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ORIGINAL ARTICLE

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-γ Pro12Ala POLYMORPHISM AND RISK OF OSTEOPENIA IN β-THALASSEMIA MAJOR PATIENTS

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□ *Genetic factors have an important role in the incidence of osteopenia in thalassemia patients. The purpose of this study was to investigate the effect of the Pro12Ala polymorphism of the* peroxisome proliferator-activated receptor-γ (PPARγ) *gene on bone mineral density (BMD) and subsequently, the rate of osteopenia in β-thalassemia major (β-TM) patients. Blood samples were obtained from 156 β-TM patients referred to the Tehran and Qazvin Thalassemia Clinics. Samples were analyzed for polymorphisms of the* PPARγ *gene using polymerase chain reaction-restriction fragment length polymorphism (RFLP)-based methods. Multivariate analysis was used to investigate the relationship between the risk of osteopenia and the* PPARγ *gene polymorphism. Correlation analysis showed that there was a significant association between homozygous wild-type genotypes with susceptibility to osteopenia in β-TM patients* ($p = 0.024$). *Logistic regression analysis showed that the risk of osteopenia was significantly* ($p < 0.05$) *higher in the homozygous wild-type genotype than carriers of the rare alleles. Furthermore, the associations were strengthened in men with a homozygous wild-type genotype after adjustment for age and body mass index (BMI)* ($p < 0.05$). *This study suggests that the Pro12Ala polymorphism of the* PPARγ *gene might be an independent factor in BMD level and osteopenia in thalassemia patients.*

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INTRODUCTION

β-Thalassemia (β-thal) disease is a type of hemoglobinopathy that is caused by a disorder in partial or complete synthesis of the β chains of hemoglobin (Hb) (1). Most patients require regular blood transfusions and chronic transfusions lead to pathologic iron accumulation. Iron overload is usually the main cause of death, heart failure, skeletal complications such as osteoporosis and osteopenia (2,3). Thalassemic patients are more prone to bone mass loss, because of both the underlying disease and complications of treatment. Anemia, overactive bone marrow, excess iron deposited in the bones, deferoxamine (DFO) therapy and endocrine related problems are the major mechanism of bone loss in β-thal major (β-TM) patients (4). Previous studies suggested that osteopenia is one of the important factors of mortality in young adults with β-thal (1). Osteopenia is characterized by reduced bone mineral density (BMD) (2,5). The best way to measure BMD is using dual energy X-ray absorptiometry (DXA), because of its high precision and accuracy (5,6). Measurements are performed on the femur neck and lumbar spine (6). In previous years, studies have proposed that genetic factors play an important role in BMD regulation (2,5).

It has been found that genetic factors influence the BMD level in patients with β-thal (6,7). Peroxisome proliferator-activated receptor-γ (PPARγ) is a subtype of the PPAR family that regulate lipid and glucose metabolism (8). Peroxisome proligerator-activated receptor-γ plays an important role in the adipocytes differentiation, insulin sensitivity and also blood glucose control in patient with type 2 diabetes (8). On the other hand, the *PPARγ* gene is located on 3p25 in humans and is composed of nine exons (9,10). Several single nucleotide polymorphisms (SNPs) have been detected in the *PPARγ* gene in humans. One of these polymorphisms is Pro12Ala (rs1801282), which has the substitution of proline to alanine at codon 12 in exon B (10). Recent studies have suggested that PPARγ plays an important role in osteogenesis (11,12). Based on this hypothesis that osteoblasts and adipocytes have a common mesenchymal precursor, recent studies have shown that the activation of PPARγ promoted adipocyte differentiation and simultaneously suppressed their activity to differentiate into osteoblasts or stimulated their apoptosis (13,14). The PPARγ activity appears to be important in regulating bone metabolism through induction of osteoblast differentiation into osteoclasts (11,15). Furthermore, studies have found that rs1801282 was significantly associated with BMD of the lumbar spine in post-menopausal women (16). In another study, in healthy Korean women, a significant association between

this polymorphism and serum osteoprotegerin (OPG) level, a key inhibitor of osteoclastogenesis, has been reported (17). However, no study has been performed on the relationship between the Pro12Ala polymorphism and BMD in β-TM patients. To test this hypothesis, we investigated the influence of the Pro12Ala polymorphism *PPARγ* gene on variation of BMD in β-TM patients.

MATERIALS AND METHODS

Subjects

In this cross-sectional study, the participants were 156 β-TM patients with an age range of 20-45 years who were referred to the blood centers in Tehran and Qazvin, between April 2009 and May 2011. The study was approved by the ethics committee of Qazvin University of Medical Sciences, Qazvin, Iran. The age of onset of anemia and features of hemolysis on a peripheral smear with increased fetal Hb was determined by a high performance liquid chromatography (HPLC) technique, used for the diagnosis of β-thal. All the patients were on oral calcium-vitamin D supplementation; patients without any history of fracture were selected for this study.

Exclusion criteria included diabetes, endocrine disorders (thyroid, parathyroid and pituitary diseases) and retardation. Patients seropositive for Hbs Ag, hepatitis C virus and human immunodeficiency virus were also excluded from the study. Subjects who were receiving Biophosphonate at the time of DXA were excluded. Serum ferritin estimation was done by an an enzyme-linked immunosorbent assay (ELISA) kit (Alpco Diagnostics, Salem, NH, USA).

Bone Mineral Density Measurements

BMD was measured in the femur neck and lumbar spine by DXA QDR 2000 (Hologic, Bedford, MA, USA).The coefficient of variability values of DXA measurements was 1.0% for lumbar spine vertebra, and 1.2% for femoral neck (5). Z-scores for the lumbar spine and femoral neck were calculated on the basis of the standardized records of β-TM patients.

Genotyping

Genetic analyses were performed on genomic DNA that was isolated from peripheral blood leukocytes by standard methods. A 295 bp sequence of the *PPARγ* gene was amplified by polymerase chain reaction (PCR) in a DNA thermal cycle (ABI PRISM™; Applied Biosystems, Foster City, CA, USA) using oligonucleotide primers F: 5'-CTG ATG TCT TGA CTC ATG GG-3' and R: 5'-GGA AGA CAA ACT ACA AGA GC-3'. The PCR condition were as follows: 15

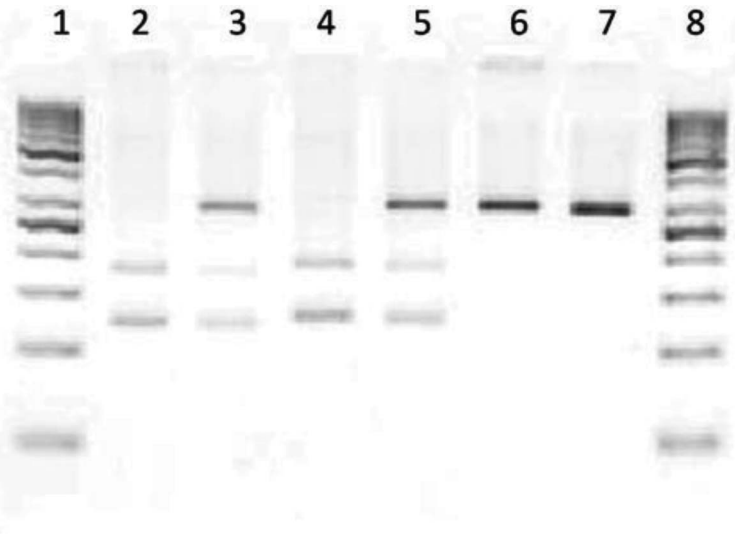

FIGURE 1 Gel picture analysis with the restriction enzyme *Hga*I of the PPARγ Pro12Ala genotypes in genomic DNAs of the study subjects. Lanes 1 and 8: molecular weight marker 50 bp; lanes 2 and 4: homozygous GG; lanes 3 and 5: heterozygous CG: lanes 6 and 7: homozygous wild-type GG.

min. initial denaturation at 95˚C, and each PCR was subjected to 35 cycles at 94˚C for 30 seconds, 53˚C for 30 seconds, 72˚C for 30 seconds, followed by 7 min. at 72˚C (18). Restriction of the PCR product with the *Hga*I enzyme generates fragments of 178/117 bp in rare homozygotes, 295/178/117 bp in heterozygotes and 295 bp in common homozygotes (Figure 1). Samples were electrophoresed on 3.0% agarose gel, and stained with ethidium bromide.

Statistical Analyses

Values were presented as mean ± SD (standard deviation), and statistical significance was defined as p values less than 0.05. Statistically significant differences in mean measurements between different PPARγ genotypes were assessed by the t-test. Logistic regression analysis was performed to evaluate associations between genotypes and osteopenia as a dependent variable. All analyses were carried out using the Statistical Package for Social Science (SPSS) for windows version 11.0 (Chicago, IL, USA).

RESULTS

The general characteristics of the study subjects, pre transfusion Hb, serum ferritin, BMD Z-score and genotypes are presented in Table 1. The mean age of the study population was 23.3 ± 8.2 years, and the mean

TABLE 1 Clinical and Biochemical Characteristics of Patients With β-Thalassemia Major

Age (years)	23.3 ± 8.2
Women (%)	51.2
BMI (kg/m^2)	20.4 ± 3.3
Pro12Ala genotypes, *n*	
•CC	121
•CG	35
Pre transfusion Hb (g/dL)	9.41 ± 1.32
Serum ferritin (μg/L) (range 55.0–8900.0)	1406.0
BMD Z-score	−2.11 ± 1.07

Values are given as mean ± SD, number or percentage with condition.

body mass index (BMI) was 20.4 ± 3.3 kg/m^2. The average Z-score of BMD was –2.11 ± 1.07. The mean serum ferritin concentration was 1406 μg/L. All patients were receiving chelation therapy with oral deferasirox (DFRA) (26.4%) or subcutaneous DFO (73.6%). All patients were classified as having normal BMD with Z-score >-1 and as osteopenic with Z-score <-1 (Table 2). Of the 156 subjects, 33 were normal and 123 had osteopenia. The frequency of the PPARγ Pro12Ala CC genotype in normal subjects was significantly higher ($p = 0.024$) than in patients with osteopenia (Table 3). Clinical and biochemical characteristics of patients according to the Pro12Ala genotype are presented in Table 4. There were no significant differences in clinical and biochemical characteristics between the two genotype groups. Furthermore, a separate analysis of women and men also showed no significant differences between clinical and biochemical characteristics (Table 5). The risk of

TABLE 2 Clinical and Biochemical Characteristics of Normal Patients *Versus* Osteopenia Patients

	Normal ($n = 33$)	Osteopenic ($n = 123$)	*p* Value
Age (years)	33.90 ± 8.2	31.80 ± 8.1	0.19
Women (%)	47.00	52.00	0.64
BMI (kg/m^2)	21.00 ± 2.9	20.20 ± 3.4	0.21
Pre transfusion Hb (g/dL)	9.53 ± 1.38	9.38 ± 1.32	0.56
Natural logarithm serum ferritin (mg/L)	6.62 ± 1.08	6.80 ± 1.01	0.39

Values are given as mean ± SD. The patients were classified as having normal BMD if the Z-score was -1 and as osteopenic if the Z-score was <-1.

TABLE 3 Genotype Distributions in Normal Patients *Versus* Osteopenic Patients

Gene	Normal ($n = 33$)	Osteopenic ($n = 123$)	*p* Value
Pro12Ala genotypes, *n*			
•CC	21	100	0.024
•CG	12	23	

TABLE 4 Clinical and Biochemical Characteristics According to Pro12Ala Genotypes

	CC	CG	*p* Value
n	121	35	
Age (years)	32.20 ± 7.9	32.70 ± 9.9	0.79
BMI (kg/m^2)	20.50 ± 3.5	20.20 ± 3.0	0.69
Pre transfusion Hb (g/dL)	9.38 ± 1.38	9.50 ± 1.14	0.66
Natural logarithm serum ferritin (mg/L)	6.81 ± 0.98	6.63 ± 1.19	0.37

Values are given as mean ± SD.

TABLE 5 Clinical and Biochemical Characteristics in Men and Women According to the Pro12Ala Genotypes

Men	CC	CG	*p* Value
n	56	21	
Age (years)	32.60 ± 8.3	32.30 ± 10.4	0.89
BMI (kg/m^2)	19.50 ± 3.1	20.00 ± 2.1	0.51
Pre transfusion Hb (g/dL)	9.57 ± 1.46	9.60 ± 1.27	0.93
Natural logarithm serum ferritin (mg/L)	6.84 ± 0.92	6.76 ± 1.16	0.76
Women	CC	CG	*p* Value
n	65	14	
Age (years)	31.90 ± 7.7	33.10 ± 9.4	0.59
BMI (kg/m^2)	21.30 ± 3.5	20.50 ± 4.1	0.47
Pre transfusion Hb (g/dL)	9.22 ± 1.30	9.34 ± 0.94	0.75
Natural logarithm serum ferritin (mg/L)	6.78 ± 1.04	6.43 ± 1.23	0.28

Values are given as mean ± SD.

osteopenia in different genetic groups was estimated by logistic regression analysis (Table 6). This analysis showed that the risk of osteopenia in subjects with a homozygous CC genotype was significantly higher [odds ratio (OR) = 2.59; $p = 0.03$]. When the men and women were analyzed separately, the association between the PPARγ genotype and osteopenia were no longer

TABLE 6 Logistic Regression Analyses of the Pro12Ala Genotypes With Respect to the Presence of Osteopenia as a Dependent Variable[a]

	95% CI	OR	*p* Value
All patients	1.11–6.05	2.59	0.03
Men	1.03–9.98	3.21	0.04
Women	0.52–7.45	1.96	0.32

Values are given as 95% confidence interval (95% CI), the odds ratio (OR) and *p* value level of significance, which shows the association between genotypes and low BMD Z-scores. The patients were classified as having normal BMD if the Z-score was -1 and as osteopenic if the Z-score was <-1.

[a]Adjusted for age and BMI.

significant in women (OR = 1.96; $p = 0.32$), while the association in men (OR = 3.21; $p = 0.04$) remained significant.

DISCUSSION

β-Thalassemia is a disease resulting from genetic defects caused by impaired synthesis of Hb β chains (1). Bone loss is one of the major complications of this disease. Various factors such as genetic, environmental, nutritional and hormonal factors play roles in the incidence of osteopenia in β-TM patients (19). Consistent with previous studies (6), our study showed a high prevalence, about 80.0%, of osteopenia in β-TM patients. Study of genetic polymorphisms associated with susceptibility to osteopenia in thalassemia patients can give us better insight into the role of genetic factors in this disease. In this study, we showed that the Pro12Ala polymorphism of the *PPARγ* gene may play an important role in the pathogenesis of thalassemic osteopenia. Our finding also showed that compared with the common homozygous genotype (CC), the risk of osteopenia is lower in the heterozygous genotype (CG). A previous study suggested that there is a significant negative correlation between age and BMD in patients with thalassemia (20). However, such a relationship was not observed in our study. Some reports showed that there were no significant differences in Z-score level between men and women (21), while other studies have shown that the Z-scores are higher in men than in women (22). Our data showed no statistically significant independent association between the PPARγ Pro12Ala genotype and BMD levels in women, whereas in men the risk of osteopenia was lower for those with the Pro12Ala polymorphism. Since women in this study were not menopausal, it was suggested that (probably) female sex hormones, especially estrogens, can be an important factor in preventing bone loss, and consequently reduce osteopenia in women.

The *PPARγ* gene was first identified in white adipose tissue. Essentially, it has an important role in the final stages of differentiation of pre adipocyte into mature adipocytes (5,23). The hypothesis that PPARγ may also be involved in bone metabolism has arisen from the fact that osteoblasts and adipocytes are grown from common mesenchymal precursor cells (23), although this conclusion still needs further research before it is accepted (24). There are several studies that have shown the role of PPARγ in differentiation and activation osteoblasts (12,13). Using heterozygous mice that had disrupted the *PPARγ* gene (PPAR+/−) and a 50.0% reduction in *PPARγ* gene expression, it has been shown that insufficiency in PPARγ activity increases bone mass *via* stimulation of osteoblastogenesis from bone marrow precursors (25,26). These studies have shown the negative role of *PPARγ* gene activation on bone formation (25). Accordingly, Mbalaviele *et al.* (15) suggested that the

activity of the PPARγ pathway by an endogenic ligand, would prevent the effects of OPG ligand, a key inhibitor of osteoclastogenesis. It was probably due to inhibition of receptor-activated nuclear factor-kappa B (NF-κB) and receptor activator of nuclear factor-kappa B ligand (RANKL), blocking the NF-κB pathway, which is essential for osteoclastic precursors (27). The Chan *et al.* (11) study showed that the addition of a PPARγ agonist in cultured human peripheral blood mononuclear cells prevents formation of multinucleated osteoclasts. Okazaki *et al.* (28) had also suggested that the reduction of the number of osteoclast cells following treatment with thiazolidinedione (PPARγ agonists) are enhanced by parathyroid hormone (PTH) and 25-hydroxy vitamin D (25-OHD), suggesting the inhibitory effect of *PPARγ* gene activation on bone resorption. In our study, patients with the Pro2Ala CG genotype had higher Z-score values. Conversely, it has been shown that the BMD values were not significantly different between the genotype groups (17). Furthermore, Masud and Ye (29) showed that subjects with the Pro12Ala genotype had significantly higher BMI or obesity than those without the alanine allele (29). In our study, this relationship was not significant. On the other hand, our findings showed that the effect of the Pro12Ala polymorphism on BMD in β-thal patients is independent of BMI. These contradictions seem to be due to racial differences in the alanine allele frequency and distribution of BMI. Our study limitations include unmeasured confounding factors such as dietary fat, 25-OHD and OPG (3).

In conclusion, the present study suggested novel evidence of significant beneficial effects of the PPARγ Pro12Ala polymorphism on BMD level, and consequently, osteopenia in β-thal patients. This relationship showed that study of potential genetic effects can offer important insight to improve the management strategy of β-TM patients at high risk for osteopenia.

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