Acta Pharmacologica Sinica (2010) 31: 455–460
 1671-4083/10 $32.00
www.nature.com/aps

Original Article

Association analysis of genetic polymorphisms and potential interaction of the osteocalcin (BGP) and ER-α genes with body mass index (BMI) in premenopausal Chinese women

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Aim: To investigate whether estrogen receptor α (ER-α) *Pvu*II and osteocalcin (also known as bone Gla protein, or BGP) *Hind*III genetic polymorphisms and their potential interactions are associated with body mass index (BMI) variation.
Methods: Data on BMI and ER-α *Pvu*II and BGP *Hind*III genotypes were obtained from 328 healthy premenopausal Chinese women in east China. The study subjects were unrelated, at least 21 years old (mean age of 33.2±5.9 years), and had an average BMI of 21.58±2.59. All subjects were genotyped at the ER-α *Pvu*II and BGP *Hind*III loci using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP).
Results: The BGP *Hind*III genotypes were significantly associated with BMI (P=0.003). Carriers of the HH and Hh genotypes had approximately 2.73% and 1.27% higher BMI than those of the hh genotype, respectively. In contrast, the ER-α *Pvu*II polymorphism was not significantly associated with BMI (P=0.454). In addition, there was no evidence of potential interactions between the ER-α and BGP genes in our subjects (P≥0.013).
Conclusion: The *Hind*III polymorphism of the BGP gene, but not the *Pvu*II polymorphism of the ER-α gene or their potential interaction, was associated with BMI in premenopausal Chinese women.

Keywords: body mass index (BMI); osteocalcin (BGP) gene; estrogen receptor-α (ER-α) gene; analysis of covariance (ANCOVA)

Acta Pharmacologica Sinica (2010) 31: 455–460; doi: 10.1038/aps.2010.1; published online 22 Mar 2010

Introduction

Obesity is a serious and complex disease worldwide[1–3]. Body mass index (BMI), defined as weight in kilograms divided by the square of height in meters, is a WHO standard index for obesity. BMI is under strong genetic determination with heritability ranging from 20% to 90%[4–8]. Although genetic factors have a clear role in determining BMI variations, the most substantial genes underlying the variation of BMI are not well identified[9].

The estrogen receptor α (ER-α) gene has been listed as one of 127 possible candidate genes associated with obesity[9]. Several lines of recent evidence suggest a potential role for ER-α in the determination of BMI. ER-α may play a critical role in adipose tissue development, metabolism, deposition[10, 11] and energy metabolism[12]. The estrogen antagonist acting on ER-α can prevent diet- and ovariectomy-induced obesity, mainly by decreasing fat deposition[13]. Higher ER-α expression in adipose tissue was observed among obese postmenopausal women[14]. The ratio of ER-α to ER-β in adipose tissue was associated with obesity in both pre- and postmenopausal women[15]. Male ER-α knockout mice have previously been reported to develop obesity after sexual maturity[16, 17]. Additionally, ER-α levels were associated with BMI in breast cancer patients[18]. ER and progesterone receptor status was used to define breast cancer risk factors[19]. Polymorphisms in the ER-α gene have been reported to be associated with BMI, though contradictory results were also reported[20–27].

Osteocalcin, also called bone Gla protein (BGP), is an osteoblast-specific protein that is known to play a role in bone growth and has recently been reported to function as a new metabolic hormone regulating the adiposity and glucose homeostasis in experimental animals[28–30]. Osteocalcin increases adiponectin and insulin expression in adipocytes and β-cells, respectively. Osteocalcin-deficient mice also display

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Received 2009-07-27 Accepted 2010-01-04

obesity[28]. Osteocalcin was suggested to be the only molecule made by osteoblasts that accounts for the osteoblast-mediated regulation of glucose metabolism[31]. Serum osteocalcin levels were associated with BMI, glucose metabolism and body fat in several clinical studies[29, 32–35].

Due to the importance of ER-α and BGP genes in regulation of adiposity and glucose homeostasis, we hypothesized that genetic polymorphisms and potential interactions of the BGP and ER-α genes were associated with BMI. The polymorphism of the *Pvu*II site in the ER-α gene is the most extensively studied genetic marker in relation to BMI variation. However, the results so far have been largely inconsistent and controversial[20–27]. In addition, no data have been provided about the associations between BGP polymorphisms and BMI variation. The most frequently seen polymorphism in the BGP gene is *Hind*III, which is located in the promoter region. BGP *Hind*III has been commonly applied in the study of complex traits[36–38]. *Hind*III is, therefore, a possible genetic marker in the search for associations between the BGP gene and BMI variation. Our study was designed to determine whether ER-α *Pvu*II and BGP *Hind*III polymorphisms were associated with BMI variation and to test whether there was an effect of the interactions between the ER-α and BGP genotypes in determining BMI variation in a population of premenopausal Chinese women.

Materials and methods

Subjects

The study was approved by the Ethical Committee of Nanchang University and People's Hospital of Jiangxi Province. The 328 subjects in this study were recruited from a local population of Nanchang City in east China. They were all of the Han ethnic, comprising greater than 93% of the total Chinese population. Subjects with diseases, treatments, or conditions that would have an apparent influence on health or contribution to abnormal obesity were excluded. Informed consent was obtained from each subject. For all subjects, a detailed medical history, including menstrual history, was recorded by nurse-administered questionnaires. Information on female history including age at menarche and menopause, years since menopause, and number of births was collected. Physical activity and smoking history were also documented. The subjects were unrelated, healthy, non-smoking and premenopausal women. Body weight was measured to the nearest 0.01 kg using a digital scale, height was measured to the nearest 0.1 cm using a wall-mounted stadiometer, and BMI was calculated as weight (kg) divided by height squared (m^2).

Genotyping

Genomic DNA was isolated from whole blood using the phenol-chloroform extraction method[39]. All subjects were genotyped by polymerase chain reaction followed by restriction fragment length polymorphism analysis (PCR-RFLP). Figure 1 shows the structure of the ER-α and BGP genes and the locations of the studied polymorphisms in the two genes. The ER-α gene is located on chromosome 6q25 and is composed of 8 exons and 7 introns. The polymorphism of ER-α *Pvu*II is a C/T single nucleotide polymorphism (SNP) in intron 1 of the ER-α gene. The forward primer (5′-CTG CCA CCC TAT CTG TAT CTT TTC CTA TTC ACC-3′) and reverse primer (5′-TCT TTC TCT GCC ACC CTG GCG TCG ATT ATC TGA-3′) were used to amplify a 1.3 kb DNA fragment in intron 1 by previously described amplification conditions[40]. The BGP gene is located on chromosome 1q25-q31 and is composed of 4 exons and 3 introns. The polymorphism of BGP *Hind*III is a C/T SNP located in the promoter region of the BGP gene at the 198th nucleotide upstream from exon 1. For the *Hind*III polymorphism of the BGP gene, a 253 bp DNA fragment was produced using the forward primer (5′-CCG CAG CTC CCA ACC ACA ATA AGC T-3′) and the reverse primer (5′-CAA TAG GGC GAG GAG T-3′) by previously described amplification conditions[36]. After amplification, 8 μL of the PCR products was digested with the respective restriction endonucleases, *Pvu*II and *Hind*III (Promega Corp, Madison, WI, USA), at 65 °C for 4 h, electrophoresed on a 2% agarose gel in 1×TAE buffer, stained with ethidium bromide, and visualized under UV light. The genotypes were designated as PP, Pp, and pp for *Pvu*II and HH, Hh, and hh for *Hind*III. Uppercase and lowercase letters represent the absence and presence of the restriction sites, respectively.

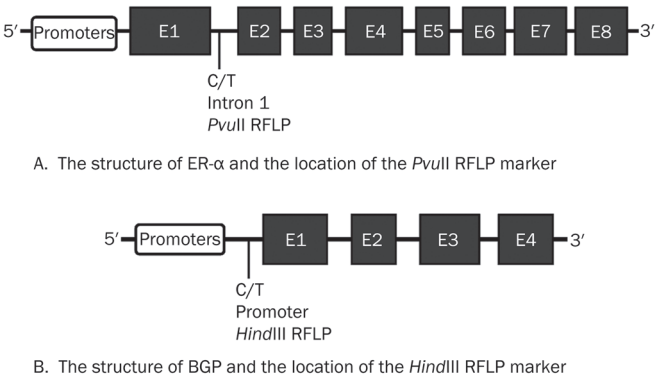

Figure 1. The structure of the ER-α and BGP genes and the locations of the studied polymorphisms in the two genes. Exons are depicted as filled boxes and introns as single lines between filled boxes.

Statistical analyses

All statistical analyses were conducted using SAS version 6.12 (SAS Institute, Cary, NC, USA). The χ^2 test was performed to examine Hardy-Weinberg equilibrium (HWE) at the studied marker loci, medical histories, genotype distributions of ER-α *Pvu*II and BGP *Hind*III polymorphisms, and associations with BMI. The phenotypic values were evaluated for the presence of a normal distribution by the Shapiro-Wilk test. Bartlett's tests were performed to test the homogeneity of variances in BMI between each BGP *Hind*III and ER-α *Pvu*II genotype. Linear regression analyses were performed to test the impact of medical history on BMI variation. The analysis of covariance (ANCOVA) was used to evaluate the relationship between BMI and each of the ER-α and BGP genetic polymorphisms. The frequency of the HH genotype in the BGP gene was low

in our subjects (7.93%). When the subjects were divided into three groups, HH, Hh and hh, the sample sizes were too small to analyze the effect of the polymorphism interaction appropriately. Therefore, the subjects were divided into two groups, those with and without the minor allele, H. To analyze the effect of a special ER-α allele, the subjects with this allele were identified as "1" and those without it were identified as "0". ANCOVA was used to test for the effect of a given ER-α gene allele associated with the special BGP genotype. When a significant result was observed, it was interpreted as an interaction between the ER-α and BGP genes. To avoid errors resulting from multiple-testing, *Bonferroni* corrections were applied to establish an empirical threshold which requires that individual tests have *P*-values <0.013 in order to achieve a global significance level of 0.05 for association and interaction analyses in the present study.

Results

Descriptive characteristics of the study subjects

The basic characteristics of the 328 unrelated premenopausal women are summarized in Table 1. The ER-α *Pvu*II allele frequencies were 38.6% for P and 61.4% for p and the BGP *Hind*III allele frequencies were 28.2% for H and 71.8% for h. The *Pvu*II polymorphism of ER-α and the *Hind*III polymorphism of BGP were in HWE (*P*>0.05). Medical histories and genotype distributions of ER-α *Pvu*II and BGP *Hind*III polymorphisms associated with BMI are listed in Table 2. Linear regression analyses indicated that only age had a significant impact on BMI variation among all the medical history (data not shown) and, thus, age was used to adjust BMI in association and interaction analyses.

Table 1. Basic characteristics of the subjects. *n*=328.

	Age (years)	Height (m)	Weight (kg)	BMI (kg/m^2)
Mean	33.2	1.568	55.11	21.58
SD	5.9	0.052	8.02	2.95
Min	21.3	1.450	39.00	16.24
Max	38.7	1.725	87.00	30.96

Note: Mean, SD, Min, and Max denote the mean, standard deviation, minimum and maximum values, respectively. BMI values are unadjusted raw data.

Association of the ER-α and BGP genes with BMI

The analyses did not reveal any significant violations of the assumptions of ANCOVA. For example, the *P* value was 0.203 for Bartlett's test for homogeneity of variances of BMI among the three BGP *Hind*III genotypes. The associations between ER-α and BGP with BMI variation are summarized in Table 3. Our analysis revealed a significant association between the BGP *Hind*III polymorphism and BMI (*P*=0.003). In our study subjects, individuals with the HH genotype had the highest BMI (21.81±0.73 kg/m^2), individuals with the Hh genotype had intermediate BMI (21.50±0.53 kg/m^2), and individuals with the hh genotype had the lowest BMI (21.23±0.63 kg/m^2), on average. Therefore, carriers of HH and Hh genotypes had approximately 2.73% and 1.27% higher BMI, respectively, than those with the hh genotype. We did not observe a significant association between the ER-α *Pvu*II polymorphism and BMI (*P*=0.454). In addition, the effect of the interactions between the ER-α *Pvu*II and BGP *Hind*III polymorphisms on BMI is listed in Table 4. We show that, with the *P* values of 0.017, 0.015, 0.020, and 0.015, the interactions between a given ER-α gene allele and the special BGP genotype did not have

Table 2. Medical history and genotype distributions of ER-α *Pvu*II and BGP *Hind*III polymorphisms associated with BMI.

	BMI (kg/m^2) Normal (<25) *n*=290	Overweight (≥25) *n*=38	*P* value
Age at menarche	13.65	13.42	>0.05
Number of births	0.57	0.58	>0.05
Exercise (%)			>0.05
Rarely/never	45.6	46.7	
<1/week	19.1	18.2	
1–3/week	24.6	26.0	
≥4/week	10.7	9.1	
ER-α *Pvu*II			0.001
PP	37	11	
Pp	137	20	
pp	116	7	
BGP *Hind*III			0.01
HH	23	3	
Hh	121	12	
hh	146	23	

Note:
1. Three subjects (30<BMI<31) in our population were classified as overweight.
2. The *P* values were obtained from *t*-tests for continuous variables and χ^2 for categorical variables.
3. '*n*' denotes the sample sizes of the normal and overweight groups.

Table 3. Association analyses for the ER-α *Pvu*II and BGP *Hind*III polymorphisms and BMI.

Marker	Genotypes	BMI (kg/m^2)	*P* value
ER-α-*Pvu*II	PP (48)	21.65±0.58	0.454
	Pp (157)	21.63±0.62	
	pp (123)	21.52±0.57	
BGP-*Hind*III	HH (26)	21.81±0.73	0.003
	Hh (133)	21.50±0.53	
	hh (169)	21.23±0.63	

Note:
1. The BMI values are displayed as mean±SD of BMI adjusted for age.
2. The values in parentheses are the number of genotypes for each marker.

significant effects on BMI values (Table 4). Thus, there was no evidence of potential interactions between the ER-α and BGP genes in our subjects ($P \geq 0.013$).

Table 4. Effects of interaction between the ER-α *Pvu*II and BGP *Hind*III genes on BMI.

		BGP *Hind*III	
		A	B
ER-α *Pvu*II			
P allele	1	21.74±0.90 (99)	21.30±0.80 (106)
	0	20.65±0.88 (60)	20.24±0.85 (63)
	P value	0.017	0.015
p allele	1	21.03±0.79 (130)	20.55±0.59 (150)
	0	22.36±0.71 (29)	22.07±0.43 (19)
	P value	0.020	0.015

Note:
1. All data are displayed as mean±SD of BMI values adjusted for age.
2. The values in parentheses indicate sample size.
3. "1" denotes carriers and "0" denotes non-carriers of the corresponding ER-α *Pvu*II allele.
4. "A" denotes the subjects with allele H (the HH and Hh genotypes) at the BGP *Hind*III locus and "B" denotes the subjects without allele H (the hh genotype) at the BGP *Hind*III locus.
5. *P* values were calculated by ANCOVA.

Discussion

In a population of healthy premenopausal Chinese Han women, we showed that the *Hind*III polymorphism of the BGP gene, but not the *Pvu*II polymorphism of the ER-α gene or their potential interaction, was associated with BMI.

A number of studies have shown that BGP plays an important role in obesity. However, no information regarding the role of BGP polymorphisms in BMI variation is available[28, 29, 31]. With an attempt to disclose the effect of BGP on BMI, we performed an association analysis of the BGP gene and BMI in a cohort of Chinese premenopausal women. The results suggest that the BGP *Hind*III polymorphism is significantly associated with BMI in our subjects. Considering that the effect of BGP *Hind*III on BMI may vary between different populations, the effect observed here has yet to be confirmed by separate analyses in different populations or ethnic groups.

Recent studies of the association between the ER-α gene polymorphism and BMI have yielded conflicting results[20–27]. Our results are inconsistent with the previous findings in non-Chinese populations, indicating significant associations between the ER-α gene and BMI variation in postmenopausal Caucasian women[20], middle-aged Japanese women[22], postmenopausal white women[21], African-American families[24], Brazilian subjects[25], and men from the Framingham Heart Study[23]. However, our findings are consistent with the two previous studies performed on Chinese females[26, 27] and a study in women from the Framingham Heart Study showing no effect of the ER-α gene on BMI variation[23]. The discrepancies between our study and the other study[23] may be due, partially, to ethnic differences, which may account for the difference in pathogenesis of BMI variation at genetic levels in different ethnic groups. Thus, testing the candidate gene(s) in different populations or ethnic groups is necessary to interpret the peculiar role of the candidate gene(s) in those populations. Additionally, differences in study subjects may partially account for the contradictory results. In the present study, the subjects were all premenopausal women, whereas in the Caucasian study[20, 21], subjects were all postmenopausal women. The effect of the ER-α genotype might vary between different periods of a woman's life.

It has been suggested that GXG (gene-by-gene) interactions are a ubiquitous phenomenon in the genetic control of complex traits[41, 42]. BMI is a complex trait determined by individual genes as well as their potential interactions. Substantial evidence indicates the ER-α and BGP genes may interact with each other to affect the BMI variation from a physiological point of view. Ovariectomy up-regulates and estrogen administration down-regulates the gene expression of osteocalcin in cancellous bone[43], bone marrow mesenchymal stem cells (MSCs) exposed to osteogenic differentiation medium[44], and periosteal cells in the long bones of rats[45]. Additionally, serum levels of osteocalcin were significantly elevated in ovariectomized animals compared to intact animals and were reduced by E_2 and the ER-α agonist[46, 47]. Intrigued by these findings, we offer insight into the relationship between the ER-α and BGP polymorphism interaction and BMI. Although no evidence of potential interaction between the ER-α and BGP genes in determining BMI was observed in our subjects, the present study represents our efforts to detect and characterize potential GXG effects that determine the genetic contribution to BMI variation that could not be derived from analyzing the individual polymorphisms. Most of the previous genetic studies have focused on the effects of individual genes on BMI[20–27] irrespective of the effects of interactions among multiple genes. Further studies are required to evaluate and dissect the potential effects of gene interactions on BMI.

It should be noted that the present study has potential limitations. First, we did not completely assess all of the factors related to BMI. Some potential confounding factors such as caloric intake and socioeconomic factors were not included. Second, only one genetic marker was analyzed in each gene in our study due to budget limitations. Further studies examining additional SNP markers that span the entire ER-α and BGP genes are necessary to draw a definitive conclusion on the importance of the ER-α and BGP genes on BMI variation. Next, statistical significance was only observed in a small sample of premenopausal Chinese women. The statistical power decreases considerably with the relatively small sample size. Finally, statistical significance was observed in a regular population association. Further studies employing robust approaches unaffected by population admixture, such as the transmission-disequilibrium test (TDT)[48], are required to confirm our finding. Despite these drawbacks, the current results

confirm findings of previous studies. Thus, our study is valid in terms of revealing potentially important associations that warrant additional investigation.

In conclusion, we show the *Hind*III polymorphism of the BGP gene, but not the *Pvu*II polymorphism of the ER-α gene or their potential interaction was associated with BMI in premenopausal Chinese women. The present study represents the first effort to simultaneously investigate the individual effects of the ER-α and BGP genes as well as their potential interactions on BMI variation with an attempt to completely understand the genetic effect underlying BMI. The statistical significance provides confirmation for the need for functional studies on molecular and cellular levels. Further studies in other populations with larger sample sizes and denser markers are required to confirm the findings reported here.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No 30760071, 30960118), the Foundation of the Education Department of Jiangxi Province (No GJJ08106), and the Foundation of Nanchang University (No J06). We thank all the study subjects for their participation in the study.

Author contribution

Hong XU and Hai-bin KUANG conceived and designed the experiments. Hong XU and Wen XIAO performed the experiments. Hong XU, Wen XIAO, Dan LUO, and Yong-ming LIU analyzed the data. Hong XU wrote the paper. Lin ZOU and Yong-ming LIU helped recruit the study subjects. Hai-bin KUANG revised the paper.

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