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Association of estrogen receptor β and estrogen-related receptor α gene polymorphisms with bone mineral density in postmenopausal women

Amira Shoukry[1] · Sally M. Shalaby[2] · Rasha L. Etewa[2] · Hanan S. Ahmed[3] ·
Hossam M. Abdelrahman[4]

Abstract The aim of the study was to investigate the possible association of *Alu*I and *Rsa*I polymorphisms of estrogen receptor β (ER-β) gene and 23-bp nucleotide repeat polymorphism of estrogen-related receptor α (ERRα) gene with bone mineral density (BMD) in postmenopausal Egyptian women. Two-hundred postmenopausal osteoporotic women as cases and 180 healthy age-matched postmenopausal women as controls were genotyped by PCR fragment length polymorphism for *Alu*I, allele-specific PCR for *Rsa*I, and by sizing of PCR products on agarose gels for ERRα repeats. sRANKL levels were estimated by ELISA. BMD measurements for spine and femoral neck were performed by dual energy X-ray absorptiometry. A significant difference between women with osteoporosis and controls regarding allele and genotype distributions of *Alu*I G/A (OR 2.37, 95 % CI 1.77–3.18 and $p < 0.001$ for A allele) and ERRα polymorphisms (for the two repeats allele OR 2.08, 95 % CI 1.09–4.00, and $p = 0.02$). Osteoporotic women with the *Alu*I AA + GA genotype or with the EERα 2,2 genotype had significantly lower BMD than did women with the other genotypes. Moreover, there was a significant increase of the mean values of sRANKL in carriers of *Alu*I A, *Rsa*I A alleles and in patients having 2,2 genotypes of ERRα ($p < 0.001$, $p < 0.001$, $p = 0.02$, respectively). We demonstrated an association of ER-β *Alu*I G/A and ERRα 23-repeats polymorphisms with BMD in postmenopausal Egyptian women. A possible effect of ER-β and ERRα polymorphisms on the levels of sRANKL was estimated.

Keywords Osteoporosis · Estrogen receptor β · Estrogen-related receptor α polymorphisms · RANKL

Introduction

Osteoporosis is a common skeletal disorder characterized by low bone mass, low bone mineral density (BMD), and deteriorated skeletal microarchitecture, with consequent increase in bone fragility and fracture risk [1]. Osteoporosis is a multifactorial disease with a strong genetic component. Although a number of factors including hormonal, environmental and nutritional have been shown to affect BMD; twin and family studies have revealed that at least 40–70 % of the variation of BMD is accounted for by genetic factors [2]. Among many candidate genes, estrogen receptor (ER) genes have been proven to regulate bone mass [3].

The concept that estrogen deficiency is critical to the pathogenesis of osteoporosis was based initially on the fact that postmenopausal women, whose estrogen levels naturally decline, are at the highest risk for developing the disease. However, the heterogeneity of osteoporosis may be due not only to differences in the production of estrogen, but also to changes in receptors, signal transduction mechanisms, nuclear transcription factors, and enzymes that produce or inactivate local regulators [3]. Estrogen acts through binding to ER-α and ER-β; both receptors are highly expressed in bone [4]. The human ER-α and ER-β

✉ Sally M. Shalaby
sallyshalaby@hotmail.com

1 Internal Medicine Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt

2 Medical Biochemistry Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt

3 Clinical Pathology Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt

4 Radiology Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt

are the products of separate genes present on distinct chromosomes (locus 6q25.1 and locus14q22-24, respectively). ER-α and ER-β genes comprise eight exons separated by seven intronic regions and spans more than 140 kb and approximately 40 kb, respectively [5].

Genetic screening of ER-α and ER-β genes has revealed the existence of several polymorphic sites. ER-α gene polymorphisms have been extensively studied for their associations with bone mass and osteoporosis risks in several populations [6–9]. A number of studies have found an association between a dinucleotide (CA) repeat polymorphism in intron 5 of ER-β gene and BMD phenotypes [10–12]. However, there are few studies about the association of the common polymorphisms, *Alu*I (1730 G/A, rs4986938) located at exon 8 or *Rsa*I (1082 G/A, rs1256049) located at exon 5 of ER-β gene and BMD [13, 14].

An orphan nuclear receptor, estrogen-related receptor α (ERRα), with sequence homology to ER-α and ER-β, was shown to be highly expressed throughout the osteoblast developmental sequence. Despite its inability to bind estrogens, this receptor may interact with ER-α and ER-β, or act directly to alter bone cell function. ERRα gene is located on chromosome 11q12 and comprises seven exons separated by six introns, which span about 20 kb [15]. Laganière et al. [16] reported a 23-base pair (bp) sequence referred to as ERRα 23 (rs3217060), located at position −682 in the promoter region of the ERRα gene. ERRα 23 is a 23-bp nucleotide repeat found in one to four copies in human chromosomes. It was mainly found in two copies of a 23-bp element; however, about 12 % of the population had three or four copies. Two studies found this variant to be associated with BMD in premenopausal women [17, 18]; however up to date, no reports concerning the role of this polymorphism in postmenopausal osteoporotic women have been reported as yet.

One of potential sites of action of estrogen/ER pathway includes effects on osteoblastic cells to alter their production of receptor activator of nuclear factor-kappa-B ligand (RANKL) [3]. It was also shown that RANKL levels were increased on the surface of bone marrow cells from early postmenopausal women who are estrogen deficient [19]. It is now clear that RANKL is the key final effector osteoclastogenic cytokine. Bone cells appear to express the membrane-bound form of RANKL, and thus, osteoblasts must physically interact with osteoclasts precursors in order to activate RANK [20]. Soluble RANKL (sRANKL) can be produced by activated T lymphocytes and is as active as membrane-bound RANKL in binding to RANK [21].

The aim of this study was to evaluate the association of *Alu*I G/A and *Rsa*I G/A polymorphisms of ER-β gene and 23-bp nucleotide repeat polymorphism of ERRα gene with BMD in Egyptian postmenopausal women, and to determine the effect of these polymorphisms on serum levels of sRANKL.

Subjects and methods

This case–control study included two-hundred postmenopausal osteoporotic women evaluated in the Internal Medicine and Radiology Departments of Zagazig University Hospitals. One-hundred and eighty age-matched healthy postmenopausal women with normal BMD were recruited from the same demographic area and taken as controls. Women were considered postmenopausal if they had no menstruation for at least 12 months. All the subjects underwent BMD measurements by dual energy X-ray absorptiometry at lumbar spine (vertebrae L1-L4) and at the femoral neck. On the basis of T score of dual energy X-ray absorptiometry, they were classified as osteoporotic or normal BMD according to the World Health Organization definition and diagnosis of osteoporosis [22]. T score of both lumbar spines and femoral neck was taken to differentiate between women with normal BMD (T score ≥ -1 at both sites) and osteoporotic women (T score ≤ -2.5 at both sites).

Women with endocrinological disorders (such as hypo- or hyperparathyroidism, hyperthyroidism, Cushing's syndrome, and diabetes mellitus) or other diseases affecting BMD such as chronic disorders of liver and kidney, rheumatoid arthritis, systemic lupus erythematosus, ankylosing spondylitis, and other skeletal diseases (e.g., Paget's disease, osteogenesis imperfecta) were excluded from the study. Women who were on medications known to affect bone density and metabolism e.g., corticosteroids, anticonvulsants, bisphosphonates, or calcitonin; and women with hormone replacement therapy, premature menopause <40 years, or underwent bilateral oophorectomy were also excluded. None of the women was a smoker and their daily work comprises usual physical activities. The study was approved by the ethics committee of Zagazig University and informed consent was obtained from all the subjects included in the study. All subjects were interviewed for their personal and medical history, including age, menarche and menopause and submitted to thorough clinical examination including height and weight measurements and assessment of body mass index (BMI) [weight in Kg/surface area in m^2]. Baseline characteristics of the studied groups are shown in Table 1.

DNA isolation and determination of ER-β and ERRα genotypes

Genomic DNA was extracted from EDTA whole blood using a spin column method according to the manufacturer recommendations (QIAamp Blood Kit; Qiagen GmbH, Hilden, Germany). The *Alu*I ER-β gene polymorphism was detected by PCR restriction fragment length polymorphism analysis according to Meng et al. [23]. PCRs were

Table 1 Baseline characteristics (mean ± SD) of osteoporotic women and control women

Parameters	Osteoporotic women ($n = 200$)	Control women ($n = 180$)	p
Age (years)	54.4 ± 4.4	54.5 ± 4.2	0.82
Body mass index (kg/m^2)	29.3 ± 3.6	29.9 ± 3.4	0.10
Age at first menarche (years)	12.3 ± 1.6	12.2 ± 1.7	0.55
Age at menopause (years)	51.1 ± 4.2	51.5 ± 3.9	0.34
No of children	3.3 ± 1.2	3.5 ± 1.0	0.08
T score lumbar spine	−2.8 ± 0.3	−0.4 ± 0.3	<0.001
T score femoral neck	−2.9 ± 0.2	−0.5 ± 0.4	<0.001
Lumbar spine BMD (g/cm^2)	0.78 ± 0.11	0.96 ± 0.08	<0.001
Femoral neck BMD (g/cm^2)	0.76 ± 0.08	0.94 ± 0.06	<0.001
Serum calcium (mg/dl)	9.6 ± 0.4	9.6 ± 0.3	1.00
Serum soluble RANKL (pmol/l)	0.48 ± 0.15	0.28 ± 0.12	<0.001

BMD bone mineral density, *RANKL* receptor activator of nuclear factor-kappa-B ligand

performed in a 25 µl volume containing 100 ng of DNA template, 12.5 µl one step PCR mixture containing 1 unit Taq polymerase, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.75), 0.1 % Triton X-100, 0.1 mg/ml BSA and 200 µM dTNPs (Bio-Basic Inc., Ontario, Canada), and 0.2 µM of each primer (Biosynthesis Inc., Lewisville, Tx, USA). The PCR product was digested with restriction endonuclease *Alu*I (New England Biolabs, Ipswich, MA, USA).

In all subjects, allele-specific PCR was performed to detect the *Rsa*I variants of ER-β according to Silva et al. [24]. Two reactions per subject were run, using a specific primer for either the polymorphic A variant or for the wild-type G variant, together with an upstream and a downstream primers. PCR conditions were established to generate both a control fragment and a shorter, allele-specific band in the presence of the variant and only the control fragment in its absence. Allele-specific PCR of the *Rsa*I polymorphism was performed in a total volume of 25 µl containing 200 ng DNA, 12.5 µl one step PCR mixture (Bio-Basic Inc., Ontario, Canada), and 0.2 µM of each of the primers *Rsa*I forward (Fw), *Rsa*I reverse (Rev), and either *Rsa*I Rev A or *Rsa*I Rev G.

The ERRα repeats polymorphism was detected by sizing of PCR products on agarose gel according to Laflamme et al. [17] with some modifications. 25 µl reaction mixtures were containing 200 ng genomic DNA, 12.5 µl one step PCR mixture, and 0.2 µM of each primer. The ER-β genotype was identified by electrophoresis of PCR products in 2 % agarose gel and ERRα genotype in 3 % agarose gel. The gel was visualized under UV trans-illuminator with a 100-bp ladder (SiZer™-100 DNA Marker, iNtRON Biotechnology, Seongnam, Korea) for both ER-β genotype and a 20-bp ladder (SiZer™-20 DNA Marker, iNtRON Biotechnology, Seongnam, Korea) for ERRα genotype. The primers, PCR conditions, and DNA fragment sizes are listed in Table 2.

Measurement of serum sRANKL

Centrifuged serum was frozen and stored at −80 °C until assay. Determinations of sRANKL levels were performed by ELISA (Immundiagnostik, Bensheim, Germany). The sensitivity, intra-assay and inter-assay coefficient of variation were 0.04 pmol/l, 5, and 7 %, respectively.

Table 2 Standard PCR conditions used in genotyping estrogen receptor β (ER β) and estrogen-related receptor α (ERRα) polymorphisms

Polymorphic site	Primer sequence	PCR conditions	Fragment size (bp)
*Alu*I G/A	Fw: 5′-GACCTGCTGCTGGAGATGCT-3′	30 cycles of 30 s at 95 °C, 1 min at 62 °C, 90 s at 72 °C	G 405
	Rev: 5′-AATGAGGGACCACACAGCA-3′		A 163 + 242
*Rsa*I G/A	Fw: 5′-ACTTGCCATTCTGTCTCTACA-3′	35 cycles of 1 min at 96 °C, 30 s at 58 °C, 3 min at 72 °C	Control 409
	Rev control: 5′-CACAGGACCCTGAATCCT-3′		A 127
	Rev A: 5′-AGCTCTCCAAGAGCCGT-3′		G 127
	Rev G: 5′-AGCTCTCCAAGAGCCGC-3′		
ERRα 23 repeats	Fw: 5′-CGTGGCCCCGCCCTTCC-3′	30 cycles of 45 s at 95 °C, 45 s at 58 °C, 45 s at 72 °C	1 repeat 175
	Rev: 5′-GTAGACCCAGTAGCCCCACAG-3′		2 repeats 198
			3 repeats 221
			4 repeats 244

Table 3 Genotype and allele frequencies of estrogen receptor β (ER β) and estrogen-related receptor α (ERRα) polymorphisms in osteoporotic women (n = 200) and control women (n = 180)

Parameters	Osteoporotic women, n (%)	Controls, n (%)	Odds ratio (95 % CI)	p value*
*Alu*I G/A				
Genotypes				
GG	52 (26.0)	85 (47.2)	0.39 (0.26–0.60)	<0.001
GA	73 (36.5)	65 (36.1)	1.02 (0.67–1.55)	0.94
AA	75 (37.5)	30 (16.7)	3.00 (1.85–4.87)	<0.001
Alleles				
G	177 (44.3)	235 (65.3)		
A	223 (55.7)	125 (34.7)	2.37 (1.77–3.18)	<0.001
*Rsa*I G/A				
Genotypes				
GG	150 (75)	144 (80)	0.75 (0.46–1.22)	0.24
GA	48 (24)	35 (19.4)	1.31 (0.80–2.14)	0.28
AA	2 (1.0)	1 (0.6)	1.80 (0.16–20.11)	0.62
Alleles				
G	348 (87.0)	323 (89.7)		
A	52 (13.0)	37 (10.3)	1.30 (0.83–2.04)	0.24
ERRα 23 repeats				
Genotypes				
2,2	186 (93.0)	154 (85.5)	2.24 (1.13–4.44)	0.02
2,3	12 (6.0)	23 (12.8)	0.44 (0.21–0.90)	0.02
2,4	1 (0.5)	2 (1.1)	0.45 (0.44–4.97)	0.50
3,3	1 (0.5)	1 (0.6)	0.90 (0.06–14.49)	0.94
Alleles				
1 or 2 repeats	385 (96.3)	333 (92.5)		
3 or 4 repeats	15 (3.7)	27 (7.5)	2.08 (1.09–4.00)	0.02

* Compared to controls

Statistical analysis

Continuous variables were expressed as means ± SD. The means of the two groups were compared by student t test. The statistical significances of differences in frequencies of variants between the groups were tested using the Chi square test. In addition, odds ratios (ORs) and 95 % confidence intervals (CIs) were calculated as a measure of the association of the ER-β *Alu*I, *Rsa*I, and ERRα 23 repeats alleles with osteoporotic women. Interaction between ER-β and ERRα genes were determined based on Bayesian algorithm using the Phase program. A difference was considered significant if p was <0.05. Each of the association studies had 80% power to detect a gene conferring a genotype relative risk of 1.5 at the 5 % significance level.

Results

Baseline characteristics of the studied groups

There were insignificant difference as regard age ($p = 0.82$) and menopause ($p = 0.34$) between osteoporotic women and controls. Osteoporotic women had statistically significant lower mean values of T score and BMD of both lumbar spine and femoral neck ($p < 0.001$) as compared to control women, but there was no statistical significance between both groups as regard BMI, age at first menarche, number of children, and serum calcium levels (Table 1).

Genotype and allele distributions of ER-β and ERRα gene polymorphisms

The genotype and allele frequencies of the ER-β and ERRα gene polymorphisms studied women are shown in Table 3. Our results showed significant differences between osteoporotic women and controls regarding the genotype and allele distributions of the ER-β *Alu*I 1730 G/A polymorphism. The AA genotype for the *Alu*I G/A polymorphism was significantly more frequent in osteoporotic women than in controls (37.5 vs. 16.7 %, $p < 0.001$), and there was a statistically significant increase in the A allele in the osteoporotic women as compared to controls ($p < 0.001$, OR 2.37 and 95 % CI 1.77–3.18). The frequency of *Alu*I GG genotype was significantly higher in controls than in osteoporotic women (47.2 vs. 26 %, respectively, $p < 0.001$).

Table 4 Baseline characteristics (mean ± SD) of the osteoporotic women ($n = 200$) according to estrogen receptor β *Alu*I, *Rsa*I and estrogen-related receptor α (ERRα) genotypes

Parameters	*Alu*I G/A genotype		p	*Rsa*I G/A genotype		p	ERRα 23 repeats genotype		p
	GG ($n = 52$)	GA + AA ($n = 148$)		GG ($n = 150$)	GA + AA ($n = 50$)		2,2 ($n = 186$)	2,3 + 2,4 + 3,3 ($n = 14$)	
Age (years)	54.3 ± 3.2	54.4 ± 3.5	0.86	54.3 ± 3.4	54.7 ± 3.4	0.47	54.4 ± 3.3	54.4 ± 3.5	1.00
Body mass index (kg/m^2)	28.9 ± 2.5	29.4 ± 4.4	0.44	29.1 ± 3.2	29.9 ± 3.6	0.14	29.1 ± 3.1	31.4 ± 1.2	0.006
Age at first menarche (years)	12.5 ± 1.2	12.2 ± 1.2	0.12	12.3 ± 1.2	12.5 ± 1.2	0.31	12.3 ± 1.2	12.4 ± 1.3	0.77
Age at menopause (years)	51.4 ± 4.2	51.0 ± 4.4	0.57	51.2 ± 4.5	50.8 ± 4.1	0.58	51.1 ± 4.3	51.1 ± 3.1	1.00
No of children	3.2 ± 1.2	3.3 ± 0.9	0.53	3.2 ± 1.2	3.5 ± 1.3	0.14	3.3 ± 1.3	3.6 ± 1.3	0.40
Lumbar spine BMD (g/cm^2)	0.81 ± 0.10	0.77 ± 0.09	0.01	0.79 ± 0.10	0.76 ± 0.09	0.06	0.77 ± 0.09	0.97 ± 0.08	<0.001
Femoral neck BMD (g/cm^2)	0.78 ± 0.07	0.75 ± 0.09	0.03	0.77 ± 0.12	0.74 ± 0.09	0.11	0.74 ± 0.09	0.95 ± 0.07	<0.001
Serum soluble RANKL (pmol/l)	0.40 ± 0.12	0.51 ± 0.17	<0.001	0.42 ± 0.17	0.66 ± 0.13	<0.001	0.49 ± 0.14	0.40 ± 0.10	0.02

BMD bone mineral density, *RANKL* receptor activator of nuclear factor-kappa-B ligand

Concerning ER-β *Rsa*I 1082 G/A polymorphism, there were no significant differences of genotype or allele frequencies between patients and controls ($p = 0.24$, OR 1.3 and 95 % CI 0.83–2.04 for the A allele).

The ERRα genotype frequencies in this sample were as follows: 1, 1 repeats (0 %), 1, 2 (0 %), 1, 3 (0 %), 1, 4 (0 %), 2, 2 (85.5 %), 2, 3 (12.8 %), 2, 4 (1.1 %), 3,3 (0.6 %), 3, 4 (0 %), and 4, 4 (0 %) for controls and 1, 1 (0 %), 1, 2 (0 %), 1, 3 (0 %), 1, 4 (0 %), 2, 2 (93 %), 2,3 (6 %), 2, 4 (0.5 %), 3, 3 (0.5 %), 3, 4 (0 %), and 4, 4 (0 %) for osteoporotic women. The 2, 2 genotype was significantly more frequent in osteoporosis patients than in controls (93 vs. 85.5 %, $p = 0.02$) and OR, and 95 % CI for the 2 repeats allele of ERRα = 2.08 (1.09–4.00), $p = 0.02$. On the other hand, the 2, 3 genotype frequency was significantly higher in the control group than in osteoporosis one (12.8 vs. 6 %, $p = 0.02$).

Interaction between ER-β and ERRα genes

We studied the osteoporosis susceptibility in relation to various combinations of genotypes. We found that patients who were carriers of haplotype (*Alu*I AA, *Rsa*I AA and ESRRα 2,3 repeats 2,2 genotypes) had an increased risk of osteoporosis (OR and 95 % CI 3.3 (1.9–5.6), $p < 0.001$).

The association of ER-β and ERRα gene polymorphisms with characteristics of osteoporotic women

Further, we analyzed the relation between ER-β and ERRα genotypes with the characteristics and the BMD measures

in osteoporotic women. There was no significant difference in age, BMI, age at menarche, age at menopause, and number of children between the different genotypes of ER-β polymorphisms. The mean values of lumbar spine BMD were significantly lower in carriers of *Alu*I A mutated alleles in comparison with GG genotype patients ($p = 0.01$). Also the average femoral neck BMD of the patients with mutated alleles *Alu*I A was significantly lower than those with GG genotypes ($p = 0.03$). For ER-β *Rsa*I polymorphism, no effect of different *Rsa*I genotypes on both lumbar spine and femoral neck BMD was found ($p = 0.06$ and 0.11, respectively) (Table 4).

For ERRα polymorphism, because of some genotypes were rare or absent and for association analyses, ERRα genotypes were regrouped into those having 2,2 genotype for contrast with those having 2,3; 2,4 and 3,3 genotypes, as in the previous report of Laflamme et al. [17]. We found that the long repeats (2,3 + 2,4 + 3,3) genotypes of ERRα 23 were associated with a higher BMI than the short repeats 2,2 genotype ($p = 0.006$). Moreover, the values of both lumber and femoral neck BMD were significantly lower in the short repeat 2,2 genotype than in the long repeats genotype patients ($p < 0.001$ for both) (Table 4).

Serum sRANKL in all studied groups

Mean values of serum sRANKL levels were significantly higher in osteoporotic women than in controls ($p < 0.001$) (Table 1). When we studied the relation between ERRα and ER-β genotypes and sRANKL levels, we found a significant increase of the mean values of sRANKL in carriers of *Alu*I A, *Rsa*I A alleles and in patients having 2,2

genotype of ERRα ($p < 0.001$, $p < 0.001$, $p = 0.02$ respectively) (Table 4).

Discussion

Since estrogens have important effects on bone mass and bone remodeling, ER genes have been tested for association with osteoporosis and fracture risk. Most of the studies that investigated the relationships between BMD and ER genes have been focused on polymorphisms in ER-α gene that is robustly expressed in reproductive tissues. The results obtained, even if compelling, for ER-α gene involvement in osteoporosis or fracture were not conclusive; likely because of confounding factors, i.e., the ethnic- and gender-distribution of ER-α gene polymorphisms [7–9]. After the identification of ER-β gene and its gene product ER-β, which is more abundant than the ER-α one in trabecular bone, several studies have also evaluated polymorphisms of ER-β gene as risk markers for osteoporosis [10–12]. The rational in studying the ER-β gene as a candidate gene for osteoporosis lays on the fact that ER-β is robustly expressed by developing human bone, especially the cancellous bone compartment [4]. As well as studies on cell cultures revealed that there is a greater than nine fold increase in ER-β expression in cultured human osteoblasts during bone mineralization, whereas ER-α levels remain unchanged during this process [25]. Furthermore, experimental studies utilizing ER-β gene knockout mouse models remarked the important involvement of ER-β in bone formation [26]. In addition, ER-β was shown to be more potent than ER-α in mediating estrogen-induced repression of tumor necrosis factor α expression, which is associated with the pathogenesis of osteoporosis [27].

In this study, we have evaluated the association of AluI 1730 G/A and RsaI 1082 G/A polymorphisms of ER-β gene with BMD in postmenopausal women. Our results showed significant differences between osteoporotic women and control women regarding the genotype and allele distributions of the ER-β AluI 1730 G/A polymorphism. The homozygous AA genotype and the A allele for the AluI G/A polymorphism were significantly more frequent in osteoporotic patients than in controls. Interestingly, the frequency of homozygous wild AluI GG genotype was significantly higher in controls than in osteoporotic women. Moreover, mean values of lumbar spine BMD and femoral neck BMD were significantly lower in carriers of AluI A mutated alleles in comparison with GG genotypes among osteoporotic patients.

Concerning ER-β RsaI 1082 G/A polymorphism, there were no significant differences of genotype or allele frequencies between osteoporotic women and controls and also there was no effect of different RsaI genotypes on both lumbar spine and femoral neck BMD. Our results are in consistence with Curro et al. [13] who found that ER-β AluI polymorphism was associated with BMD in a cohort of Caucasian osteopenic and postmenopausal women living in Sicily (Italy). In particular, they observed a significant, negative correlation between the mutated homozygous genotype AA1730 and lumbar spine BMD values in comparison to subject with GA or GG genotypes. Interestingly, 85 % of patients with homozygous genotype AA1730 in their study presented the smallest lumbar spine BMD values, thus suggesting that this genetic variant may likely contribute to bone loss worsening in postmenopausal women. Moreover, they demonstrated that the negative effect of AA1730 genotype on BMD has been reinforced by longer times since the onset of menopausal status, accordingly with a progressive reduction in estrogen synthesis. However, several previous investigations did not find any significant association of ER-β AluI 1730 G/A [28–30] and RsaI 1082G/A polymorphisms with either spine or hip BMD [14, 28].

In our study, the recruited participants were a homogenous population regarding age, race, ethnic origin, and menopausal status. The human ER-β synthesis shows variability in a gender, age, and cell-type dependent manner. Specifically, it has been suggested that the worsening effects of ER-β polymorphisms on female bone mass become more evident at lower estrogen concentrations, as occurring in postmenopausal women, than at higher estrogen concentrations, as seen in premenopausal women [13, 29]. To date, several ER-β gene polymorphisms have been tested for their association with BMD and changes in bone turnover markers. However, only few studies reported a positive association between ER-β gene variants and BMD or fracture risk [10–12], while other results were inconclusive [28, 30] or showed a dissimilar effect at different ER-β gene loci [29]. The contradictory results conferred by different studies on a specific candidate gene could be attributed to different sample size, cross-sectional study approaches as well as differences in race/ethnicity, age, and gender in the populations studied; and also, the heterogeneity of selected populations encompassing healthy individuals with significantly different ages and hormonal milieu, ranging from fertile to pre or peri-menopausal status [28, 29]. The crucial role of hormonal status is also supported by the findings on the lack of association of ER-β AluI polymorphism with BMD in a population of Caucasian men, underlining the gender-related differences effects of estrogens [30]. Thus, different genetic backgrounds and environmental effects and/or their interaction could explain the diversity in the results reported by different study groups. Although the exact mechanism behind the observed associations with BMD is still not fully understood, it has been hypothesized that the ER-β AluI polymorphism, consisting in a G/A1730 transition in the 3′-Untraslated region, could alter mRNA stability and protein levels [31], leading, in turn, to a

reduced synthesis of ER-β. This would indicate that this *Alu*I ER-β gene variant is likely implicated in the distribution and density of ER-β in woman body organs, primarily affecting the interaction of estrogen to ERs on trabecular bone. In this context, the use of natural molecules, i.e., soy isoflavone derivatives, mainly targeting ER-β receptor has already been demonstrated successfully in improving both BMD and biochemical bone markers in osteopenic postmenopausal women [32, 33].

In this study, the association of 23-bp nucleotide repeat polymorphism in ERRα gene promoter with BMD was evaluated as well. We found that the 2,2 genotype was significantly more frequent in osteoporotic women than in controls. On the other hand, the 2,3 genotype frequency was significantly higher in the control group than in osteoporosis one. Also, we found that the values of both lumber and femoral neck BMD were significantly lower in the short repeat 2,2 genotype than in the long repeats (2,3 + 2,4 + 3,3) genotypes among osteoporotic women. Our results are in agreement with Laflamme et al. [17] who reported that this polymorphism was associated with a significant difference in lumbar spine BMD in a sample of premenopausal women. They also found that women with long variants (three repeats) of the ERRα promoter polymorphism showed a higher lumbar spine BMD, whereas the short variant genotype (two repeats) which is more common and present in about 89 % of the population is associated with lower BMD and likely increased risk of bone fracture. However, contradictory to our results, Giroux et al. [18] found no association of 23-bp nucleotide repeat polymorphism in ERRα gene promoter and BMD. A functional study done by Laganière et al. [16] implicated that the 23-nucleotide-long sequence includes a functional ERR-response element. Indeed, they showed that an interdependent ERRα/PGC-1α (peroxisome proliferator activated receptor γ coactivator-1α)-based transcriptional pathway targets this ERRα polymorphic element to dictate the level of ERRα expression. Also, they had shown that a higher number of repeats lead to a higher expression of ERRα. Interestingly, ERRα is highly expressed in bone at all developmental stages, from the earliest precursors to the most mature osteoblasts in mineralized nodules. Furthermore, in vitro studies using rat calvaria cells indicated that inhibition of ERRα expression through antisense oligonucleotide treatment results in a significant decrease in mineralized bone nodule number. In the same manner, ERRα overexpression by transient transfection with CMV-ERRα construct resulted in a significant increase in the number of mineralized bone nodules formed [15]. Despite significant amino acid homology in the ligand binding domain with the ERs, ERRα does not bind estradiol or other natural ligands and is therefore still considered as an orphan receptor. However, the synthetic ER agonist diethylstilbestrol was identified as an ERRα antagonist [34] and the structural basis for the antagonistic action of this synthetic compound has

recently been solved showing that ERRα can be a target for therapeutic intervention [35].

The evidence is now extremely strong supporting a final effector role of the RANK/RANKL/OPG system in osteoclast formation and the regulation of bone resorption. The binding of RANKL to its receptor (RANK) induces differentiation, activation, and the prevention of osteoclast apoptosis, leading to enhanced bone resorption and bone loss [36]. In the present study, serum sRANKL was significantly higher in osteoporotic women than controls. Oelzner et al. [37] demonstrated that high-serum levels of RANKL were associated with osteoporosis in patients with rheumatoid arthritis. Gurban et al. [38] showed that levels of sRANKL and RANKL$_{OBL}$ in primary cultures of osteoblasts are significantly higher in postmenopausal osteoporotic women demonstrating osteoclast activation. A monoclonal antibody against RANKL was shown to produce prolonged inhibition of bone resorption in postmenopausal women [39].

We also studied the effect of ER-β and ERRα polymorphisms on the serum levels of sRANKL (as one of effectors molecules of the estrogen/ER pathway). To the best of our knowledge, our study is the first to demonstrate the influence of the ER polymorphisms on serum RANKL concentrations. We found a significant increase of sRANKL levels in carriers of *Alu*I A, *Rsa*I A alleles and in women having 2,2 genotype of ERRα.

There are a few limitations of our study. Identifying significant associations of genetic variants with a complex qualitative trait, such as osteoporosis, may need a larger sample size. In the current study, a replication sample is lacking to further confirm the association between ER-β and ERRα polymorphisms and osteoporosis.

In conclusion, we demonstrated an association of ER-β *Alu*I G/A and ERRα 23-repeats polymorphisms with BMD in postmenopausal Egyptian women. These polymorphisms can be considered one of the genetic markers for predicting osteoporosis and can be used to make possible early therapeutic interventions in women at high risk for osteoporosis. Also, we suggested the possible relation of ER-β and ERRα polymorphisms with the levels of sRANKL; the final effect or osteoclastogenic molecule.

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