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Original Article

Association between *VDR* and *ESR1* gene polymorphisms with bone and obesity phenotypes in Chinese male nuclear families

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Aim: The goal of this study was to determine whether polymorphisms in the vitamin D receptor (*VDR*) and estrogen receptor alpha (*ESR*1) genes are associated with variations of peak bone mineral density (BMD) and obesity phenotypes in young Chinese men.
Methods: A total of 1215 subjects from 400 Chinese nuclear families were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and allele-specific multiple PCR (ASM-PCR) analysis at the *Apa*I, *Fok*I, and *CDX*2 sites in the *VDR* gene and the *Pvu*II and *Xba*I sites in the *ESR*1 gene. BMD at the lumbar spine and hip, total fat mass, and total lean mass were measured using dual energy X-ray absorptiometry. The associations between *VDR* and *ESR*1 gene polymorphisms with peak BMD, body mass index (BMI), total fat mass, total lean mass, and percentage fat mass (PFM) were determined using quantitative transmission disequilibrium tests (QTDTs).
Results: Using QTDTs, no significant within-family associations were obtained between genotypes or haplotypes of the *VDR* and *ESR*1 genes and peak BMD. For the obesity phenotypes, the within-family associations were significant between *CDX*2 genotypes and BMI (P=0.046), fat mass (P=0.004), and PFM (P=0.020). Further, *Pvu*II was significantly associated with the variation of fat mass and PFM (P=0.002 and P=0.039, respectively). A subsequent 1000 permutations were in agreement with these within-family association results.
Conclusion: Our findings showed that *VDR* and *ESR*1 polymorphisms were associated with total fat mass in young Chinese men, but we failed to find a significant association between *VDR* and *ESR*1 genotypes and peak BMD. These findings suggested that the *VDR* and *ESR*1 genes are quantitative trait loci (QTL) underlying fat mass variation in young Chinese men.

Keywords: *VDR*; *ESR*1; polymorphism; bone mineral density; fat mass; lean mass; transmission disequilibrium test

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Introduction

Osteoporosis and obesity are two common complex diseases and major health problems. It is also known that obesity and osteoporosis are closely related diseases[1]. Human body composition generally changes with aging, mainly including a reduction in lean body mass, an accumulation of fat body mass, and a loss of bone mass[2]. These changes may correspondingly lead to osteoporosis and obesity. Both bone mass and obesity phenotypes are known to be under strong genetic regulation[3, 4]. In the past decade, a number of candidate genes have been identified that may contribute to bone mineral density (BMD), but far less is known about genes affecting obesity phenotypes such as lean mass and fat mass[5, 6]. Recently, Tang *et al*[7] conducted a bivariate whole-genome linkage scan and identified several genomic regions shared by obesity and osteoporosis.

The vitamin D receptor (*VDR*) is one of the most extensively studied genes in relation to BMD, due to the important role of vitamin D in bone metabolism[8]. A functional genetic polymorphism in *VDR* could be involved in other tissues that respond to vitamin D, such as muscle cells and adipocytes. Indeed, it has been demonstrated that vitamin D stimulates the differentiation of preadipocytes to adipocytes in the OB 17 cell line[9]. Some studies have also suggested that *VDR* genotype may function as a determinant of body composition[3].

Estrogen receptor alpha (*ESR*1) is also an important potential candidate gene for osteoporosis, and extensive studies have been performed on the relationship between polymorphisms

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of this gene and BMD variation. The *ESR*1 gene is known to be involved in metabolic pathways influencing body growth, which may correlate with body mass index (BMI)[10, 11]. One study demonstrated the association of *ESR*1 polymorphisms with body fat distribution in Japanese women[12]. Another study in Caucasians found *Pvu*II polymorphisms within the *ESR*1 gene to be associated with BMI, with the *Pvu*II genotype giving rise to the highest BMI values in postmenopausal women[13].

All of the above data indicate that the *VDR* and *ESR*1 genes could be pleiotropic genetic factors influencing both osteoporosis and obesity phenotypes. However, until now, the clear relationship between polymorphisms in the *VDR* and *ESR*1 genes with peak BMD and obesity phenotypes have not been elucidated. In this study, we investigated three *VDR* loci and two *ESR*1 loci with peak BMD, BMI, total fat mass and total lean mass in Chinese male nuclear families. Dual-energy X-ray absorptiometry (DXA) is a precise, accurate, non-invasive, safe, and convenient technique, found on a three compartment model separating the body into total body mineral mass, fat mass, and lean mass[14,15]. Most association studies use traditional association approaches in random population, and such study designs are prone to population stratification/admixture, which produces false positive/negative results. In addition, the linkage approach often lacks statistical power with the currently used sample. However, the transmission disequilibrium test (TDT), a family-based association approach, is immune to population stratification, much more powerful compared with the traditional linkage approach, and can be used in nuclear families with or without parental phenotypes. Thus, in this study, we used quantitative transmission disequilibrium tests (QTDTs) to determine whether *VDR* and *ESR*1 polymorphisms were associated with peak BMD and obesity phenotypes in a relatively large sample of Chinese male nuclear families.

Materials and methods

Subjects

All subjects involved in the study were collected by the Department of Osteoporosis of Shanghai Jiao Tong University Affiliated Sixth People's Hospital from the local population of Shanghai City (located on the mid-east coast of China) and signed informed consent documents before entering the project. Between 2004 and 2007, we recruited 1296 individuals from 427 male nuclear families whose offspring were sons. Of these, samples from 15 individuals could not be amplified and discriminated genotypes due to the poor quality of the DNA, and 12 sons deviated from Mendelian inheritance. Thus, there were a total of 400 male nuclear families composed of both parents and at least one healthy male child (1215 individuals) whose ages were largely between 20 to 40 years old. The average family size was 3.03; 385 families had one child and 15 families had 2. For each study subject, we also collected information on age, sex, medical history, family history, marital status, physical activity, alcohol use, diet habits and smoking history. The recruited sons were healthy. The exclusion criteria for the study subjects were a history of: (1) serious residual effects of cerebral vascular disease; (2) diabetes mellitus, except for easily controlled, non–insulin-dependent diabetes mellitus (defined as adult asymptomatic hyperglycemia controlled by diet or oral agents); (3) chronic renal disease manifested by a serum creatinine level of 11.9 mg/dl; (4) chronic liver disease or alcoholism; (5) chronic lung disease; (6) 12 weeks of corticosteroid therapy at pharmacologic levels; (7) 16 months of treatment with anticonvulsant therapy; (8) evidence of other metabolic or inherited bone diseases (*eg*, hyper- or hypoparathyroidism, Paget's disease of bone, osteomalacia, or osteogenesis imperfecta); (9) rheumatoid arthritis or collagen disease; (10) major gastrointestinal disease (*eg*, peptic ulcer, malabsorption, chronic ulcerative colitis, regional enteritis, or any significant chronic diarrhea state); (11) significant disease of any endocrine organ that would affect bone mass (*eg*, diabetes, hyperthyroidism, *etc*); (12) any neurologic or musculoskeletal condition that would be a nongenetic cause of low bone mass; and (13) any disease, treatment, or condition that would be a nongenetic cause of low bone mass[16] .

Phenotype measurements

The BMD (g/cm^2) of the lumbar spine and left proximal femur including lumber spine 1−4 (L1-4), femoral neck, total hip, total fat mass(kg), and total lean mass (kg) were measured by a Lunar Prodigy DXA densitometer (Lunar Corp, Madison, WI), and data were analyzed by Prodigy encore software (ver.6.70, standard-array, mode). The percentage of fat mass (PFM) was calculated as the ratio of fat mass to body weight[17]. The DXA scanner was on fan-beam mode. The machine was calibrated daily, and the coefficient of variability (*CV*) values of the DXA measurements (which were obtained from 15 individuals repeatedly measured three times) were: for lumbar spine, 1.39%; for femoral neck, 2.22%; for total hip, 0.70%; and for trochanter, 1.41% [18]. For body composition, the *CV*s were 1.18% and 3.72% for total lean mass and fat mass, respectively. The long-term reproducibility of our DXA data during the trial, based on weekly repeated phantom measurements, was 0.45%[4]. Body mass index (BMI) was defined as the weight/height^2 in units of kg/m^2.

Genotyping

Genomic DNA was isolated using the phenol-chloroform extraction method. Genotypes were detected using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and allele-specific multiple PCR (ASM-PCR). The primers are shown in Table 1. A 265 bp fragment containing the *Fok*I (rs10735810) polymorphism in the start codon of the *VDR* gene was amplified by PCR[19]. The *Fok*I genotypes were identified by electrophoresis of the DNA samples in 1.5% agarose gels. The *Fok*I genotypes were named as follows: FF (absence of the restriction site); ff (presence of the restriction site); Ff (heterozygous for the restriction site). A 740 bp fragment containing the *Apa*I (rs7975232) polymorphism near the 3′-end of the *VDR* gene was amplified by PCR[20]. The *Apa*I genotypes were named as follows: AA (absence of

Table 1. Information of the analyzed SNPs in this study.

Snp name	Loci		
*Fok*I	rs10735810	upstream primer 5′-AGCTGGCCCTGGCACTGACTCTGCTCT-3′	downstream primer 5′-ATGGAAACACCTTGCTTCTTCTCCCTC-3′
*Apa*I	rs7975232	upstream primer 5′-CAGAGCATGGACAGGGAGCAA-3′	downstream primer 5′-GCAACTCCTCATGGCTGAGGTCTC-3′
*CDX*2	rs11568820	G-upstream 5′-AGGATAGAGAAAATAATAGAAAACATT-3 G-downstream 5′-AACCCATAATAAGAAATAAGTTTTTAC-3′	A-upstream 5′-TCCTGAGTAAACTAGGTCACAA-3′ A-downstream 5′-ACGTTAAGTTCAGAAAGATTAATTC-3′
*Pvu*II	rs2234693	upstream primer 5′- CTGCCACCCTATCTGTATCTTTTCCTATTCACC-3′	downstream primer 5′- TCTTTCTCTGCCACCCTGGCGTCGATTATCTGA-3′
*Xba*I	rs9340799	upstream primer 5′- CTGCCACCCTATCTGTATCTTTTCCTATTCACC-3	downstream primer 5′- TCTTTCTCTGCCACCCTGGCGTCGATTATCTGA-3′

the restriction site); aa (presence of the restriction site); Aa (heterozygous for the restriction site). The *CDX*2 (rs11568820) polymorphism was determined using allele-specific multiple PCR (ASM-PCR) according to the method established by Fang *et al*[21]. The position of the *CDX*2 polymorphism is located in the promoter region of the *VDR* gene. Two sets of primers were designed for the ASM-PCR tests: These four primers generate three PCR fragments: primer set G-upstream and G-downstream specifically amplifies the G allele with a size of 110 bp; A-upstream and A-downstream specifically amplify the A-allele with a size of 235 bp; and the out-primer pair (G-upstream and A-downstream) amplifies the internal control PCR fragment with a size of 297 bp. Therefore, the GG genotype produced 297 bp and 110 bp fragments, the AA genotype produced 297 bp and 235 bp fragments, and the AG genotype produced 297 bp, 235 bp and 110 bp fragments[22]. PCR products were size-separated on a 2.5% agarose gel. A 1.3 kb fragment containing the *Pvu*II (rs2234693) and *Xba*I (rs9340799) polymorphisms in intron 1 of the *ESR*1 gene was amplified by PCR[23]. The PCR products were digested with *Pvu*II and *Xba*I, respectively, and separated by 2% agarose gel electrophoresis with ethidium bromide staining. The genotypes are represented as PP, Pp, and pp for *Pvu*II and XX, Xx, and xx for *Xba*I[24].

Statistical analyses

To test the population homogeneity of the study subjects, the genotype frequencies for each of the five polymorphic sites were tested against the Hardy-Weinberg equilibrium by the χ^2 test. To ensure unrelated individual samples, only genotype data from parents of each nuclear family were used in the statistical analysis[25]. The heritability estimates were done using the linear regression of parents' mean value and offspring's value for every phenotype (described at www.heritability.com). Statistical power was estimated using Piface Software (version 1.65) (http://www.math.uiowa.edu/~rlenth/Power/) in our current sample size, according to the MAF(minor allele frequency) of every genotype and the variation of BMD and obesity genotypes. The QTDT program, using the orthogonal model, was used to test for population stratification, total association, linkage and within-family association between SNPs and haplotypes and BMD phenotypes, BMI, fat mass and lean mass. The QTDT software package is available at: http://www.sph.umich.edu/csg/abecasis/QTDT/. This method, as implemented in the QTDT software[4, 26, 27], extends the trio-based TDT to quantitative trait data and uses genotypes data from available sibling and parents. Because in our nuclear families, all of the children were sons and the effects of parents' phenotypes were excluded in the QTDTs, sex was not used as a covariate to adjust the sons' bone phenotype variation[4, 25]. Of course, raw BMD values were adjusted by age, height and weight as covariates. The BMI, fat mass and lean mass were adjusted by age as a covariate. Because false-positive results can be generated in multiple tests (as in the present study) permutations (1000 simulations) were performed to generate the empirical p values to assess the reliability of the results[28–31]. The QTDT program generates P values for various tests via asymptotic χ^2 distribution. $P<0.05$ was considered significant for all analyses.

In unrelated sons, differences in BMD among the genotype and haplotype groups were tested using one-way ANOVA and general linear model-ANOVA (GLM-ANOVA), adjusting for confounding variables such as age, height and weight. Differences in BMI, fat mass, lean mass and PFM among the genotypes and haplotypes groups were determined using GLM-ANOVA, adjusting for age. Statistical analyses were performed using the SPSS package, version 11.5 (SPSS, Chicago, IL, USA).

Results

There were 400 nuclear families with 1215 individuals in this study, including 800 parents and 415 sons. The general characteristics of male nuclear families are shown in Table 2, and the basic characteristics of the sons are shown in Table 3. All of the subjects were genotyped at the *Apa*I, *Fok*I, *CDX*2, *Pvu*II, and *Xba*I polymorphic sites. Pearson correlation analysis

Table 2. Basic characteristics of the subjects (mean±SD).

Variables	Father (*n*=400)	Mother (*n*=400)	Son (*n*=415)
Age (years)	61.1±7.1	58.4±6.3	30.4±6.1
Height (cm)	167.8±6.0	155.7±5.5	172.9±5.9
Weight (kg)	69.7±9.5	58.2±8.2	70.7±10.8
BMI (kg/m^2)	25.2±2.7	24.0±3.1	24.2±3.2
Spine BMD (g/cm^2)	1.139±0.171	0.992±0.168	1.138±0.137
Femoral neck BMD (g/cm^2)	0.892±0.132	0.796±0.144	0.995±0.141
Total hip BMD (g/cm^2)	0.958±0.138	0.852±0.162	1.008±0.142
FM (kg)	–	–	16.31±7.56
LM (kg)	–	–	51.43±5.76
PFM (%)	–	–	21.9±7.3

confirmed that both fat mass and lean mass were positively correlated with BMD. According to the Pearson's coefficient (*r*), lean mass was more strongly related to BMD (with correlations range from 0.389 to 0.508), and fat mass was related to BMD (with correlations range from 0.198 to 0.243). To determine the relative contributions of lean mass and fat mass to BMD at various sites, we performed logistic regression analysis. In agreement with the Pearson analysis, both lean mass and fat mass showed positive regression coefficients, but lean mass had a significantly greater effect on BMD than fat mass. The r^2 between lean mass and BMD ranged from 0.149 to 0.249 and was from 0.056 to 0.068 between fat mass and BMD. The distribution of all of the genotypes was in Hardy-Weinberg equilibrium. The linkage disequilibrium (LD) between *Pvu*II and *Xba*I was not very strong (D′=0.684, r^2=0.256). For the *VDR* gene, the LDs between *Apa*I, *Fok*I, *CDX*2 were all weak (D′<0.1, r^2<0.01). Peak BMD is thought to be under strong genetic control. In our sample, the heritability estimates for peak BMD in the spine, femoral neck, and total hip were 0.565, 0.702, and 0.693, respectively.

We investigated the association between every genotype and BMD, BMI, fat mass and lean mass in 400 unrelated sons (randomly selected from 415 sons) using ANOVA. There was a significant association between *CDX*2 genotypes and BMI (*P*=0.006), and adjusted BMI values were 5.4% higher in the AA genotype compared to the AG genotype (*P*=0.002). A significant association between *CDX*2 genotypes and fat mass (*P*=0.004) was found, and adjusted fat mass values were 18.8% higher in the AA genotype compared to the AG genotype (*P*=0.002). There was a significant association between *CDX*2 genotypes and FPM (*P*=0.002), and adjusted PFM values were 14.8% higher in the AA genotype compared to the AG genotype (*P*=0.001). However, no significant association was found between *CDX*2 genotypes and peak BMD. In addition, marginally significant association was found between the *Pvu*II genotypes and BMD at the total hip (*P*=0.049). Moreover, a significant association between *Pvu*II genotypes and fat mass was observed (*P*=0.022); adjusted fat mass values were 14.3% higher in the pp genotype compared to the Pp genotype (*P*=0.006). A significant association between the *Pvu*II genotypes and PFM was also observed (*P*=0.020), and adjusted PFM values were 10.0% higher in the pp genotype compared to the Pp genotype (*P*=0.006). (Tables 4 and 5) Analysis of these SNPs showed that genetic variations in *CDX*2 explained 2.40%, 3.08%, and 3.40% of the variation in BMI, fat mass, and PFM, respectively; while *Pvu*II explained 1.86%, 2.18%, and 2.12% of population variance in BMI, fat mass, and PFM, respectively.

Association between *VDR* and *ESR*1 genotypes and peak BMD were performed using the QTDT program for nuclear families. The MAF of five SNPs was >30%, and they had high

Table 3. The bacic characteristics of the sons (*n*=415).

		n	Age (years)	Height (cm)	Weight (kg)	BMI	Lumber spine BMD (g/cm^2)	Femoral neck BMD (g/cm^2)	Total hip BMD (g/cm^2)	Fat mass (kg)	Lean mass (kg)	PFM (%)
*Apa*I	AA	36	29.5±6.0	172.0±6.3	70.9±11.1	23.9±3.3	1.147±0.148	1.009±0.145	1.018±0.155	16.9±7.0	50.8±6.4	23.1±6.8
	Aa	165	30.3±6.0	173.0±6.3	70.7±11.1	23.6±3.4	1.134±0.132	0.995±0.132	1.012±0.134	16.1±6.8	51.2±5.6	22.2±7.1
	aa	214	30.5±6.2	173.0±5.6	70.8±10.5	23.7±3.4	1.141±0.140	0.998±0.151	1.017±0.138	15.6±7.0	51.4±5.6	21.4±7.3
*Fok*I	FF	111	30.2±6.4	172.3±5.5	71.1±10.4	23.9±3.2	1.129±0.131	0.992±0.130	1.003±0.129	16.5±6.6	50.8±5.4	22.6±6.6
	Ff	211	30.3±6.1	172.8±6.0	70.8±11.3	23.7±3.5	1.146±0.132	1.008±0.141	1.025±0.135	16.1±6.8	51.2±5.8	22.2±7.0
	ff	93	30.7±5.8	173.8±6.1	70.1±10.3	23.2±3.2	1.132±0.156	0.981±0.160	1.005±0.153	14.9±7.4	52.0±5.6	20.4±8.0
CDX2	AA	108	30.1±6.5	172.2±5.7	72.8±10.4	24.5±3.2	1.142±0.126	1.001±0.142	1.013±0.131	17.9±6.6	51.5±5.3	24.0±6.6
	AG	184	30.2±5.7	173.1±6.2	69.8±11.5	23.3±3.6	1.128±0.143	0.998±0.150	1.014±0.145	15.1±7.3	51.1±6.1	20.9±7.5
	GG	123	30.9±6.2	173.3±5.7	70.4±9.8	23.5±3.0	1.151±0.138	0.995±0.134	1.017±0.132	15.4±6.2	51.3±5.2	21.4±6.8
*Pvu*II	PP	47	31.0±6.4	171.9±5.9	69.4±10.2	23.4±3.1	1.171±0.131	1.017±0.149	1.035±0.152	15.2±6.8	51.0±5.2	21.4±6.6
	Pp	197	30.4±6.2	173.4±5.9	69.9±10.7	23.2±3.4	1.140±0.138	0.996±0.140	1.013±0.133	15.1±7.0	51.0±5.5	21.0±7.6
	pp	171	30.2±5.9	172.6±5.9	72.1±10.9	24.2±3.3	1.128±0.138	0.995±0.145	1.010±0.139	17.2±6.7	51.7±5.9	23.1±7.0
*Xba*I	XX	15	28.3±5.6	174.0±6.6	70.7±11.6	23.3±3.4	1.200±0.153	1.046±0.203	1.022±0.163	15.5±8.5	51.7±4.7	21.6±8.5
	Xx	142	31.1±6.0	173.1±5.2	71.0±10.4	23.7±3.2	1.141±0.138	0.987±0.141	1.016±0.139	16.0±6.7	51.2±5.0	21.9±7.2
	xx	258	30.1±6.1	172.7±6.3	70.6±11.0	23.6±3.4	1.134±0.136	1.001±0.140	1.014±0.136	15.9±6.9	51.3±6.0	21.9±7.1
	Total	415	30.4±6.1	172.9±5.9	70.7±10.8	23.7±3.4	1.139±0.137	0.998±0.143	1.015±0.137	15.9±6.9	51.3±5.6	21.9±7.2

Table 4. Association of the three SNPs of *VDR* gene with phenotypic values in sons from one offsping (mean±SD, *n*=400). Values for BMD are raw values (means±SD); *P* values are results of analysis of variance (ANOVA) test for the least square mean of BMD among various genotypes after adjusting for significant covariates of age, weight, and height. *P* values are results of ANOVA test for the lease square mean of BMI, fat mass, lean mass among various genotypes after adjusting for significant covariates of age. Bold indicates significant *P* values (*P*<0.05). [c]*P*<0.01 *vs* AG genotype. [f]*P*<0.01 *vs* GG genotype..

Genotype	*n*	Age (years)	Height (cm)	Weight (kg)	BMI	Lumber spine BMD (g/cm^2)	Femoral neck BMD (g/cm^2)	Total hip BMD (g/cm^2)	Fat mass (kg)	Lean mass (kg)	PFM (%)
*Apa*I											
AA	36	29.5±6.0	172.0±6.3	70.9±11.1	23.9±3.3	1.147±0.148	1.009±0.145	1.018±0.155	16.9±7.0	50.8±6.4	23.1±1.3
Aa	155	30.1±6.0	173.0±6.4	70.8±11.1	23.6±3.4	1.137±0.131	0.997±0.127	1.017±0.132	16.1±6.8	51.2±5.4	22.1±0.6
aa	209	30.5±6.2	173.1±5.6	70.7±10.6	23.6±3.4	1.141±0.141	0.995±0.152	1.015±0.140	15.6±7.1	51.4±5.6	21.4±0.5
P		0.678	0.64	0.992	0.83	0.893	0.938	0.964	0.574	0.823	0.383
*Fok*I											
FF	107	30.2±6.4	172.4±5.5	71.0±10.5	23.9±3.2	1.127±0.132	0.989±0.131	1.002±0.129	16.4±6.6	50.7±5.4	22.5±6.6
Ff	204	30.1±6.1	172.9±6.0	70.8±11.2	23.7±3.4	1.148±0.132	1.006±0.140	1.025±0.135	16.2±6.8	51.2±5.7	22.2±7.0
ff	89	30.6±5.8	173.7±6.3	70.2±10.5	23.3±3.3	1.137±0.156	0.986±0.160	1.013±0.153	14.8±7.5	52.1±5.6	20.1±8.2
P		0.855	0.311	0.855	0.419	0.425	0.415	0.174	0.243	0.224	0.054
*CDX*2											
AA	103	29.8±6.5	172.4±5.7	72.9±10.4	24.5±3.2[c]	1.143±0.125	1.004±0.143	1.017±0.130	18.0±6.6[cf]	51.7±5.2	24.0±6.6[cf]
AG	180	30.2±5.8	173.1±6.2	69.7±11.7	23.3±3.6	1.129±0.144	0.995±0.147	1.014±0.144	15.1±7.4	51.1±6.1	20.9±7.6
GG	117	30.8±6.2	173.2±5.7	70.3±9.6	23.4±3.0	1.154±0.137	0.994±0.134	1.019±0.135	15.4±6.1	51.2±5.1	21.3±6.8
P		0.496	0.579	0.053	**0.006**	0.287	0.879	0.445	**0.004**	0.712	**0.002**

Table 5. Association of the two SNPs of *ESR*1 gene with phenotypic values in sons from one offsping (mean±SD, *n*=400). Values for BMD are raw values (means±SD); *P* values are results of analysis of variance (ANOVA) test for the least square mean of BMD among various genotypes after adjusting for significant covariates of age, weight, and height. *P* values are results of ANOVA test for the lease square mean of BMI, fat mass, lean mass among various genotypes after adjusting for significant covariates of age. Bold indicates significant *P* values (*P*<0.05). [b]*P*<0.05 *vs* pp genotype, [c]*P*<0.01 *vs* pp genotype. [e]*P*<0.05, [f]*P*<0.01 *vs* 2.

Genotype	*n*	Age (years)	Height (cm)	Weight (kg)	BMI	Lumber spine BMD (g/cm^2)	Femoral neck BMD (g/cm^2)	Total hip BMD (g/cm^2)	Fat mass (kg)	Lean mass (kg)	PFM (%)
*Pvu*II											
PP	46	30.9±6.5	171.8±5.9	69.5±10.3	23.5±3.1	1.175±0.129[c]	1.019±0.150	1.041±0.151[b]	15.5±6.8	50.9±5.2	21.4±6.6
Pp	189	30.2±6.2	173.4±6.0	69.9±10.6	23.3±3.4	1.143±0.136	0.997±0.139	1.016±0.133	15.0±7.0[c]	51.0±5.4	21.0±7.6[c]
pp	165	30.1±5.9	172.7±5.9	71.9±11.2	24.1±3.4	1.126±0.140	0.991±0.143	1.009±0.139	17.1±6.8	51.6±6.0	23.1±7.0
P		0.752	0.209	0.158	0.057	**0.010**	0.073	**0.049**	**0.022**	0.620	**0.020**
*Xba*I											
XX	15	28.3±5.6	174.0±6.6	70.7±11.6	23.3±3.4	1.200±0.153	1.043±0.210	1.024±0.170	16.1±8.5	51.3±4.7	21.6±8.5
Xx	134	30.9±6.0	173.0±5.2	71.1±10.3	23.7±3.2	1.145±0.137	0.990±0.142	1.021±0.140	15.9±6.7	51.3±5.0	21.9±7.1
xx	251	30.0±6.2	172.8±6.3	70.5±11.1	23.6±3.4	1.134±0.136	0.998±0.138	1.013±0.135	15.9±7.0	51.2±6.0	21.9±7.1
P		0.166	0.729	0.891	0.936	0.201	0.633	0.667	0.983	0.969	0.997
Haplotype containing px											
none	43	30.8±6.3	172.0±6	69.9±10.5	23.6±3.1	1.176±0.130[f]	1.012±0.146	1.036±0.148[e]	15.6±6.9	51.4±4.9	21.4±6.8
1	221	30.6±6.7	173.3±6.1	70.8±11.0	23.6±3.4	1.145±0.135	0.997±0.144	1.021±0.140	15.7±7.3	51.4±5.7	21.4±7.5
2	146	29.9±5.9	172.8±5.9	71.8±11.2	24.0±3.5	1.123±0.141	0.990±0.142	1.006±0.136	17±6.9	51.6±6.1	22.9±6.9
P		0.514	0.4	0.558	0.427	**0.011**	0.159	**0.048**	0.229	0.962	0.176

heterozygosis in our population, so we obtained 290, 309, 296, 283, and 246 informative nuclear families for the QTDT analysis for the *Apa*I, *Fok*I, *CDX*2, *Pvu*II, and *Xba*I genotypes, respectively. In this study, using 400 nuclear families has more than 80% of the power to test a candidate gene as a QTL, which can explain about 10% of the BMD or obesity phenotypes' varia-

tion. The results of the QTDT analysis are presented in Table 6. There was no population stratification for the genotypes at any sites of BMD. For the total association, *Pvu*II was associated with the variation of the lumbar spine, femoral neck and total hip BMD (P=0.008, P=0.006, and P=0.016, respectively). *Xba*I genotypes were associated with the variation of the lumbar spine BMD (P=0.040). For the within-family association, we did not obtain significant association evidence at any bone sites between *VDR* or *ESR*1 genotypes and peak BMD at any site.

Table 6. *P* value of tests for population stratification and within-family association between *VDR*, *ESR*1 genotypes and peak BMD. BMD values were adjusted by significant covariate effects of age, height and weight. Bold indicates significant *P* values (P<0.05).

	*Apa*I	*Fok*I	*CDX*2	*Pvu*II	*Xba*I
Tests of population stratification					
Lumber spine BMD	0.478	0.728	0.725	0.873	0.450
Femoral neck BMD	0.935	0.504	0.187	0.177	0.621
Total hip BMD	0.554	0.428	0.288	0.457	1.000
Test of total association					
Lumber spine BMD	0.175	0.973	0.241	**0.008**	**0.040**
Femoral neck BMD	0.932	0.279	0.497	**0.006**	0.105
Total hip BMD	0.544	0.937	0.634	**0.016**	0.107
Test of within-family association					
Lumber spine BMD	0.969	0.776	0.817	0.255	0.098
Femoral neck BMD	0.911	0.947	0.381	0.919	0.752
Total hip BMD	0.822	0.517	0.472	0.608	0.446
P 1000 permutation of within-family association					
Lumber spine BMD	0. 964	0.774	0.766	0.195	0. 079
Femoral neck BMD	0.902	0.943	0.354	0. 907	0. 782
Total hip BMD	0.801	0.531	0.469	0. 634	0. 485

Table 7. *P* value of tests for population stratification and within-family association between *VDR*, *ESR*1 genotypes and obesity phenotypes; BMI, total fat mass, total lean mass and PFM values were adjusted by age. Bold indicates significant *P* values (P<0.05).

	*Apa*I	*Fok*I	*CDX*2	*Pvu*II	*Xba*I
Tests of population stratification					
BMI	0.442	0.761	0.114	0.435	0.634
Fat mass	0.195	0.919	0.329	0.080	**0.008**
Lean mass	0.960	0.375	0.379	0.397	0.456
PFM	0.306	0.903	0.595	0.346	0.099
Test of total association					
BMI	0.851	0.900	0.194	0.079	0.544
Fat mass	0.165	0.069	**0.001**	**0.009**	0.900
Lean mass	0.566	0.120	0.700	0.144	0.541
PFM	0.082	**0.030**	**0.003**	**0.047**	0.807
Test of within-family association					
BMI	0.553	0.742	**0.046**	0.129	0.889
Fat mass	0.059	0.193	**0.004**	**0.002**	**0.049**
Lean mass	0.738	0.089	0.668	0.104	0.335
PFM	0.067	0.120	**0.020**	**0.039**	0.158
P 1000 permutation of within-family association					
BMI	0.536	0.741	**0.038**	0.129	0.888
Fat mass	0.110	0.290	**0.012**	**0.013**	0.127
Lean mass	0.748	0.063	0.626	0.108	0.354
PFM	**0.049**	0.087	**0.009**	**0.023**	0.153

The results of the associations between the five genotypes and obesity phenotypes in the QTDT analyses are presented in Table 7. There was population stratification between *Xba*I and fat mass (P=0.008). For the total associations, *CDX*2 and *Pvu*II were associated with fat mass (P=0.001 and P=0.009, respectively) and PFM (P=0.003 and P=0.047, respectively). For the within-family associations, *CDX*2 genotypes were associated with BMI (P=0.046), fat mass (P=0.004), and PFM (P=0.020), and *Pvu*II genotypes were associated with the variations of fat mass and PFM (P=0.002 and P=0.039, respectively). Considering multiple parameters were tested, 1000 permutation tests were performed, and subsequent permutations were in agreement with these significant within-family association results.

There were eight different haplotypes of the *VDR* gene and four haplotypes of the *ESR*1 gene. The frequencies of the haplotypes for all parents are shown in Table 8. We further observed the associations between *VDR* and *ESR*1 haplotypes and peak BMD using QTDTs. There was no significant population stratification, total association, or within-family association between the *VDR* or *ESR1* haplotypes and BMD. We also observed associations between *VDR* haplotypes and BMI, fat mass, lean mass, and PFM using QTDTs. For haplotype 1 (aFG) and fat mass, there was population stratification (P=0.008), significant total association (P=0.022), and within-family association (P=0.001 and P=0.010 of 1000 permutation tests). For haplotype 1 (aFG) and PFM, there was significant total association (P=0.012) and within-family association (P=0.010 and P=0.008 of 1000 permutation tests). For haplotype 6 (AFA) and fat mass, there was significant total association (P=0.018) and within-family association (P=0.012 and P=0.041 of 1000 permutation tests). Also, there was significant total association (P=0.017) and within-family association (P=0.040 and P=0.041 of 1000 permutation tests) for haplotype 6 (AFA) and PFM. Moreover, we investigated the association between *ESR*1 haplotypes and BMI, fat mass, lean mass, and PFM using QTDTs. For haplotype 1 (px) and fat mass, there was population association (P=0.011) and significant within-family association (P=0.002 and P=0.009 of 1000 permutation tests) (data not shown).

In addition, we also investigated associations between the most common haplotypes of the two genes (aFG for *VDR* and px for *ESR*1) and BMD, BMI, fat mass, lean mass and PFM in 400 unrelated sons using GLM-ANOVA. There was no

significant association between the aFG haplotype and BMD, BMI, fat mass, lean mass or PFM (data no shown). There was, however, significant association between the px haplotype and BMD at the lumbar spine (P=0.011) and total hip (P=0.048). Subjects carrying two copies of the px haplotype had higher BMD at the lumbar spine and total hip compared with those carrying no copy of the px haplotype (P=0.004 and P=0.032, respectively) (Table 5). No relationship between such combinations of genotypes and peak BMD or obesity phenotypes in 400 unrelated sons was found using two-way analysis of variance.

Table 8. Frequencies of *VDR* and *ESR*1 haplotypes for all parents in the study.

VDR gene				*ESR*1 gene			
Index	Haplotype	*n*	Frequency	Index	Haplotype	*n*	Frequency
1	aFG	325	0.203	1	px	966	0.604
2	aFA	278	0.174	2	pX	77	0.048
3	afG	272	0.170	3	Px	269	0.168
4	afA	238	0.149	4	PX	288	0.180
5	AFG	131	0.082				
6	AFA	121	0.076				
7	AfG	128	0.080				
8	AfA	107	0.067				

Discussion

Extensive population-based association studies have been performed in different ethnic groups to test the relationships between *VDR* and *ESR*1 genotypes and BMD variation[32, 33]. However, the results have been inconsistent or even contradictory. Our previous study did not find any relationship between *Apa*I genotypes and BMD in Chinese nuclear families[34], and this did not agree with Dundar's findings which indicated that the *VDR Apa*I polymorphism may be responsible for an important part of the heritable component of lumbar spine BMD in postmenopausal women[35]. Lau *et al*[36] reported that the *VDR Fok*I polymorphism may have a weak effect on the BMD of elderly Chinese women, but Remes *et al*[37] failed to find any association between *VDR FokI* and *ApaI* polymorphisms on BMD in middle-aged men. We found that *ESR*1 *Pvu*II and *Xba*I polymorphisms may have minor effects on peak BMD in Chinese women[24, 25], and another study[38] also found that the *ESR*1 *Pvu*II and *Xba*I genotypes may modulate the relationship between BMD in men. However, Finnish researchers failed to support the opinion that the *ESR*1 *Xba*I and *Pvu*II polymorphisms have a substantial impact on the development of peak bone mass in young men[39]. This may be due to the ethnic difference of the participants, to some specific effects of the gene mutation, and/or different genetic architecture and allele frequencies. In this study, we collected 400 Chinese male nuclear families, and used TDTs to simultaneously test linkage and/or association of the *VDR* and *ESR*1 gene polymorphisms with peak BMD. We found that *VDR* and *ESR*1 genotypes were not associated with lumbar spine and hip BMD in young Chinese males. Moreover, no significant within-family association was found between the *VDR* and *ESR*1 haplotypes and peak BMD. Long *et al*'s study[40] suggested that the *ESR*1 haplotypes, not single markers, may be associated with BMD variation at some skeletal sites in Chinese male samples. This result is in partial agreement with our findings. Therefore, further studies in other ethnic male populations are needed to better define the relationship between *VDR* and *ESR*1 genotypes and BMD.

Although the importance of the *VDR* and *ESR*1 genes to bone biology is widely acknowledged, their importance to obesity is seldom reported. Obesity is an excess proportion of total body fat. Several studies have shown that body weight is the most common index of obesity, but it cannot be used to distinguish body fat from lean mass. More defined phenotypes have been proposed for studying obesity, such as fat mass, lean mass, and percentage fat mass. Genetic variation in *VDR* is associated with muscle strength, fat mass, and body weight in Swedish women[3]. Recently, however, Moreno *et al*[41] failed to find an association between *VDR* genotypes and haplotypes with fat-free mass in postmenopausal Brazilian women. Roht's research[42] showed that *VDR* genotype is associated with fat-free mass in elderly Caucasian men. In the present study, we measured whole body fat mass, lean mass, and FPM as indices of the degree of obesity, using DXA. Using ANOVA and QTDT, we all found that *CDX*2 was significantly associated with BMI, fat mass, and PFM, and *Pvu*II was significantly associated with fat mass and PFM. Haplotype analysis also supported the above findings. These results are inconsistent with findings in Caucasian populations; evidence of a possible link between genetic variation in *ESR*1 and obesity is provided by the observation that the *Pvu*II polymorphism is associated with fat mass in women but not men[43]. However, Grundberg *et al*[44] found that a TA-repeat polymorphism in the *ESR*1 gene did not correlate with muscle strength or body composition in young adult Swedish women. This may be due to the difference of the participants, especially sex and menopause-associated changes.

The present study differs from most other studies in the following three characteristics. First, most of the studies mentioned above were based on the traditional population association approach, which is susceptible to population structure and with which it is easy to generate spurious results. In this study, we applied a more robust method, QTDT, to estimate the relationship between polymorphisms in the *VDR* and *ESR1* genes with spine and hip BMD. Peak BMD is thought to be under strong genetic control. In our sample, the heritability estimates for peak BMD in the spine, femoral neck, and hip are 0.565, 0.702, and 0.693, respectively. Second, we have now learned that lean mass and fat mass may be important determinants of the BMD[45, 46]. Many previous studies used BMD as a surrogate marker of bone strength, which did not take into account determinants of bone strength (*eg*, fat mass, lean mass and PFM) other than bone mass. Additionally, few studies have focused on the association of genetic polymorphisms with fat mass and lean mass. To our knowledge, this is the first study to investigate the possible influence of *VDR* and

ESR1 genotypes and haplotypes to BMD, BMI, fat mass, lean mass, and FPM variation in Chinese males. Third, we used a relatively large sample of male offspring nuclear families.

Of course, our study has several limitations. We tested only five common loci in the two genes, so we cannot rule out the possibility that association may exist between other polymorphisms in the genes with bone and obesity phenotypes. Therefore, further studies using denser markers are needed to test the effects of the *VDR* and *ESR*1 genes (and other candidate genes) in the Chinese or other populations. In addition, because all nuclear families were represented by only two generations in this study, no sibling pairs were informative for the linkage analyses. As a result, no linkage for the *VDR* or *ESR1* genotypes and haplotypes with BMD, BMI, fat mass, lean mass, and PFM were detected.

In conclusion, our results showed that the *VDR* and *ESR*1 polymorphisms were associated with fat mass in young Chinese men, but we failed to find a significant association between these polymorphisms and peak BMD. These findings suggested that the *VDR* and *ESR*1 genes are the QTL underlying fat mass variation in young Chinese men. Confirmation of our results is needed in other populations and with more functional markers of the two genes.

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Author contribution

Zhen-lin ZHANG designed research; Jie-mei GU, Wen-jin XIAO, Jin-wei HE, and Wen-zhen FU performed research; Hao ZHANG, Wei-wei HU, Yun-qiu HU, Miao LI, Yu-juan LIU, Jin-bo YU, Gao GAO, Hua YUE, and Yao-hua KE recruited subjects; jie-mei GU and Zhen-lin ZHANG wrote the paper.

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