LEU432VAL POLYMORPHISM IN CYP1B1 GENE AND ASSOCIATION WITH OSTEOPOROSIS IN POSTMENOPAUSAL WOMEN

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Abstract
Osteoporosis is a multifactorial disease of bones that leads to an increased risk of fracture. Sex steroids are important regulators of bone mass, and genes regulating sex steroid production and metabolism are obvious as candidate genes for osteoporosis susceptibility. Estrogens are crucial to bone health as demonstrated by the loss that follows after menopause. The analysis of biochemical markers has proved useful in the diagnosis of different disorders of bone and especially in the prognosis of postmenopausal osteoporosis and osteoporotic fractures. The purpose of this study was to determine whether Leu432Val polymorphism in the CYP1B1 gene is present also in Slovak population and subsequently, if it is associated with osteoformation markers - alkaline phosphatase (ALP), osteocalcin (OC). We examined associations between Leu432Val polymorphism of mentioned gene and bone remodeling markers in 338 women. We found that this polymorphism is present in Slovak population and the genotype frequencies were 34.6 % for CC, 46.7 % for CG and 18.6 % for GG. We found nonsignificant association between CYP1B1 genotype and bone turnover markers.

Key words: Osteoporosis, CYP1B1 gene, Polymorphism, Bone remodeling markers

1 Introduction
Bone is a dynamic tissue that provides mechanical support, physical protection, and enables movement. Bone also serves as a storage site for minerals and is where blood cells are produced (Rho et al., 2004). Bone, similar to other tissues, undergoes constant renewal as a dynamic process of continual resorption and formation throughout the life. Osteoporosis is characterized by an imbalance between these processes, bone resorption and formation (Stančíková et al., 1997). Osteoporosis is the most prevalent metabolic bone disease and a major clinical and public health problem. Heredity plays an important and well-established role in determining the lifetime risk of this disease (Zmuda et al., 2006). One of the major applications of bone turnover markers in the field of osteoporosis is to detect high bone turnover in postmenopausal women. This could allow identification of individuals who should be preferentially targeted for prevention or curative therapy, as there is strong evidence that the higher bone turnover, the greater future bone loss and/or fracture risk (Garnero et al., 1996). Markers of bone formation include bone alkaline phosphatase, osteocalcin, and the C- and N-terminal propeptides of type I collagen. Bone resorption markers include breakdown products of type I collagen such as pyridinium crosslinks (pyridinoline [PYR], deoxypyridinoline [D-PYR]) and the C- and N-telopeptides of type I collagen (CTx and NTx) (Bonnick and Shulman, 2006).

Estrogens exert diverse biological effects such as female sexual differentiation and development, arterial vasodilatation, neuroprotective actions and maintenance of bone density (Tsuchiya et al., 2005). Estrogen deficiency remains the most important risk factor for bone loss (Barrett-Connor et al., 2000). Since ovarian activity is minimal after menopause, most circulating estrogen in postmenopausal women is derived from the aromatization of androstenedione to estrone, which can be reversibly oxidized to estradiol (Napoli et al., 2009). Estrogen is metabolized by the CYP450 group of enzymes namely, CYP1A1, CYP1A2, CYP1B1 and CYP3A4, each encoded by a different gene (Badawi et al., 2001).
Cytochrome P4501B1 (CYP1B1) is important estrogen-metabolizing enzyme (Wen et al., 2005) and has been implicated in women’s health conditions, including lower femoral bone mineral density (De Vivo et al., 2002). CYP1B1 is a major enzyme catalyzing the formation of 4-hydroxyestradiol, a catechol estrogen metabolite with significant estrogenic activity (Hayes et al., 1996). The CYP1B1 gene is located in chromosome 2p21-p22 and contains three exons (Tang et al., 1996). In the Leu432Val polymorphism is Leucine (Leu) substituted for the nonpolar, hydrophobic amino acid valine (Val) at amino acid position 432 (Miyoshi and Noguchi, 2003).

The aim of the study was to determine whether Leu432Val polymorphism in the CYP1B1 gene is present also in Slovak population and subsequently, if it is associated with bone remodeling markers - alkaline phosphatase (ALP), osteocalcin (OC).

2 Material and Methods

The study sample consisted of 338 healthy and postmenopausal women (63.4 ±7.5 years). Subjects were selected according to strict inclusion criteria.

Genomic DNA was extracted from leukocytes, blood samples (300 µl) collected in ethylenediamine tetraacetate (EDTA) by a standard phenol-chloroform extraction procedure and by using the Simax Genomic DNA Extraction kit (SBS Genetech, China).

Genotypes were detected using polymerase chain reaction (PCR) followed by RFLP method. The PCR was performed using primers by Wen et al. (2005). After initial denaturation at 95 °C for 5 minutes followed 35 cycles, each consisting of denaturation at 94 °C for 45 seconds, annealing at 60 °C for 45 seconds, and extension at 72 °C for 45 seconds. The PCR was completed by a final extension cycle at 72 °C for 7 minutes.

Each PCR product (20 µl) was digested with AcuI restriction enzyme (New England Biolabs, Inc.) at 37 °C for night.

The DNA fragments were then separated and visualized by electrophoresis on 4 % agarose gel containing ethidium bromide.

The differences between the genotypes were analysed by GLM procedure and covariance analysis after correction of the measurements for age and BMI. Standard $\chi^2$ statistics was used for determining whether CYP1B1 genotype and allele frequencies were in Hardy-Weinberg equilibrium. Statistical significance level was set at $P<0.05$. For data processing and statistical evaluation we used Statistica 4.3 (1993), GraphPad (2005) and SPSS v. 8.0 (1997). All procedures were approved by the Ethical Committee of the Specialized Hospital of St. Svorad in Nitra.

3 Results and discussion

3.1 CYP1B1 genotypes detection

To study the genotypes we first amplified a region covering the polymorphic site. Representative results of PCR-RFLP are shown in Figure 1. In the case, that the AcuI enzyme recognizes specific DNA sequence on one allele (CG genotype) the fragments 294 bp, 187 bp and 107 bp in length are produced. If the enzyme recognizes specific sites on both alleles (CC genotype), fragments of size 187 bp, 107 bp are present. If the enzyme has no recognition site (GG genotype), there is one unchanged 294 bp long fragment after the PCR product digestion.
3.2 Allele and genotype frequencies of the CYP1B1 genetic polymorphism

We found that CYP1B1/Leu432Val polymorphism is present in Slovak population. The distributions of the genotypes and the alleles of the studied polymorphism are summarized in Table 1. In the analyzed population CG genotype (46.7 %) was the most frequent, the least frequent was GG genotype (18.6 %). The frequency of C allele for Leu432Val was 0.58 and of G the frequency allele was 0.42. The $\chi^2$ test showed that the genotype distributions were in agreement with Hardy-Weinberg equilibrium.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Frequencies</th>
<th>Percent</th>
<th>$\chi^2$</th>
<th>Allele frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>117</td>
<td>34.6</td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>CG</td>
<td>158</td>
<td>46.7</td>
<td>0.6471</td>
<td>0.58</td>
</tr>
<tr>
<td>GG</td>
<td>63</td>
<td>18.6</td>
<td></td>
<td>G</td>
</tr>
</tbody>
</table>

Our findings are supported by the study of Salinas-Sánchez et al. (2011). In their study, the most common was CG genotype (42.2 %), less CC genotype (38.3 %) and at least was GG genotype (19.5 %). Similar genotypes distribution was found in study of Napoli et al. (2009): CG genotype (52.3 %), CC genotype (28.3 %), and GG genotype (19.4 %). Wen et al. (2005) observed that in the CYP1B1 gene, contrary to our findings, the most frequent genotype (75.5 %) was CC followed by CG (22.3 %) and GG (2.2 %) genotypes.

3.3 Association analyzes of the CYP1B1 genetic polymorphism with bone remodeling markers

We did not find statistically significant associations between genotypes of Leu432Val polymorphism in CYP1B1 gene and bone remodeling markers.

As seen in Figure 2, the GG genotype had insignificantly highest mean concentration (0.604 ± 0.085 µkat/l) of ALP, lower one was found in CC genotype (0.583 ± 0.062 µkat/l) and the CG genotype (0.545 ± 0.055 µkat/l) had the lowest mean concentration of this marker.
Fig. 2 The association of the CYP1B1 genotypes with ALP concentration

The association between genotypes and marker of bone formation - osteocalcin is shown in Figure 3. Similar to the results for ALP the differences were not significant. The highest mean concentration was seen in CG genotype (3.886 ± 0.082 µg/l), lower mean concentration was in CC genotype (3.880 ± 0.094 µg/l) and the lowest in GG genotype (3.805 ± 0.128 µg/l).

Fig. 3 The association of the CYP1B1 genotypes with OC concentration

The Leu432Val polymorphism of CYP1B1 gene has not been analyzed as a candidate one for bone remodeling process intensity yet, so our results seem to be primary ones. We can propose that including further remodeling markers (CTx – marker of osteoresorption) into the analyses have a potential to bring more accurate results maybe with a manifestation of significant differences.

The CYP1B1 gene has been analyzed in an association with other osteoporosis – related traits. Results from study of Napoli et al. (2009) indicate that the Leu432Val polymorphism of the CYP1B1 gene may represent as one of the determinants of postmenopausal BMD through its effect on estrogen metabolism certain racial groups, in this particular study, American women.

Among the women who experienced natural menopause, the three non-synonymous SNPs were significantly associated with menopausal age, years of menstruation, and total number of menstrual cycles. The Gly and Ser alleles of Arg48Gly and Ala119Ser were associated with later menopause, more years of menstruation and more menstrual cycles, while women with allele Val at Leu432Val had a 0.9 year earlier menopause, 1.0 year shorter reproductive span, and 12.6 fewer menstrual cycles than those women without this allele (Long et al., 2006). From the literature we can also mention some studies of other polymorphisms in CYP1B1 gene as candidate ones for the other disease.
Homozygous subjects with Ala in Ala119Ser polymorphism had twice the risk of renal cell carcinoma as homozygous for Ser or heterozygotes (Salinas-Sánchez et al., 2011).

Kocabas et al. (2002) reported that carriers of Val allele (Val/Leu + Val/Val) of Leu432Val polymorphism in Turkish women had a higher risk of breast cancer than those with the Leu/Leu genotype.

Rylander-Rudqvist et al. (2004) investigated the association of CYP1B1 genotype and endometrial cancer risk in a population-based case-control study of postmenopausal women. They found no evidence for this association.

4 Conclusion
In this population-based study of CYP1B1 genotypes in association with bone remodeling markers (ALP, OC), we found no overall association among analyzed postmenopausal women. We can propose that including further remodeling markers (CTx – marker of osteoresorption) into the analyses have a potential to bring more accurate results maybe with a manifestation of significant differences. The results of this study can be used within the screening of risk individuals in term of osteoporosis.

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5 References


