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The association between CYP 2C9 polymorphism and bone health

Kanitpong Phabphal [a,*], Alan Geater [b], Kitti Limapichat [a], Pornchai Sathirapanya [a], Suwanna Setthawatcharawanich [a], Rattana Leelawattana [c]

[a] Neurology Unit, Department of Internal Medicine, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand
[b] Epidemiology Unit, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand
[c] Endocrinology and Metabolism Unit, Department of Internal Medicine, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand

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ABSTRACT

Purpose: There is a strong scientific rationale to support the view that cytochrome P450 (CYP P450) enzyme-inducing AEDs induce bone loss in patients with epilepsy. However, no study has investigated the association between CYP 2C9 polymorphism and bone mineral density (BMD), 25-hydroxyvitamin D or parathyroid hormone levels in patients with epilepsy. This study sought to determine the association between BMD and CYP 2C9 polymorphism.
Methods: Ninety-three patients taking phenytoin as monotherapy were examined for CYP 2C9 polymorphism, vitamin D level and parathyroid hormone level and underwent basic chemistry testing. The bone mineral density of the lumbar spine and left femur were measured using dual-energy X-ray absorptiometry.
Results: The results indicated that about 18.3% of the patients with epilepsy were positive for CYP2C9*3. Furthermore, bone mineral density was associated with CYP 2C9 polymorphism epileptic patients. Specifically, patients with 2C9 polymorphism had higher *T*-scores and *Z*-scores of the femoral neck ($p = 0.02$ and 0.04, respectively), but not of the lumbar spine ($p = 0.27$ and 0.06, respectively). There was also a trend of having higher serum PTH levels and statistically significantly lower 25-hydroxyvitamin D levels in patients with wild type than in those compared with CYP 2C9 polymorphism ($p = 0.05$ and 0.03, respectively). Additionally, the patients with CYP 2C9 polymorphism had higher plasma levels of phenytoin, particularly when compared with those with wild type ($p = 0.01$). However, there was no association between serum levels of phenytoin and low BMD at femoral neck or lumbar spine.
Conclusion: CYP 2C9 polymorphism is associated with higher BMD, independent of plasma levels of phenytoin.

Previous studies of bone health in epilepsy patients have reported significant variability in the prevalence of low bone mineral density, ranging from 26% to 75% depending on ethnicity.[1–4] There is a strong scientific rationale to support the view that cytochrome P450 (CYP P450) enzyme-inducing AEDs increase the catabolism of the active forms of vitamin D to inactive metabolites. This, in turn, should lead to an increase in parathyroid hormone (PTH) levels, which is required for the body to convert more vitamin D into its active forms, and this increase in PTH would then cause an increase in bone turnover, with resultant bone loss over time.[5] Other possible mechanism in respect to the production of osteopenia by phenytoin could be the direct effects of local factors that regulate bone turnover.[6] Previous studies have reported an association between serum phenytoin level and BMD or the blood bone biomarkers.[7,8] Nakade et al. have reported direct evidence that low doses of phenytoin can stimulate the proliferation, differentiation, and maturation of osteoclasts in in vitro human craniofacial bone cells.[8] Moreover, Lau et al., who conducted a study on phenytoin-treated patients, found that serum levels of osteocalcin, skeletal alkaline phosphatase, and procollagen peptides were significantly elevated in phenytoin-treated patients compared to age-matched subjects; in each case these biochemical markers were significantly correlated with serum phenytoin level.[7]

Cytochrome P450 has been identified as a major catabolic enzyme for phenytoin; it converts phenytoin to its hydroxylated form in the liver. Recently, two single nucleotide polymorphisms (SNPs) in the coding region of CYP2C9 were identified in the most

* Corresponding author. Tel.: +66 74451452; fax: +66 74429385.
E-mail address: pkanitpo@medicine.psu.ac.th (K. Phabphal).

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important CYP superfamily.[7] The most common polymorphisms that have significant clinical importance are CYP2C9*2 and CYP2C9*3; CYP2C9*1 refers to the wild-type gene. The identified mutant alleles of CYP2C9, CYP2C9*2 and CYP2C9*3 have respectively a 70% and 3–5% enzymatic activity compared to the wild-type gene.[9] Additionally, these gene polymorphisms, especially 2C9*2 and 2C9*3, have been found to affect bone mineral density (BMD).

However, the previous studies were conducted on the facial bone[8] and measured only serum markers of osteogenesis, not bone mineral density.[6,7] To our knowledge, no studies focusing on the association between phenytoin level and bone mineral density at other sites, specifically the lumbar spine and femoral neck, and investigating their susceptibility to fracture, have been reported.[1–4] Furthermore, to date, no study has investigated the association between CYP 2C9 polymorphism and BMD or 25-hydroxyvitamin D or parathyroid hormone levels in young adults taking phenytoin.

This study was conducted to evaluate whether 2C9 polymorphism has any effect on phenytoin metabolism and subsequent serum phenytoin concentration, and to examine the association between 2C9 polymorphism and BMD.

1. Subjects and methods

1.1. Subjects

We performed a cross-sectional study and recruited young Thai ambulatory adults with focal onset epilepsy, who had attended the general medical or neurological clinics of Songklanagarind Hospital between October 2010 and April 2012. Before recruitment, the patients completed a questionnaire focusing on age, age at onset of epilepsy, sex, drug use, medical history including epilepsy characteristics, and presence of conditions known to affect bone turnover and BMD. The information obtained from the patients was rechecked with their medical records and, in some cases, by contacting their physicians.

The inclusion criteria were: a Thai national with focal onset epilepsy and aged 20–40 years; use of phenytoin for at least 2 years before enrollment, regular menstruation (for women), stable weight (over the previous 6 months), stable dosage of phenytoin (in the previous 6 months), no chronic medical illness other than epilepsy, taking no medication except antiepileptic(s), with an active daily life (able to perform activities of daily living without assistance), no history of amenorrhoea, hysterectomy or oophorectomy, and not consuming alcohol or smoking. Patients taking other AEDs before inclusion, but who had stopped taking them at least 2 years prior to the study period, were also included. The exclusion criteria were: pregnancy; having significant disability such as mental retardation, ataxia, paresis, or other motor disabilities, learning disability, language disorder, hearing or visual disability; psychiatric disease; having a significant medical disorder other than epilepsy known to affect bone metabolism such as hepatic, hematological, rheumatologic, renal or gastrointestinal disorder, hyperparathyroidism, hyperthyroidism, hypogonadism, osteogenesis imperfecta, and fracture over the last year prior to the study period; and taking medications known to affect bone turnover, e.g., glucocorticoids, bisphosphonates, thiazides, anticoagulants, GnRH analogs, vitamin D or A, calcium supplement, hormonal replacement and steroids. Also, patients with a family history of osteoporosis were excluded from the study.

The weight and height of the patients were measured and their body mass index (BMI) was calculated [body weight (kg)/height $(m)^2$]. A body mass index in the range of 18.5–23.0 was considered normal and one higher than 23.0 deemed as overweight, in accordance with the World Health Organization (WHO) recommendations.[7] Blood samples were taken for albumin, calcium and phosphate level determination on a HITACHI 971 automatic analyzer during the sample hospital visit. Additionally, the 25-hydroxyvitamin D and parathyroid hormone levels were measured.

1.2. Serum 25-hydroxyvitamin D

The serum 25-hydroxyvitamin D level was measured in duplicate via the HPLC method with UV detection (High Performance Lipid Chromatography, HPLC; Agilent 1100, USA). The intra-assay variability was less than 1.48% within a concentration range of 64.74 ng/ml–68.70 ng/ml (normal range, 30 ng/ml–80 ng/ml). Vitamin D deficiency was defined as a 25-hydroxyvitamin D level < 20 ng/ml and insufficiency as a level between 20 ng/ml and 29 ng/ml.[10]

1.3. Serum parathyroid hormone

The intact parathyroid hormone level was measured in duplicate using electrochemiluminescence immunoassay (ECLIA; Molecular Analytics E170, Germany). The detection limits were 1.20 pg/ml–5000 pg/ml. The intra-assay variability was less than 0.6% within a concentration range of 52.37 pg/ml–53.64 pg/ml (normal range, 15 pg/ml–65 pg/ml).

1.4. Dual-energy absorptiometry

The bone mineral densities of the lumbar spine (L1–L4) and left femur were measured by dual-energy X-ray absorptiometry (DXA) (DXP MD Software version: 4.6, Lunar Corporation, USA) and reported as a *T*-score (the difference in standard deviation units between the measured bone density value and the peak bone density in the normal reference population of Japanese population, as supplied by the manufacturer, because no Thai reference was available, and a *Z*-score [SD from age-sex-specific score in the reference population]). The areas selected for the determination of BMD were the neck of the left femur and the lumbar (L1–L4) vertebrae. The WHO defines normal bone mineral density as a *T*-score greater than -1.0, osteopenia as a *T*-score between > -2.5 and < -1.0, and osteoporosis as a *T*-score < -2.5. All of the patients who had measurements taken at both sites were included in the analysis.[11]

1.5. CYP2C9 genotyping

Genomic DNA was isolated from leukocyte nucleoids using a QIAamp DNA Mini Kit (QIAGEN). Realtime HRM PCR (realtime polymerase chain reaction high resolution melting) was performed in a strip tube with a reaction volume of 20 μl, containing 2 μl of SsoFast Evagreen Supermix "BioRad" (ready to use reactionmix), 0.15 μM of the forward and reverse primers for CYP2C9*2 and CYP2C9*3, and 100 ng/μl of genomic DNA. The sequences of the forward and reverse primers used were 5′ TACAAATACAATGAAAA-TATCATG 3′ and 5′CTAACAACCAGA CTCATAATG 3′ for the CYP2C9*2 (Arg144Cys) genotype, and the forward primer 5′ AATAATAATATGCACGAGGTCCAGAGGTAC 3′ and the reverse primer 5′ GATACTATGAATTTGGGACTT C 3′ for the CYP2C9*3 (Ile359Leu) genotype (these primers were described by Ramasamy et al.[12] and synthesized by BioDesign Co., Ltd.).

Realtime PCR amplification to detect CYP2C9*2 and/or CYP2C9*3 was performed using CFX 96 Connect Realtime-PCR (Bio-Rad Laboratories, USA) with an initial denaturation at 95.0 °C for 3 min, followed by 40 cycles of denaturation at 95.0 °C for 10 s, annealing at 59.0 °C for 15 s, and final extension at 95.0 °C for 10 s. A melt curve was plotted from 73.0 °C to 85.0 °C, with an increment of 0.2 °C every10 s. The analysis of the melt curves used the

high-resolution melt curve (HRM) method on Precision Melt Analysis software version 1.1 (Bio-Rad Laboratories, USA). The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method used the same primer sets as the Realtime PCR technique. PCR was performed at a reaction volume of 20 μl, consisting of 2 μl of Top Taq Master Mix ready-to-use reaction mix (QIAGEN), 0.2 μM each of the forward and reverse primers for CYP2C9*2 and CYP2C9*3, and 100 ng/μl of genomic DNA. PCR was performed in a S1000-PCR thermal cycler (Bio-Rad Laboratories, USA) with an initial denaturation at 94.0 °C for 4 min, followed by 40 cycles of denaturation at 95.0 °C for 30 s, annealing at 56.0 °C for 30 s, extension at 72.0 °C for 45 s and final extension at 72.0 °C for 10 min. Eight microliters of alliquot from each PCR product were digested with 5 U of restriction enzymes AvaII (Promega) and 5 U of Kpn I (Takara) for CYP2C9*2 and CYP2C9*3, respectively. Incubation was done at 37.0 °C overnight. The detection of the digested product was performed with 12% polyacrylamide gel electrophoresis, using 120 V for 2.5 h. The PCR-RFLP results were randomly selected to be confirmed by direct nucleotide sequencing on an ABI 3130 Genetic Analyzer and 3130 Collection Software.

We classified the patients into two groups: (1) the CYP2C9 polymorphism gene group, which included patients who were homozygous or heterozygous CYP2C9*3 and (2) the wild-type gene of 2C9*1.

The Ethics Review Committee of the Faculty of Medicine, Prince of Songkla University, approved the study and informed consent was sought and obtained from all of the patients.

1.6. Statistical analysis

The characteristics of the study patients were described in terms of mean and standard deviation for continuous variables, and number and percentage for categorical variables. Comparisons of continuous variables between two subgroups of subjects were performed using a two-tailed *t*-test or Mann–Whitney test if Shapiro–Wilks test revealed evidence of non-normality within either group. Correlations between serum phenytoin, serum PTH, serum 25-hydroxyvitamin D and BMD parameters in the group with CYP2C9 polymorphism were explored using the Pearson product movement correlation coefficient. The independent effect of CYP2C9 polymorphism on BMD was explored by multivariate linear regression modeling in which sex, age, BMI, and serum levels of phenytoin, 25-hydroxyvitamin D level and parathyroid hormone were included as covariates. *p*-Values of <0.05 were considered to be statistically significant. All statistical analyses were performed using Stata version 7.0 (Stata Statistical Software: version 7.0, College Station, TX, USA).

2. Results

All of the 93 young adult epilepsy patients who were invited to participate in the study agreed to participate. All of the participants had focal epilepsy with or without generalized tonic clonic epilepsy and were taking phenytoin as monotherapy. The basic demographic and clinical characteristics of the patients are shown in Table 1. The mean ± SD age was 31.93 ± 4.60 years (range: 20–40 years), 45.2% were male and 54.8% female. The mean duration of treatment ± SD with antiepileptic drugs for the overall group at the time of the study was 5.4 ± 1.7 years (range: 2.5–9 years). Concerning the frequency of each genotype of CYP P450 2C9, about 14% (13 out of 93) of epilepsy patients were found to be CYP2C9*3 heterozygous carriers (wt/mt) and 4.3% (4 out of 93) were CYP2C9*3 homozygous (mt/mt), which is characterized by the phenotype with poor phenytoin metabolism. No homozygous or heterozygous CYP2C9*2 carriers were found.

Table 1
Baseline demographics, bone mineral density, and blood chemistry of the study population.

Variable	CYP 2C9 genotype	
	Wild type (76)	CYP 2C9 polymorphism (17)
Age		
20–25 years	4	2
26–30 years	26	6
31–40 years	46	9
Sex (n)		
Male	36	6
Female	40	11
Type of seizure		
Simple with or without generalization	35	9
Complex partial with or without generalization	41	8
Serum PHT level mean (range)	16.6 (10–24)	18.9 (11–24)
10–20 mg/l	64	9
>20 mg/l	12	8
Seizure control		
Seizure free[a]	54	12
Well-controlled[b]	22	5
Body mass index		
<18.5	0	0
18.5–23.0	23	7
>23.0	53	10
Etiology (n)		
Unknown	65	14
Head injury	5	1
CNS infection	6	2
Duration		
<5 years	38	8
>5 years	38	9
Phosphate level		
>4.5 mg/dl	1	1
2.5–4.5 mg/dl	73	16
<2.5 mg/dl	2	0
Calcium level		
>10.2 mg/dl	0	0
9–10.2 mg/dl	53	11
<9 mg/dl	23	6
25-Hydroxyvitamin D level		
<20 ng/ml	2	0
>20–29 ng/ml	12	1
>29 ng/ml	62	16
Parathyroid hormone level		
<11 pg/ml	0	0
11–62 pg/ml	75	17
>62 pg/ml	1	0
Femoral neck T-score		
<−2.5	1	0
−2.5 to −1	33	10
>−1	42	7
Lumbar spine T-score		
<−2.5	3	0
−2.5 to −1	32	4
>−1	41	13

CYP, cytochrome P450; PHT, phenytoin.
[a] Defined as seizure free for >24 months under AED therapy.
[b] Defined as seizure free for 12 months under AED therapy.

In the epilepsy patients with CYP 2C9 polymorphism, of the total 17 subjects, 6 were male and 11 female, having a mean ± SD age of 31.7 ± 5.2 years (range 20–39). Shapiro–Wilks test revealed no evidence of non-normality of any variables within either group and 2-tailed *t*-test was therefore used for all comparisons. There was no significant difference between the patients with wild type and the

Table 2
Clinical characteristics, mean levels of biochemical parameters, parathyroid hormone and 25-hydroxyvitamin D levels, and BMD according to the genotype of the vitamin D receptor.

Variable	CYP 2C9 genotype				*p*-value
	Wild type (76)		CYP 2C9 polymorphism (17)		
	Mean ± SD	95% CI	Mean ± SD	95% CI	
Age (years)	32.0 ± 4.5	30.9–33.0	31.7 ± 5.2	29.0–34.4	0.83
Sex (male:female) (*n*)	36:40		6:11		0.43
Duration of phenytoin (years)	5.4 ± 1.7	5.0–5.8	5.5 ± 1.7	4.6–6.4	0.73
25-Hydroxyvitamin D level (ng/ml)	38.3 ± 10.6	35.9–40.8	44.8 ± 13.5	37.9–51.7	0.03
Serum PHT level (mg/l; mean ± SD)	16.6 ± 3.3	15.9–17.4	18.9 ± 4.0	16.9–21	0.01
Parathyroid hormone level (pg/ml)	49.0 ± 13.4	45.9–52.1	42.2 ± 9.6	37.2–47.1	0.05
Phosphate level (mg/dl)	3.34 ± 0.53	3.22–3.46	3.24 ± 0.54	2.97–3.52	0.47
Calcium level (mg/dl)	9.11 ± 0.37	9.02–9.19	9.11 ± 0.35	8.94–9.30	0.91
BMI	24.2 ± 2.2	23.7–24.8	23.8 ± 2.4	22.6–25.1	0.49
Femoral neck *Z*-score	−0.77 ± 0.76	−0.94 to (−0.59)	−0.36 ± 0.6	−0.67 to (−0.05)	0.04
Lumbar spine *Z*-score	0.03 ± 1.07	−0.22 to 0.27	0.60 ± 1.47	−0.15 to 1.350	0.06
Femoral neck *T*-score	−0.86 ± 0.78	−1.04 to (−0.68)	−0.30 ± 1.14	−0.89 to 0.29	0.02
Lumbar spine *T*-score	0.02 ± 1.09	−0.23 to 0.27	0.38 ± 1.52	−0.40 to 0.16	0.27

CYP, cytochrome P450; PHT, phenytoin; BMI, body mass index.

patients with CYP 2C9 polymorphism in terms of sex and age ($p = 0.43$ and $p = 0.83$, respectively). Likewise, the duration of treatment ± SD did not differ significantly between the groups; the patients with CYP 2C9 polymorphism and the patients with the wild type had means durations treatment of 5.5 ± 1.7 years (range: 3–8 years) and 5.4 ± 1.7 years (range: 2.5–9 years), respectively ($p = 0.73$). The mean ± SD BMI in the patients with CYP 2C9 polymorphism was 23.8 ± 2.4; 7 had a normal value for BMI, no patient had a low BMI and 10 were overweight. The mean ± SD BMI of patients with wild type was 24.2 ± 2.2; 23 had a normal value for BMI, no patient had a low BMI and 53 were overweight. Overall, there was no significant difference between the two groups in BMI ($p = 0.49$). The means ± SD of phosphate and calcium levels were 3.24 ± 0.54 vs. 3.34 ± 0.53 and 9.11 ± 0.35 vs. 9.11 ± 0.37 in the patients with CYP 2C9 polymorphism and the patients with wild type, respectively. The other basic demography characteristics are shown in Tables 1 and 2. None of the demographic variables showed any significant difference between the two groups. The patients with CYP 2C9 polymorphism had higher plasma levels of phenytoin when compared with the patients with wild type ($p = 0.01$).

2.1. Blood chemistry and bone mineral density

The mean ± SD serum 25-hydroxyvitamin D levels in the patients with CYP 2C9 polymorphism was 44.8 ± 13.5 ng/ml. No patient had a level of <20 ng/ml, which would have indicated 25-hydroxyvitamin D deficiency. One patient (5.9%) had a level between >20 ng/ml and 29 ng/ml, suggesting 25-hydroxyvitamin D insufficiency. Overall, we found an association between low 25-hydroxyvitamin D and low BMD at the femoral neck. Moreover, the mean ± SD of serum 25-hydroxyvitamin D levels in the wild-type patients was 38.3 ± 10.6 ng/ml. Two patients (2.6%) had levels of <20 ng/ml, which indicated 25-hydroxyvitamin D deficiency and 12 others (15.8%) had levels between >20 ng/ml and 29 ng/ml, suggesting 25-hydroxyvitamin D insufficiency. There was a statistically significant ($p = 0.03$) lower mean serum 25-hydroxyvitamin D levels among patients with wild type when compared with their counterparts with CYP 2C9 polymorphism (Table 2). Furthermore, there was marginal evidence of higher serum PTH levels in patients with wild type compared patients with CYP 2C9 polymorphism ($p = 0.05$). Serum calcium concentrations and serum phosphate levels were not significantly different between the two groups ($p = 0.91$ and $p = 0.47$, respectively).

The bone mineral density results are shown in Table 2. BMD was associated with CYP 2C9 polymorphism in the epilepsy patients. Specifically, patients with CYP 450 2C9*3 (homozygotes and heterozygotes) had higher *T*-scores ± SD and *Z*-scores ± SD of the femoral neck than those with wild type ($p = 0.02$ and 0.04, respectively). However, this was not the case with *T*-scores ± SD and *Z*-scores ± SD of the lumbar spine ($p = 0.27$ and 0.06, respectively). The wild-type patients' mean *T*-scores ± SD and *Z*-scores ± SD of the femoral neck were -0.86 ± 0.78 and -0.77 ± 0.76, respectively. Osteopenia at the femoral neck was detected in 58.8% of patients with CYP 2C9 polymorphism and 43.4% of patients with wild type. Additionally, we found osteoporosis in the wild-type patients at the rate of 1.3% (1 of 76 patients). CYP2C9 polymorphism remained the only variable significantly related to femoral neck *T*- and *Z*-scores in multivariate modeling after adjusting for duration of therapy, sex, age, BMI, and serum levels of phenytoin, 25-hydroxyvitamin D, and parathyroid hormone. Surprisingly, no correlation between serum phenytoin levels and BMD at the femoral neck or at the lumbar spine was detected.

3. Discussion

Epilepsy patients receiving the CYP P450 enzyme-inducing AED, phenytoin, and who had the CYP 2C9 polymorphism had higher *T*-scores and *Z*-scores of the femoral neck ($p = 0.02$ and 0.04, respectively), but not of the lumbar spine ($p = 0.27$ and 0.06, respectively) than the wild-type patients. However, we did not detect any correlation between serum phenytoin level and BMD.

Nevertheless, the mean serum PTH level tended to be lower among patients with CYP 2C9 polymorphism when compared with the patients with wild type ($p = 0.05$). Interestingly, the mean serum 25-hydroxyvitamin D levels was statistically significantly lower in wild-type patients than in those with 2C9 polymorphism ($p = 0.03$).

The CYP P450 super family represents one group of important Phase-I drug-metabolizing enzymes that oxidize a number of endogenous compounds and xenobiotics, including more than 90% of the current therapeutic drugs.[13] Approximately 40% of the white and 5% of the Asian or black populations are heterozygous for either CYP2C9*2 or CYP2C9*3.[9] In Thailand, Kuanprasert et al. conducted a study on valvular heart disease in Northern Thailand and found that 95% of the 242 patients under investigation had CYP2C9*1/*1; CYP2C9*1/*3 was found in the remaining 5%. Neither the mutant CYP2C9*2 allele nor the individual homozygote for CYP2C9*3 was found.[14] However, our study found that the CYP 2C9 mutation had a prevalence of 18.3%. About 14% (13 out of 93) of our epilepsy patients were found to be CYP2C9*3 heterozygous carriers

(wt/mt) and 4.3% (4 out of 93) were CYP2C9*3 homozygous (mt/mt). No homozygous or heterozygous CYP2C9*2 carriers were found. Our study was conducted in a location in Southern Thailand near the Malaysian border. The Gan et al. study in patients who live in Malaysia (91 Malays, 96 Chinese, and 46 Indian patients) found that the prevalence of CYP2C9 polymorphism in that population ranged from 8% to 15%.[15] The higher prevalence of CYP2C9 polymorphism in our study compared with that from Northern Thailand; may be explained by the history of ethnic mixing between the Thais and Malaysian in the southern region. There has been no study of the prevalence of CYP 2C9 polymorphism in the healthy general population in Thailand.

Our study found that epilepsy patients who had heterozygous or homozygous CYP2C9*3 had higher serum phenytoin levels than those who had the wild type. A few investigators have reported an association between serum antiepileptic drug level and BMD or blood bone biomarkers. The dose of phenytoin related to effects on bone health has been identified by Nakade et al.[8] They conducted a study on the effects of phenytoin on human-mandible-derived bone cells and noted that the osteogenic effects of phenytoin on human-mandible-derived bone cells were biphasic. However, the possibility that the dose-dependent situation in vivo could be different from the in vitro one is real. Lau et al., who conducted a study in male epilepsy patients taking phenytoin, also found that the serum levels of osteocalcin, skeletal alkaline phosphatase, and procollagen peptides were significantly correlated with the serum phenytoin level, but not with the dose or duration of phenytoin treatment.[7] Moreover, we found a higher BMD at the femoral neck in epilepsy patients who had CYP2C9*3 (heterozygous or homozygous) than those carrying the wild type allele. In this study, we did not detect a correlation between serum phenytoin and BMD at the femoral neck in CYP 2C9*3 polymorphism (homozygous or heterozygous) as well as wild type after adjusting for potentially confounding factors such as age, sex, duration of treatment with phenytoin, 25-hydroxyvitamin D, and parathyroid hormone.

Previous studies have reported that epilepsy patients taking antiepileptic drugs had low 25-hydroxyvitamin D levels, ranging from 11% to 77%.[16,17] An earlier study has suggested that phenytoin induces bone loss via the CYP P450 enzyme-inducing antiepileptic drugs, which leads to an increased metabolism of 25-hydroxyvitamin D.[18] This mechanism is supported by our findings because they indicate that low serum 25-hydroxyvitamin D levels were associated with low BMD in epilepsy patients taking phenytoin. In our study, we found that epilepsy patients with wild-type CYP 2C9 had lower 25-hydroxyvitamin D levels than epilepsy patients with CYP 2C9 polymorphism ($p = 0.03$). Reductions in 25-hydroxyvitamin D can result in malabsorption of calcium and secondary hyperparathyroidism. According to previous clinical studies, the incidence of hypocalcemia in patients being treated with anticonvulsants is 4–30%.[19,20] Nevertheless, in previous experimental studies, the cause of hypocalcemia in rats treated with various doses of phenytoin (1–150 mg/kg/day) was not determinable.[21] Our study found low 25-vitamin D levels and hypocalcemia at the rates of 18.4% and 30.3% in the wild-type individuals and 5.9% and 35.3% in those with CYP 2C9 polymorphism. Furthermore, we did not find a significant serum calcium level difference between the two groups ($p = 0.91$). Likewise, we did not observe any association between serum calcium or 25-hydroxyvitamin D levels and serum phenytoin level. Typically, hyperparathyroid conditions are characterized by a depletion of the cortical bone and conservation of the trabecular bone.[22] That study resulted in reduced BMD level, which, in respect of patients with wild-type CYP 2C9, primarily affects the femoral neck that is fundamentally composed of cortical bone. Yet, we found no significant alteration of BMD in the lumbar spine where the composition is mostly trabecular bone. Moreover, the present study confirmed that parathyroid hormone levels in epilepsy patients taking phenytoin are not associated with low BMD at either site. Again, we did not detect any correlation between serum phenytoin and BMD at either site. Overall, the serum parathyroid hormone level was not significantly different between epilepsy patients with wild type and CYP 2C9 polymorphism. Hence, the possible mechanism at hand is something other than a hyperparathyroid state. This finding is supported by those of a previous study that was also conducted in a tropical Asian country on both CYP P450-inducer and non-inducer AEDs among young adult epilepsy patients, which reported that the parathyroid hormone level was not associated with low BMD.[4] This result contrasts with those from studies conducted in Western countries. This is probably explained by the fact that our patients were on an ambulatory regimen and not institutionalized as they would have been in the past. Another important factor is the differences in the diet between the study populations. The nutritional contribution together with other sources of vitamin D, like exposure to the sun, may contribute to the maintenance of some normal deposits of this vitamin despite the possible negative metabolic effect of hepatic degradation of vitamin D produced by phenytoin.[17] Another suggested possible mechanism for low BMD is the direct action of phenytoin on bone resorption and bone formation.[7]

This investigation has a number of important limitations due to a lack of data on calcium intake; dietary intake estimated using the recall technique over a short period of time may not be a good representation of usual or long-term calcium intake. Reports of previous studies, however, have failed to demonstrate a statistically significant effect of dietary calcium on parameters of bone health.[23] Another limitation is our failure to provide bone remodeling markers in this study; however, we believe the results are of interest and contribute to the body of knowledge on the effects of AEDs on the skeleton in the setting of CYP 2C9 polymorphism.

The following strengths of this study are that; firstly, it is the first study to explore the relationship between CYP 2C9 polymorphism, serum phenytoin concentration and BMD in young adult epilepsy patients taking phenytoin as monotherapy, allowing on investigation of the association between CYP 2C9 gene polymorphism and BMD, 25-hydroxyvitamin D or parathyroid hormone levels. We also included a homogenous sample population. Secondly, the measurement of 25-hydroxyvitamin D and parathyroid hormone levels as well as those of CYP 2C9 gene polymorphism and BMD used modern techniques and appropriate sites (femur neck and lumbar spine). We excluded patients with potential confounding factors that are known to affect BMD, especially fracture within 1 year before inclusion, smoking, alcohol consumption, abnormal menstruation, use of hormonal drugs, and recent weight change. Thus, our study should be able to evaluate a true association between CYP 2C9 gene polymorphism and BMD, 25-hydroxyvitamin D or parathyroid hormone levels in young adult epilepsy patients.

In conclusion, this is the first time that the association between young epilepsy patients taking phenytoin and CYP 2C9 gene polymorphism or parathyroid hormone or 25-hydroxyvitamin D levels has been investigated. Our study confirms that epilepsy patients carrying the CYP2C9*3 polymorphism (heterozygous or homozygous) had higher serum phenytoin levels than those with the wild-type allele (CYP2C9*1). Moreover, we found a higher BMD in epilepsy patients with CYP2C9*3 (heterozygous or homozygous) than wild-type ones. However, we did not detect a correlation between serum phenytoin levels and BMD at the femoral neck or

lumbar spine. In a future study, we will evaluate the best choice AED for long term treatment of epilepsy patients with CYP2C9*3.

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References

1. Lado F, Spiegel R, Masur JH, Boro A, Haut SR. Value of routine screening for bone demineralization in an urban population of patients with epilepsy. *Epilepsy Research* 2008;**78**:155–60.
2. Farhat G, Yamout B, Mikati MA, Demirjian S, Sawaya R, El-Hajj Fuleihan G. Effect of antiepileptic drugs on bone density in ambulatory patients. *Neurology* 2002;**58**:1348–53.
3. Pack AM, Olarte LS, Morrell MJ, Flaster E, Resor SR, Shane E. Bone mineral density in an outpatient population receiving enzyme-inducing antiepileptic drugs. *Epilepsy & Behavior* 2003;**4**:169–74.
4. Phabphal K, Geater. Leelawattana R, Sathirapanya P, Sattawacharawanich S, Limapichat K. Prevalence and risk factors of low bone mineral density and 25-hydroxyvitamin D status in young healthy epileptic adult patients in a tropical Asian country taking antiepileptic drug. *Bone* 2009;**45**:232–7.
5. Mintzer S, Mattson RT. Should enzyme-inducing antiepileptic drugs be considered first-line agents? *Epilepsia* 2009;**50**:42–50.
6. Nakade O, Baylink DJ, Lau KH. Osteogenic actions of phenytoin human bone cells are mediated in part by TGF-B. *Journal of Bone and Mineral Research* 1996;**11**:1880–8.
7. Lau KH, Nakade O, Barr B, Taylor AK, Houchin K, Baylink DJ. Phenytoin increases markers of osteogenesis for the human species in vitro and in vivo. *The Journal of Clinical Endocrinology and Metabolism* 1995;**80**:234–53.
8. Nakade O, Baylink DJ, Lau KH. Phenytoin at micromolar concentrations is an osteogenic agent for human-mandible-derived bone cells in vitro. *Journal of Dental Research* 1995;**74**:331–7.
9. Anderson GD. Pharmacogenetics and enzyme induction/inhibition properties of antiepileptic drug. *Neurology* 2004;**63**:S3–8.
10. Holic MF. Vitamin D deficiency. *The New England Journal of Medicine* 2007;**357**:226–81.
11. Alekel DL, Mortillaro E, Hussain EA, West B, Ahmed N, Perterson CT, et al. Lifestyle and biologic contributors to proximal femur bone mineral density and hip axis length in two distinct ethnic groups of premenopausal woman. *Osteoporosis International* 1999;**9**:327–38.
12. Ramasamy K, Narayan SK, Chanolean S, Chandrassekaran A. Severe phenytoin toxicity in a CYP2C9*3*3 homozygous mutant from India. *Neurology India* 2007;**55**:408–9.
13. Rendic S. Summary of information on human CYP enzymes: human P450 metabolism data. *Drug Metabolism Reviews* 2002;**34**:83–448.
14. Kuanprasert S, Dettrairat S, Palacajornsuk P, Kunachiwa W, Phorommintikul A. Prevalence of CYP2C9 and VKORC1 mutation in patients with valvular disease in Northern Thailand. *Journal of the Medical Association of Thailand* 2009;**92**:1597–601.
15. Gan GG, Phipps ME, Lee MM, Lu LS, Subramaniam RY, Bee PC, et al. Contribution of VKORC1 and CYP2C9 polymorphisms in the interethic variability of warfarin dose in Malaysian populations. *Annals of Hematology* 2011;**90**:635–41.
16. Andress DL, Ozuna J, Tirschwell D, Grande L, Johnson M, Jacobson AF, et al. Antiepileptic drug-induced bone loss in young male patients who have seizure. *Archives of Neurology* 2002;**59**:781–6.
17. El-Hajj Fuleihan G, Dib L, Yamout B, Sawaya R, Mikati MA. Predictors of bone density in ambulatory patients on antiepileptic drugs. *Bone* 2008;**29**:1586–93.
18. Verrotti A, Copola G, Parisi P, Mohn A, Chiarelli F. Bone and calcium metabolism and antiepileptic drugs. *Clinical Neurology and Neurosurgery* 2010;**112**:1–10.
19. Moro-Alvarez MJ, Díaz Curiel M, de la Piedra C, Mariñoso ML, Carrascal MT. Bone disease induced by phenytoin therapy: clinical and experimental study. *European Neurology* 2009;**62**:219–30.
20. Feldkamp J, Becker A, Witte OW, Scharff D, Scherbaum WA. Long-term anticonvulsant therapy leads to low bone mineral density evidence for direct drug effects of phenytoin and carbamazepine on human osteoblast-like cells. *Experimental and Clinical Endocrinology & Diabetes* 2000;**108**:37–43.
21. Ohta T, Wergedal JE, Gruber HE, Baylink DJ, William Lau KH. Low dose phenytoin is an osteogenic agent in the rat. *Calcified Tissue International* 1995;**56**:42–8.
22. Stephen LJ, McLellan AR, Harrison JH, Shapiro D, Dominiczak MH, Sills GJ, et al. Bone density and antiepileptic drugs: a case-controlled study. *Seizure* 1999;**8**:339–42.
23. Stevenson JC, Whitehead MI, Padwick M, Endacott JA, Sutton C, Banks LM, et al. Dietary intake of calcium and postmenopausal bone loss. *BMJ (Clinical research ed)* 1988;**297**:15–7.