Association of vitamin D receptor and estrogen receptor-α gene polymorphism with peak bone mass and bone size in Chinese women

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ABSTRACT

AIM: To investigate if vitamin D receptor (VDR) gene *Apa* I polymorphism and estrogen receptor-α (ER-α) gene *Pvu* II, *Xba* I polymorphisms are related to bone mineral density (BMD), bone mineral content (BMC) and bone size in premenopausal Chinese women. **METHODS:** The VDR *Apa* I genotype and ER-α *Pvu* II, *Xba* I genotype were determined by PCR-restriction fragment length polymorphism (RFLP) in 493 unrelated healthy women aged 20-40 years of Han nationality in Shanghai city. BMD (g/cm^2), BMC (g), and bone areal size (BAS, cm^2) at lumbar spine 1-4 (L_{1-4}) and proximal femur (femoral neck, trochanter and Ward's triangle) were measured by duel-energy X-ray absorptionmetry. **RESULTS:** All allele frequencies did not deviate from Hardy-Weinberg equilibrium. After phenotypes were adjusted for age, height, and weight, a significant association was found between VDR *Apa* I genotype and BMC variation at L_{1-4} and Ward's triangle ($P<0.05$), but not in BMD or BAS at lumbar spine and proximal femur. ER-α *Pvu* II, *Xba* I genotype was not related to BMC, BMD, and BAS at all sites. **CONCLUSION:** The study suggested that *Apa* I polymorphism in VDR gene may influence on attainment and maintenance of peak bone mass in premenopausal Chinese women.

INTRODUCTION

Osteoporosis is characterized by low bone mineral density (BMD) and microarchitectural deterioration of bone tissue leading to increased bone fragility and susceptibility to fracture. The risk of osteoporotic fracture in later life is determined by the peak bone mass (PBM) achieved in early adulthood as well as the rate of bone loss with aging. PBM is a quantitative trait determined by interaction of genetic and environmental factors. Previous studies have shown that genetic factors make a strong contribution to PBM variation[1,2], the heritability of PBM at spine and hip is 0.70 and 0.80, respectively[1].

Up to now, about sixty candidate genes polymorphism were investigated, among which the most studies were related to vitamin D receptor (VDR) gene and estrogen receptor-α (ER-α) gene. Vitamin D, by interacting with its receptor, plays an important role in calcium homeostasis by regulating bone cell growth and

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differentiation, intestinal calcium absorption. Estrogen as the key regulator of skeletal growth and maturation is required for the attainment of PBM, its deficiency is the major cause of age-related bone loss in women. Generous studies have been performed to test the VDR and ER-α gene polymorphism underlying BMD variation[3,4]. While BMD is determined by bone mass and bone size, bone size is an independent determinant of bone strength, deficit in bone size may partly account for the increased bone fragility[5,6]. However, few studies were performed to identify the relationship of candidate gene polymorphism underlying peak bone mass and bone size variation. This study investigated the association about *Apa* I polymorphism within VDR gene and *Pvu* II, *Xba* I polymorphisms within ER-α with peak bone mineral content and bone size in 493 premenopausal Chinese women.

MATERIALS AND METHODS

Subjects From 1997 to 2002, total 493 aged 20-40 unrelated female volunteers of Han nationality lived in Shanghai more than ten years were recruited. After a health examination for each subject, we collected the information including age, sex, medical history, family history, marital status, menses history, obstetrical history, physical activity, alcohol use, diet habits, smoking history, *etc*. We excluded peri- or postmenopausal women and those subjects who took any medicine which will influence bone mass and turnover.

Measurement The BMD (g/cm^2), bone mineral content (BMC, g), and bone area size (BAS, cm^2) at the lumber spine 1-4 (L_{1-4}) and proximal femur (femoral neck, trochanter and Ward's triangle) were measured by dual-energy X-ray aborptionmetry (DEXA, Hologic QDR-2000, Hologic Corporation, Waltham, MA) in each subject. The machine is calibrated daily, and the coefficient of variability values at L_{1-4}, femoral neck, trochanter, and Ward's triangle are 0.97 %, 1.93 %, 1.48 %, and 3.85 %, respectively.

Genotyping Genomic DNA was isolated using the phenol-chloroform extraction method. A 740 bp fragment containing the *Apa* I polymorphism in the 3'-end region of the VDR gene was amplified by polymerase chain reaction (PCR) using the upstream primer: 5'-CAGAGCATGGACAGGGAGCAA-3' and the downstream primer: 5'-GCAACTCCTCATGGCTGAGGTCTC-3'. PCR was amplified as Morrison *et al* described[7]. The *Apa* I genotypes were identified by electrophoresis of the DNA samples in 1.5 % agarose gels. The *Apa* I genotype was named as follows: AA (absence of the restriction site); aa (presence of the restriction site); Aa (heterozygous for the restriction site).

The ER-α gene polymorphism identification was conducted based on the method of Kobayashi *et al*[8]. Products of 1.3 kb long were obtained with a pair of forward primer: 5'-CTGCCACCCTATCTGTATCTTTTCCTATTCTCC-3', and reverse primer: 5'-TCTTTCTCTGCCACCCTGGCGTCGATTATCTGA-3'. PCR products were digested with restriction endonuclease (*Xba* I, *Pvu* II). The ER-α genotype was identified by electrophoresis of the DNA samples in 2.0 % agarose gels. XX or PP (absence of the restriction site *Xba* I or *Pvu* II, respectively); xx or pp (presence of the restriction site); Xx or Pp (heterozygous for the restriction site).

Statistical analysis Statistical analyses were performed with the SPSS 8.0 software package. In order to test the population homogeneity of the study subjects, the allele frequencies of *Apa* I, *Pvu* II, and *Xba* I were tested against Hardy-Weinberg equilibrium by the χ^2 test. After the raw BMD, BMC and BAS values were adjusted for age, weight, and height as covariates, the association between VDR *Apa* I genotype and ER-α *Pvu* II, *Xba* I genotype and BMD, BMC and BAS were tested using analysis of covariance (ANOVA). Differences were considered to be significant if $P<0.05$.

RESULTS

Allele and genotype frequencies The VDR *Apa* I genotype and ER *Pvu* II, *Xba* I genotype were determined by the PCR-RFLP (Fig 1-3). The distribution of alleles and genotypes frequencies for *Apa* I, *Pvu* II, and *Xba* I in this population are shown in Tab 1. All allele frequencies did not deviate from Hardy-Weinberg equilibrium.

Association between genotypes and BMD, BMC and BAS Neither *Apa* I polymorphism within VDR gene, nor *Pvu* II , *Xba* I polymorphism within ER gene was related to BMD and BAS variation at all sites. As to BMC, a significant association was found between *Apa* I genotype and L_{1-4} and Ward's BMC variation ($P<0.05$). The subjects with AA genotype had lower trend in BMC at all sites as compared with those with Aa and aa genotypes, although it was significant at the L_{1-4} and Ward's triangle only. No significant association was detected between the *Pvu* II or *Xba* I genotype and BMC variation at lumbar spine and any site of proximal femur (Tab 2-4).

Tab 1. Frequencies distribution of genotypes and alleles for ER-α and VDR in 493 Shanghai women.

Gene	Genotype			Allele	
ER (*Pvu* II)	PP 64 (0.130)	Pp 242 (0.491)	pp 187 (0.379)	P 370 (0.375)	p 616 (0.625)
ER (*Xba* I)	XX 21 (0.042)	Xx 200 (0.406)	xx 272 (0.552)	X 242 (0.245)	x 744 (0.754)
VDR (*Apa* I)	AA 28 (0.06)	Aa 203 (0.41)	aa 262 (0.53)	A 259 (0.26)	a 727 (0.74)

The number in parentheses is the frequency

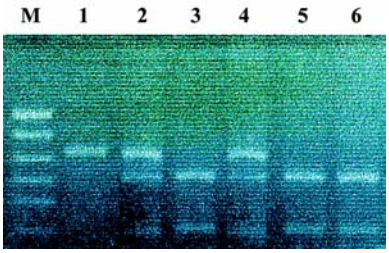

Fig 1. VDR Apa I genotype was determined by PCR-RFLP. M: marker; Lane 1: AA genotype; Lane 2, 4: Aa genotype; Lane 3, 5, 6: aa genotype.

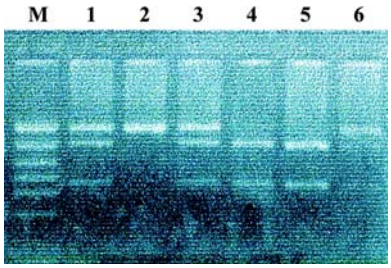

Fig 2. ER-α Pvu II genotype was determined by PCR-RFLP. M: marker; Lane 2: PP genotype; Lane 1, 3, 6: Pp genotype; Lane 4, 5: pp gennotype.

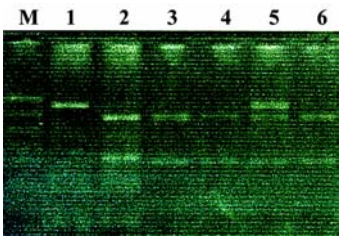

Fig 3. ER-α Xab I genotype was determined by PCR-RFLP. M: marker; Lane 1: XX genotype; Lane 5: Xx genotype; Lane 2, 3, 4, 6: xx gennotype.

DISCUSSION

In the study, we found that Chinese have higher frequencies of "a", "p", and "x" allele (73.7 %, 62.5 %, and 75.4 %, respectively), similar to Korean and significant difference compared with Caucasians[3,9-11]. The difference of genotype frequencies may contributed to various ethnics.

In 493 premenopausal Shanghai women, no significant association was found in BMD variation with *Apa* I, *Pvu* II, and *Xba* I genotypes at all sites. Our results about *Pvu* II and *Xba* I genotypes underlying BMD variation were supported by recent findings in 104 premenopausal Korean women[9]. However, a significant association was found between *Pvu* II genotype and femoral neck BMD variation in 216 premenopausal British women, but not in *Xba* I genotype[12]. Willing *et al* [10] reported a marked relationship between *Pvu* II and *Xba* I polymorphisms and lumbar spine BMD in 253 premenopausal Caucasians women, and pp and xx genotypes showed lower BMD than other genotypes. So these contradictory findings suggested that genetic and environment may influence the attainment and maintenance of peak bone mass in different population.

Our study did not observe a significant relationship between BMD and *Apa* I polymorphism. These findings are in agreement with the recent studies in premenopausal Caucasian, Israeli Jewish, and Southern Chinese women[11,13,14]. In contrast, the reports from Australia, USA supported a relationship between the AA genotype and low BMD in postmenopausal women [7,15]. However, none of these previous studies had investigated the association between VDR, ER-α genotypes, BMC, or bone size in women.

In fact, BMD is determined by two factors, BMC

Tab 2. Clinical features of the 493 women in relation to *Apa* I genotype. Age, height and weight were expressed as Mean±SD. BMD, BMC and BAS were expressed as Mean±SEM adjusted for age, height and weight. The differences of BMD, BMC and BAS in genotypes were analyzed by ANCOVA, the differences of age, height and weight were analyzed by ANOVA. [b]*P*<0.05, AA genotype *vs* Aa or aa genotype at L_{1-4} BMC. [e] *P*<0.05, AA genotype *vs* Aa or aa genotype at Ward's BMC.

	AA	Aa	aa	*P* value
n	28	203	262	
Age/a	31±5	31±6	31±5	0.775
Height/cm	160±4	160±5	160±5	0.938
Weight/kg	55±7	55±8	55±8	0.808
L_{1-4} BMD	0.924±0.018	0.966±0.007	0.965±0.006	0.072
L_{1-4} BMC	52.2±1.4	56.1±0.5[b]	55.6±0.5[b]	0.033
L_{1-4} BAS	56.2±0.7	57.86±0.26	57.46±0.23	0.083
Neck BMD	0.746±0.018	0.786±0.007	0.780±0.006	0.109
Neck BMC	3.57±0.08	3.726±0.031	6.80±0.027	0.173
Neck BAS	4.81±0.06	4.751±0.022	4.720±0.019	0.294
Troch BMD	0.607±0.015	0.639±0.006	0.636±0.005	0.141
Troch BMC	5.87±0.20	6.14±0.08	6.15±0.07	0.413
Troch BAS	9.70±0.20	9.58±0.07	9.64±0.06	0.746
Ward's BMD	0.666±0.023	0.721±0.008	0.711±0.007	0.072
Ward's BMC	0.80±0.03	0.881±0.011[e]	0.864±0.010[e]	0.035
Ward's BAS	1.197±0.014	1.221±0.005	1.214±0.005	0.234

Tab 3. Clinical features of the 493 women in relation to *Pvu* II genotype. Age, height and weight were expressed as Mean±SD. BMD, BMC and BAS were expressed as Mean±SEM adjusted for age, height and weight. The differences of BMD, BMC, and BAS in genotypes were analyzed by ANCOVA, the differences of age, height and weight were analyzed by ANOVA.

	AA	Aa	aa	*P* value
n	64	242	187	
Age/a	31±5	31±5	31±5	0.770
Height/cm	159±6	160±5	160±5	0.425
Weight/kg	55±8	55±8	55±8	0.937
L_{1-4} BMD	0.966±0.012	0.963±0.006	0.963±0.007	0.975
L_{1-4} BMC	55.8±0.9	55.76±0.48	55.53±0.55	0.947
L_{1-4} BAS	57.5±0.5	57.71±0.24	57.41±0.27	0.705
Neck BMD	0.78±0.12	0.782±0.006	0.777±0.007	0.826
Neck BMC	3.7±0.06	3.708±0.028	3.666±0.032	0.569
Neck BAS	4.7±0.04	4.748±0.020	4.727±0.023	0.800
Troch BMD	0.639±0.010	0.635±0.005	0.635±0.006	0.913
Troch BMC	6.069±0.135	6.16±0.07	6.095±0.078	0.723
Troch BAS	9.442±0.131	9.69±0.07	9.593±0.076	0.209
Ward's BMD	0.720±0.015	0.714±0.008	0.707±0.009	0.733
Ward's BMC	0.876±0.020	0.870±0.010	0.858±0.012	0.628
Ward's BAS	1.126±0.009	1.218±0.005	1.211±0.005	0.636

and BAS. An increase in bone size would protect against fracture[16,17], whereas a deficit increase in BMC led to low PBM and increased the risk of osteoporosis in the later life. Our results showed a significant association between the *Apa* I genotype and BMC at lumbar spine and Ward's triangle. Spine or Ward's triangle is com-

Tab 4. Clinical features of the 493 women in relation to *Xba* I genotype. Age, height and weight were expressed as Mean±SD. BMD, BMC and BAS were expressed as Mean±SEM adjusted for age, height and weight. The differences of BMD, BMC, and BAS among genotypes were analyzed by ANCOVA, the differences of age, height and weight were analyzed by ANOVA.

	XX	Xx	xx	*P* value
n	21	200	272	
Age/a	33±6	31±5	31±5	0.403
Height/cm	159±4	160±5	160±5	0.624
Weight/kg	54±5	54±8	55±8	0.299
L_{1-4} BMD	0.960±0.021	0.968±0.007	0.961±0.006	0.735
L_{1-4} BMC	55.5±1.6	56.07±0.53	55.4±0.5	0.623
L_{1-4} BAS	57.8±0.8	57.72±0.26	57.5±0.23	0.712
Neck BMD	0.777±0.021	0.789±0.001	0.775±0.006	0.287
Neck BMC	3.64±0.10	3.715±0.031	3.680±0.027	0.594
Neck BAS	4.7±0.07	4.716±0.022	4.759±0.019	0.287
Troch BMD	0.650±0.018	0.638±0.006	0.632±0.005	0.492
Troch BMC	6.11±0.24	6.16±0.08	6.10±0.06	0.849
Troch BAS	9.39±0.23	9.62±0.07	9.64±0.06	0.576
Ward's BMD	0.714±0.027	0.717±0.009	0.708±0.007	0.758
Ward's BMC	0.87±0.04	0.873±0.012	0.861±0.010	0.721
Ward's BAS	1.214±0.017	1.217±0.005	1.214±0.005	0.930

prised predominantly with trabecular bone, and femoral neck and trochanter comprised predominantly cortical bone. However, no significant relationship was found in BMC with three tested genotypes at femoral neck and trochanter in our study. Indeed, the surface ratio of cancellous bone is eight- or to ten- fold greater than that of cortical bone, since the turnover of bone is a surface-based event, this activity is greater on cancellous than on cortical surfaces. Puberty is terminated by epiphyseal plate closure, when volumetric BMC has reached about 90 %–95 % of PBM. The mineralization process brings the skeleton to its maximal values by continued periosteal apposition and possibly by trabecular thickening. Vitamin D is required for normal bone mineralization. It affected the absorption of calcium from the gut and controlled calcium and phosphate homeostasis. Although *Apa* I marker loci located in intron VIII of the VDR gene and represented silent mutation that do not alter the protein sequence of the VDR[18], we supposed that *Apa* I polymorphism was in linkage disequilibrium with other functional sequence variation. Therefore, the *Apa* I genotype may exert an influence on attainment and maintenance of PBM, especially for cancallous bone. We did not found that ER-α *Pvu* II, *Xba* I genotypes influenced on BMC at trabecular bone or cortical bone in premenopausal Chinese women, although some evidence suggested that estrogen increased osteoblast formation, differentiation, and proliferation.

However, BMD or BMC is not the only determinant of skeletal fragility, polymorphism of gene may influence other factors such as bone size to increase fracture risk[19]. The bone size and geometry also determine its mechanical strength, which is independent of BMD and can predict the risk of fracture[20]. About 60 %–80 % of the variation in measures of proximal femur geometry (such as femoral neck area size and hip axis length) in population may be caused by genetic [21]. Our data did not support that VDR *Apa* I genotype or ER-α *Pvu* II, *Xba* I genotypes potentially influenced bone size. The results indicated that *Apa* I genotype may contribute to the genetic regulation of BMC at lumbar spine and Ward's triangle, but not BMD and BAS. These observations are consistent with the hypothesis that the bone size and BMC may be controlled by different genes[22].

There is a statistics limitation in present study, because we used population association approach to test the association between the gene polymorphism and phenotypes. Association approach is also most employed to disentangle genetic bases underlying complex trait (such as BMD). But the association study approach

may yield false positive/negative results between a complex trait and candidate gene polymorphism, when there is a population admixture[23,24]. It is a valuable approach but limited in the results from this approach alone[25]. While the transmission disequilibrium test (TDT) is a powerful family-based test and robust to population admixture and/or stratification[26]. The TDT has been widely employed in practice, with great success in resolving controversies regarding the results obtained from association and traditional linkage studies of candidate genes with quantitative trait locus (QTL)[27]. Nuclear families are being recruited in our study group, we will employ the TDT approach to test whether the VDR and ER-α genes as a putative QTL underlying the variation of peak bone mass in Chinese women.

In conclusion, *Apa* I polymorphism within VDR gene have a significant association with peak BMC in premenopausal Chinese women, but no relation to bone size and BMD. No significant association was found between ER-α gene *Pvu* II, *Xba* I polymorphisms and BMD, BMC, and BSA variation. The study suggests that *Apa* I polymorphism may influence attainment and maintenance of peak bone mass in premenopausal Chinese women.

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