Polymorphism of Cyp1a1 (T6235C) is not a significant risk factor of osteoporosis in postmenopausal Indonesian woman

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Abstract. Osteoporosis is an increasingly common disease resulting in reduced bone mineral density (BMD) and elevated likelihood of bone fracture, and particularly affected are postmenopausal women with additional risk factors including genetic predisposition. The CYP1A1, is one of the candidate genes that have been suggested to be associated with the pathogenesis of osteoporosis. This work aimed to evaluate the distribution of a selected polymorphism of this gene (T6235C) with respect to the BMD status in postmenopausal Indonesian women. The results show that osteoporosis is associated with age and menopause, as expected, but not with the tested polymorphism of CYP1A1 in the Indonesian sample population. It is suggested that other P450 cytochrome enzymes and their polymorphisms could provide more significant indicators of the future health of postmenopausal women.

1. Introduction

Osteoporosis is an increasingly common silent disease of the bone, characterized by reduced bone mineral density (BMD), destruction of bone tissue and elevated risk to fracture. There is no known cure, only ways to slow it down [1]. At greatest risk are elderly people over 50, and women are five times more likely to suffer it than men [2]. In Indonesia, 15.5 million women over the age of 50 had osteoporosis in 2005, and this is predicted to increase to 24 million in 2015 [3].

Many factors can influence the risk of osteoporosis, including diet, physical activity, medication, and co-existing disease; however, one of the most important clinical risk factor is thought to be a positive family history, emphasizing the importance of genetics in the pathogenesis of osteoporosis [4-7]. One of the candidate genes associated with osteoporosis is the CYP1A1 gene that resides in the chromosome 15 [8].

Polymorphism of CYP1A1 is thought to play a role in estrogen metabolism. Estrogen is metabolized predominantly via two competing pathways, the 2-hydroxyl (nonestrogenic) and the 16alpha-hydroxyl (estrogenic) pathways. Studies have indicated that these pathways are important determinants of bone mineral density (BMD) in postmenopausal women. Women with predominant metabolism through the 2-hydroxyl pathway have accelerated postmenopausal bone loss and lower BMD compared to those with predominant 16alpha-hydroxylation pathway who are protected from...
bone loss. Increased 2-hydroxylation has been observed in women with a positive family history of osteoporosis suggesting that the increased risk of osteoporosis may be partly related to inherited differences in estrogen metabolism. Polymorphisms in the cytochrome P450 (CYP450) enzymes that metabolize estrogen are believed to result in alteration in the activity of these enzymes leading to differences in estrogen hydroxylation. It is the resulting "estrogen tone" generated from the variable accumulation of metabolic products with divergent estrogenic activity that has been hypothesized to modify the risks for hormone-dependent disorders associated with these polymorphisms, for example, osteoporosis [9-18]. This work aimed to evaluate the distribution of selected polymorphism of CYP1A1 gene (T6235C) with respect to the BMD status in postmenopausal Indonesian women.

2. Materials and Methods
2.1. Study population
In total, 190 consenting postmenopausal Indonesian women were included in the study, with an age range of 40-70 years. The ethical clearance was granted by the Ethical Committee of the Faculty of Dentistry, University of Indonesia. To survey the genotype-phenotype variation related to the T6235 (rs4646903) polymorphism of CYP1A1, the polymorphism status was determined from DNA samples isolated from peripheral blood stored in a freezer at -20°C after collection.

Bone mineral density (BMD) of the subjects was measured from calcaneus by using Sonost 3000 ultrasonic device. Osteoporosis was assumed for BMD less than 2.5 standard deviations below mean of young adult reference, i.e. with T-score < -2.5. Normal status was assumed for T-score ≥ -1, and osteopenia for -1 > T-score ≥ -2.5. With these criteria and by grouping subjects with indicated osteoporosis and osteopenia together (as "osteoporosis" below) resulted in 130 cases (68.4%) of osteoporosis and 60 cases (31.6%) of normal (control) subjects.

2.2. Genotype analysis
The DNA samples were evaluated for polymorphism of CYP1A1 (T6235C) by PCR-RFLP, using the forward primer 5’-CAGTGAAGAGGTGTAGCCGCT-3’ and reverse primer 5’-TAGGAGTCTTGTCTCATGCCT-3’, with the expected PCR product of 340 bp in size. The PCR reaction was performed using an initial denaturation at 94°C for 5 min (1 cycle), and then 35 cycles consisting of denaturation at 94°C for 5 s, annealing at 53°C for 30 s and extension at 72°C for 30 s. The final extension was done at 72°C for 7 min, and the product was stored at 4°C. The polymorphism status was analyzed by RFLP using the restriction enzymes MSPI (5’…CCGG…3’ and 3’…GGCC…5’) for cutting at the sites of polymorphism. The results were incubated at 37°C for 6 hours and enzyme inactivated at 65°C for 20 minutes. Fragments were separated by electrophoresis on 3% agarose gel added with 2 μL GelRed™ Nucleic Acid Gel Stain (10000X in water) and visualized with GelDoc. An undigested 340 bp product is shown for the TT genotype, fragments of 200 and 140 bp for the CC genotype, and three bands of 340, 200 and 140 bp for the TC genotype.

3. Results
The PCR product consistently indicated the expected 340 bp band in electrophoresis with 50 bp ladder shown in Figure 1. Figure 2 shows an example of the RFLP results after cutting with MSPI and subjecting the fragments to electrophoresis.

In Figure 2, the samples 1 and 2 represent the homozygous wildtype or TT genotype as indicated by a single band at 340 bp. Heterozygous genotype TC in sample number 3 shows three bands at 340 bp, 200 bp and 140 bp, and the homozygous mutant genotype CC genotype in the samples 4 and 5 is indicated by two bands at 200 bp and 140 bp. The observed distribution of CYP1A1 genotypes is shown in Table 1, and the corresponding distribution of alleles in Table 2. Chi-square testing showed no significant association of the genotype or allele frequency with the status of osteoporosis in the studied population (p > 0.05).
Figure 1. PCR product electrophoresis results, indicating a single band of 340 bp

Figure 2. RFLP products after electrophoresis of the fragments

Table 1. CYP1A1 genotype and allele frequencies in postmenopausal Indonesian women

| Group       | Genotype | CC      | TC      | TT      | Total |
|-------------|----------|---------|---------|---------|-------|
|             |          | N       | %       | N       | %     | N     | %     | N     | %     |
| Control     | CC       | 4       | 6.67    | 37      | 61.67 | 19    | 31.66 | 60    |
| Osteoporosis| TC       | 14      | 10.77   | 77      | 59.23 | 39    | 30.00 | 130   |
| Total       | TT       | 18      | 9.47    | 114     | 62.11 | 58    | 28.42 | 190   |

Table 2. CYP1A1 allele frequency in postmenopausal women

| Groups       | Allele | C      | T      |
|--------------|--------|--------|--------|
|              |        | N      | %      | N      | %      |
| Normal       | 45     | 37.5   | 75     | 57.5   |
| Osteoporosis | 105    | 40.4   | 155    | 59.6   |
| Total        | 150    | 39.5   | 230    | 60.5   |
4. Discussion
It has been previously reported that the same polymorphism (T6235C) of CYP1A1 is significantly associated with increased transcript half-life and therefore increased enzyme activity resulting in an elevated level of activated metabolites of estrogen. This is not unexpected assuming that hydroxylation of estrogen is driven by CYP1A1 (Figure 3) [19].

Nevertheless, the results of the present work showed no association of this polymorphism with osteoporosis (BMD) status of postmenopausal Indonesian women. The results are in agreement with those of Quan et al (2009) who reported that of seven tested SNPs of CYP1A1 and CYP1B1 genes in postmenopausal Japanese women on hormone therapy (HT), only the polymorphism L432V of CYP1B1 was significantly associated with BMD; in particular, the GG genotype showed reduced HT response in terms of BMD and low-density lipoprotein cholesterol [20]. In total, more than 100 genes have been considered as candidates for osteoporosis-related association, based on studies on selected SNPs of one or few genes, or more recently on genome-wide studies (GWS) [19,21-23].

Although the sensitivity of GWS work may be sometimes lower than that of testing the status of single SNPs, in general, the conclusion has been the same as from the results of the present work on T6235 (rs4646903) polymorphism: there seems to be no significant association between the status of CYP1A1 and osteoporosis, and this also appears to apply to postmenopausal Indonesian women. CYP1A1 is generally well conserved in vertebrates and apparently exhibiting relatively little variation in function and homeostatic activity [24]. However, with a network of regulatory proteins (Figure 4), CYP1A1 can mediate important adverse responses to the environment, for example on exposure to
pollutants such as polyaromatic hydrocarbons (PAH), dioxins, furans, organotins and other toxic compounds that can interfere at low concentrations with bone development and differentiation [25]. Such effects are often due to the xenobiotic metabolism induced by CYP1A1 in liver, producing potentially toxic, carcinogenic, and possibly synergistic intermediary products [26]. Exposure to PAH or dioxins can result in strong expression of the metabolizing cytochrome liver enzymes that apart from the detoxifying clearance of harmful compounds also contribute to the carcinogen-mediated DNA damage [27-29]. It is in these processes that CYP1A1 polymorphisms, and those of other cytochrome enzymes, have been suggested to be significantly involved [30-32]. Considering the observations of the present work, it is suggested that other cytochrome enzymes than CYP1A1, and their polymorphisms, may offer more significant indications on the subject health for future study.

5. Conclusion
In conclusion, the results show that osteoporosis is associated with age and menopause, as expected, but not with the tested polymorphism of CYP1A1 in the Indonesian sample population. It is suggested that other P450 cytochrome enzymes and their polymorphisms could provide more significant indicators of the future health of postmenopausal women.

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