ORIGINAL ARTICLE

Changes in bone biological markers after treatment of Iranian diabetic patients with pioglitazone: No relation to polymorphism of PPAR-γ (Pro12Ala)

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Background: Thiazolidinediones (TZDs) improves insulin sensitivity by activating the peroxisome proliferator-activated receptor γ (PPAR-g). We aimed to study any association between variation in bone biochemical markers and single nucleotide polymorphism (SNP) in PPAR-γ (Pro12Ala) and investigate if these genetic variants affect bone turnover markers in Iranian diabetic population before and after treatment with pioglitazone. **Materials and Methods:** A total of 101 patients (type 2 diabetic (T2D) were treated for 12 weeks with pioglitazone (15 mg/day). Bone Biological markers, osteocalcin, and C-terminal telopeptide of type 1 collagen (CTx) were measured before and after pioglitazone therapy. We genotyped 128 nondiabetic controls and 101 T2D patients as well. Pro12Ala polymorphism in PPAR-γ was done by real-time polymerase chain reaction (RT-PCR) using TaqMan assay. **Results:** There were statistically significant differences in allele frequencies of Pro12Ala while comparing the controls with T2D subjects. Ala frequency was 7 vs 3%, $P = 0.036$ and genotypic frequency of Pro/Ala was 5.94 vs 14.06%, $P = 0.04$. After treatment, the homeostasis model of assessment of insulin resistance (HOMA-IR) as a maker of insulin resistance was significantly decreased ($P < 0.001$). In respect of bone turnover markers, CTx values decreased and osteocalcin significantly increased. ($P < 0.001$). **Conclusion:** Our findings did not reveal a significant association between this polymorphism and bone turnover markers after pioglitazone treatment. The reduced insulin resistance might be the reason that CTx values decreased and osteocalcin increased significantly after short-term pioglitazone treatment. These findings suggest the need for further studies on the possible role of insulin in regulation of bone metabolism.

Key words: Bone biological markers, diabetic patient, insulin resistance, Iranian population, osteoporosis, polymorphisms PPAR-γ, pioglitazone

INTRODUCTION

The nuclear hormone receptor, peroxisome proliferator-activated receptor gamma (PPAR-γ), has important effects on insulin sensitivity, atherosclerosis, inflammation, endothelial cell function, and the pathogenesis of insulin resistance.[1-4] PPAR-γ protein was detected in primary osteoblasts. It has three subtypes, but the PPAR-γ is the major one for terminal differentiation of adipocyte.[5] PPAR-γ activation inhibits adipocytes differentiation into osteoblasts[6,7], and also regulates bone metabolism by affecting their differentiation into osteoclasts.[8,9]

It is reported that the long term treatment of diabetic women with thiazolidinedione (TZD) is associated with a significant 50% increase in the whole body bone loss.[10,11] Several assessment on bone biological markers have also been done in order to clarify the underlying pathophysiological mechanisms with contradictory results.[12]

The highly-prevalent Pro12Ala polymorphism in PPAR-γ gene was first identified by Yen *et al*.[13]. The substitution of alanine for proline in codon 12 of exon B is due to a CCA→GCA base exchange. This polymorphism decreases the risk of insulin resistance[14] and is associated with weight regulation,[15] as well as with a protective effect against obesity,[16,17] type 2 diabetes (T2D) and its complications,[18-20] and myocardial infarction.[21] A study on the association between bone density and a genetic polymorphism of PPAR-γ in postmenopausal women has shown the involvement of PPAR-γ in bone loss.[22] In this regard, Ogawa *et al*.,[22] have shown a significant association between a polymorphism of PPAR-γ gene and bone mineral density (BMD) in postmenopausal Japanese women. In the literature, there are few reports that

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evaluated the effect of genetic polymorphism and bone markers. Rhee *et al*.,[23] have founded significant association of Pro12Ala polymorphisms with serum osteoprotegerin levels, as a bone formation marker in Korean females.

Recent studies have demonstrated links between bone metabolism and glucose intolerance. Insulin is known to play important anabolic roles in the bone,[24] and deficiency of insulin signaling is associated with osteopenia in both mice and humans.[24,25] Osteocalcin, which is secreted by osteoblast, is an important protein in the bone tissue after collagen. It participates in mineralization and calcium ion homeostasis. The activation of the insulin receptor in osteoblasts resulted in an increase in undercarboxylated osteocalcin level and serum osteocalcin concentration was inversely associated with metabolic syndrome.[26] On the other hand, osteocalcin is a regulator of glucose metabolism and elevated levels of osteocalcin were associated with improved glucose tolerance.[27]

Type 1 collagen is the only collagen type found in mineralized bone. C-terminal telopeptide of type 1 collagen (CTx) is the carboxyterminal telopeptide region of type 1 collagen. It comes to blood circulation as the product of bone resorption and degradation of loose connective tissues. Hence, the increased level of CTx in the blood is associated with increased lysis of the bone. Few studies provided evidence that insulin signaling in osteoblasts leads to increased bone resorption[12] and there was a positive association between insulin resistance and CTx concentration.[28] The findings described in these studies suggests insulin's involvement in bone metabolism. However, more research is needed.

The aims of the present study were to investigate the effects of short term treatment with pioglitazone as insulin synthesizers on insulin resistance and biological bone markers and to determine whether PPAR-γ gene variants can modulate the responses in bone turnover markers in patients with T2D.

MATERIALS AND METHODS

One hundred and one patients from Shiraz, Iran, with T2D, diagnosed according to the 2009 World Health Organization criteria[21], were enrolled. The patients consisted of 21 men and 80 women, aged 30-70 years (mean ± SD = 51.44 ± 7.7 years). They were treated with pioglitazone (15 mg/day) during 12 weeks with no changes in their previous medications. They had no history of receiving PPAR-γ agonist, insulin, and other medications that might affect bone metabolism. Patients with type 1 diabetes (T1D) and pregnant or lactating women and also those with diseases affecting bone metabolism were excluded from the study.

One hundred and twenty-eight samples of blood from healthy volunteer donors were obtained from Shiraz Blood Transfusion Organization as a control group just for genotype study.

The study was approved by the Ethics Committee of Shiraz University of Medical Sciences. All the participants gave their written informed consent.

Laboratory tests

Anthropometric measurements were made with standard techniques before and after treatment. Blood samples were taken between 7.00 am and 9.00 am, after the patients had fasted overnight.

The serum was separated to determine fasting blood sugar (FBS). The serum insulin and C-peptide concentration were analyzed with a radioimmunoassay kit (Monobind, Lake Forest, CA, USA). Hemoglobin (Hb) A1C was measured with a boronate affinity assay (Nycocard, Oslo, Norway).

Measurements of bone turnover markers

As a bone formation marker, serum levels of osteocalcin and CTx level as a bone resorption marker were measured using a radioimmunoassay kit (Orion diagnostic, Espo, Finland).

Genotyping

We used buffy coat taken from the blood samples of each participant and stored at -80°C and genomic DNA was isolated using the Cinagen Kit dNp protocol (DNG plus DNA Extraction Kit, Cinagene Company, Tehran, Iran). For the analysis of the Pro12Ala polymorphisms of PPAR-γ gene, we used real-time polymerase chain reaction (RT-PCR). The allelic discrimination assay was performed based on the procedure we used in our previous work,[29] using TaqMan probe (MetaBion, Germany).

Statistical analysis

Genotype distribution and allele frequencies were calculated and compared between groups using Chi-square (χ^2) test or Fisher's exact test. The Hardy-Weinberg equilibrium (HWE) was tested with Arlequin 313 software in the control group. The data are shown as the mean ± standard deviation (SD). Clinical characteristics before and after drug therapy were compared with paired *t*-tests. Continuous variables were compared between genotypes with analysis of variance (ANOVA). All statistical analyses were done with SPSS software (version 15.0, Chicago. IL, USA) and $P \leq 0.05$ were considered as statistically significant.

RESULTS

A total of 101 patients with T2D (n = 101; 21 men, 80 women) aged 30-70 years (mean 51.44 ± 7.7) with a mean body weight of 69.86 ± 12.40 kg were enrolled in the study. The change in serum FBS, insulin concentration, C-peptide level, HbA1C, homeostasis model assessment of insulin resistance (HOMA-IR), osteocalcin, and CTx before and after pioglitazone treatment was measured [Table 1]. FBS, insulin, C-peptide, HbA1C values were significantly decreased after pioglitazone treatment ($P < 0.001$).

The HOMA-IR as a maker of insulin resistance was also significantly decreased ($P < 0.001$). In respect of bone turnover markers, CTx values decreased and osteocalcin increased significantly after 12 weeks treatment ($P < 0.001$). Mean body weight and waist-hip ratio did not change significantly ($P > 0.05$).

The biochemical characteristics before pioglitazone therapy (baseline) according to Pro12Ala genotypes are presented in Table 2. There was no significant difference between clinical parameters such as BMI, waist-hip ratio with Pro12Ala genotype ($P > 0.05$).

The baseline level of FBS, insulin, C-peptide, HbA1C, and HOMA-IR index was not different between genotypes ($P > 0.05$). No significant difference between bone turnover markers and Pro12Ala genotypes was observed ($P > 0.05$).

Table 1: Patients' clinical characteristics before and after treatment with pioglitazone

	Before	After	*P* value
CTx	52.87±84.15	13.73±15.89	<0.001
Osteocalcin	7.29±6.54	16.04±13.84	<0.001
FBS (mg/dL)	196.66±65.30	152.12±56.55	<0.001
Insulin	13.41±7.81	9.59±3.78	<0.001
HOMA-IR	6.49±4.52	3.63±2.02	<0.001
C-Peptide	2.10±0.95	1.18±0.57	<0.001
HbA1C	9.26±2.08	8.29±1.73	<0.001

CTx=C-terminal telopeptide of type 1 collagen; FBS=Fasting blood sugar; HOMA-IR=Homeostasis model assessment of insulin resistance; HbA1C=Haemoglobin A1C

Table 2: Baseline characteristics of the patients before pioglitazone treatment

Characteristic	Genotypes		*P* value
	Pro/Pro	Pro/Ala+Ala/Ala	
BMI (kg/m^2)	27.45±4.23	25.51±2.98	0.271
Waist-hip ratio	0.89±0.053	0.90±0.02	0.672
FBS (mg/dl) baseline	196.47±66.92	184.67±40.82	0.671
Insulin baseline	12.88±7.14	21.73±13.18	0.162
C-peptide baseline	2.06±0.95	2.67±0.85	0.132
HOMA-IR baseline	6.25±4.15	10.32±8.06	0.032
CTx baseline	53.74±84.84	39.07±77.72	0.681
Osteocalcin baseline	7.14±6.30	9.77±10.04	0.342
HbAIC D (%) baseline	9.22±2.06	9.93±2.57	0.420

BMI=Body mass index; FBS=Fasting blood sugar; HOMA-IR=Homeostasis model assessment of insulin resistance; CTx=C-terminal telopeptide of type 1 collagen; HbA1C=Haemoglobin A1C

Genotyping

The PPAR-γ (rs1801282) genotype and allele frequencies in both groups are shown in Table 3. Distributions of genotypes were 0.86 for Pro/Pro, 0.14 for Pro/Ala, and 0.00 for Ala/Ala in the control group. The allelic frequencies were 0.93 and 0.07 for Pro and Ala, respectively. Distributions of genotypes in the patient group were 0.94 for Pro/Pro, 0.06 for Pro/Ala, and 0.00 for Ala/Ala. The allelic frequencies were 0.97 and 0.03 for Pro and Ala, respectively [Table 3]. The Ala substitution allele was significantly less frequent among patients. The Ala phenotype was negatively associated with diabetes, with an odds ratio of 0.4048 (95% confidence interval (CI) =0.1576–1.0395) [Table 4].

Patients with Ala allele had lower BMI and FBS, although these differences in comparison to patients with the Pro/Pro genotype did not reach the statistical significance. There was no association between the Pro/Pro and Pro/Ala variants in the PPAR-γ gene and mean serum change of CTx and osteocalcin level. There was no significant difference in bone turnover markers among subjects with different genotypes [Table 5].

DISCUSSION

The PPAR-γ in humans which is a key regulator of adipocyte differentiation in bone metabolism was identified as the receptor for TZD insulin-sensitizing drugs in 1995.[29] Patients with diabetes who have been treated with TZD

Table 3: Genotypes and allele frequencies of PPAR-γ in patients with diabetes and healthy volunteers

Genotype/allele	Patients (n=101); n (%)	Control (n=128); n (%)	*P* value
Genotypes			
Pro/Pro	95 (94.06)	110 (85.94)	0.036
Pro/Ala	6 (5.94)	18 (14.06)	
Ala/Ala	0 (0.00)	0 (0.00)	
Alleles			
Pro	196 (97.02)	238 (92.97)	0.040
Ala	6 (2.97)	18 (7.03)	

PPAR-γ=Peroxisome proliferator-activated receptor γ

Table 4: Association of the Pro12Ala variant with type 2 diabetes mellitus

	Diabetic patients (n=97) n (%)	Controls (n=128) n (%)	*P* value (A)	Odds ratio (95% CI)
Pro	6 (2.97)	18 (7.03)	0.040	2.4706 (0.9621-6.3443)

CI=Confidence interval

Table 5: Patients' baseline characteristics before and after treatment with pioglitazone

Characteristic	Genotypes		*P* value
	Pro/Pro	Pro/Ala+Ala/Ala	
BMI (kg/m^2)	27.45±4.23	25.51±2.98	0.271
Waist hip ratio	0.89±0.053	0.90±0.02	0.672
FBS (mg/dL)			
Baseline	196.47±66.92	184.67±40.82	0.671
After pioglitazone	153.25±57.92	134.33±22.76	0.430
Mean change	−44.17±48.37	−50.33±53.33	0.764
Insulin			
Baseline	12.88±7.14	21.73±13.18	0.162
After pioglitazone	9.31±3.26	14.05±7.75	0.195
Mean change	−3.57±6.66	−7.68±6.33	0.145
C-peptide			
Baseline	2.06±0.95	2.67±0.85	0.132
After pioglitazone	1.152±0.56	1.59±0.65	0.069
Mean change	−0.91±0.96	−1.07±1.29	0.688
HOMA-IR			
Baseline	6.25±4.15	10.32±8.06	0.032
After pioglitazone	3.58±2.00	4.5±2.31	0.280
Mean change	−2.67±3.84	−5.82±5.94	0.064
CTx baseline	53.74±84.84	39.07±77.72	0.681
After pioglitazone	13.92±16.31	10.86±6.40	0.631
Mean change	−39.82±84.47	−28.38±78.51	0.748
Osteocalcin			
Baseline	7.14±6.30	9.77±10.04	0.342
After pioglitazone	15.60±12.88	23.00±25.47	0.205
Mean change	8.46±15.60	13.23±33.37	0.505
HbAIC (%)			
Baseline	9.22±2.06	9.93±2.57	0.420
After pioglitazone	8.24±1.74	9.10±1.53	0.238
Mean change	−0.99±1.40	−0.83±1.65	0.798

BMI=Body mass index; FBS=Fasting blood sugar; HOMA-IR=Homeostasis model assessment of insulin resistance; CTx=C-terminal telopeptide of type 1 collagen; HbA1C=Haemoglobin A1C

and experienced bone loss were compared with non-TZD users.[30] Japanese studies which focused on subjects with T2D treated with troglitazone, showed significant reductions in markers of both bone formation and resorption.[31,32] In another study, diabetic subjects taking TZDs (pioglitazone, troglitazone, and rosiglitazone) showed bone loss in over 4 years.[33]

Contrary to data from previous studies that suggest TZDs may promote bone loss, we did not reach such a conclusion in patients with T2D after treatment with pioglitazone for 12 weeks.[34,35] It should be emphasized that the bone loss effect of these drugs was observed after long term treatment and low dose pioglitazone did not show any apparent detrimental effects on bone formation and resorption markers. This variation in results may be attributable to these differences when we compared results of our study to another studies.

It is known that pro12Ala polymorphism of PPAR-γ reduces the transcriptional activity of PPAR and is involved in the pathogenesis of insulin resistance, decreased risk of T2D, atherosclerosis, adipocyte differentiation, lipid metabolism, inflammation, and osteoporesis.[36] According to our study, there was a significant difference in the allele and genotype frequency of Pro12Ala between patients with T2D and healthy control participants. We found that the Ala phenotype frequency was lower in patients with T2D.

We also found no evidence of an association between bone turnover markers, CTx, and osteocalcin and the genetic variants in a sample of Iranian population. Currently, there is few data available on the bone skeletal actions of Pro12Ala in humans. Rhee *et al*.,[23] have reported that Pro12Ala polymorphism is significantly associated with lower serum OPG level as a key inhibitor of osteoclastogenesis.[23] In line of our study Yue *et al*.,[33] have shown that the Pro12Ala polymorphism is not associated with BMD at the lumbar spine and femoral neck in Chinese women.

Recent observations suggest that circulating osteocalcin is a regulator of glucose metabolism and elevated levels of osteocalcin were associated with improved glucose tolerance in humans.[37-39] Pittas *et al*.,[37] found that serum osteocalcin concentration was inversely associated with FPG, insulin, and BMI.[37]

In various models of obesity (diet-induced or hyperphagia), osteocalcin was protective against obesity and T2D.[40]

Mbalaviele *et al*.,[35,36] have showed that activation of PPAR-γ pathway by an endogenous PPAR-γ ligand inhibited the formation and activation of osteoclasts in human mesenchymal stem cells.

In animal model, a lack of one allele of the insulin receptor in osteoblasts resulted to increase glucose resistance and decrease in osteocalcin bioactivity.[27] TZDs are insulin sensitizer agents. It is possible that these commonly used drugs can break the insulin resistance. Therefore, reduced insulin resistance in peripheral tissues and improved glucose tolerance, might be the reason that CTx values decreased and osteocalcin increased significantly after 12 weeks of treatment in our study.

These results were in line of other studies that showed insulin signals in osteoblasts led to increase in bone resorption, and the resorptive process which in turn, increases osteocalcin biologic activity, which favors insulin secretion and sensitivity.[27]

Potential weaknesses in our study are that the other bone turnover markers; hydroxyproline, hydroxylysine, type I collagen crosslinks (pyridinoline and deoxypyridinoline); were not measured in this study. These markers

are dynamic variables that are affected by several uncontrollable and controllable factors which include preanalytical, analytical, and postanalytical phases.[41] Factors such as age, sex, time of day, food intake, physical activity, recent fracture, smoking or alcohol consumption, climate, and BMI are the main variables. Appropriate reference intervals must be used for the optimum interpretation of results, which is affected with type of assay.[41]

Therefore in this study sampling was done in early morning in fasting state in diabetic patients who has no physical exercise for last 24 h. To preserve stability of markers, temperature was controlled during sample processing, storage and transport with avoiding repeated freezing and thawing cycles.

The major finding of our study is the improved glucose tolerance after short term treatment with pioglitazone and possible improvement of bone metabolism by decrease in insulin resistance.

Limitations of the present study are small sample size with short duration of drug therapy; it may be after long term use of pioglitazone (more than 3 months) that we could reach the effects of bone complications.

Therefore, we suggest that this could be tested in larger samples with considering all bone turnover markers in patients using TZD for long term.

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