Informed consent was obtained from all individual participants included in the study. Procedures have been approved by the ethics board of Akdeniz University, and were performed in accordance with the Declaration of Helsinki.

**Mutational analyses of PI3K binding sites of IRS1 in Type 2 diabetes patients**

Genomic DNA was extracted from whole blood of patients and controls by using Qiagen DNA isolation kit. Genetic analysis of the PI3K binding site of IRS1 gene was performed by PCR amplification. The following primers were used for PCR amplification: Primer 1/1 (forward, 5'g gag gtg gca gtg gag gcc gac tgc c3'; reverse, 5'cct cag ggc cgt agt agc agt c3') Primer 1/2 (forward, 5'ctg gag ccc agc ctt cca catc3'; reverse, 5'ccc tgg gca ggc tca cct cctc3') PCR was performed in total volume of 25 µl, containing 1X Qiagen Taq polymerase buffer, 2 mM MgCl<sub>2</sub>, 6 mM dNTPs, 0,5 uM of each primer, 0.2 units Qiagen Taq DNA polymerase and 50 ng genomic DNA. PCR conditions were 5 minutes at 94 °C, followed by 35 cycles of 94 °C for 30 seconds, 58 °C for 1 minutes, 72 °C for 45 seconds, and one cycle of 72 °C for 10 minutes. PCR products were purified by the Invitrogen PCR Purification Kit (Carlsbad, CA), and the Big dye-terminator sequencing kit (Applied Biosystems, Foster City, CA) was used for the primers during amplification. Sequencing fragments were detected by capillary electrophoresis using the ABI Prism 3130 DNA analyzer (Applied Biosystems, Foster City, CA). In all cases, samples harboring mutations were reamplified and re-sequenced using the same experimental conditions. Sequence chromatograms were analyzed by Finch TV.

**Cell culture**

Total mononuclear cells were isolated from peripheral blood using ficoll-hypaque (Sigma) gradient method. WBC (white blood cells) were counted by Beckman cell counter and each samples were equally divided to two flasks. Cells were grown in 5% CO<sub>2</sub> at 37 °C in Roswell Park Memorial Institute medium (RPMI-1640) (Biochrome) supplemented with 10% FBS (Biochrome), 2 mM L-glutamine, 100 µg/ml penicillin, 50 µg/ml streptomycin. Cells were initially starved overnight in RPMI-1640, treated with 100µM Sodium orthovanadate one hour prior to addition of insulin (100 ng/ml) for five minutes.

**Immunoprecipitation (IP) and Western Blot analysis**

Reagents were obtained from the following sources: monoclonal anti-IRS1, antiphosphotyrosine antibodies and protein A/G-agarose beads from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-rabbit HRP and anti-mouse HRP were purchased from BioRad (Hercules, CA).

WBC were homogenized in Triton X-100 buffer containing 50 mM HEPES, pH 7.0, 150 mM NaCl, 10% glycerol, 1.2% Triton X-100, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 0.15 units/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml pepstatin A. Two milligrams of cell lysates were pre-cleared with protein A/G agarose for 1 hr, centrifuged at 10,000 rpm for 1 minute, the supernatants were transferred to another tube and 2 mg of anti-IRS1 antibody was added and samples were shaken for 3 h at 4°C, then 100 µl Protein A/G agarose beads were added and the mixture was shaken overnight at 4°C. Samples were centrifuged at 10,000 rpm for 1 min. at 4 °C, and the pellets were washed three times with lysis buffer, beads were resuspended in 100 µl of Laemmli buffer, boiled for 5 min, centrifuged and 50 µl of supernatants were fractioned using 7.5 % polyacrylamide gels and transferred to PVDF membranes. The blots were first labeled with anti-phosphotyrosine antibody. Labeled blots were then stripped off and re-labeled with anti-IRS1 antibody. Signal intensity on blots were determined using the enhanced chemiluminescent detection system.

**Statistical analysis**

The statistical software SPSS was used. Comparison of parameter was performed using Wilcoxon Signed Ranks Test. A p value of <0,05 was considered to be statistically significant.

**Results**

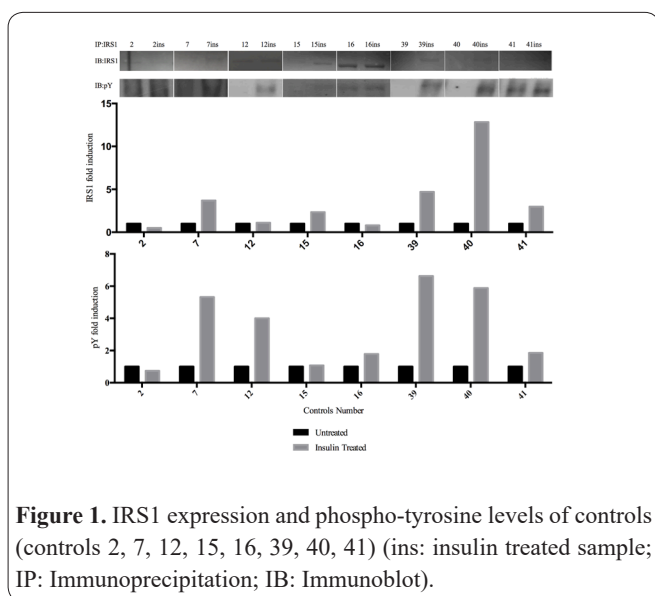
The clinical features of our patients are summarized in Table 1. Since phosphorylation of IRS1 at YXXM motif is the main event in insulin signaling we thought that alterations at these motifs would affect insulin response. Therefore, initially we want to determine the genomic changes at or around YXXM motifs of IRS1. To test our hypothesis, we sequenced coding region of IRS1 between the amino acids 561-1028.

The sequenced region spans GYMPMS<sub>616</sub>P, DYMPMS<sub>636</sub>P, GYMMMS<sub>666</sub>P, DYMNMS<sub>736</sub>P motifs and mutational analysis of these regions revealed no mutation.

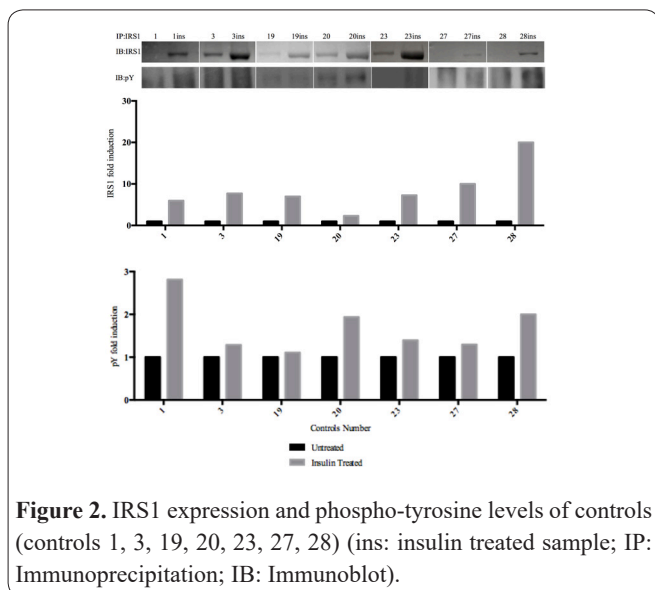
Since we could not detect any mutations at above mentioned regions of IRS1, we thought that expression or tyrosine phosphorylation of IRS1 could be different in these patients compared to healthy controls. Therefore, we determined phosphorylation status and expression levels of IRS1. Most of the mononuclear cells of healthy controls (number 1, 7, 12, 16, 20, 28, 39, 40, 41) responded to insulin as judged by increased tyrosine phosphorylation and/or stabilization of IRS1, and only six controls (numbers 2, 3, 15, 19, 23, 27) did not respond to insulin significantly by increased tyrosine phosphorylations of IRS1 however five of them (numbers 3, 15, 19, 23, 27) responded to insulin through

**Table 1.** Clinical features of patients (M: Male; F: Female; BMI: Body Mass Index).

Patient No	Gender	Age	BMI	Clinical History
4	M	28	34,1	None
5	M	33	24,6	Schizophrenia
6	M	28	29,7	Type 2 diabetes history in the first generation
9	F	33	23,9	None
10	F	29	24	None
13	F	26	27,2	Type 2 diabetes history in the first-second generation
14	F	25	26,6	Thalassemia carrier
17	F	52	24	Rheumatoid arthritis, Hashimoto's thyroiditis, Hyperlipidemia
18	M	23	24	None
21	M	24	29	None
22	M	38	27,01	None
25	M	37	36,8	Hypercholesterolemia
26	F	32	24	None
37	F	32	19,1	Type 2 diabetes history in the first-second generation
38	M	32	23,7	Type 2 diabetes history in the first-second generation
42	M	38	26,9	None
43	F	24	20,6	Lipodystrophy
44	M	33	30	None



**Figure 1.** IRS1 expression and phospho-tyrosine levels of controls (controls 2, 7, 12, 15, 16, 39, 40, 41) (ins: insulin treated sample; IP: Immunoprecipitation; IB: Immunoblot).

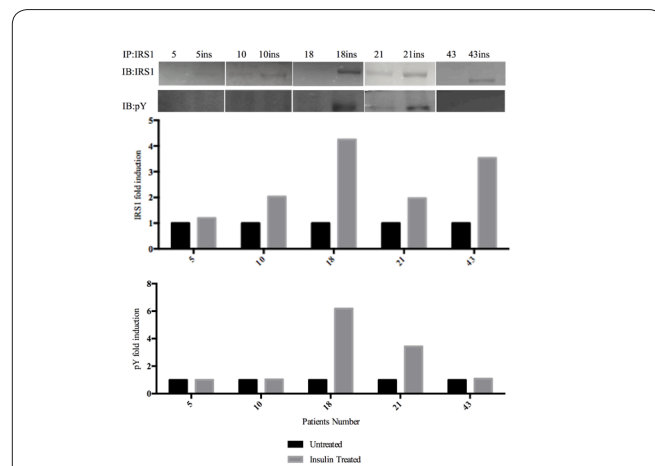


**Figure 2.** IRS1 expression and phospho-tyrosine levels of controls (controls 1, 3, 19, 20, 23, 27, 28) (ins: insulin treated sample; IP: Immunoprecipitation; IB: Immunoblot).

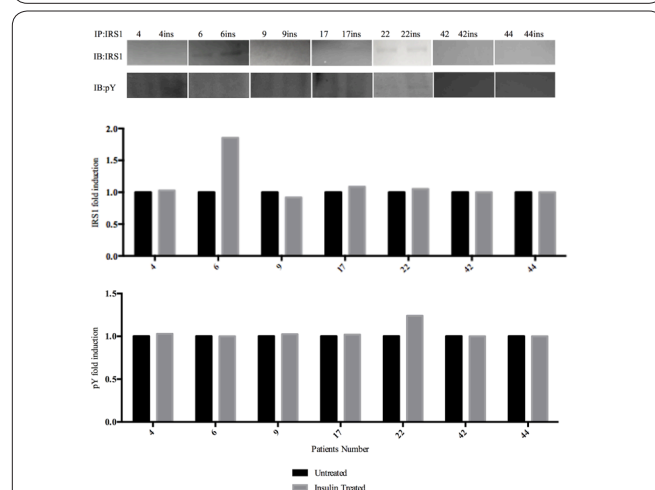
increased IRS1 expression levels Figure 1 and Figure 2.

Contrary to these, 2 of 18 patients (patients 18, 21) responded to insulin similar to healthy controls

as judged by increased tyrosine phosphorylation, expression and stabilization of IRS1 and patients 10 and 43 responded to insulin only by increased expression level of IRS1, Figure 3. However, different than healthy

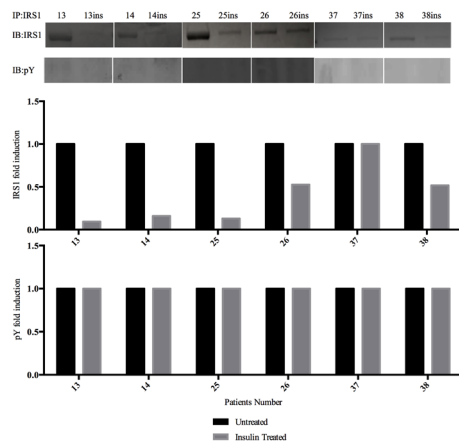


**Figure 3.** IRS1 expression and phospho-tyrosine levels of patients (patients 5, 10, 18, 21, 43) (ins: insulin treated sample; IP: Immunoprecipitation; IB: Immunoblot).



**Figure 4.** IRS1 expression and phospho-tyrosine levels of patients (patients 4, 6, 9, 17, 22, 42, 44) (ins: insulin treated sample; IP: Immunoprecipitation; IB: Immunoblot).





**Figure 5.** IRS1 expression and phospho-tyrosine levels of patients (patients 13, 14, 25, 26, 37, 38) (ins: insulin treated sample; IP: Immunoprecipitation; IB: Immunoblot).

controls, expression level of IRS1 did not change in 2 patients (patients 22, 37), Figure 4 and Figure 5, and immediately degraded in 5 patients (patients 13, 14, 25, 26 and 38), Figure 5 after insulin stimulation, and remaining 6 patients (patients 4, 5, 9, 17, 42, 44) did not show detectable level of IRS1 expression, Figure 4.

It was shown in the overall statistical analysis that IRS1 and pIRS1 levels of controls were statistically significant compared to the patients in terms of response to insulin.

## Discussion

Type 2 diabetes is a multifactorial disorder characterized by insulin resistance and failure of secretion insulin from pancreatic beta cells (35). IRS1 protein is an adaptor molecule which act as a docking molecule that connect receptor activation to the downstream targets. Regulation of IRS1 activity by post-translational modifications and/or genomic changes is crucial to understand the molecular mechanism of Type 2 Diabetes and insulin resistance (16,21,36). Several studies showed that G972A change is the most common alteration of Type 2 diabetes patients in Western world (20,37,38). However, mutations or at/or around YXXM motifs have not been reported. Esposide et al. suggested that missense mutations of these sites can be the most critical sites, and changes at these region would have deleterious effect on activation or inactivation of the insulin signaling (39). In this respect, we sequenced the region covering YXXM motifs of IRS1, however, we did not find any changes in these sites.

Up to date, most of the studies on IRS1 protein have been done on cell lines and animals and sometimes their results did not correlated with humans (40). Several previous studies have shown decrease in expression or phosphorylation levels of IRS1 in muscles and adipocytes of type 2 diabetes patients compared to the healthy controls (41-43). Similar to these findings, Pasini et al. demonstrated that while expression levels of IRS1 did not change its tyrosine phosphorylation levels decreased in lymphocytes of patients with metabolic syndromes (44). Level of IRS1 in any cells can vary depending on the presence and amount of various growth factors and cytokines (23,45,46). Most of the

studies performed in this respect suggests that level of IRS1 protein decreases after chronic insulin treatment via proteasomal degradation (47-50). Therefore, insulin-mediated degradation of IRS1 seem to be one of the mechanism causing metabolic disorders or insulin resistance.

In line with previous studies, we did not detect any IRS1 protein in six patients (patients 4, 5, 9, 17, 42, 44), and level of IRS1 did not change in three patients (patients 6, 22, 37) after insulin stimulation. Contrary to this, mononuclear cells of patients 18, 21 responded to insulin as good as cells of healthy controls as judged by increased tyrosine phosphorylation of IRS1. In these cases, IRS1 may not be responsible for development of insulin resistance, and other signaling components need to be studied. One of the possible mechanism could be increased S/T phosphorylation of IRS1, which may interfere with binding of adaptors proteins (such as Grb2, Shc) to IRS1 and inhibits down stream signaling events.

In patients 13, 14, 25, 26 and 38 we did not observe any tyrosine phosphorylation, however, IRS1 levels were reduced after insulin stimulation. This is opposite to what we observed in most of the control samples where insulin induces stabilization of IRS1. Among these patients 13, 37 and 38 have the same clinical history implying that behavior of IRS1 may be considered as a biomarker for some patients. The reason for immediate degradation of IRS1 in these patients may be the consequence of aberrant phosphorylation of IRS1 which may facilitate rapid proteasomal degradation.

Overall, our study shows that phosphorylation and expression levels of IRS1 is different in mononuclear cells of Type 2 Diabetes patients and this non-invasive method may be used to assess the effects of interventions with specific therapeutic strategies. Similar study can also be done after treatment to see whether *in vitro* results will correlate with *in vivo* data.

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