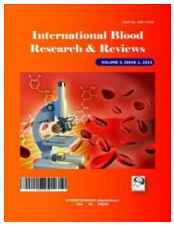

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Preliminary Report on the Frequency of Pro12Ala Polymorphism of the Peroxisome Proliferator-Activated Receptor-gamma Gene in Egyptian β-Thalassemia Major Patients

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Authors' contributions

This work was carried out in collaboration between all authors. Author NAMH designed the study and contributed in writing protocol and manuscript. Authors MED and OG contributed in writing manuscript. Author DE did the laboratory work and contributed in writing manuscript. Author MK collected samples and data and contributed in writing manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Osteoporosis represents an important cause of morbidity in adult thalassemic patients. Peroxisome proliferator-activated receptor-γ (PPAR γ) is a master transcriptional regulator involved in expression of probably hundreds of genes. Recent studies have suggested that PPAR-γ plays an important role in osteogenesis. Furthermore, PPARγ inhibition in mice, increased bone formation with no effect on bone resorption. Our aim was to investigate the frequency of Pro12Ala polymorphism (substitution of proline to alanine at codon 12 in exon B) of PPARγ gene in Egyptian β-thalassemia major (β-TM) patients and its influence on their bone mineral density (BMD). Blood samples from 30 β-TM

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patients and 10 healthy controls matched for age, sex and body weight were analyzed for PPARγ gene polymorphism using polymerase chain reaction-restriction fragment length polymorphism. BMD were measured in all patients and controls by a dual energy X-ray absorptiometry at the lumbar spine. Low BMD (Z score is -1 or lower) was present in all thalassemic cases. There was no statistically significant difference between BMD in thalassemic males (-3.43±-1.08) and females (-2.78±-0.81) (p=0.265). Pro12Ala polymorphism was present in 2 out of 30 (6.67%) β-TM patients with osteoporosis. One patient had heterozygous 12Ala polymorphism and the other had homozygous 12Ala polymorphism. Both had normal body mass index, lipid profile, ejection fraction and elevated serum ferritin. Only one male control (10%) has homozygous 12Ala polymorphism. This study suggests that Pro12Ala polymorphism is unrelated to BMD level in Egyptian thalassemic patients. Further studies on a larger population of patients are still needed to confirm this finding.

Keywords: Thalassemia major; osteoporosis; PPAR gamma; Pro12Ala polymorphism.

1. INTRODUCTION

β-Thalassemia (β-TM) is a hemoglobinopathy resulting from defective β chains of hemoglobin synthesis. Most patients require regular blood transfusions leading to pathologic iron overload [1]. Osteoporosis is found in approximately 40–50% of β-TM patients. It is an important cause of morbidity in adult thalassemic patients [2]. Patients commonly present with bone deformities, scoliosis, chronic bone pain, osteoporosis, fractures especially of the lumbar spine, growth failure or nerve compression [3]. Unbalanced bone turnover and seriously diminished bone mineral density (BMD) continues despite hemoglobin levels normalization, adequate hormone replacement and effective iron chelation [1].

Several genetic and acquired factors are implicated in bone destruction [1,4]. Elevated osteoclast activity and deregulated osteoblasts have been detected in thalassemia osteoporosis [2]. Bone marrow expansion, endocrine dysfunction (mainly hypogonadism), iron overload, over-chelation and under-chelation, vitamin C/ Vitamin D deficiency, inappropriately high doses of desferrioxamine and reduced physical activity all adversely affect bone density [1].

Peroxisome proliferator–activated receptor (PPAR)- γ is a transcription factor belonging to the same family of nuclear receptors as steroid and thyroid hormone receptors. PPAR-γ is a master transcriptional regulator involved in the expression of probably hundreds of genes [5]. The PPAR-γ gene is located on chromosome 3 p25 in humans [6]. There are two distinct isoforms, PPARγ1 and PPARγ2. PPAR-γ1 is expressed in most tissues and PPAR-γ2 is specific for adipose tissue, where it plays a key role in regulating adipogenic differentiation [7]. One of PPARγ gene polymorphisms is Pro12Ala (rs1801282), in which there is substitution of proline to alanine at codon 12 in exon B as a result of a cytosine to guanine substitution [6]. Pro12 allele is present in at least 80% of humans [8]. A significant association between PPAR-γ polymorphism and serum osteoprotegerin (OPG) level, a key inhibitor of osteoclastogenesis, has been reported in healthy Korean women [9].

Sahmani et al. [10] suggested significant beneficial effects of Pro12Ala polymorphism on BMD level, and osteopenia in β-thalassemia patients independent of BMI. It was hypothesized that osteoblasts and adipocytes have a common mesenchymal precursor [11]. Activation of PPARγ promoted adipocyte differentiation and simultaneously suppressed their activity to differentiate into osteoblasts or stimulated their apoptosis [12].

Identification of new markers in the serum of patients with bone disease has greatly contributed to a better understanding of its pathophysiology. So, we aimed at determining the frequency of Pro12Ala polymorphism of PPARγ gene in Egyptian adult β-thalassemic patients and its influence on their BMD if present.

2. METHODOLOGY

Study population: 30 β-TM patients were the subject of the study (group 1). They were followed up in the "outpatient clinic of Hematology unit, at Alexandria main university hospital" between December 2014 and August 2015. Seventeen were males and thirteen were females. Their ages range was 16 – 39 years (21.53±5.44). In addition, 10 healthy volunteer

subjects matched for age, sex and body weight to the patients were considered group 2. Five were males and five were females. Their mean age was 23.5±5.19 years.

All patients were diagnosed as β-TM since early age based on cellulose acetate Hemoglobin electrophoresis. Thorough history taking and clinical examination was done in all cases. Seventeen (56.67%) patients had hepatomegaly and 20 patients were splenectomized (66.67%). All patients received blood transfusion and desferrioxamine since early childhood. Patients with endocrine disorders (thyroid disease, other), malabsorption, steroid and anticonvulsant use, spinal radiological abnormalities (scoliosis, and others), smokers and HIV infected subjects were excluded from the study.

The study was approved by local research ethics committee of Alexandria Faculty of Medicine according to "Declaration of Helsinki". Written informed consent was obtained from every case.

2.1 Laboratory Investigations

A detailed medical review of the patients was performed to ascertain the presence of bone pain and previous fractures if any. BMD was measured in all patients and controls by dual energy X-ray absorptiometry (DEXA lunar DPX), at the lumbar spine (L2–L4) in A-P projection. Subjects on biphosphonate at the time of DXA were also excluded. BMD data were expressed as grams per centimeter squared and compared with BMD values of normal subjects of the same age and sex. BMD is considered normal if Z score is above -1 and low if Z score is -1 or lower.

Fasting whole blood on EDTA samples were taken for measurement of Hb while serum samples were used for measuring ferritin, serum alanine aminotransferase (ALT), AST, albumin, calcium, urea, creatinine and lipid profile. Samples were taken before giving patients their regular blood transfusion. All were determined by automated routine procedures (CBC by Sysmex KX-21N, lipid profile by Cobas c 501, serum ferritin by Advia Centeur and by dimension RXL for other parameters). Fasting blood samples of patients and controls were also taken for measurement of Pro12Ala polymorphisms by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) [13].

2.2 Assay of PPAR-γ Pro12Ala Polymorphisms

Genetic analyses were performed on genomic DNA isolated from peripheral blood leukocytes using thermoscientific extraction kit (made in EU Lithuania) by Biometra. A 295 base pair (bp) sequence of the PPARγ gene was amplified by PCR in a DNA thermal cycler using oligonucleotide primers F: 5'-CTG ATG TCT TGA CTC ATG GG-3' and R: 5'- GGA AGA CAA ACT ACA AGA GC-3'[10]. PCR condition was as follows: initial denaturation at 95℃ for 15 minutes, followed by 35 cycles at 94℃ for 30 seconds, 53℃ for 30 seconds, 72℃ for 30 seconds, followed by 7 minutes at 72℃. Restriction of the PCR product was detected after digestion with the HgaI enzyme (thermoscientific, EU Lithuania). Samples were electrophoresed on 3.0% agarose gel, and stained with ethidium bromide. The PCR product size is 178/117 bp in rare homozygotes, 295/178/117 bp in heterozygotes and 295 bp in common homozygotes [14].

2.3 Statistical Analysis

Data were analyzed using IBM SPSS software package version 20.0. (SPSS 20.0 Inc. Chicago, IL, USA). Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Qualitative data were compared using chi square test and Fisher's Exact test or Monte Carlo correction (when more than 20% of the cells have expected count less than 5). Comparisons between the cases and controls were performed by Student's *t*-test for normally distributed variables and Mann Whitney test for abnormally distributed variables. *p* value of ≤0.05 was considered significant.

3. RESULTS

Table 1 shows the clinical and laboratory data of the studied 30 β TM cases and their matched controls. Table 2 shows echocardiographic and Z score findings in both groups. Normal BMD (Z score above -1) was present in controls, low BMD (Z score -1 or lower) was present in all thalassemic cases (100%) cases. There was no statistically significant difference between BMD in males (-3.43 ±-1.08) and females (-2.78±-0.81) (t= 1.295, p= 0.265).

Table 1. Clinical and laboratory data of the studied cases and controls

Parameter	Cases (n=30)	Controls (n=10)	p value
Age (yrs)	21.53±5.44	23.50 ±5.19	0.323
Sex			0.731
Males	17 (56.70%)	5 (50%)	
Females	13 (43.30%)	5 (50%)	
Hb (g/dL) **	6.85(6.77 ± 0.85)	13.50 (13.40 ±1.20)	<0.001
Ferritin (ng/L)**	4855 (4976.30 ± 2216.41)	103.50 (102.60 ± 12.69)	<0.001
Fasting blood glucose (mg/dL)**	92.50 (100.03 ± 37.36)	89.50 (88.40 ± 9.16)	0.444
ALT (U/L)**	79.50 (88.03 ± 48.96)	27 (27.80 ± 7.11)	<0.001
AST (U/L)**	92.50 (91.60 ± 48.36)	28 (29.40 ± 4.84)	<0.001
Blood urea (mg/dL) **	27(27.10 ± 5.32)	29.50(28.70 ±5.01)	0.409
Serum creatinine (mg/dL)**	0.40 (0.44 ± 0.19)	0.75 (0.72 ± 0.21)	0.001
PPARγ			0.585
Absent	28 (93.30%)		
Homozygous	1(3.33%)	1 (10%)	
Heterozygous	1(3.33%)		

** *values are expressed as median (mean±SD), p is considered significant if <0.05*

Table 2. Radiological findings of the studied cases and controls

Parameter	Cases (n=30)	Controls (n=10)	p value
Ejection fraction (%)	62.23 ± 3.46	63.80 ± 4.34	t=1.163 p=0.252
Z score	-3.33 ± - 1.20	0.64 ± 0.87	t= 4.687* p<0.001
Above -1	-	10 (100%)	X^2=40*
-1 or lower	30 (100%)		p=0.000

Z score above -1: normal BMD, Z score -1 or lower: low BMD, p is considered significant if <0.05

Pro12Ala polymorphism was present in 2 out of 30 (6.67%) β-TM patients with low BMD. Heterozygous 12Ala polymorphism was present in an 18 year old female patient and homozygous 12Ala polymorphism was present in 20 year old male case. The former patient had BMI: 20.2 kg/m^2, BMD:-2.4, ferritin: 4923 ng/l, fasting blood sugar: 87 mg/dl, ejection fraction: 70% and the latter case had BMI: 19.8 kg/m^2, BMD:-6, ferritin: 4886 ng/l, fasting blood sugar: 79 mg/dl, ejection fraction: 68%. Only one male among controls (10%) had homozygous 12Ala polymorphism. No statistically significant difference was detected on comparing the frequency of 12Ala polymorphism between cases and controls (x^2 = 1.509, ^{MC}**p** =0.585).

4. DISCUSSION

Approximately 75% of individual variance in BMD is genetically determined [15]. PPARγ has been identified as the pivotal transcription factor involved in the differentiation of bone marrow mesenchymal cells into adipocytes [16].

Homozygous 12Ala polymorphism was present in one male among our controls. The frequency of 12Ala in our healthy population was 10% (1/10). Across all studies, the frequency of 12Ala allele in control groups ranged from 1.7% to 21.6% (median, 9.5%). 12Ala allele frequency ranged from 5.9% to 21.6% (median, 12.7%) in Caucasian controls and ranged from 1.7% to 9.3% (median, 4.5%) in East Asian controls [8].

A 12Ala allele frequency in our thalassemic patients was 6.67% (2 out of 30) while it was (~10 and 11%) in South Asians and in Caucasians respectively [17]. A similar 12Ala allele frequency of~11% was also reported in South Asians who migrated to Singapore [18]. Caucasian group living in Dallas have an allele frequency of ~10% which is in concordance with the published literature [19,20]. No difference between Asians and Caucasian was suggested by another study [17].

Pro12Ala polymorphism was present in 2 β-TM patients. Heterozygous Pro12Ala polymorphism

was present in one female patient and homozygous 12Ala polymorphism was present in one male case. Thalassemia men are more commonly and more severely affected with low bone mass than females. The reverse of what happen in the general population [21]. A relation was observed between the polymorphism and higher BMI in men and not in women [22,23].

The mean age of our thalassemic patients was 21.53±5.44. About 1% of bone is lost yearly in both sexes from the age of 30 years in optimally treated thalassemia patients [24]. Low BMD was found in children as early as 12 years of age, suggesting reduced reserve for use in adulthood [21]. Both our cases are of normal BMI.

The alanine isoform leads to less efficient stimulation of PPARγ target genes and predisposes people to lower levels of adipose tissue mass accumulation [8]. Insufficient PPARγ activity increases bone mass via stimulation of osteoblastogenesis from bone marrow precursors [25]. In contrast, PPARγ promotes osteoblast activity in other studies [26,27].

In β-TM, PPAR-γ polymorphism and PPARγ pathway activity may prevent the effects of OPG ligand [26]. PPARγ can antagonize the transcription factor nuclear factor-kappa B, which is a fundamental pathway for RANKL (Receptor–activator of nuclear factor-kappa B ligand) signaling and osteoclast development, survival and function [28]. PPARγ activation also inhibited tumor necrosis factor-α induced osteoclastogenesis independent of RANKL [27].

The lower frequency of 12Ala in our thalassemic patients (6.67%) compared to other population can be explained by gene-gene interaction and environment-gene interaction, including diet and exercise. Lifestyle, drugs, and dietary modifications, including consumption of foods can activate PPAR-α, and affect its metabolic responses [29]. The “protective” role of allele 12Ala for diabetes risk may have both genetic and environmental origins [17].

Low BMD (Z score –1 or lower) was present in all thalassemic cases. Osteopenia-osteoporosis syndrome is seen in 50–80% of adult β-thalassemia patients worldwide and is considered a major cause of bone pain and fragility fractures especially of the lumbar spine [30]. Osteoporosis was observed only at the lumbar level in thalassemic patients without evidence of hypogonadism. In hypogonadic patients, osteoporosis was more severe and also affected the femoral neck, [31].

No statistically significant difference was detected in our study between 12Ala polymorphism frequency between cases and controls [32]. PPARγ Inhibition in mice caused increase bone formation with no effect on bone resorption. This was partly related to enhanced differentiation of bone marrow precursor cells into mature osteoblasts. There is significant reduction in marrow fat infiltration and decreased transcription of adipogenic and increased transcription of osteogenic genes [32].

5. CONCLUSION

This study suggests that the Pro12Ala polymorphism of the PPARγ gene might be unrelated to BMD level and osteopenia in Egyptian β-TM patients. Still further studies on a larger population of patients are needed to confirm this finding.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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