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Estrogen receptor α gene relationship with peak bone mass and body mass index in Chinese nuclear families

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Abstract Estrogen receptor alpha (ER- α) plays an important role in mediating estrogen signaling. Studies in Caucasian populations have shown that it is involved in endocrine-related diseases such as osteoporosis and obesity. In the present study, we first used a quantitative transmission disequilibrium test (QTDT) to examine the relationship between this gene and both the osteoporosis-related phenotype bone mineral density (BMD), and the obesity-related phenotype body mass index (BMI), in 384 Chinese nuclear families. We genotyped a dinucleotide repeat marker (TA)_n, and a long-range haplotype was reconstructed using this marker and two other restriction fragment length polymorphism (RFLP) markers at *PvuII* and *XbaI* loci. Although we found significant total association [allele (TA)₂₁ with hip BMD ($P=0.001$), and haplotype Px(TA)₂₁ with spine ($P=0.0007$) and hip ($P=0.0006$) BMD], the more reliable within-family associations were not significant between these phenotype pairs. No linkage signal was obtained for either spine BMD or hip BMD. We found no association or linkage between any of the three studied polymorphisms and the long-range haplotypes of the ER- α gene and BMI. Our study does not support

an association of the ER- α gene with BMD and BMI in the Chinese population.

Keywords Association · Linkage · Estrogen receptor alpha gene · Bone mineral density · Body mass index

Introduction

Estrogen acts on a variety of aspects of physiological processes in addition to its well-known effect on reproduction. A dramatic decrease in estrogen level is associated with a series of diseases such as obesity, osteoporosis, insulin resistance, cardiovascular disease, and loss of muscle mass (Labrie et al. 1998). The estrogen receptor (ER) is a ligand-activated transcription factor comprising ER- α and ER- β , both of which play a central role in the estrogen signaling that may be related with endocrine-related diseases such as osteoporosis and obesity.

Bone mineral density (BMD), the most powerful measurable factor for osteoporosis, is under strong genetic control (Jian et al. 2005; Recker and Deng 2002). The ER- α gene is an important potential candidate gene for BMD variation, and extensive studies have been performed on the relationship between polymorphisms of this gene and BMD. In Caucasians, results have been somewhat inconsistent, with some studies finding significant association between the ER- α gene and BMD, and others coming to contradictory conclusions (Bagger et al. 2000; Becherini et al. 2000; Van Meurs et al. 2003). In Japanese populations, a large sample study (2,230 subjects) suggested that the ER- α gene is a susceptibility locus for BMD in elderly Japanese women (Yamada et al. 2002). Several studies performed in postmenopausal Chinese women, using a relatively small sample size, found an almost consistent association between BMD and the ER- α gene (Chen et al. 2001; Liu et al. 2003). Body mass index (BMI) is a WHO standard index for obesity. BMI is also under strong genetic control,

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with a heritability of 20–90% (Deng et al. 2001; Maes et al. 1997; Rice et al. 1999). The ER- α gene is known to be involved in metabolic pathways influencing body growth, which may correlate with BMI (Giuffrida et al. 1992; Joyner et al. 2001). One study demonstrated association of polymorphisms in the ER- α gene with body fat distribution in Japanese women (Okura et al. 2003). Another study in Caucasians found *PvuII* polymorphisms within the ER- α gene to be associated with BMI, with the PP (absence of the *PvuII* site is designated P allele) genotype giving rise to the highest BMI values in postmenopausal women (Deng et al. 2000).

To date, traditional linkage or association approaches have commonly been used to search for genes underlying complex disease. These approaches have limitations as well as merits. The regular association approach may yield spurious results due to population stratification/admixture. The linkage approach is often short on statistical power with currently used sample sizes. An alternative approach, quantitative transmission disequilibrium test (QTDT), is useful in identifying genes underlying complex traits (Abecasis et al. 2000a, b). It is immune to population stratification, and can be used in nuclear families, with or without parental genotypes.

In this study, using a QTDT method, we aim to test association and linkage between BMD, BMI and three polymorphisms in the ER- α gene, including a dinucleotide thymine–adenine repeat locus (TA)_n located 1,174 bp upstream of the first exon, and two RFLP (restriction fragment length polymorphism) loci, *PvuII* and *XbaI*. Since our previous study (Qin et al. 2003) studied the relationship between *PvuII* and *XbaI* RFLP loci and BMD using the sample from which the present study sample was selected, we did not study the linkage and association between BMD and these two polymorphisms.

Materials and methods

Subjects

The subjects in this study came from our previous study (Qin et al. 2003), which was approved by the Research Administration Departments of the Shanghai Sixth People's Hospital and Hunan Normal University. Each nuclear family comprised both parents and at least one female child aged between 20 and 45 years. In total, 384 nuclear families with 1,207 individuals were analyzed. The average family size was 3.14, in which 333, 48, 2, and 1 families had 1, 2, 3, and 4 children, respectively. All subjects were recruited from Shanghai local residents of Han ethnicity (greater than 93% of the total Chinese population are of Han descent). The enrollment work was completed within 1 year (January 2001–February 2002). For each study subject, we also collected information on age, gender, gynecological and medical history, etc. The daughters were all pre-menopausal. All subjects signed informed consent documents before entering the project. Exclusion criteria were chosen to

minimize any known potential confounding factors on BMD, as detailed by Deng et al. (2002), and subjects were assessed by gaining information from nurse-administered questionnaires and/or medical records.

Measurement

The BMDs (g/cm²) at lumbar spine (L_{1–4}) and total hip were measured using a Hologic QDR 2000+ dual-energy X-ray absorptiometry (DXA) scanner (Hologic, Bedford, MA). For lumbar spine, the quantitative phenotype was the combined BMD of L_{1–4}. For total hip, the quantitative phenotype was combined BMD of femoral neck, trochanter, and intertrochanteric region. The machine was calibrated daily. The coefficients of variation (CV) of BMDs obtained by five repeated measurements on seven individuals were 0.9% for lumbar spine, and 0.8% for total hip. Weight and height were measured at the time of DXA measurement by standard methods; BMI was calculated as weight (in kilograms) divided by [height (in meters) squared].

Genotyping

Genomic DNA was extracted by a standard phenol–chloroform extraction procedure (Lu 1999). *PvuII* and *XbaI* RFLP polymorphisms were distinguished using a polymerase chain reaction (PCR)-RFLP method. A 1.3-kb fragment containing the *PvuII* and *XbaI* polymorphisms in intron 1 of the ER- α gene was amplified by PCR using previously described primers and amplification conditions (Kobayashi et al. 1996). PCR products were digested with *PvuII* and *XbaI* restriction endonucleases (Promega, Madison, WI), separated by 1.5% agarose gel electrophoresis, and stained with ethidium bromide. The alleles were represented as P and p for *PvuII*, and X and x for *XbaI*. Upper and lower case letters represent absence and presence of restriction sites, respectively. The (TA)_n microsatellite was genotyped using an ABI 377 sequencer (Applied Biosystems, ABI, Foster City, CA). The primers for this marker were 5'-GACGCATGATATACTTACC-3' (forward, 5' terminus labeled with a fluorescent tag) and 5'-GTCCTACAACTCGATCTTCTC-3' (reverse). The genotype was determined using GENESCAN and GENOTYPER software (ABI) and GenoDB (Li et al. 2001). The program PedCheck was employed to verify Mendelian inheritance of all the marker alleles within each family (O'Connell and Weeks 1998).

Statistical analyses

Linkage disequilibrium analysis

Linkage disequilibrium (LD) coefficient (*D'*) between pairs of the three markers was calculated from the

disequilibrium measure D . The allele frequency-independent D' was calculated, where $D' = D/D_{\max}$. If $D < 0$, then $D_{\max} = pq$, and if $D > 0$, then $D_{\max} = p(1-q)$.

Haplotype reconstruction

The haplotypes at the three loci for all individuals were determined by applying the program SimWalk2 (available at <http://www.genetics.ucla.edu/home/software.htm>). SimWalk2 estimates the most likely set of fully typed maternal and paternal haplotypes of the marker loci from each individual in the pedigree. This analysis uses simulated annealing algorithms and Markov chain Monte Carlo (MCMC) to deduce the haplotype of each subject.

Quantitative transmission disequilibrium test analysis

Normality of the phenotype values were tested using the Shapiro–Wilks test before QTDT analysis. The program QTDT (<http://www.sph.umich.edu/csg/abecasis/QTDT>) was used to test population stratification, linkage, and association for $(TA)_n$ and haplotypes. The association test is based on the method described by Abecasis et al. (2000a). Evidence for association can be evaluated by maximization of likelihood (L) value with the constraint $\beta_a = 0$ (null-hypothesis likelihood, L_0) and without constraints on the parameters (alternative-hypothesis likelihood, L_1), where β_a is a coefficient of additive genetic value for the observed marker. Asymptotically, the quantity $2[\ln(L_1) - \ln(L_0)]$ is distributed as χ^2 , with df equal to the difference in number of parameters estimated. The means model is $\hat{Y} = \mu + \beta_b b_i + \beta_w w_{ij}$, in which association effects ($\beta_a g_{ij}$) are partitioned into two components, orthogonal between-family ($\beta_b b_i$) and within-family ($\beta_w w_{ij}$) association effects, where g_{ij} is the genotype score for the j th offspring in the i th family. The between-family association is sensitive to population structure, while the within-family association is immune to confounding population substructure effects and is significant only in the presence of LD. When the within-family association is observed, the approximate phenotypic variation due to the detected marker is calculated as $2p(1-p)a^2/V_p$, where V_p is the total phenotypic variance, p is the allele frequency of the marker and a is the estimate of additive effect. To assess the reliability of the within-family association, permutations (1,000 times) were performed. In all the statistical analyses, raw BMD was adjusted by covariates of age, weight, and height, and raw BMI was adjusted by age. Since the phenotypes of parents were excluded in QTDT analyses, and all the offspring in our sample were daughters, and all were premenopausal, gender and menopausal status were not used as covariates. Taking the multiple testing problem into account, 1,000 repetitions of Monte Carlo permutation tests were performed to establish an empirical threshold, which was $P \leq 0.002$ for an individual test to

achieve a global significance level of 0.05 for our analyses.

Results

Table 1 shows the basic characteristics of all phenotypes in 439 female offspring of the nuclear families. The mean age of the daughters was 31.4 years. BMD (mean \pm SD) at the spine and hip were 0.96 ± 0.10 and 0.85 ± 0.11 g/cm², respectively. BMI (mean \pm SD) was 21.5 ± 2.9 kg/m². The values are basic statistics representing raw values. All phenotype values in daughters were agreement with normal distribution when tested by the Shapiro–Wilks method.

Allele and haplotype frequencies and LD analysis

The most frequent alleles of $(TA)_n$ in our random sample of parents were alleles of 14–16 repeats. When analyzed by chi-square test, the allele distribution in our sample differed significantly from Dutch and Japanese populations (Table 2) (Ban et al. 2000; Van Meurs et al. 2003). In our population, the most common haplotypes were $px(TA)_{15}$, $px(TA)_{16}$ and $px(TA)_{14}$, with frequencies of 16.1, 15.3, and 11.0%, respectively. LD was observed between the *PvuII* and *XbaI* RFLP loci and $(TA)_n$ locus with $D' = 0.42$.

QTDT analysis

The level of empirical threshold for statistical significance in our study is $P = 0.002$. Significant total association was observed between the $(TA)_{21}$ allele of the ER- α gene and hip BMD ($P = 0.001$). Haplotype analysis showed significant total association between the $Px(TA)_{21}$ haplotype and BMD for spine ($P = 0.0007$) and total hip ($P = 0.0006$). Suggestive within-family association was obtained between $(TA)_{18}$ and spine BMD ($P = 0.035$), which explained only about 2.42% of total variation even if we assume the within-family association to be significant. For BMI, we obtained suggestive within-family association at the $(TA)_{15}$ ($P = 0.043$) and $(TA)_{24}$ ($P = 0.021$) alleles, and suggestive total association with

Table 1 Basic characteristics in 439 female offspring. All basic statistics were analyzed using raw data from all daughters in nuclear families. *BMI* Body mass index, *BMD* bone mineral density

Phenotype	$\bar{x} \pm$ SD
Age (years)	31.4 \pm 5.7
Height (cm)	159.9 \pm 5.2
Weight (kg)	55.1 \pm 8.1
BMI (kg/m ²)	21.5 \pm 2.9
BMD at L ₁₋₄ lumbar spine (g/cm ²)	0.96 \pm 0.10
BMD at total hip (g/cm ²)	0.85 \pm 0.11

Table 2 Allele frequency comparison for the (TA)_n locus between our Chinese population and two other populations

Allele	Number [frequency (%)]		
	Our sample ^a	Dutch (Van Meurs et al. 2003)	Japanese (Ban et al. 2000)
(TA) ₁₀	1 (0.07)	–	–
(TA) ₁₂	2 (0.13)	–	24 (6.3)
(TA) ₁₃	140 (9.11)	153 (7.87)	88 (23.2)
(TA) ₁₄	262 (17.06)	543 (27.93)	58 (15.3)
(TA) ₁₅	334 (21.74)	194 (9.98)	39 (10.3)
(TA) ₁₆	315 (20.51)	54 (2.78)	13 (3.4)
(TA) ₁₇	56 (3.65)	63 (3.24)	12 (3.2)
(TA) ₁₈	43 (2.80)	25 (1.29)	23 (6.1)
(TA) ₁₉	62 (4.04)	78 (4.01)	16 (4.2)
(TA) ₂₀	39 (2.54)	59 (3.03)	23 (6.1)
(TA) ₂₁	58 (3.78)	187 (9.62)	15 (3.9)
(TA) ₂₂	67 (4.36)	170 (8.74)	37 (9.7)
(TA) ₂₃	60 (3.91)	237 (12.19)	18 (4.7)
(TA) ₂₄	63 (4.10)	144 (7.41)	10 (2.6)
(TA) ₂₅	19 (1.24)	37 (1.90)	2 (0.5)
(TA) ₂₆	9 (0.59)	–	1 (0.3)
(TA) ₂₇	6 (0.39)	–	1 (0.3)
<i>P</i> value	< 0.001	< 0.001	

^aOur sample here denotes all parents of our nuclear families

the P_X(TA)₁₅ ($P=0.037$) and P_X(TA)₁₉ ($P=0.045$) haplotypes. We found no association between the *PvuII*, *XbaI* and *PvuII/XbaI* haplotypes and BMI. For results on the relationship between polymorphisms of *PvuII* and *XbaI* loci, and *PvuII/XbaI* haplotypes and BMD, please refer to a previous study of our Chinese sample (Qin et al. 2003). In the present study, no significant linkage was found with either BMD or BMI (Table 3).

Discussion

Extensive population-based association studies have been performed in different ethnic groups to test the relationship between the ER- α gene and BMD (Albagha

et al. 2001; Becherini et al. 2000; Liu et al. 2003; Van Meurs et al. 2003). However, the results have been inconsistent or even contradictory. 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 ER- α gene and BMD/BMI. Second, among those studies performed with Chinese individuals, most tested the *PvuII* and *XbaI* loci and BMD, and few detected the effect of (TA)_n polymorphism and long-range haplotype on BMD, except one study with a very

Table 3 All suggestively significant results of association analyses^a

Alleles and haplotypes	BMD chi-square value (<i>P</i> value)		BMI chi-square value (<i>P</i> value)
	L ₁₋₄ spine	Total hip	
Population stratification	–	–	–
(TA) ₂₁	5.42 (0.020)	–	–
p _X (TA) ₁₅	–	–	4.26 (0.039)
Total association	–	–	–
(TA) ₂₁	6.22 (0.013)	10.68 (0.001*)	–
(TA) ₂₅	4.60 (0.032)	–	–
p _X (TA) ₂₁	11.41 (0.0007*)	11.73 (0.0006*)	–
P _X (TA) ₂₁	–	5.06 (0.025)	–
P _X (TA) ₁₈	–	4.76 (0.030)	–
p _X (TA) ₁₄	–	3.93 (0.047)	–
P _X (TA) ₁₅	–	–	4.37 (0.037)
P _X (TA) ₁₉	–	–	4.02 (0.045)
Within-family association	–	–	–
(TA) ₁₈	3.93 (0.047) ([0.035])	–	–
(TA) ₂₄	–	–	5.36 (0.033) ([0.021])
(TA) ₁₅	–	–	4.07 (0.036) ([0.043])

^aAll values are chi-square values (*P* values in parentheses) of test of comparison for null-hypothesis and alternative-hypothesis likelihood for different individual alleles or haplotype tests. – $P>0.05$, * significant at the level of empirical threshold $P\leq 0.002$. Values in square brackets indicate *P* values from 1,000 permutation tests. All other values are *P* values for suggestively significant results

small sample size that demonstrated that subjects with genotype 18+ ($n=4$) at the $(TA)_n$ locus had lower BMD values and a 54.5-fold greater risk for osteoporosis when compared with subjects with genotype 18- ($n=170$) at the lumbar spine (Liu et al. 2003). To obtain more information on polymorphism of the ER- α gene, we analyzed the $(TA)_n$ locus as well as *PvuII* and *XbaI* loci in this study. Third, in the present study, instead of the mixed populations of pre-, peri-, and post-menopausal women analyzed in other studies, we focused on the phenotypes of pre-menopausal daughters in our nuclear families, thus avoiding the confounding effect of menopause on BMD. In addition, the ages of the daughters in our nuclear families ranged from 20 to 45 years, which is approximately within the age range for peak BMD in Chinese women (Liao et al. 2002). Since peak bone mass attainment in adulthood is an important factor for osteoporosis risk in later life, our study focused on the relationship between peak BMD and the ER- α gene. To our knowledge, the present study is the first to test family-based association between peak BMD and polymorphisms of $(TA)_n$, long-range haplotype reconstructed by *PvuII*, *XbaI*, and $(TA)_n$ loci of the ER- α gene using the QTDT method in Chinese populations.

Total association was observed between the $Px(TA)_{21}$ haplotype and the $(TA)_{21}$ allele with hip BMD. The haplotype $Px(TA)_{21}$ was also associated with spine BMD. When multiple markers are included in LD, analysis based on haplotypes may be more efficient and powerful than separate analyses of individual markers. This has been demonstrated by both simulation studies (Morris and Kaplan 2002) and empirical studies (Martin et al. 2000). However, since the sample size for haplotype $Px(TA)_{21}$ (17 subjects) was relatively small, we should interpret the results of our haplotype analysis with caution. Unexpectedly, we found no within-family association for the $(TA)_{21}$ allele and the $Px(TA)_{21}$ haplotype, despite the fact that total association was observed between these two markers and hip BMD. Since within-family associations are not subject to population admixture/stratification, they are more reliable than total association results. Although our results indicated significant total association between the $Px(TA)_{21}$ haplotype and the $(TA)_{21}$ allele with hip BMD, the insignificant results of within-family association and linkage analyses do not support any real association between the $(TA)_n$ locus and BMD in our sample. Our results are in accordance with the conclusion of a recent meta-analysis demonstrating that none of the three polymorphisms or haplotypes had any statistically significant effect on BMD, but that *XbaI* polymorphism determines fracture risk by mechanisms independent of BMD (Ioannidis et al. 2004).

Studies to evaluate the relationship between BMI and 5' polymorphisms of the ER- α gene have seldom been performed. This is the first study to test association of the 5' polymorphisms and long-range haplotypes of the ER- α gene with BMI variation based on QTDT. We found no apparently significant association

between BMI and polymorphisms of the *PvuII*, *XbaI* and *PvuII/XbaI* haplotypes in our Chinese nuclear families. Suggestive association was observed with $(TA)_n$ alleles and long-range haplotypes. These results are inconsistent with findings in Caucasian populations, in which *PvuII* polymorphism is significantly associated with BMI, explaining 6.2% of BMI variation (Deng et al. 2000). Such inconsistency may be due to the following potential reasons. First, the genetic factors accounting for BMI variation may be ethnically heterogeneous; the effect of the ER- α gene on BMI may be not the same in different ethnic populations. Second, the effect of ER- α genotype might vary in different periods of a woman's life. In the present study, the phenotyped daughters were aged 20–45 years and all were pre-menopausal, whereas in the Caucasian study, subjects were all postmenopausal and aged 65–84 years. Although we found no significant association in our sample, we cannot rule out a role for the ER- α gene in BMI. Since we tested only three loci, further studies using denser markers are needed in future studies to test the effect of the ER- α gene in Chinese populations. Unexpectedly, there was no indication of significant linkage for either BMD or BMI. This may largely be due to the relatively small sample of informative sib pairs for linkage analysis. In our nuclear families, there were only 58 sib pairs, i.e., too few informative sib pairs for a linkage test. As quantitative trait loci (QTLs) with small effect are difficult to detect by regular linkage test (Weeks and Lathrop 1995), another possible reason is the relatively small effect of the ER- α gene on the phenotypes tested. A potential limitation of our study that should be mentioned is the relatively small sample size for within-family association. Since only those families including at least one heterozygous parent were informative in QTDT analysis, the negative within-family association between the ER- α gene and BMD and BMI may be overstated in this study.

In conclusion, the present study indicates that 5' polymorphism of the ER- α gene may be not associated with total hip and spine BMD and BMI in our Chinese population. Further studies with larger sample size are needed to better define the relationship between the ER- α gene and BMD and BMI. If possible, more direct phenotypes for osteoporosis, such as osteoporotic fractures, should be included in future genetic analyses.

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