The *CYP3A4*18* Genotype in the Cytochrome P450 3A4 Gene, a Rapid Metabolizer of Sex Steroids, Is Associated With Low Bone Mineral Density

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Osteoporosis is influenced by genetic factors. The interindividual variability in the activity of CYP3A, the metabolic enzyme of sex hormones, may result from genetic polymorphisms. In a study of 2,178 women of ages 40–79 years, the presence of the *CYP3A4*18* variant was found to be significantly associated with low bone mass. *In vitro* functional analyses indicate that *CYP3A4*18* is a gain-of-function mutation in sex steroid metabolism, resulting in rapid oxidation of estrogens and testosterone; *in vivo* pharmacokinetics using midazolam (MDZ) verify the altered activity of the CYP3A4*18, showing lower metabolic turnover in the mutant than in the wild type. Molecular modeling reveals the structural changes in the substrate recognition sites of CYP3A4*18 that can cause changes in enzymatic activity and that potentially account for the difference between the catalytic activities of estrogen and MDZ, depending on the genotype. The results indicate that a genetic variation in the *CYP3A4* gene—as a gain-of-function mutation in the metabolism of certain CYP3A substrates, including sex steroids—may predispose individuals to osteoporosis.

Osteoporosis is a multifactorial disease with a strong genetic component. Genetic factors influence bone mass, bone size, bone quality, and bone turnover, and they may modulate the risk of osteoporosis.[1] Many candidate genes have thus far been suggested, but none has yet been supported strongly and consistently by subsequent studies.

The members of the cytochrome P450 3A (CYP3A) subfamily are the major enzymes in the nicotinamide adenine dinucleotide phosphate-oxidase–dependent oxidative metabolism of various endogenous and exogenous compounds, including sex hormones. A wide interindividual variability in the expression and catalytic activity of CYP3A has been reported in the general population.[2] The interindividual variation, exceeding 30-fold in some populations, may influence the circulating levels of endogenous sex steroids and thereby mediate the risk of certain estrogen-associated diseases such as osteoporosis.[3–5] This variation is, at least partly, caused by multiple environmental factors, including induction by drugs, chemicals, and endogenous compounds, but genetic factors are also among the most plausible mechanisms.

The CYP3A activity of the adult human liver is the sum activity of at least two CYP3A family members: CYP3A4 and CYP3A5. To date, approximately 40 allelic variants in the *CYP3A4* gene have been reported as showing marked ethnic differences in allele frequencies.[6,7] CYP3A5, the second-most important CYP3A protein in the liver, has characteristic polymorphic expression caused by genetic variation; certain genetic variations, such as *CYP3A5*3* and *CYP3A5*6*, give rise to an aberrantly spliced mRNA with a premature stop codon, which produces a nonfunctioning protein.[8,9]

We therefore hypothesized that genetic variations of CYP3A proteins, the important metabolizing enzymes of estrogen, might be among the major determinants in the development of osteoporosis. To identify the candidate genetic variations in the *CYP3A4* gene, we sequenced the entire coding region and performed detailed structural and functional studies, including

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Received 19 May 2008; accepted 11 September 2008; advance online publication 19 November 2008. doi:10.1038/clpt.2008.215

Table 1 Clinical characteristics and bone density data of the subjects ($n = 2,178$)

	CYP3A4			*CYP3A5*			
	*CYP3A4*1*	*CYP3A4*18*	*P* value	**1/*1*	**1/*3*	**3/*3*	*P* value
Number	2,125	53		80	733	1,365	
Age (years)	54.0 ± 6.4	54.7 ± 7.1	0.435	54.6 ± 7.2	54.3 ± 6.6	53.9 ± 6.3	0.279
Weight (kg)	57.1 ± 7.1	56.1 ± 6.6	0.271	56.1 ± 7.1	56.9 ± 7.2	57.3 ± 7.0	0.148
Height (cm)	155.8 ± 5.0	156.6 ± 3.6	0.214	155.6 ± 4.4	155.9 ± 5.0	155.7 ± 5.0	0.671
BMI (kg/m^2)	23.5 ± 2.7	22.9 ± 2.6	0.061	23.2 ± 2.8	23.4 ± 2.7	23.6 ± 2.6	0.064
Age at menopause (years)	48.6 ± 3.7	49.5 ± 4.2	0.088	47.8 ± 3.7	48.6 ± 3.8	48.7 ± 3.6	0.104
YSM (years)	5.7 ± 6.0	5.4 ± 6.0	0.677	6.5 ± 7.2	6.0 ± 6.1	5.5 ± 6.0	0.177
Spine BMD (g/cm^2)	0.894 ± 0.142	0.834 ± 0.136	0.003 (0.014)[a]	0.886 ± 0.147	0.888 ± 0.140	0.896 ± 0.144	0.394 (0.833)[b]

Values are means ± SD.

BMD, bone mineral density; BMI, body mass index; YSM, years since menopause.

[a]*P* value adjusting for age, BMI, and *CYP3A5*. [b]*P* value adjusting for age, BMI, and *CYP3A4*.

both *in vitro* and *in vivo* analyses for candidate genotypes. We also assessed the *CYP3A5*3* alleles that are known to contribute to the reduction of CYP3A5 activity. We analyzed the association of *CYP3A* genotypes with bone mineral density (BMD) in Korean women.

RESULTS

CYP3A4 and *CYP3A5* genotyping analysis

A screening search for base changes in all 13 exons in the *CYP3A4* gene in 225 Koreans identified two mutations: a T → C point mutation (L293P) at codon 293, named *CYP3A4*18*, and a silent mutation (L295L) at codon 295. Among 2,178 Korean women of ages ranging from 40 to 79 years, *CYP3A4*18* was detected in 53 women, with an allelic frequency of 1.2%: 2,125 were wild type (WT), 52 were heterozygotes, and 1 was a homozygote. For *CYP3A5*, the genotyping revealed that 62.7% of the subjects were the *CYP3A5*3/*3* genotype and the allelic frequency of *CYP3A5*3* was 79.5%. Significant linkage disequilibrium was shown between the two genotypes in our study ($D' = 0.80$, $P < 0.001$), as presented in the previous data.[10]

CYP3A4 polymorphism and BMD

The clinical characteristics of the study groups in relation to *CYP3A4* and *CYP3A5* genotypes are listed in Table 1. The groups were all balanced with regard to clinical variables. Subjects with the *CYP3A4*18* genotype were significantly associated with low lumbar spine BMD after adjusting for age, body mass index (BMI), and *CYP3A5* genotype ($P = 0.014$). However, in analyses of BMD according to *CYP3A5* genotype, no BMD difference was observed in women with deficient CYP3A5 activity as compared to those with whole CYP3A5 activity, thereby indicating that CYP3A5 has no significant influence on bone metabolism (Table 1). Subsequent analyses to assess the differences between the *CYP3A4* genotypes, adjusted for age, years since menopause, BMI, or *CYP3A5* genotype, did not elucidate any significant interaction effects between the *CYP3A4* genotype and each covariate. The relationship between lumbar spine BMD and years since menopause in subjects with the *CYP3A4* genotype is shown in Figure 1a. No significant differences in slope were found between genotypes. To assess the femoral BMD, we analyzed 1,353 subjects. Their BMD was measured by dual-energy X-ray absorptiometry using the QDR-2000 (Figure 1b). Adjusted BMD for the total hip was significantly lower in *CYP3A4*18* subjects than in *CYP3A4*1* subjects ($P = 0.027$). We also observed a tendency

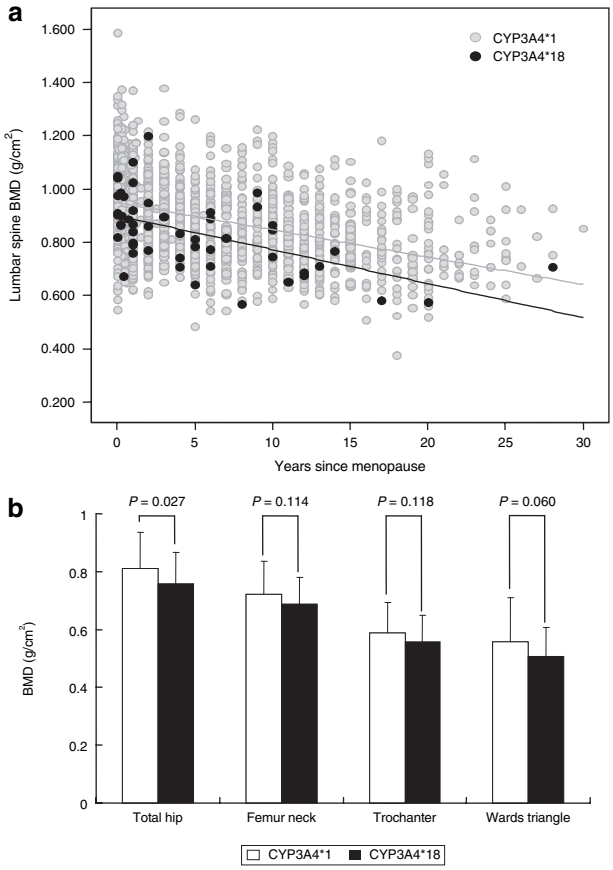

Figure 1 The association of *CYP3A4* genotype with lumbar and femur bone mineral density (BMD). (**a**) Relationship of lumbar spine BMD to years since menopause (YSM), relative to *CYP3A4* genotype, in 2,178 women of ages 40–79 years. The slope relating to the *CYP3A4*18* genotype did not differ from that of the *CYP3A4*1* (wild type) genotype ($P = 0.437$). YSM values <6 months were arbitrarily set to YSM = 0. (**b**) Comparison of femur BMD by *CYP3A4* genotype in 1,353 women in whom femur BMD measurements were performed using a QDR-2000. BMD comparisons were adjusted for age, body mass index, and *CYP3A5* genotype.

toward association of *CYP3A4*18* with a low adjusted BMD at the femoral neck, trochanter, and Ward's triangle; however, this tendency did not reach statistical significance.

In vitro functional analysis of CYP3A4 enzyme activity

To compare CYP3A4 enzyme activity for metabolism of sex hormones *in vitro*, we obtained both types of recombinant enzymes using CYP3A4*1 or CYP3A4*18 prepared in a baculovirus system. Western blotting and CO spectra indicated that both were present in the form of their holoproteins (data not shown). The levels of the individual metabolites (16α-OHE_1, 2-OHE_1, and 4-OHE_1) in the oxidations of estrone (E_1) tended to be higher with the mutant enzyme, CYP3A4*18, than with the CYP3A4*1 enzyme (**Table 2**). The oxidative metabolite of testosterone, 6β-hydroxytestosterone, was present in significantly higher quantities by the CYP3A4*18 enzyme (**Table 2**). These results suggest that CYP3A4*18 has a higher catalytic efficiency for both estrogen and testosterone metabolism than the CYP3A4 WT enzyme does.

In vivo pharmacogenetics for *CYP3A4* polymorphism

Midazolam (MDZ) is the drug that is most widely used as an *in vivo* probe for phenotyping CYP3A activity.[11,12] We carried out a pharmacokinetic test to compare MDZ metabolic activity in 13 *CYP3A4*18*$^+$ subjects and 26 *CYP3A4*1*$^+$ subjects, each matched for age, sex, BMI, and *CYP3A5* genotype (**Figure 2a** and **b**). The concentration–time plot revealed that the mean plasma concentrations of MDZ after a 7.5 mg oral administration of MDZ tended to be lower in subjects with the *CYP3A5*3* genotype, but that this difference was not statistically significant (data not shown). On the other hand, plasma concentrations were significantly higher in *CYP3A4*18*$^+$ subjects. The *CYP3A4*18*$^+$ subjects showed diminished MDZ clearance and increased MDZ area under the plasma concentration curve as compared to *CYP3A4*1*$^+$ subjects ($P = 0.029$, $P < 0.001$, respectively), thereby suggesting that *CYP3A4*18* is associated with decreased catalytic activity for MDZ. To confirm the contradictory data resulting from the MDZ pharmacokinetic tests, we tested the *in vitro* MDZ kinetics with recombinant enzymes (**Figure 2c**). The maximum reaction velocity value for 1-OH MDZ in the case of CYP3A4*1 was greater than the maximum reaction velocity value for CYP3A4*18. These results were consistent with the results from *in vivo* pharmacokinetic tests: metabolic turnover for MDZ was significantly lower in CYP3A4*18 than in CYP3A4 WT.

Table 2 Estrone and testosterone hydroxylase activity with CYP3A4 constructs

	CYP3A4*1	CYP3A4*18	*P* value
Estrone			
16α-OHE_1	181.9 ± 4.9	232.9 ± 22.5	0.018
2-OHE_1	234.0 ± 40.0	318.0 ± 55.8	0.100
4-OHE_1	98.5 ± 16.4	126.5 ± 30.0	0.230
Testosterone			
6β-OHT	131.4 ± 1.8	140.1 ± 3.5	0.018

Values are means ± SD of three experiments. Concentration is expressed as ng/ml. Substrate concentrations were as follows: estrone 200 μmol/l and testosterone 250 μmol/l.

6β-OHT, 6β-hydroxytestosterone; E_1, estrone.

Molecular modeling construction for the CYP3A4 polymorphism

To investigate the structural behavior of the proteins, molecular modeling studies and molecular dynamic simulation were carried out, for the WT and CYP3A4*18 proteins. As shown in **Figure 3**, both proteins have centrally located hemes; however, there is a structural difference between the WT and

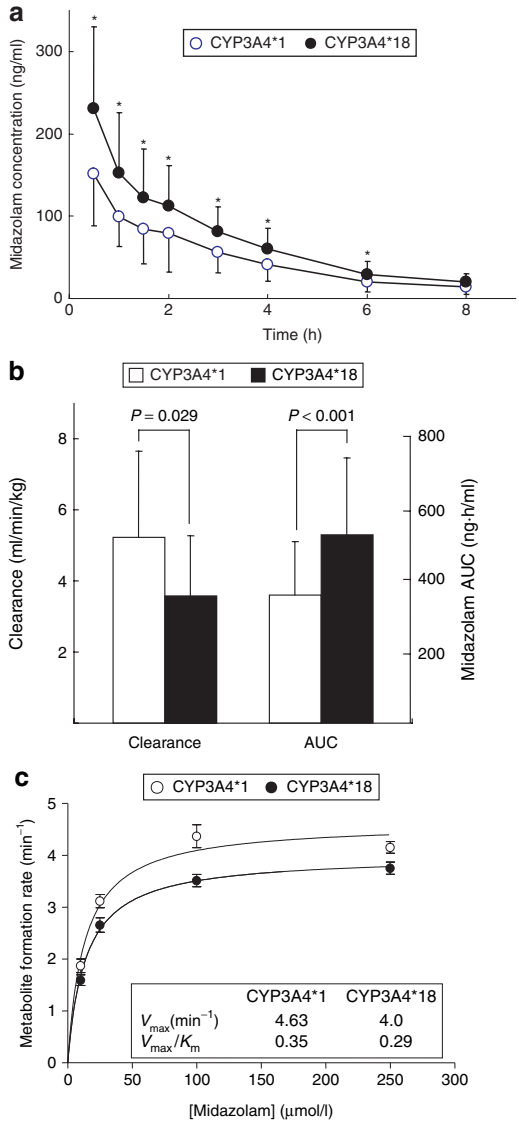

Figure 2 *In vivo* pharmacokinetics and *in vitro* enzyme kinetics to assess the differences in midazolam (MDZ) oxidation relative to the *CYP3A4* genotype. (**a**) The mean MDZ plasma concentration and (**b**) MDZ clearance and area under the plasma concentration curve after a 7.5-mg oral dose of MDZ indicated that CYP3A4*18 is associated with decreased metabolism of MDZ as compared to CYP3A4*1. *$P < 0.05$, *P* values by linear mixed-effects model. (**c**) Kinetic characteristics of MDZ biotransformation *in vitro*. Kinetics of 1-hydroxy (OH) MDZ was determined using recombinant CYP3A4*1 and CYP3A4*18. Assays were performed at substrate concentrations between 10 and 250 μmol/l for MDZ. Data were analyzed using the Michaelis–Menten equation.

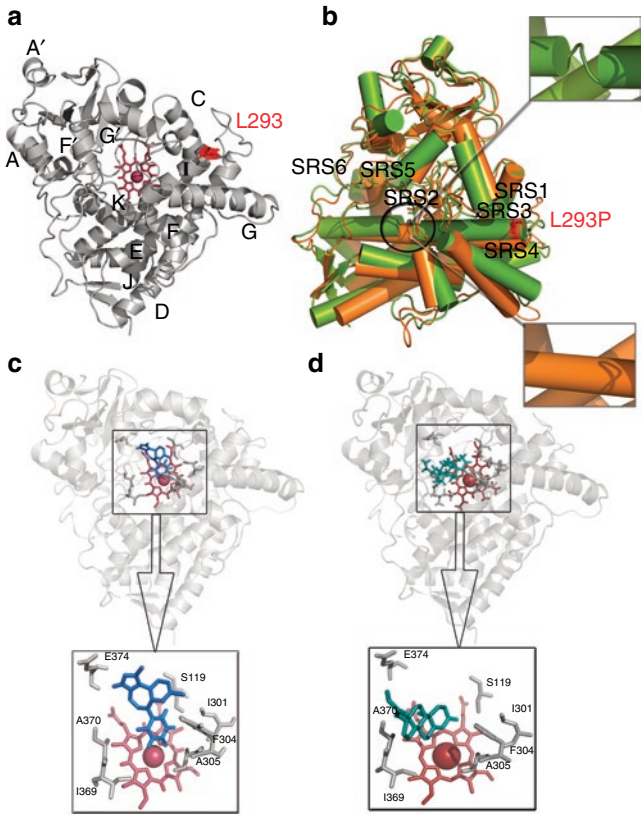

Figure 3 Molecular dynamic simulation and molecular docking results for CYP3A4*1 (wild type) and CYP3A4*18. (**a**) Secondary structural details of CYP3A4*1 (illustration in gray) where the heme is shown as ruby-colored sticks. (**b**) Snapshots taken from the simulations at 2 ns, CYP3A4*1 (orange) superimposed with CYP3A4*18 (green) (RMSD = 2.13Å). Comparison of the secondary structural changes on helix I between CYP3A4*1 (lower box) and CYP3A4*18 (upper box) are presented in an enlarged view in the small boxes. Docking mode of (**c**) midazolam (blue) and (**d**) testosterone (aqua green) in the CYP3A4*1 active site cavity. Residues are represented as gray sticks.

CYP3A4*18 proteins. Codon 293 is located at the start of the highly conserved helix I, which has been known to play an essential role in substrate specificity (**Figure 3a**).[12] The most important change in the L293P secondary structural elements was observed in the conserved helix I: a long, straight α helix in the WT was modified into two small α helices separated by a short loop in the final conformation of CYP3A4*18 (**Figure 3b**, small boxes). This change reduces stability, and the consequent fluctuation on the separated helices could lead to changes in the other substrate recognition sites (SRSs) through SRS4 of helix I. SRS3 and SRS1 were closer to SRS4 in the CYP3A4*18 than in the WT protein. Furthermore, the conformational shifts on the G and F helices observed in CYP3A4*18 could affect all the SRS regions except SRS6. To uncover the possible conformation of substrate–enzyme binding in the active site, molecular docking was carried out using GOLD, version 3.1.1 (CCDC Software Ltd., Cambridge, UK), with the MDZ and testosterone structures (**Figure 3c** and **d**). The active site pocket is mostly hydrophobic with neutral residues. One side of the substrate is brokered by nonpolar residues (I301, F304, A305, I369, and A370), and the other side is occupied by negative (E374) and polar (S119) residues. Although a more refined study using molecular dynamic simulation would be necessary to confirm this issue, our results showed that the docking mode of MDZ bonding to the active site is different from that of testosterone, suggesting compatibility with our enzyme kinetic studies.

DISCUSSION

This study shows that the *CYP3A4*18* variant of the *CYP3A4* gene is associated with low BMD in Korean women, causing the conformational changes in the SRS regions of the mutant protein that lead to change in enzymatic activity.

The major CYP3A family members expressed in the adult human liver, CYP3A4 and CYP3A5, were targeted in our study as candidate genes for osteoporosis. Large race-related differences in the *CYP3A4* gene have been reported. For instance, *CYP3A4*3* (M445T) and *CYP3A4*17* (F189S) are found only in Caucasians, *CYP3A4*15* (R162Q) is found only in Africans, and *CYP3A4*10* (D174H) is found in both Caucasians and Africans.[14,15] We have therefore screened all the exons of the *CYP3A4* gene in Koreans and identified two single-nucleotide polymorphisms: L293P and the silent mutation, L295L, which is a novel single-nucleotide polymorphism detected first in our study. *CYP3A4*18* has been identified only in some Asian populations, including Japanese and Chinese people, but not in Caucasians or African Americans. The certain mutations reported in Chinese or Japanese people, such as *CYP3A4*3* (M445T), *CYP3A4*16* (F189S), *CYP3A4*4* (I118V), *CYP3A4*5* (P218R), and *CYP3A4*6* (A17776), were not identified in our screening.[10,16] These findings might be explained by the presence of ethnic differences even among Asians.

CYP3A4*18 has shown a significantly higher turnover activity for both testosterone and insecticide chlorpyrifos *in vitro*.[14] However, the functional significance of CYP3A4*18 in drug metabolism or disease pathogenesis has not been clarified *in vivo*. The data we present in this study suggest, for the first time in the field of osteoporosis genetics, that gene mutation possibly contributes to disease vulnerability, and that the *CYP3A4*18* genotype is one of the genetic risk factors for low bone mass. For CYP3A5, the second CYP3A family member in the adult human liver, we focused on the *CYP3A5*3* genotype because the *CYP3A5*3/*3* homozygote is common in Koreans[17] and is not able to make functioning CYP3A5 by alternative splicing. No BMD difference was observed between women with whole CYP3A5 activity and those with deficient CYP3A5 activity, thereby indicating that CYP3A5 has no significant influence on bone metabolism.

The molecular modeling strongly supports our hypothesis, showing the significant secondary structural change in CYP3A4*18. Surprisingly, change of a single amino acid, L293P, at the beginning of helix I has an influence on the overall protein structure and leads to the modification of the arrangement of SRS regions, the important sites for substrate recognition, and substrate access to the active site. Our *in vitro* and *in vivo* pharmacokinetic studies using a conventional probe drug, MDZ, also indicate that *CYP3A4*18* is a functional mutation.

In our *in vivo* pharmacokinetic study, subjects with *CYP3A4*18* exhibited lower enzyme activity in MDZ hydroxylation as compared to the *CYP3A4*1* group matched for the *CYP3A5* genotype. This result was confirmed by our *in vitro* enzyme kinetic assay with different doses of MDZ. The data obtained from both *in vitro* and *in vivo* experiments with MDZ are contradictory to ours and to previous data using sex steroids as substrates. Molecular docking studies with the WT structure showed that the pattern of MDZ bonding to the active site is different from that of testosterone. Therefore, structural changes and variable docking patterns detected in our modeling studies suggest that the conformational change in CYP3A4*18 may lead to the alteration of metabolic activity, depending on substrate types. CYP3A4*18, therefore, seems to be a two-faced mutation in metabolic activity: it acts as a rapid metabolizer of sex steroids, but, on the other hand, it is a poor metabolizer of some drugs, such as MDZ.

CYP3A4 is thought to be responsible for the phase I metabolism of numerous structurally diverse exogenous and endogenous molecules, including steroids, fatty acids, prostaglandins, and lipid-soluble vitamins.[18] As the critical role of estrogen in the maintenance of skeletal health has been well demonstrated, and the degree of estrogen exposure is a determining factor for bone mass,[19,20] we evaluated whether CYP3A4*18 could affect estrogen metabolism. Estrogens are metabolized to a large number of oxidated (2-, 4-, 6α-, 6β-, 12β-, 15α-, 16α-, and 16β-hydroxylated) metabolites, mainly by CYP3A enzymes.[21,22] CYP3A4 has a catalytic activity predominantly for the 2-hydroxylation (which is devoid of estrogen activity) rather than for the 4-hydroxylation of estrogen.[23,24] In our study, CYP3A4 had high catalytic activity for the oxidation of both estrogen and testosterone. Although it is difficult to obtain statistically significant changes in estrogen metabolism by CYP3A4*18 due to the small and multiple peaks of metabolites, the oxidation of major metabolites of estrone by CYP3A4*18 had a tendency to be greater than that associated with the WT protein. As previous data show,[14] the production of 6β-hydroxytestosterone by CYP3A4*18 from the metabolism of testosterone was significantly higher than that produced by CYP3A4*1.

Based on the data from our experiments, the plausible explanation for low BMD in *CYP3A4*18*$^+$ women might be that a gain-of-function mutation for sex steroids on codon 293 in the *CYP3A4* gene results in the rapid metabolic clearance of sex hormones, including estrogens, leading to a relative sex-hormone deficiency and consequent rapid bone turnover. To confirm our hypothesis more clearly, it would be helpful to assess the circulating estrogen concentrations, estrogen metabolites, or the skeletal responsiveness (such as bone turnover and BMD) to the hormone therapy, in relation to the various genotypes in a large population. Our study involving a less common genotype found only in Asians might have limitations in its clinical implications, but our pharmacogenetic approach focusing on metabolizing enzymes was able to identify one of the oligogenic determinants in osteoporosis. Searching for and grouping of oligogenic determinants in serial metabolic pathways of candidate molecules can be a powerful tool to predict future osteoporosis.

The genotype-specific differences in BMD were shown first in our study involving the CYP3A4*18 protein. The results of an additional search for the kinetics of metabolism and construction of enzyme structure suggest a possible mechanism to explain the effect of CYP3A4*18 on bone mass. This finding suggests that the *CYP3A4* polymorphism may be a predictor for osteoporosis in some Asian populations. Genetic markers in estrogen metabolism could be clinically useful for identifying subjects at risk for osteoporosis and also for estrogen-related diseases such as breast cancer.

METHODS

Subjects and measurement of BMD. The subjects in our study were a hospital-based series of 2,178 healthy women of ethnic Korean background who had visited the hospital for a general checkup between 1994 and 2004. The protocol for the study was approved by the Chell General Hospital Institutional Review Board, and informed consent was obtained from the participants. We enrolled women aged 40–79 years, and those who had a history of chronic medical disease or were taking medications that could affect bone and calcium metabolism were excluded from the study. Each patient was clinically examined, and routine biochemical tests were performed to exclude underlying diseases.

BMD at the lumbar spine (L2–L4) was measured by dual-energy X-ray absorptiometry using a QDR-2000 (Hologic, Bedford, MA), or XR-36 (Norland, Fort Atkinson, WI). The precision errors (the coefficients of variation for the *in vivo* BMD measurements) were 0.65 and 0.7%, respectively. The scores measured by XR-36 were converted to those of QDR-2000 in accordance with the conversion equation used in a previous study[25]: QDR-2000 = (0.876 × XR-36) + 0.124. Data were obtained in 1,353 women for BMD at the proximal femur, measured by dual-energy X-ray absorptiometry using the QDR-2000. The precision error was 1.2%.

DNA genotyping in *CYP3A* genes. To determine the presence of genetic variations in the *CYP3A4* gene, the 596 bp 5′-upstream and all 13 exons were amplified and sequenced in DNA from 225 randomly selected, unrelated Koreans. The identified single-nucleotide polymorphism, *CYP3A4*18* (L293P), was detected by PCR and the restriction fragment length polymorphism method, using forward primer (5′-TGATGCCCTACATTGAT CTGA-3′) and reverse primer (5′-GTGGTGAGGAGGCATTTTTG-3′) and restriction enzyme *Msp* I (NEB, Beverly, MA). On the basis of the published *CYP3A5* variant alleles, we developed specific PCR–restriction fragment length polymorphism tests for *CYP3A5*3* (6986A>G). The sequences of the primers are as follows: forward primer (5′-TGGCATAGGAGATACCCACG-3′) and reverse primer (5′-GT GGTCCAAACAGGGAAGAAATA-3′).

CYP3A4 enzyme activity *in vitro*. The human *CYP3A4*1* cDNA in the vector pUV1 was a generous gift from Dr Gonzales (National Cancer Institute, National Institutes of Health). A mutant containing *CYP3A4*18* was made using the GeneEditor *in vitro* site-directed mutagenesis kit (Promega, Madison, WI). Recombinant CYP3A4*1 and CYP3A4*18 were expressed using a baculovirus expression system purchased from Clontech Laboratories (Mountain View, CA). For western blot analysis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis was used to separate the recombinant proteins, after which the proteins were transferred onto nitrocellulose membranes. The membranes were incubated with anti-CYP3A4 primary antibody for 1 h at room temperature. An enhanced chemiluminescent kit (Pierce, Rockford, IL) was used for immunodetection. Enzyme content was monitored by means of the reduced CO spectrum, using a DW-2000 Spectrophotometer. Protein concentration was determined

by the Bradford method.[26] Next, we compared the catalytic activities of CYP3A4*1 and CYP3A4*18 for estrone, testosterone, and MDZ in accordance with previously described methods.[14] Metabolites were analyzed using a liquid chromatographic–tandem mass spectrometric system (API 2000; MDS Sciex, Concord, Ontario, Canada). Formation data for 1-hydroxy (OH)-MDZ for MDZ were fitted to a Michaelis–Menten model. All experiments were performed in triplicate.

***In vivo* pharmacokinetics using MDZ as a probe.** Thirteen subjects with the *CYP3A4*18* allele and 26 normal healthy volunteers matched for age (±2 years), sex, and *CYP3A5* genotype were enrolled in the *in vivo* pharmacokinetic study using MDZ as a phenotypic probe drug. After an overnight fast, all subjects received a single 7.5-mg oral dose of MDZ (time 0). Blood samples were obtained before the drug was administered and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, and 24 h afterward. Pharmacokinetic parameters included MDZ concentration in plasma, area under the plasma concentration curve, and total clearance. Weight-normalized oral MDZ clearance was calculated by dividing total clearance by body weight (in kilograms).

Protein structure preparation for molecular modeling and molecular dynamics simulations. Molecular modeling studies were used for investigating the structure of WT CYT3A4 and the effects of its mutation (CYP3A4*18) on structural stability. For this study, the three-dimensional coordinates of the CYP3A4 protein were obtained from the Protein Data Bank (PDB ID: 1TQN).[27] The X-ray crystal structure of the WT protein was resolved, and the missing part was recovered using the HOMOLOGY module in the INSIGHTII program (Insight II, version 2005.3L; Accelrys., San Diego, CA). The CYP3A4*18 mutation model was constructed by the INSIGHTII BIOPOLYMER module.

The molecular dynamic simulations were performed using GROMACS Simulation Software, version 3.3.1, (a web-based software available at http://www.gromacs.org/)[28] to study protein structural behavior in the polar environment. To compare the binding conformation of MDZ and testosterone, the study of the molecular docking of the substrates to the active site of the protein was carried out using GOLD, version 3.1.1. (CCDC Software Ltd., Cambridge, UK)[29]

Statistical analysis. The data were presented as mean values ± SD, and compared using Student's unpaired *t*- or Mann–Whitney *U*-tests and one-way analysis of variance or Kruskal–Wallis test as appropriate. Multiple linear regression analysis was used to adjust BMD for confounding factors such as age, years since menopause, BMI, and *CYP3A5* genotype. The interactions between genotype and several covariates, such as age, years since menopause, BMI, and *CYP3A5* genotype, were examined using linear regression analysis. Comparison of MDZ clearance and area under the plasma concentration curve between the CYP3A4*18 and CYP3A4*1 proteins was performed using a linear mixed-effects model. Underlying assumptions regarding linear regression and mixed-effects models were checked by residual plot, normal probability plot of the residuals, and the absolute residual plot, and no relevant violations were found. A P value <0.05 was considered to indicate a significant difference. Statistical analysis was performed using commercially available software, SPSS 11.0 for Windows (SPSS, Chicago, IL) and the Statistical Analysis System program, version 9.1 (SAS Institute, Cary, NC).

Linkage analysis between the *CYP3A4* and *CYP3A5* genotypes was performed using SNPstats, a web-based software available at http://bioinfo.iconcologia.net/SNPstats. Linkage disequilibrium results are presented as D′ and P values. We defined a D′ ≥ 0.70 as high linkage disequilibrium and a P value <0.05 as a significant value (e.g., the calculated D′ value is significant).[30]

ACKNOWLEDGMENTS

This study was supported by grants from the Korea Healthcare technology project, Ministry for Health, Welfare and Family Affairs, Republic of Korea (A080016), and the MOST/KOSEF for the Environmental Biotechnology National Core Research Center (R15-2003-012-02001-0).

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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