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

A R T I C L E  I N F O
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

A B S T R A C T
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/jg 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.

© 2016 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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 predisperse 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]. ERα, specifically ERα, are involved in bone cells’ early
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 estrogen 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.

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. 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 osteoporosis is a T-score below −2.5. If 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 were 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 expression 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′-GATTTGCTCTGCTGGG-3′ and 5′-ATGCGCTCATACATTTC-CC-3′ for ERα and 5′-GACCTTGGAGCCCTGTAAT-3′ and 5′-TCCAAGATCACAATCAGGCT-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 (Ct) values were calculated by determining the point at which the fluorescence exceeded the threshold limit. Ct 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 ΔCt 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.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n = 31)</th>
<th>Osteopenia (n = 45)</th>
<th>Osteoporosis (n = 45)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>55.04 ± 10.31*</td>
<td>60.90 ± 10.06</td>
<td>75.53 ± 10.41</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>156.3 ± 4.56</td>
<td>154.21 ± 5.23</td>
<td>149.38 ± 6.12</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>60.95 ± 5.50</td>
<td>58.58 ± 10.77</td>
<td>54.52 ± 10.75</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.98 ± 2.55</td>
<td>24.63 ± 4.42</td>
<td>24.45 ± 4.73</td>
<td>0.672</td>
</tr>
<tr>
<td>LBMD (g/cm²)</td>
<td>1.19 ± 0.13</td>
<td>0.93 ± 0.09</td>
<td>0.69 ± 0.13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HBMD (g/cm²)</td>
<td>1.01 ± 0.12</td>
<td>0.84 ± 0.09</td>
<td>0.64 ± 0.13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ERα mRNA</td>
<td>250.39 ± 550.56</td>
<td>50.35 ± 173.81</td>
<td>9.35 ± 32.77</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>E2 (pg/ml)</td>
<td>55.35 ± 56.01</td>
<td>46.86 ± 33.97</td>
<td>36.98 ± 11.81</td>
<td>0.442</td>
</tr>
<tr>
<td>T-score-lumbar</td>
<td>0.43 ± 0.91</td>
<td>−1.67 ± 0.45</td>
<td>−3.60 ± 0.81</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Z-score-lumbar</td>
<td>0.75 ± 1.14</td>
<td>−0.74 ± 0.70</td>
<td>−1.6 ± 0.79</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>T-score-hip</td>
<td>0.44 ± 0.91</td>
<td>−1.02 ± 0.68</td>
<td>−2.36 ± 1.22</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Z-score-hip</td>
<td>0.95 ± 0.91</td>
<td>0.03 ± 0.80</td>
<td>−0.28 ± 0.52</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BAP</td>
<td>1.83 ± 1.37</td>
<td>2.21 ± 1.64</td>
<td>1.64 ± 1.05</td>
<td>0.383</td>
</tr>
<tr>
<td>CTX</td>
<td>0.27 ± 0.18</td>
<td>0.24 ± 0.18</td>
<td>0.39 ± 0.39</td>
<td>0.328</td>
</tr>
</tbody>
</table>

BMD: body mass index (kg/m²).
LBMD: lumbar bone mineral density (g/cm²).
HBMD: hip bone mineral density (g/cm²).
BAP: bone-specific alkaline phosphates.
CTX: carboxy-terminal telopeptide.

*: Mean ± standard deviation.
2.4. Statistical analysis

In this study, the data were presented as mean ± SD. We determined the differences in ERα mRNA between the control and disease groups using a Mann–Whitney test. The correlations between ERα mRNA, estrogen, age, T-scores, and Z-scores were calculated using the Pearson correlation method for all subjects. To determine whether ERα mRNA could be used as an independent risk factor for osteoporosis, a multiple unconditional logistic model was used to obtain the adjusted odds ratios (ORs) for the difference in ERα mRNA and estrogen status, and a corresponding 95% CI was used for the T-score after adjusting for the effect of potential confounding factors. The factors used in the multivariate were analyzed via univariate analysis first; if the p value was less than 0.05, they were subjected to multivariate analysis. In order to determine the change in the age, body height, body weight, serum estrogen levels, and ERα mRNA in PBCs, the area under the curve per year (AUC/year) was calculated. All the statistical analyses were performed using SAS® 9.1 statistical software (SAS Institute, Inc., Cary, NC, USA). All p values were calculated from two-tailed statistical tests.

3. Results

3.1. Clinical data for control, osteopenia, and osteoporosis groups at the time of diagnosis

We enrolled 107 women older than 45 years without menstruation, and the study subjects were stratified into three groups (31 control, 31 osteopenia, and 45 osteoporosis groups) depending on their T-score. As shown in Table 1, the age (p < 0.0001), body height (p < 0.0001), body weight (p = 0.020), BMI (p < 0.0001), E2 (p < 0.0001), Z-score (p < 0.0001), lumbar and hip T-score (p < 0.0001), and lumbar and hip Z-score (p < 0.0001) of these three groups were significantly different. No significant differences were found for BMI, serum estrogen levels, BAP, and CTX (p = 0.672 for BMI; p = 0.442 for E2; p = 0.383 for BAP; p = 0.328 for CTX).

3.2. ERα mRNA in PBCs and serum estrogen levels in the control, osteopenia, and osteoporosis groups

To determine whether the ERα mRNA in the PBCs and in serum estrogen levels was different between the control, osteopenia, and osteoporosis groups of menopausal women, the expression levels of ERα mRNA in the PBCs were detected via real-time RT-PCR. As shown in Fig. 1, the expression levels of ERα mRNA in the PBCs of the menopausal control group were significantly higher than in the osteopenia and osteoporosis groups (PBCs ERα mRNA: 250.39 ± 550.56 for the control group, 46.86 ± 33.97 for the osteopenia group, and 9.54 ± 32.77 for the osteoporosis group, p < 0.0001, Fig. 1A). The data demonstrate that ERα mRNA in PBCs plays a role in osteoporosis progression.

3.3. ERα mRNA in PBCs was correlated with age, serum estrogen levels, and BAP in the disease groups, but not in the control group

To further verify whether the role of ERα mRNA in the PBCs in the control and disease groups (the osteopenia and osteoporosis groups) was different, the correlations between ERα mRNA levels in the PBCs

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unfavorable/favorable OR</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>&lt;55/65</td>
<td>9.188</td>
<td>2.211–38.177</td>
</tr>
<tr>
<td>ERα mRNA</td>
<td>&lt;250.39/250.39</td>
<td>31.25</td>
<td>1.357–1000</td>
</tr>
<tr>
<td>(Copies/μg RNA)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body height (cm)</td>
<td>&lt;156.3/156.3</td>
<td>3.472</td>
<td>0.792–15.15</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>&lt;60.95/60.95</td>
<td>1.153</td>
<td>0.264–5.025</td>
</tr>
</tbody>
</table>

Fig. 1. PBL ERα mRNA (A) and estrogen (B) levels in the control, osteopenia, and osteoporosis groups.
and age, body weight, height, T-scores, and Z-scores in the control and the disease groups were analyzed. As shown in Table 2, in the disease group, ERα mRNA had a negative correlation with age \((r = -0.263, p = 0.002)\) and a positive correlation with serum estrogen levels \((r = 0.458, p = 0.001)\) and BAP \((r = 0.411, p < 0.0001)\). There was no correlation with the other factors, including body height \((r = 0.130, p = 0.261)\), body weight \((r = -0.054, p = 0.642)\), T-score in the lumbar area \((r = 0.181, p = 0.118)\), T-score in the hip area \((r = 0.137, p = 0.328)\), BMI \((r = -0.110, p = 0.342)\), lumbar Z-score \((r = 0.082, p = 0.479)\), hip Z-score \((r = -0.011, p = 0.937)\), and CTX \((r = -0.055, p = 0.638)\). The results for the disease groups were different than in the control group. In the control group, the ERα mRNA levels in the PBCs were not correlated with any clinical parameters, including age \((r = -0.174, p = 0.377)\), serum estrogen levels \((r = 0.197, p = \ldots\)

![Graphs](image_url)
showed that ERoporosis groups (Fig. 1A). In addition, the analysis results of ROC curve quantitative PCR is applied to rapidly detect nucleic acids that are diagnostic for the non-invasive and real-time molecular monitoring of diagnosis and treatment effects for this disease. In this study, the expression levels of ERα mRNA in PBCs were an important risk factor for menopausal osteoporosis. Our data showed that older persons (≥55 years old), and those with low ERα mRNA levels in PBCs (≥250.39 copies/μg DNA) had an approximately 9.188- and 31.250-fold risk of osteoporosis as compared with healthy persons (55 years old; 95% CI = 2.211–38.177, p = 0.002), and those with high ERα mRNA levels in PBLs (>250.39 copies/μg DNA; 95% CI = 1.357–1000, p = 0.032) (Table 3). Thus, ERα mRNA levels in PBLs are correlated with age as independent risk factors for menopausal osteoporosis.

3.5. Expression of ERα mRNA could be used as a biomarker of osteoporosis

To further confirm whether the changes in the expression levels of ERα mRNAs could be used as a useful biomarker in osteoporosis, we analyzed the receiver operating characteristic (ROC) curve. As shown in Fig. 2, age (AUC = 0.761, p < 0.0001), body height (AUC = 0.729, p = 0.001), body weight (AUC = 0.669, p = 0.017), and ERα mRNA levels (AUC = 0.768, p < 0.0001) have good predictability, but not serum estrogen (AUC = 0.554, p = 0.460). Thus, the expression levels of ERα mRNA in the PBCs of osteoporosis patients acted as a significant risk biomarker for the development of osteoporosis.

4. Discussion

The decline in estrogen function is the main cause of menopausal osteoporosis. The action of estrogen is mediated by its binding to ERα and entry into the cell to activate the ERα-related signaling pathway. In this study, we found that the levels of ERα mRNA in PBCs are more important than estrogen in increasing the risk of osteoporosis and that they are promising markers for the early diagnosis of postmenopausal osteoporosis.

It is well-known that cells of monocytic lineage differentiate into osteoclasts [8–11]. In addition, changes in ER mRNA in the osteoblast have been reported in disease processes [8–11]. In present study, we found that the expression levels of ERα mRNA in the PBLs of the menopausal control group were significantly higher than in the osteopenia and osteoporosis groups (Fig 1A). In addition, the analysis results of ROC curve showed that ERα mRNA (AUC = 0.768, p < 0.0001) have good predictability (Table 3). Thus, the expression levels of ERα mRNA in the PBCs of osteoporosis patients acted as a significant risk biomarker for the development of osteoporosis. However, the detection of these biomarkers using the osteoblasts of osteoporosis patients is difficult to apply in the clinic in order to monitor the disease. The detection of ER mRNA in the peripheral blood, either cell-associated or cell-free, holds great potential for the non-invasive and real-time molecular monitoring of diagnosis and treatment effects for this disease. In this study, the expression levels of ERα mRNA in PBCs were detected via real-time RT-PCR. Diagnostic quantitative PCR is applied to rapidly detect nucleic acids that are diagnostic of several types of disease. This assay is high-throughput and rapid. In clinical settings, examining ERα mRNA levels is easy. In addition, an ideal biomarker of a disease is released into the systemic circulation or another body fluid, where it can be detected in a blood-based assay or an assay of another accessible body fluid. Thus, we believe that the expression levels of ERα mRNA in PBCs can be used as an important biomarker for osteoporosis progression and therapy.

Lv et al. (2011) showed that the frequency of promoter hypermethylation of ERα in postmenopausal women was significantly higher than in menopausal women [19]. In addition, several reports showed that the genetic polymorphism of ERα is a risk factor for osteoporosis in postmenopausal women [20,21]. Both promoter hypermethylation and genetic polymorphism reduce ERα mRNA transcription. No previous study has focused on the expression levels of ERα mRNA in osteoporosis using human subjects. Our data from menopausal women indicate that the expression of ERα mRNA is decreased in PBLs from patients with osteoporosis as compared with patients without osteoporosis. The decreased ERα mRNA expression in PBCs in patients with menopausal osteoporosis could reflect either a predisposing factor or a consequence of the disease. Our data also showed that menopausal women with low ERα mRNA expression in PBLs (>250.39 copies/μg) had an approximately 31.250-fold risk of osteoporosis as compared with women with high ERα expression (>250.39 copies/μg). ERα mRNA expression in PBCs could be used as a biomarker and indicate that estrogen has therapeutic benefits in menopausal women.

Selective estrogen receptor modulators (SERMs) are a class of compounds that interact with intracellular ERs in target organs as estrogen agonists and antagonists [22]. They include chemically diverse molecules that lack the steroid structure of estrogens but possess a tertiary structure that allows them to bind to ERα and/or ERβ [23]. In this study, we demonstrated that ERα mRNA levels in PBCs were an important risk factor for osteoporosis. Therefore, we suggested that ERα mRNA levels in PBCs may use to predict the therapeutic response of SERMs.

5. Conclusion

In conclusion, our findings support the view that both estrogen and ERα mRNA levels play a role in human postmenopausal osteoporosis. Abundance of residual ERα mRNA leading to effective utilization of estrogen E2 might be important in slowing osteoporosis progression. New approaches to the development of anti-bone-resorptive medicine, particularly those designed to increase the expression of ERα, should be studied further. Genetic analyses of ERα gene polymorphisms may predict which patients are at a higher or lower risk of postmenopausal osteoporosis. The evaluation of ERα mRNA expression in PBCs could help to improve the assessment of fracture risk.

Contribution of authors

Cheng YW and Tsai LH designed the study and wrote the paper; Cheng YW, Chou CW, and Chiang TI, conceived the experiments, wrote the paper, and prepared the figures; Chou CW, Chang IC, and Chiang TI, collected the colorectal tumor samples. All authors gave final approval for the manuscript to be submitted for publication. Chou CW and Chang IC contributed equally to this work.

Conflicts of interest and funding

This work was supported by grants from the National Science Council (100-2314-B-040-012) and Health and welfare surcharge of tobacco products (MOHW105-TDU-B-212-134001) of Taiwan.

Transparency document

The Transparency document associated with this article can be found, in online version.

References


