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Sex-Specific Association of the Glucocorticoid Receptor Gene With Extreme BMD

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ABSTRACT: To study the role of the *GR* gene on BMD regulation in the Chinese, a sex-specific association study was performed. The results indicated that *GR* variation contributed to the extreme BMD variation in the Chinese.

Introduction: The *glucocorticoid* (*GC*) *receptor* (*GR*) gene is an important candidate gene for BMD regulation in GC-induced osteoporosis (GIO). However, no study has explored the genetic effects of the *GR* gene on BMD variation in the Chinese population.
Materials and Methods: Our sample consisted of 800 unrelated subjects (400 women and 400 men) with extreme age-adjusted hip BMD Z-scores selected from a population composed of 1988 normal adult Chinese Han. Four single nucleotide polymorphisms (SNPs) in the *GR* gene were genotyped. Both single SNP and haplotype association analyses were conducted.
Results: SNP rs1866388 ($p_c = 0.028$) was found to be significantly associated with extreme BMD only in men. In both sexes, haplotypes involving rs1866388 and rs2918419 were found to have different frequency distributions in extremely low and high BMD groups ($p_p = 0.024$, 0.001, and 0.002 in women and 0.002, 0.003, and 0.003 in men for window sizes of two, three, and four SNPs, respectively). Most shared haplotypes showed opposite effects between women and men.
Conclusions: For the first time, our study suggested the possible role of the *GR* gene on BMD regulation and sex specificity in the association of *GR* with extreme BMD in the Chinese.
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Key words: glucocorticoid receptor, single nucleotide polymorphism, haplotype, association, sex specificity

INTRODUCTION

GLUCOCORTICOIDS (GCs) HAVE been widely used in treating various diseases. However, long-term treatment using GCs will cause severe bone loss[1] predisposing to osteoporosis. Approximately 50% of GC-treated patients with ≥6 mo therapy will develop osteoporosis and suffer osteoporotic fractures ultimately.[2] GC-induced osteoporosis (GIO) is the most common drug-related osteoporosis and the third leading cause of osteoporosis.[3,4]

The GC receptor (GR), a ligand-activated transcription factor, regulates the activation or repression of target genes involved in osteoblastogenesis and osteoclastogenesis, largely by binding to either positive or negative GC response elements (GREs),[5–7] which will cause the GC-induced decrease of BMD.[8–11] The human *GR* gene, located at chromosome 5q31.3, is widely expressed in a number of bone cells including osteoblasts, osteocytes, and chondrocytes.[12] The *GR* transcripts were also detected in the stromal-like tumor cells, macrophage-like cells (putative osteoclast precursors), and multinucleated osteoclast-like cells.[13]

Previous studies have suggested that the genetic variations of the *GR* gene were correlated with BMD variation in whites. Huizenga et al.[14] found that a polymorphism in codon 363 of the *GR* gene, resulting in an asparagines-to-serine change, was associated with an increased sensitivity to GC. van Rossum et al.[15] reported that a C/G substitution in intron 2 of the *GR* gene was associated with hypersensitivity to GCs, and the *G* allele female carriers had lower BMD at the lumbar spine. In addition, our group previously performed a comparative gene expression study of circulating monocytes using the Affymetrix HG-U133A GeneChip array and first confirmed that the *GR* gene was upregulated in white premenopausal women with lower BMD.[16]

The authors state that they have no conflicts of interest.

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However, the generality of the importance of the genetic variations in the *GR* gene on BMD variation, particularly in the Chinese, is still largely unknown. Here, we conducted sex-specific association analyses between the *GR* gene and extreme BMD phenotypes using both single nucleotide polymorphisms (SNPs) and their haplotypes in the Chinese population.

MATERIALS AND METHODS

Study subjects

This study was approved by the necessary Institutional Review Boards of the involved institutions. The subjects were randomly recruited from the resident Han population of Changsha and its surrounding areas. All the participants signed informed consent documents before entering the study. The detailed designs of questionnaire and the exclusion criteria have been published elsewhere.[17,18] The whole sample contained 1988 unrelated healthy subjects (1110 men and 878 women).

Areal BMD (g/cm^2) at the total hip (femoral neck, Ward's triangle, trochanter, and intertrochanter) for each subject was measured by a Hologic QDR 4500W DXA scanner (Hologic, Bedford, MA, USA). The machine was calibrated daily. The CV of the DXA measurements for BMD at the hip was 1.33%. Weight (kg) and height (cm) were measured using standard methods.

Based on the distribution of the hip-BMD Z-scores adjusted for age, in each sex population, 400 subjects with extreme phenotypes (200 from the bottom and 200 from the top), were selected for genotyping in this study. Eventually, a total of 735 subjects were successfully genotyped, containing 370 men (185 subjects for each group) and 365 women (184 and 181 subjects for the low and high BMD groups, respectively). Xiong et al.[18] found no existing population stratification in the same sample.

SNP selection and genotyping

Genomic DNA was extracted from whole blood using the phenol-chloroform standard method. We selected SNPs mainly according to the information available in the public databases dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) and HapMap (http://www.hapmap.org/). Five SNPs within the *GR* gene were selected on the basis of the following criteria: (1) validation status in the Chinese, (2) degree of heterozygosity (i.e., minor allele frequencies [MAFs] >0.05), (3) functional relevance and importance, and (4) reported to dbSNP by various sources.

All the subjects were genotyped by allelic discrimination using the TaqMan SNP genotyping assays (ABI, Foster City, CA, USA).[19] The final reaction volume was 5 μl including 0.25 μl (20× concentration) TaqMan SNP genotyping assay mix, 2.5 μl (2× concentration) TaqMan universal master mix without AmpErase UNG, and 20 ng genomic DNA. The reaction conditions consisted of an initial step at 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. All reactions were run by a Prism 384-well 7900HT Fast Real-Time PCR System.

Statistical analyses

The basic characteristics of the study subjects were assessed by the statistical software SPSS v13.0 (SPSS, Chicago, IL, USA). Hardy-Weinberg equilibrium (HWE) was checked by Fisher exact tests implemented in the program PowerMarker.[20] Single SNP associations were also tested through Fisher exact tests by comparing allele and genotype frequency distributions between the low and high BMD groups. To avoid multiple testing, p values for single SNP associations were corrected by multiplying the number of the tests compared (Bonferroni adjustment).

Pairwise linkage disequilibrium (LD) of the SNPs measured by both standardized D' (D/D_{max}) and LD coefficient r^2 was estimated in the entire sample, using the program Haploview.[21] The haplotype associations were explored using a sliding window approach with window sizes ranging from two to four consecutive SNPs. The software PHASE v2.1.1[22] based on Bayesian statistical methods was used to infer haplotypes, and likelihood ratio tests were used to compare global haplotype frequency distributions between the high and low BMD groups. p values for haplotypes of each window were corrected for multiple testing by 1000 permutations (p_p) automatically by PHASE.

Additionally, for each window with significantly different global haplotype distributions, χ^2 tests with 1 degree of freedom were performed to evaluate individual haplotype association by comparing the frequency of the haplotype with that of the reference group, which was formed by pooling the remaining haplotypes, between the extremely high and low groups (SPSS). To improve the efficiency, the rare haplotypes with frequencies <5% in both two groups were excluded from the analyses according to general application. p values were also corrected by Bonferroni correction.

p values in our study were two-tailed; the p values of permutations (p_p) and the corrected p values (p_c) <0.05 were considered nominally significant.

Bioinformatic analysis

Putative transcription factor binding sites were analyzed by the web-based software TFSEACH v1.3 (http://www.cbrc.jp/research/db/TFSEARCH.html): searching transcription factor binding sites (putative scores > 0.85).[23]

RESULTS

Descriptive characteristics of the studied sample are summarized in Table 1. All of the female subjects were premenopausal. The mean Z-scores in the low and high BMD groups were −1.48 and 0.59 in women and −0.98 and 1.88 in men, respectively.

The marker information of five SNPs in the *GR* gene is presented in Table 2. Because the genotype distributions of rs852977 did not follow HWE, only four SNPs were involved in further analyses.

Table 3 shows the results of single SNP association analyses. Significant association was detected for rs1866388 in men after Bonferroni correction, with the minor allele *G* overrepresented in the high BMD group ($p = 0.007$, $p_c = 0.028$).

TABLE 1. DESCRIPTIVE CHARACTERISTICS OF STUDY PARTICIPANTS (MEANS ± SD OR RANGES)

	Women		*Men*	
	Low BMD (n = 184)	*High BMD (n = 181)*	*Low BMD (n = 185)*	*High BMD (n = 185)*
Age (yr)	26.26 ± 3.95	28.03 ± 5.40	27.87 ± 4.63	26.61 ± 3.71
Age range (yr)	19.60–41.67	20.76–44.88	19.69–43.28	20.76–43.09
Height (cm)	156.55 ± 4.81	159.53 ± 5.12	167.93 ± 5.37	171.09 ± 5.11
Weight (kg)	47.84 ± 5.28	53.82 ± 6.34	57.56 ± 7.37	66.76 ± 8.49
Z-score (hip)*	–1.48 ± 0.35	0.59 ± 0.45	–0.98 ± 0.40	1.88 ± 0.54

* Z-score was adjusted for age.

TABLE 2. PROPERTIES OF SNPS IN THE *GR* GENE

						p values for HWE		
SNP	*Allele*	*Role*	*Position**	*MAF*†	*MAF*‡	*Low BMD*	*High BMD*	*Whole*
rs1866388	G → A§	Intron 2	142739978	0.05	0.09	0.612	0.704	1.000
rs2918419	C → T	Intron 2	142702546	0.10	0.09	0.402	0.546	0.392
rs852977	G → A	Intron 4	142667687	0.50	0.12	<0.001	<0.001	<0.001
rs6194	A → G	Exon 6 (synonymous)	142658554	0.08	0.09	0.497	1.000	0.348
rs258750	G → A	Intron 8	142642082	0.10	0.12	0.757	0.782	0.416

* Position is based on NCBI (http://www.ncbi.nlm.nih.gov) reference.
† Minor allele frequency (MAF) was calculated from all 735 unrelated samples.
‡ MAFs reported by HapMap.
§ The former allele was the minor allele.

TABLE 3. SINGLE-SNP ASSOCIATION RESULTS FOR THE *GR* GENE

SNP	*Allele (%)*		*P**	P_c†	*Genotype (%)*			*P**	P_c†
rs1866388	G	A			GG	GA	AA		
Female-low	29 (8.1)	329 (91.9)			0	29 (16.2)	150 (83.8)		
Female-high	25 (7.7)	301 (92.3)	0.884	>1	1 (0.6)	23 (14.1)	139 (85.3)	0.510	>1
Male-low	9 (2.6)	339 (97.4)			0	9 (5.2)	165 (94.8)		
Male-high	24 (6.8)	330 (93.2)	0.007	0.028‡	1 (0.6)	22 (12.4)	154 (87.0)	0.017	0.069
rs2918419	C	T			CC	CT	TT		
Female-low	32 (8.7)	336 (91.3)			0	32 (17.4)	152 (82.6)		
Female-high	31 (8.7)	325 (91.3)	1.000	>1	1 (0.6)	29 (16.3)	148 (83.2)	0.778	>1
Male-low	41 (11.3)	321 (88.7)			5 (2.8)	31 (17.1)	145 (80.1)		
Male-high	36 (9.9)	326 (90.1)	0.485	>1	3 (1.6)	30 (16.6)	148 (81.8)	0.747	>1
rs6194	A	G			AA	AG	GG		
Female-low	32 (8.7)	336 (91.3)			1 (0.5)	30 (16.3)	153 (83.2)		
Female-high	31 (8.7)	325 (91.3)	1.000	>1	1 (0.6)	29 (16.3)	148 (83.2)	0.941	>1
Male-low	29 (8.1)	327 (91.9)			0	29 (16.3)	149 (83.7)		
Male-high	31 (8.5)	335 (91.5)	0.802	>1	1 (0.6)	29 (15.8)	153 (83.6)	0.947	>1
rs258750	G	A			GG	AG	AA		
Female-low	40 (10.9)	326 (89.1)			0	40 (21.9)	143 (78.1)		
Female-high	37 (10.4)	319 (89.6)	0.900	>1	0	37 (20.8)	141 (79.2)	0.815	>1
Male-low	28 (8.0)	320 (92.0)			2 (1.1)	24 (13.8)	148 (85.1)		
Male-high	39 (11.0)	317 (89.0)	0.201	0.805	3 (1.7)	33 (18.5)	142 (79.8)	0.465	>1

Number (frequency) is given for each allele or genotype.
* p for Fisher exact tests by comparing allele or genotype frequency distributions between extremely low and high groups.
† p values were corrected by the number of SNPs analyzed (four SNPs) shown as p_c.
‡ Significant p_c.

Pairwise D′ and r^2 values between the four SNPs in the whole sample are listed in Table 4. Significant global haplotype association results were discovered through the sliding window approach (Table 5). Haplotypes involving rs1866388 and rs2918419 were all detected with significantly different frequency distributions between the two groups in both sexes (p_p = 0.024, 0.001, and 0.002 in women and 0.002, 0.003, and 0.003 in men for window sizes ranging from two to four SNPs, respectively). However, the frequencies of the two-locus haplotype from rs6194–rs258750 were significantly differently distributed between the two extreme groups only in the male samples (p_p = 0.025).

TABLE 4. PAIRWISE D' AND r^2 VALUES BETWEEN SNPS IN THE *GR* GENE

	D′ (r^2)			
SNP	*rs1866388*	*rs2918419*	*rs6194*	*rs258750*
rs1866388	—	0.55 (0.20)	0.53 (0.21)	0.51 (0.16)
rs2918419	0.55 (0.20)	—	0.97 (0.83)	0.79 (0.57)
rs6194	0.53 (0.21)	0.97 (0.83)	—	0.82 (0.57)
rs258750	0.51 (0.16)	0.79 (0.57)	0.82 (0.57)	—

D′ and r^2 values were calculated to estimate LD in the whole sample.

TABLE 5. GLOBAL HAPLOTYPE ASSOCIATION RESULTS FOR *GR* WITH DIFFERENT WINDOW SIZES

	Window size 2		*Window size 3*		*Window size 4*	
SNP	p_P *(female)*	p_P *(male)*	p_P *(female)*	p_P *(male)*	p_P *(female)*	p_P *(male)*
rs1866388						
rs2918419	0.024*	0.002*				
rs6194	0.423	0.508	0.001*	0.003*		
rs258750	0.813	0.025*	0.451	0.070	0.002*	0.003*

p values are shown at the last SNP for each window and they are corrected for multiple testing by 1000 permutations (p_p).

* Significant p_p.

For each window with significant global association, haplotypes distributing differently between the groups were identified (Table 6). In women, for rs1866388-rs2918419-rs6194 SNPs, haplotype A-C-G showed significant increased frequencies in the low BMD group (p_c = 0.002), whereas A-C-A and G-T-G were more frequent in the high BMD group (p_c = 0.012 and 0.046, separately); for the haplotypes formed by all the four SNPs, G-C-A-G was overrepresented (p_c = 0.004), whereas G-T-G-A and A-C-A-G showed decreased frequencies (p_c = 0.046 and 0.030, respectively) in the low BMD group. In men, only the haplotype G-C-A-G was still significantly more distributed in the high BMD group after conserved Bonferroni correction (p_c = 0.010).

DISCUSSION

In this study, we attempted to explore the relationship between *GR* gene polymorphisms and extreme BMD phenotypes in a Chinese Han population, using both single SNP and haplotype analyses. Haplotypes can provide more information between genetic variation and phenotypes than single SNP markers.[24] However, when there exists strong LD between the causal locus and single SNP, the haplotypes can not be more informative because of the additional degrees of freedom with haplotype-based tests.[25] Therefore, it is necessary and informative to test both.

At the single SNP level, the frequency of SNP rs1866388 differs significantly between the low and high BMD group in men. It is noted that this SNP was also included in most of the significant haplotype associations in both sexes. Therefore, rs1866388 is suggested to be important for variation in extreme BMD. Furthermore, several putative transcription factor binding sites in the sequence flanking rs1866388 were found by TFSEARCH. Remarkably, the sequence contains two putative binding sites for transcription factor CCAAT/enhancer-binding proteins α (*CEBP-α*), which is a critical factor responsible for normal cellular differentiation, proliferation, and cell type–specific gene expression.[26] Combining with the effect of runt-related transcription factor 1 (*RUNX1*), *CEBP-α* can activate the macrophage-colony stimulating factor (M-CSF) receptor promoter in a synergistic manner for osteogenesis.[27] Moreover, it has been confirmed that the interaction between *GR* and *CEBP-α* by complex formation mediated GC actions on lymphocytic and mesenchymal cell proliferation.[28]

The haplotype analyses in this study were conducted using the variable-sized sliding window approach. Because of the variable LD patterns, it is difficult to predefine an optimal window size for the analyses. Therefore, it was necessary to analyze all possible sliding windows of all possible sizes. The windows of two, three, and four SNPs involving rs1866388 and rs2918419 all showed significant global associations in both sexes. These significant findings were caused by the imbalance in the distributions of several specific common or rare haplotypes between the groups, as shown by individual haplotype associations. The sliding window approach did not rely on the predefinition of the block, which is problematic for us to practice because of several different criteria proposed for defining a block. All possible sized windows captured the full genetic information within the region, and it is statistically sufficient to detect the common or rare genetic risk polymorphisms.[29]

Our findings also suggested that *GR* displayed sex-specific association effects at both SNP and haplotype levels. SNP rs1866388 showed significant association in men, which cannot be found in women. Most of the shared haplotypes showed opposite effects between women and men; for example, haplotype A-C for rs1866388-rs2918419 seemed to be protective for BMD in women, whereas it had contrary effects in men, because it was less distributed in women but more frequent in men in the low BMD group (Table 6). Previous studies also found sex-dependent sensitivity to GC; women with high-dose GC therapy had significantly lower femur BMD than men,[30] and prenatal rats exposed to dexamethasone showed sex-specific bone growth patterns,[31] which was partially caused by the genetic variations in the *GR* gene. Some evidence indicated that sex specificity of *GR* might be caused by the effects of estrogen. Estrogen, which was often used to prevent GC-induced bone loss,[32] is a key regulator of bone metabolism. It has been shown that *GR* gene expression was subjected to estrogen regulation.[33] Adult male rats had lower *GR* mRNA levels than female rats in the hippocampus, and there were also sex-dependent differences in the gene expression of *GR*.[34] In addition, *GR* expression was down-regulated by estrogen in breast cancer cells, with subsequent suppression of *GR*-mediated transcriptional activity.[35]

Our sample was chosen from the bottom and top parts of the population distribution of BMD. Because much of the genetic information resides in individuals with extreme phe-

TABLE 6. INDIVIDUAL HAPLOTYPE ASSOCIATION RESULTS FOR THE SIGNIFICANT WINDOWS OF *GR*

	Women					*Men*			
	Low (%)	*High (%)*	*p**	p_c[†]		*Low (%)*	*High (%)*	*p**	p_c[†]
rs1866388-rs2918419					rs1866388-rs2918419				
A-T	87.3	84.2	0.250	>1	A-T	88.7	88.9	0.906	>1
G-T	4.6	8.1	0.059	0.653	A-C	8.4	4.1	0.015	0.149
A-C	4.0	7.2	0.068	0.752	G-C	2.8	5.7	0.044	0.435
rs1866388-rs2918419-rs6194					rs6194-rs258750				
A-T-G	88.4	84.5	0.135	>1	G-A	88.6	88.4	0.909	>1
A-C-A	2.4	7.7	0.001	0.012‡	A-G	5.2	7.9	0.136	>1
G-T-G	2.4	6.9	0.004	0.046‡	rs1866388-rs2918419-rs6194				
A-C-G	5.7	0.9	<0.001	0.002‡	A-T-G	88.3	88.8	0.909	>1
rs1866388-rs2918419-rs6194-rs258750					A-C-A	5.8	2.3	0.017	0.171
A-T-G-A	85.6	82.3	0.228	>1	A-C-G	2.4	6.0	0.017	0.171
G-T-G-A	2.4	6.8	0.004	0.046‡	rs1866388-rs2918419-rs6194-rs258750				
A-C-A-G	2.4	7.1	0.003	0.030‡	A-T-G-A	86.1	86.3	0.917	1
G-C-A-G	5.4	0.9	<0.001	0.004‡	G-C-A-G	1.4	5.6	0.001	0.010‡

Only the haplotypes with frequencies >5% at least in one group were included in the analysis.

* p for χ^2 tests by comparing haplotype frequency distributions between the low and high BMD groups.

† p values were corrected by the number of haplotypes tested: 11 and 10 in the female and male group (p_c).

‡ Significant p_c.

notypes, which will reduce the possibility of sharing common genetic effects between two groups, our study had a higher power to detect the real genetic effects.[36] The statistical power of the sample sizes in our study was estimated by the web-based program Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/qcc.html).[37] Under the condition of the significant level of $\alpha = 0.01$, assuming a marker is in LD ($D' = 0.80$) with a quantitative trait locus (QTL) accounting for 5% variations of a phenotype, and the MAFs of the marker and QTL are both 0.10, we can achieve >90% power to detect the additive genetic effects in the male and female subgroups.

Several aspects of our study need to be further discussed. First, Bonferroni correction was overconservative and inevitably caused a great loss of power for individual haplotype association[38] because of the highly correlated haplotypes induced by the overlapping of adjacent SNPs among the sliding windows. Second, the actual trait values were ignored, and the true genetic effect sizes could not be estimated, because the individuals with extreme values were treated like cases or controls, and methods similar to case-control methods were used in this study.

In conclusion, this is the first study to reveal a significant association between the human *GR* gene and extreme hip BMD in a Chinese sample. Furthermore, our results suggested sex specificity in the association of *GR* with extreme BMD.

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