BBA Clinical 5 (2016) 124–129

Contents lists available at ScienceDirect

BBA Clinical

journal homepage: www.elsevier.com/locate/bbaclin

Expression levels of estrogen receptor α mRNA in peripheral blood cells are an independent biomarker for postmenopausal osteoporosis☆

Chi-Wen Chou [a,b,1], Tsay-I Chiang [a,b,c,d,1], I-Chang Chang [a,b], Chung-Hung Huang [a], Ya-Wen Cheng [d,*]

[a] Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan
[b] Department of Orthopedic Surgery, National Taiwan University Hospital Yun-Lin Branch, Taiwan
[c] Department of Nursing, College of Medicine & Nursing, Hung Kuang University, Taichung, Taiwan
[d] Graduate Institute of Cancer Biology and Drug Discovery, College of Medical Technology, Taipei Medical University, Taichung, Taiwan

ARTICLE INFO

Article history:
Received 13 November 2015
Received in revised form 23 February 2016
Accepted 4 March 2016
Available online 11 March 2016

Keywords:
Bone mineral density
Estrogen receptor
Estrogen
Osteoporosis

ABSTRACT

Background: The up- and down-regulation of the osteoclastogenesis response depends on the estrogen/estrogen receptor (ER) signaling pathway. Previous reports have shown that the promoter hypermethylation and gene polymorphism of ERα are risks for menopausal osteoporosis. No previous study has evaluated the expression levels of ERα mRNA in menopausal osteoporosis using human subjects. We hypothesized that ERα mRNA expression may show less resistance to postmenopausal osteoporosis.
Methods: In this study, we enrolled 107 women older than 45 years without menstruation and classified them into control, osteopenia, and osteoporosis groups depending on their T-scores. The ERα mRNA levels in peripheral blood cells (PBCs) were analyzed via quantitative real-time reverse-transcription polymerase chain reaction (QRT-PCR), and estrogen in the serum was detected via ELISA.
Results: ERα mRNA levels in PBCs had a negative correlation with age and a positive correlation with estrogen and BAP in the osteopenia and osteoporosis groups, but not in the control group. Additionally, multivariate analysis showed that older age (>55 years), and low ERα mRNA levels in PBLs (≦250.39 copies/μg DNA) were associated with an approximately 9.188-, and 31.25-fold risk of osteoporosis.
Conclusion: We conclude that ERα mRNA levels in PBLs could be used as an independent risk factor for postmenopausal osteoporosis.
General significance: Our findings suggested that ERα mRNA levels in PBLs may be more important than age and serum estrogen levels.

1. Introduction

Osteoporosis is a silent and progressive systemic skeletal disorder characterized by compromised bone strength. Osteoporosis in the aged population has become increasingly important due not only to an increase in the number of postmenopausal women because of the aging population worldwide but also to the fact that more women are living longer and are, therefore, more prone to osteoporosis [1]. Osteoporosis may predispose these individuals to consequential increases in fracture risk, and osteoporosis is often only diagnosed after a fracture. Fracture risk refers to the resilience of the bone to trauma; bone strength is, in turn, dependent on mineral quantity and bone quality.

Estrogen is essential for bone growth and for the development and maintenance of bone health in adulthood [2,3]. The cellular responses of osteoblasts and osteoclasts to estrogen are initiated via the high-affinity receptor. Osteoblasts synthesize the receptor activator of the NF-κB ligand (RANKL) and the decoy receptor osteoprotegerin (OPG), which is necessary for modulating osteoclast formation and function [4,5]. Because the inhibition of osteoclastogenesis is one of the main mechanisms by which estrogen (estradiol) prevents bone loss, it is likely that estradiol may regulate either the production of RANKL or target cell responsiveness to RANKL [6]. Thus, estrogen may down-regulate osteoclastogenesis via a differential decrease in the responsiveness of osteoclast precursors to RANKL and by directly suppressing RANKL-induced osteoclast differentiation. The lack of estrogen decreases the differentiation of cells of monocytic lineage into mature osteoclasts. In 2003, Bord et al. first demonstrated estrogen-induced changes in OPG and RANKL that were mediated by ER expression [6]. Hertrampf et al. suggested that ERα but not ERβ agonists of estrogen exerted bone-protective effects by modulating the activity of osteoclasts and osteoblasts [7]. ERs, specifically ERα, are involved in bone cells' early

☆ The authors have no proprietary or financial interest in any material or device mentioned.

* Corresponding author at: Graduate Institute of Cancer Biology and Drug Discovery, College of Medical Science Technology, Taipei Medical University, 12F., No.3, Sec. 4, Bade Rd., Nangang Dist., Taipei 115, Taiwan.
E-mail address: ywc@tmu.edu.tw (Y.-W. Cheng).

[1] Equally contributed to this study.

http://dx.doi.org/10.1016/j.bbacli.2016.03.001

responses to strain both in vitro and in vivo [8–10]. The levels of ERα in osteoblasts and osteocytes are regulated by estrogen [11]. In addition, aromatase inhibitor use in postmenopausal breast cancer patients potentially impairs their bone quality, implying that the interaction between ERα and even tiny amounts of serum E2 in postmenopausal women is a valid signaling system in bone health [12–14]. Therefore, the down-regulation of ERα associated with low levels of estrogen may reduce the effectiveness of bone cells' ERα-mediated responses to strain [15,16].

The up- and down-regulation of the osteoclastogenesis response depends on three mechanisms: the number of estrogen receptors, the binding of the estrogen in an effective manner, and the transduction of the signal in the cell [10,15]. If any of these three components is flawed, estrogen binding to the receptor may not prevent bone loss. Therefore, we postulated that the expression of ERα mRNA in PBCs may serve as a barometer of gene activity of monocytic cell lineage in osteoclasts.

Therefore, we investigated whether (1) the expression levels of ERα mRNA in PBCs were different between the control, osteopenia, and osteoporosis groups of menopausal women, (2) the ERα mRNA expression levels were correlated with clinical markers of osteoporosis, and (3) ERα mRNA expression levels could be used as an independent biomarker for osteoporosis.

Table 1
Clinical parameters of control, osteopenia, and osteoporosis groups in this study.

Parameters	Control	Osteopenia	Osteoporosis	*p* value
	(n = 31)	(n = 45)	(n = 45)	
Age (years)	55.04 ± 10.31[a]	60.90 ± 10.06	75.53 ± 10.41	<0.0001
Height (cm)	156.3 ± 4.56	154.31 ± 5.23	149.38 ± 6.12	<0.0001
Weight (kg)	60.95 ± 5.50	58.58 ± 10.77	54.52 ± 10.75	0.020
BMI (kg/m^2)	24.98 ± 2.55	24.63 ± 4.42	24.45 ± 4.73	0.672
LBMD (g/cm^2)	1.19 ± 0.13	0.93 ± 0.09	0.69 ± 0.13	<0.0001
HBMD (g/cm^2)	1.01 ± 0.12	0.84 ± 0.09	0.64 ± 0.13	<0.0001
ERα mRNA	250.39 ± 550.56	50.35 ± 173.81	9.35 ± 32.77	<0.0001
E2 (pg/ml)	55.35 ± 56.01	46.86 ± 33.97	36.98 ± 11.81	0.442
T-score-lumbar	0.43 ± 0.91	−1.67 ± 0.45	−3.60 ± 0.81	<0.0001
Z-score-lumbar	0.75 ± 1.14	−0.74 ± 0.70	−1.6 ± 0.79	<0.0001
T-score-hip	0.44 ± 0.91	−1.02 ± 0.68	−2.36 ± 1.22	<0.0001
Z-score-hip	0.95 ± 0.91	0.03 ± 0.80	−0.284 ± 0.92	<0.0001
BAP	1.83 ± 1.37	2.31 ± 1.64	1.64 ± 1.05	0.383
CTX	0.27 ± 0.18	0.24 ± 0.18	0.39 ± 0.39	0.328

BMI: body mass index (kg/m^2).
LBMD: lumbar bone mineral density (g/cm^2).
HBMD: hip bone mineral density (g/cm^2).
BAP: bone-specific alkaline phosphates.
CTX: carboxy-terminal telopeptide.
p values were analyzed by two-independent sample tests.
[a] Mean ± standard deviation.

2. Materials and methods

2.1. Subjects

In a cross-sectional study conducted from 2010 to 2012, we enrolled 107 women older than 45 years without menstruation and classified them into control, osteopenia, and osteoporosis groups depend on their T-scores. The definition of osteoporosis was based on the categories created by the World Health Organization (WHO) for bone density in white women. The WHO definition of osteopenia is a T-score below −1.0 and above −2.5; for osteoporosis, it is a T-score less than −2.5. We excluded patients who were bedridden, using steroids, on hormone therapy, or dependent on alcohol or who had a history of renal disease or cancer. The patients enrolled in this study being diagnosed with osteoporosis for the first time. The blood samples were collected before clinical treatment. All patients were subgroups of experimental and control groups based on our previous report in 2010 [17]. We determined and recorded demographic data (age, height, weight, BMI) and clinical data (bone mineral density [BMD], ERα and estrogen levels, bone-specific alkaline phosphates [BAP], carboxy-terminal telopeptide [CTX], T- and Z-scores) for the three groups at the time of diagnosis (Table 1). The definition of high and low expression of estrogen, ERα mRNA and clinical parameters were dependent on the mean value of the control group. Values higher than the mean were defined as high. Expression levels lower than the mean were defined as low.

The BMD for all candidates was determined via dual-energy X-ray absorptiometry of the lumbar spine, femoral neck, and Ward's triangle. All participants submitted written informed consent, and the study was approved by institutional review board of Chung Shan Medical University Hospital. This was a pilot study. Based on our sample size, the value of the power analysis of this case–control study was >0.9.

2.2. ELISA of estrogen levels

The levels of estrogen in the serum were calculated using the enzyme-linked immunosorbent assay (ELISA) method with TiterZyme® estrogen-detection ELISA kits (DRG International, Inc., Mountainside, NJ, USA). The plates were incubated with phosphate-buffered saline (PBS)-Tween® containing 1% fetal calf serum (Gibco, Carlsbad, CA, USA) at 37 °C for 60 min to block nonspecific binding. Estrogen standards and serum samples were added to the plate. After incubation at 37 °C for 60 min, 100 μL of yellow antibody was added, but not to the blank, and the plates were incubated at room temperature for 60 min. After a PBS wash, 100 μL of blue conjugate was added, but not to the blank, and the plates were incubated at room temperature for 30 min. The plates were washed, and the substrate solution and color were added and measured at 570 and 590 nm via a microtiter plate reader (Model 550; BIO-RAD, Hercules, CA, USA). The concentration of estrogen was calculated using linear graph paper, plotting the average net optical density for each standard versus the human estrogen concentration in each standard. An approximation of a straight line was drawn through the points. The concentration of human estrogen in the serum was determined via interpolation.

2.3. QRT-PCR for ERα mRNA expression

The ERα mRNA expression in the PBCs was detected via QRT-PCR. The total RNA was isolated from the PBCs. Cells were isolated using 1 mL Trizol® reagent (Invitrogen, Carlsbad, CA, USA), followed by chloroform re-extraction and isopropanol precipitation. Three micrograms of total RNA from lung cancer cells were reverse transcribed using Moloney murine leukemia virus reverse transcriptase (M-MLV; Promega, Madison, WI, USA) and oligo d(T)15 primer. QRT-PCR was performed in a final volume of 25 μL, containing 1 μL cDNA template, 0.2 μM primer, and 12.5 μL SYBR® green master mix (Molecular Probes, Inc., Eugene, OR, USA). The primers were designed using the ABI Primer Express 3.0 Software (Applied Biosystems, Foster City, CA, USA). The sequences of the primers used were 5′-TGATTGGTCTCGTCTGGCG-3′ and 5′-CATGCCCTCTACACATTTTC-CC-3′ for ERα and 5′-GACTCTTTCGAGGC CCTGTAATT-3′ and 5′-TCCCAAGATCCAACTACGAGC-T-3′ for the 18S gene. Quantification was carried out using the comparative CT method, and water was used as the negative control. An arbitrary threshold was chosen on the basis of the variability of the baseline. Threshold cycle (C_T) values were calculated by determining the point at which the fluorescence exceeded the threshold limit. C_T was reported as the cycle number at this point. The average of the target gene was normalized to 18S rRNA as an endogenous housekeeping gene. After cycling, the relative quantification of ERα mRNA against an internal control, 18S, was conducted via the ΔC_T method [18]. The definition of high and low expression of ERα mRNA was dependent on the mean value of gene expression in the control group. Expression levels higher than the mean were defined as high. Expression levels lower than the mean were defined as low.