The association between sex hormone-binding globulin gene polymorphism with bone mineral density

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ABSTRACT

To investigate the impact of single nucleotide polymorphisms (SNPs) of SHBG gene the neighboring genes on SHBG levels, bone mineral density (BMD) and osteoporosis in Chinese males. A group of Chinese men, aged >45 years were included in the analysis. BMD was measured with dual-energy X-ray absorptiometry (DXA), SHBG and total testosterone (TT) was measured using chemiluminescent immunoassay, and free testosterone (FT) was calculated. SNPs of SHBG gene and the neighboring genes were studied by means of improved multiple ligase detection reaction (iMLDR). A total of 404 men were included in our study. In the single locus analysis, significant associations were found between SHBG levels and four polymorphisms (rs11078701, rs9901675, rs9898876 and rs2541012) in age- and BMI-adjusted models. In addition, statistically significant difference was found between osteoporosis patients and control subjects in genotype distributions of rs9898876, rs2541012, rs6259 and rs3853894. In the models with or without adjustment for confounders (age, BMI, SHBG and free testosterone (FT) levels), carriers of variant genotype of rs9898876, rs2541012 and rs6259 had lower BMD and were more likely to suffer from osteoporosis, as compare to carriers of common genotype. Subjects with variant genotype of rs3853894 had higher BMD and were less likely to suffer from osteoporosis, as compared to subjects with common genotype. In the haplotypes analysis, CCGGT (constituted by rs11078701C, rs1017163C, rs9898876G, rs62059836G and rs2541012T) and haplotype CGGT (constituted by rs858521C, rs858518G, rs6259G and rs727428T) was associated with a significant risk effect for osteoporosis. Polymorphisms of SHBG or the neighboring genes were associated with SHBG levels or BMD and osteoporosis, suggesting the involvement of genetic variation of SHBG in bone health.

1. Introduction

Osteoporosis is a disease characterized by low bone mass and structural deterioration of bone tissue, leading to bone fragility and an increased susceptibility to fractures. Evidence from twins and family studies have shown that approximately 50–80% of individual variance in bone mineral density (BMD) is genetically determined [1–3].

Sex hormone-binding globulin (SHBG), a plasma glycoprotein that binds with high affinity to sex steroids and regulating their bioavailability and access to target cells, may play an important role in the pathophysiology of osteoporosis. Some cross-sectional studies and longitudinal studies among male populations have demonstrated significant associations between SHBG levels and bone health, high SHBG correlated with greater bone loss, and the presence of vertebral and peripheral fractures [4–8]. In addition, a number of clinical studies have suggested that SHBG had an independent impact on bone strength [9–12]. Our previous study [13] also showed that an increasing SHBG level was a risk factor for osteoporosis, independent from age, BMI, total testosterone (TT) and free testosterone (FT) level, which indicated that SHBG may have an independent role on bone. Apart from the metabolic and hormonal influences [14], circulating SHBG levels are also influenced by genetic variation, which may either lead to the increase in production or decrease clearance of SHBG [15–18]. Given the clinical significance of SHBG, a number of studies have

Abbreviations: SHBG, sex hormone-binding globulin; SNP, single nucleotide polymorphism; BMD, bone mineral density; DXA, dual-energy X-ray absorptiometry; TT, total testosterone; FT, free testosterone; iMLDR, improved multiple ligase detection reaction; BMI, body mass index; CV, coefficient of variation; CGAS, candidate gene association study; GWAS, genome-wide gene association study; RANK, receptor activator of nuclear factor kappa B; RANKL, receptor activator of nuclear factor kappa B ligand; PTH, parathyroid hormone; MAF, minor allele frequency; LD, linkage disequilibrium; HWE, Hardy–Weinberg equilibrium; OR, odds ratio; CI, confidence interval.

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examined the potential associations between polymorphisms of SHBG gene and serum SHBG levels that could be involved in the development of several hormone-related disorders like polycystic ovary syndrome (PCOS) [19,20], breast cancer [21,22] and prostate cancer [23,24].

Osteoporosis is a polygenic disease and BMD is affected by several genes. In the last decade, quite a number of studies (candidate gene association studies (CGAS) or genome-wide association studies (GWAS)) have focused on identifying genetic variants which may have potential effects on bone, such as estrogen [25,26] and androgen [27,28] receptor genes and PPAR gamma gene [29,30]. However, few studies discussed the role of SHBG gene variation on bone, especially in men. And results reported were controversial [31–34]. Our current research aims to explore the association between SHBG gene polymorphisms and BMD, presence of osteoporosis and their possible influence on circulating SHBG levels among middle-aged and elderly Chinese men.

2. Materials and methods

2.1. Study design and subjects

This was a cross-sectional and candidate gene association study. The study subjects were recruited from the health checkup population of Zhongshan Hospital, Fudan University from June 2012 to May 2013. As they all live in Shanghai, their lifestyles are considered to be similar and the cohort is fairly representative of the Chinese males in big city. The purposes and procedures of the study were explained in details to the participants by researchers. Participants were asked to fill in a comprehensive questionnaire regarding personal lifestyle, risk factors for osteoporosis and personal and family disease history. Inclusion criteria: men aged ≥45 years and with willingness to participate in the study and with ability to use complete questionnaire and provided informed consent. Exclusion criteria: those who had a history or evidence of metabolic bone diseases (Paget’s disease, osteomalacia, renal osteodystrophy); those who suffered tumor or bone metastases; those who had taken medications such as steroids which could affect bone metabolism; those who had used any anti-osteoporosis drugs such as bisphosphonates and calcitonin; those who had severe liver, kidney impairment; those who have been recently bedridden for more than 3 months; those who had both hips fractured or replaced. This study was approved by the Ethics Committee of Zhongshan Hospital, Fudan University, all of the study data and information were collected after participants gave informed consent.

2.2. Measurements

2.2.1. Anthropometric measurements

Anthropometric and DXA measurements were obtained for all participants during the same visit. Height (cm) and weight (kg) was measured without shoes in light indoor clothing using a Stadiometer and body mass index was calculated as the ratio of weight (kg) to height squared (m²).

All DXA measurements were performed by a well-trained technician using dual energy X-ray absorptiometry (Discovery A, Hologic, USA, with CV < 1%) on left hip (femoral neck, trochanter, intertrochanteric, Ward’s triangle, and total hip) and lumbar spine (L1–L4, L-Total). T score was obtained by comparison to white males (Source: NHANES). We used the WHO diagnostic criteria for osteoporosis to classify our patients into three categories: osteoporosis (T-score < –2.5 SD), osteopenia (–1.0 < T-score > –2.5 SD), or normal (T-score > –1.0 SD). The lowest T score at the femoral neck, intertrochanteric, total hip, or lumbar spine, was used for diagnosis.

2.2.2. Biochemical analyses

A single fasting morning venous blood sample was obtained from all subjects. Serum was separated immediately after phlebotomy and stored at –80 °C until assay at the end of the baseline study. Measurement of total testosterone (TT) and SHBG were carried out by Access Immunoassay Systems using the chemiluminescent immunoassay (Beckman Coulter, Brea, USA); total testosterone (analytical range 0.1–16 ng/ml [0.35–55.5 nmol/L]; intra-assay coefficient of variation (CV) < 3%; inter-assay CV < 5%), and SHBG (analytical range 0.33–200 nmol/l; intra-assay CV < 4.8%). Free testosterone (FT) was calculated from TT and SHBG using the Vermeulen equation [35], taking the concentration of TT, and SHBG into account and assuming a fixed albumin concentration of 43 g/l.

2.2.3. Questionnaire

A questionnaire was used to record the lifestyle and disease history of the participants, including smoking habit, alcohol consumption, etc. Participants who had smoked less than 100 cigarettes in the past five years were defined as non-smokers and the others as smokers. Participants who had consumed alcoholic beverages at least once per week for at least one year in the past five years were categorized as alcohol drinkers and the others as non-alcohol drinkers.

2.3. Selection of polymorphisms and genotyping

SNP selecting: The SNPs were selected from the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP) and the International HapMap Project database (http://hapmap.ncbi.nlm.nih.gov/) using “SHBG” as the target gene, in accordance with the following criteria: (1) minor allele frequency (MAF) of at least 5% in Chinese populations, (2) with low linkage disequilibrium (LD) using an r² threshold of <0.8 for each other, and the tagging SNPs are selected to constitute a minimal set of highly informative markers while minimizing redundant data. Although the MAF of rs13894 is only 3.3% in Chinese population according to HapMap data, it was genotyped in our study because it was a missense mutation (Arg126Cys). As a result, 12 SNPs of SHBG gene, which captured other 4 SNPs in the same gene, were selected. Finally we searched the literature for previous reports on SNPs of other genes influencing circulating SHBG levels [16], and included additional four SNPs (rs3853894 of ZBTB4 gene, rs9303218 of POLR2A gene, rs9901675 of CD68 gene, and rs8077824 of DNAH2 gene). Therefore, a total of 16 SNPs were chosen for analysis, including rs3853894, rs9303218, rs9901675, rs9898876, rs2541012, rs13894, rs858521, rs6259, rs727428, rs1641537, rs1017163, rs11078701, rs858518, rs59524396, rs62059836, and rs8077824.

Linkage disequilibrium: Genotype data was obtained from HapMap database and the linkage disequilibrium (LD) between SNPs in SHBG gene was examined by pairwise comparisons of r² using Haplovie version 4.2 (Fig. 1a).

Genotyping: Blood samples were collected from patients in EDTA tubes and stored at –80 °C. The genomic DNA was extracted from peripheral blood leukocytes by salting-out method, using Relax Gene Blood DNA System (TIANGEN BIOTECH, BEIJING, China), according to the manufacturers’ protocol. Study subjects were genotyped for a total of 16 SNPs carried out by Shanghai Genesky Biotech Co., Ltd. (http://biotech.geneskies.com) using the improved multiple ligase detection reaction (imMLDR) assay on 3730xl genetic analyze sequencer (Applied Biosystems, Foster City, CA, USA). For each SNP, the alleles were distinguished by different fluorescent labels of allele-specific oligonucleotide probe pairs. Different SNPs were distinguished by different extended lengths at 3 end. The primers for both PCR and LDR reactions were all designed by Primer3 online software v.0.4.0 (http://primer3.wi.mit.edu).
In brief, the PCR reactions were performed with 1 μL DNA sample (5–10 ng/μL), 1× GC-I buffer (TaKaRa, Japan), 3.0 mM Mg²⁺ (TaKaRa, Japan), 0.3 mM dNTP (Generay Biotech, China), 1 U HotStarTaq polymerase (Qiagen, Germany), 1 μL multiple PCR primers (Sangon, China) and ddH₂O in a total volume of 10 μL. The PCR cycling program was: 95 °C for 2 min, followed by 11 cycles of 94 °C for 20 s, 65 °C (decreased 0.5 °C per cycle) for 40 s, 72 °C for 90 s plus 24 cycles of 94 °C for 20 s, 59 °C for 30 s, 72 °C for 90 s, with a final extension at 72 °C for 2 min, and kept at 4 °C. Then 5U shrimp alkaline phosphatase (Promega, USA) and 1U Exonuclease I (Epicenter, USA) were added into the 15 μL PCR products for purification. The LDR reactions were performed in a final volume of 50 μL.

![Diagram of LD pattern among selected SNPs](image1)

**Fig. 1.** Pairwise linkage disequilibrium (LD) pattern among selected SNPs. (a) LD pattern of part of the selected SNPs in SHBG gene which captured the other four untapped SNPs (because genotypes of rs1017163, rs11078701, rs858518, rs59524396, and rs62059836 could not be obtained from HapMap, we didn’t show their LD with other SNPs here). Asterisks referred to tag SNPs. (b) LD pattern of the 12 selected SNPs in SHBG gene in our study population (n = 404). The value within each diamond represented the pairwise correlation between SNPs (measured as r²) defined by the upper left and the upper right sides of the diamond. The red-to-white gradient reflected higher to lower LD values. Two blocks were identified based on levels of LD. Numbers in bracket indicate the length of block.
10 μL containing 1 μL 10× ligase reaction buffer (New England Bios labs, USA), 0.25 μL Taq DNA ligase (New England Bios labs, USA), 0.4 μL 5’ ligase primer (1 μM) mixture (Sangon, China), 0.4 μL 3’ ligase primer (2 μM) mixture (Sangon, China), 2 μL purified PCR product and 6 μL ddH2O. The LDR reactions were cycled as: 38 cycles of 94 °C for 1 min and 56 °C for 4 min, and kept at 4 °C. 0.5 μL LDR product was then sequenced with ABI3730XL sequencer (Applied Biosystems, USA). Finally the raw data was analyzed by GeneMapper 4.1 (Applied Biosystems, USA). The primers for the 16 target SNPs were shown in Supplementary Table 1.

Quality control: Sample and assay quality control thresholds were set to 90%. The genotyping was carried out in a blind way to group status. A random sample accounting for 5% (n = 20) of the total subjects was genotyped twice by different researchers for quality control, yielding a reproducibility of 100%. Allele frequencies were also tested for deviation from Hardy–Weinberg equilibrium (HWE). Allele frequencies were also checked for consistency with HapMap data.

2.4. Statistical analysis

Allele frequencies were estimated by gene counting. Agreement with Hardy–Weinberg equilibrium was tested using Haploview vision 4.2 for each SNP [36].

Data distribution was analyzed by Kolmogorov–Smirnov test. Descriptive statistics were computed and results are expressed as mean (standard deviation) for normally distributed data and as median (25–75th percentile) for skewed data, and as proportions for enumeration data. Serum TT, FT and SHBG level are skewed data in our study. In further analyses, variables were logarithmic transformed to approach normality when deemed necessary. Student test or analysis of covariance (ANCOVA) was used for analysis of normal data (age, BMI and BMD) and the Mann–Whitney U-test was used for analysis of skewed data (TT, FT and SHBG levels). SHBG were transformed to approach normality and ANCOVA analysis was performed in the analysis of comparison of SHBG levels in the genotypes of SNPs. \( t \)-test was used for analysis of proportions for enumeration data (proportions of smokers and drinkers). Comparison of SHBG levels among different genotypes of SNPs were adjusted for age and BMI (Table 4), and comparison of BMD among different genotypes of SNPs were adjusted for age, BMI, SHBG and FT (Fig. 2). The associations of SNPs genotypes with osteoporosis risk were estimated by computing odds ratios (ORs) and their 95% confidence intervals (CIs) from logistic regression models, and these analysis were performed with or without adjusting age, BMI, SHBG, FT and lifestyle (smoking or alcohol) (Table 5). In the haplotypes analysis, haplview 4.2 software was used to infer haplotype frequencies based on the observed SHBG genotypes. Haplotype analyses were based on the maximum likelihood model and using the EM-algorithm as implemented in gc.em [37].

The association between haplotypes and presence of osteoporosis were estimated using computing odds ratios (ORs) and their 95% confidence intervals (CIs) from \( X^2 \) test (Table 6). Significance was determined using a two-sided test at \( \alpha \) level <0.05. All statistical analyses were performed using SPSS version 20.0.

3. Results

3.1. Basic characteristics of the study population

A total of 404 men aged 45 or over were eligible for the current study. The mean age of the study sample was 78.1 years (range 45–97 years), the median age was 79 and the percentage of subjects aged 70 or over was 82.4%. The percentage of men with osteoporosis was 26.0%. The distribution of selected characteristics between osteoporosis patients and non-osteoporosis patients is summarized in Table 1. In the selected characteristics, significant difference existed between osteoporosis group and non-osteoporosis group for BMI, SHBG level, FT level, BMD and smoking. Osteoporotic subjects had a statistically significant lower BMI (P < 0.001), FT (P < 0.019), BMD (all P < 0.001) and higher levels of SHBG (P < 0.001) than the non-osteoporosis group, while there was no significant difference in age (P = 0.230) and TT (P = 0.411).

3.2. Information of SNPs analyzed in this study

As mentioned above, 16 SNPs, which located in the coding region, 5’ flanking of the SHBG gene and the other genes, were chosen. 16 SNPs were successfully genotyped among all 404 subjects. The minor allele frequencies for all polymorphisms in our study are similar to those from the HapMap database and the NCBI SNP database (Table 2). All polymorphisms in our study cohort were found to be in Hardy–Weinberg equilibrium (all \( P > 0.05 \), Table 3). rs13894 only had GG and GA genotype, but no AA genotype. Therefore, MAF (A allele) was 2.35% in this population, reflecting the rare occurrence of this genotype. LD pattern of the selected SNPs in SHBG gene was showed in Fig. 1b. No strong LD value were observed among the selected SNPs (all \( r^2 < 0.8 \)).

3.3. Comparison of SHBG levels among different genotypes of SNPs (Table 4)

After adjustment for age and BMI, in the overall ANCOVA analysis, SHBG levels were significantly different among the genotypes of 3 SNPs (difference of SHBG level in the genotype of rs9898876 was close to significant (\( P = 0.06 \))). In further analysis of post hoc tests, compared with subjects with common homozygous genotype (C/C), subjects carrying one (C/R) or two copies (R/R) of variant allele of rs2541012 had significantly higher SHBG levels (49.30

<table>
<thead>
<tr>
<th>Variables</th>
<th>Osteoporosis group (n = 105)</th>
<th>Non-osteoporosis group (n = 299)</th>
<th>P values</th>
<th>All participants (n = 404)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>78.8 ± 0.9</td>
<td>77.8 ± 0.5</td>
<td>0.230b</td>
<td>78.1 ± 8.5</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.4 ± 0.3</td>
<td>24.1 ± 0.2</td>
<td>( &lt;0.001 )</td>
<td>23.7 ± 2.9</td>
</tr>
<tr>
<td>SHBG (nmol/L)</td>
<td>49.50 (41.20,69.80)</td>
<td>45.15 (34.25,57.65)</td>
<td>0.001b</td>
<td>47.15 (36.20, 65.0)</td>
</tr>
<tr>
<td>TT (nmol/L)</td>
<td>13.19 (10.19,15.98)</td>
<td>12.62 (10.14,15.90)</td>
<td>0.411b</td>
<td>12.78 (10.16, 15.91)</td>
</tr>
<tr>
<td>FT (nmol/L)</td>
<td>0.205 (0.156,0.233)</td>
<td>0.210 (0.175,0.251)</td>
<td>0.019b</td>
<td>0.209 (0.168, 0.247)</td>
</tr>
<tr>
<td>Smoker (%)</td>
<td>28 (26.7%)</td>
<td>47 (15.7%)</td>
<td>( 0.013 )</td>
<td>75 (18.6%)</td>
</tr>
<tr>
<td>Drinker (%)</td>
<td>22 (21.0%)</td>
<td>46 (15.4%)</td>
<td>0.190b</td>
<td>68 (16.8%)</td>
</tr>
</tbody>
</table>

Normally distributed data were expressed as mean (standard deviation), and skewed data as median (25–75th percentile), and enumeration data as proportions. Bold referred to significant P value (\( P < 0.05 \)). BMI = body mass index; SHBG = sex hormone binding globulin; BMD = bone mineral density; TT = total testosterone; FT = free testosterone.

* P referred to student t test.

b P referred to Mann–Whitney U-test.

c P referred to \( X^2 \) test.
vs. 43.10 nmol/L, \( P = 0.009 \); 47.40 vs. 43.10 nmol/L, \( P = 0.012 \), while subjects carrying variant allele (C/R or R/R) of rs11078701 had lower SHBG levels (46.05 vs. 47.60 nmol/L, \( P = 0.023 \); 37.90 vs. 47.60 nmol/L, \( P = 0.007 \)). In post hoc tests, difference of SHBG level only reached significance between subjects with the common homozygous genotype (CC) and subjects with the variant heterozygous genotype (CR) for rs9901675 (46.15 nmol/L vs. 56.75 nmol/L, \( P = 0.006 \)). Meanwhile, we didn’t find significant association between the other SNPs and SHBG levels.

Difference of testosterone (included TT and FT) among different genotypes of 4 SNPs mentioned above (rs11078701, rs2541012, rs9901075 and rs9898876) were also analyzed in our study, no significant difference was found (data not shown).

3.4. Comparison of BMD among different genotypes of SNPs (Fig. 2)

In the stratified analyses of BMD among each genotype of SNPs, after adjustment for age, BMI, SHBG and FT, in overall ANCOVA, there was a significant association between BMD (either femoral neck [FN-], total hip [TH-], or total lumbar [TL-] BMD) and four SNPs (rs9898876, rs2541012, rs6259 and rs3853894) (Fig. 2). FN-BMD (\( P = 0.022 \)) and TH-BMD (\( P = 0.008 \)) were significantly different among different genotypes of rs9898876. Similarly, the difference of FN-BMD (\( P = 0.049 \)) and TH-BMD (\( P = 0.037 \)) among genotypes of rs6259 were statistically significant. TL-BMD (\( P = 0.028 \)) among genotypes of rs2541012 was also significantly different, and the same as FN-BMD (\( P = 0.008 \)) and TH-BMD (\( P = 0.003 \)) among different genotypes of rs3853894. In further analysis of post hoc tests, significance mentioned above was obtained only when comparing subjects with the common homozygous genotype to those with the variant heterozygous genotype for rs9898876 (FN-BMD: 732.8 vs. 705.4 mg/cm\(^2\), \( P = 0.010 \); TH-BMD: 902.1 vs. 872.7 mg/cm\(^2\), \( P = 0.004 \)), rs6259 (FN-BMD: 731.2 vs. 707.8 mg/cm\(^2\), \( P = 0.027 \); TH-BMD: 899.4 vs. 876.9, \( P = 0.022 \)) and rs3853894 (FN-BMD: 709.9 vs. 746.7 mg/cm\(^2\), \( P = 0.002 \); TH-BMD: 873.0 vs. 917.5 mg/cm\(^2\), \( P = 0.001 \)). Similarly, for rs2541012, difference of TL-BMD
Comparison of SHBG levels (nmol/L) in the genotypes of SNPs (adjusted for age and BMI).

Table 4

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Gene</th>
<th>Location</th>
<th>Functional change</th>
<th>Alleles (common/variant)</th>
<th>MAF (this study)</th>
<th>MAF (HapMap and dbSNP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1017163</td>
<td>SHBG</td>
<td>5'-flanking</td>
<td></td>
<td>C/G</td>
<td>0.2661</td>
<td>0.2680</td>
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<tr>
<td>rs11078701</td>
<td>SHBG</td>
<td>5'-flanking</td>
<td></td>
<td>C/T</td>
<td>0.2277</td>
<td>0.2268</td>
</tr>
<tr>
<td>rs858518</td>
<td>SHBG</td>
<td>5'-flanking</td>
<td></td>
<td>A/G</td>
<td>0.3230</td>
<td>0.3505</td>
</tr>
<tr>
<td>rs59524396</td>
<td>SHBG</td>
<td>5'-flanking</td>
<td></td>
<td>G/A</td>
<td>0.1980</td>
<td>0.1546</td>
</tr>
<tr>
<td>rs62095836</td>
<td>SHBG</td>
<td>5'-flanking</td>
<td></td>
<td>G/A</td>
<td>0.1881</td>
<td>0.1598</td>
</tr>
<tr>
<td>rs8988876</td>
<td>SHBG</td>
<td>5'-flanking</td>
<td></td>
<td>G/T</td>
<td>0.1918</td>
<td>0.2120</td>
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<tr>
<td>rs2541012</td>
<td>SHBG</td>
<td>5'-flanking</td>
<td></td>
<td>T/C</td>
<td>0.4443</td>
<td>0.4850</td>
</tr>
<tr>
<td>rs13894</td>
<td>SHBG exon1</td>
<td></td>
<td></td>
<td>p.Arg126Cys</td>
<td>0.6023</td>
<td>0.6030</td>
</tr>
<tr>
<td>rs858521</td>
<td>SHBG intron4</td>
<td></td>
<td></td>
<td>C/G</td>
<td>0.2611</td>
<td>0.2330</td>
</tr>
<tr>
<td>rs6259</td>
<td>SHBG nonsynon_exon8</td>
<td></td>
<td></td>
<td>p.Asp356Asn</td>
<td>0.1745</td>
<td>0.1860</td>
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<tr>
<td>rs727428</td>
<td>SHBG 3'-flanking</td>
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<td></td>
<td>T/C</td>
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<td>0.4620</td>
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<tr>
<td>rs1614537</td>
<td>SHBG</td>
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<td>G/A</td>
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<tr>
<td>rs3853894</td>
<td>ZBTB4</td>
<td>exon2</td>
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<td>0.6030</td>
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<td>0.1000</td>
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</table>

HWE = Hardy–Weinberg equilibrium.

Table 5

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Common type N (%)</th>
<th>Variant type N (%)</th>
<th>Variant type N (%)</th>
<th>HWE P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1017163</td>
<td>CC (224, 55.4)</td>
<td>GG (47, 10.8)</td>
<td>GA (25, 5.3)</td>
<td>0.0136</td>
</tr>
<tr>
<td>rs11078701</td>
<td>CC (241, 59.6)</td>
<td>CT (42, 15.2)</td>
<td>TT (33, 7.4)</td>
<td>0.5954</td>
</tr>
<tr>
<td>rs858518</td>
<td>AA (182, 45.1)</td>
<td>GA (19, 4.9)</td>
<td>GG (23, 5.7)</td>
<td>0.348</td>
</tr>
<tr>
<td>rs59524396</td>
<td>GC (261, 64.6)</td>
<td>GA (20, 5.1)</td>
<td>AA (19, 4.7)</td>
<td>0.0012</td>
</tr>
<tr>
<td>rs62095836</td>
<td>GC (267, 66.1)</td>
<td>GA (20, 5.1)</td>
<td>AA (19, 4.7)</td>
<td>0.0012</td>
</tr>
<tr>
<td>rs8988876</td>
<td>GC (267, 66.1)</td>
<td>GT (20, 5.1)</td>
<td>AA (19, 4.7)</td>
<td>0.0012</td>
</tr>
<tr>
<td>rs2541012</td>
<td>TT (140, 34.7)</td>
<td>TC (169, 41.8)</td>
<td>CC (95, 23.5)</td>
<td>0.1317</td>
</tr>
<tr>
<td>rs13894</td>
<td>GG (385, 93.5)</td>
<td>GA (4, 1.0)</td>
<td>AA (1, 0.3)</td>
<td>1</td>
</tr>
<tr>
<td>rs858521</td>
<td>CC (227, 56.2)</td>
<td>CG (134, 34.3)</td>
<td>GC (34, 8.4)</td>
<td>0.2235</td>
</tr>
<tr>
<td>rs6259</td>
<td>GC (278, 68.8)</td>
<td>GA (11, 2.7)</td>
<td>AA (1, 0.3)</td>
<td>0.4931</td>
</tr>
<tr>
<td>rs727428</td>
<td>TT (125, 30.9)</td>
<td>TC (195, 48.3)</td>
<td>CC (84, 20.8)</td>
<td>0.8048</td>
</tr>
<tr>
<td>rs1614537</td>
<td>CC (190, 47.1)</td>
<td>CG (195, 48.3)</td>
<td>CC (95, 23.5)</td>
<td>0.1317</td>
</tr>
<tr>
<td>rs5833849</td>
<td>GC (237, 58.7)</td>
<td>GA (149, 36.9)</td>
<td>AA (18, 4.4)</td>
<td>0.1317</td>
</tr>
<tr>
<td>rs9033218</td>
<td>TT (349, 86.4)</td>
<td>TC (53, 13.1)</td>
<td>CC (2, 0.5)</td>
<td>1</td>
</tr>
<tr>
<td>rs8077824</td>
<td>GC (333, 82.4)</td>
<td>GA (4, 1.0)</td>
<td>AA (1, 0.3)</td>
<td>0.5336</td>
</tr>
<tr>
<td>rs90901675</td>
<td>GC (382, 94.6)</td>
<td>GA (20, 4.9)</td>
<td>AA (2, 0.5)</td>
<td>0.6647</td>
</tr>
</tbody>
</table>

SHBG were transformed to approach normality. C/C indicates common homozygous genotype, C/R indicates variant heterozygous genotype, R/R indicates variant homozygous genotype.

* P value referred to ANCOVA analysis adjusted for age and BMI.

** P referred to post hoc test for SHBG levels in the genotypes of SNPs.

3.5. Associations between variant genotypes and osteoporosis (Table 5)

Allele frequencies of rs6259, rs9898876, rs2541012 and rs3853894 were statistically different between osteoporosis group and non-osteoporosis group (P = 0.001, 0.001, 0.047, 0.001, respectively). The association between SNPs and osteoporosis were performed using binary logistic regression model and it was found that compared with rs9898876 GG genotype, subjects carrying rs9898876 GT (OR [95%CI] = 2.221[1.376–3.559]) and combined variant genotypes GT/TT (OR [95%CI] = 2.098[1.328–3.313]) were more likely to suffer from osteoporosis. Similarly, compared with rs6259 GG common genotype, rs6259 GA genotype (OR [95%CI] = 2.134[1.344–3.390]) were more likely to suffer from osteoporosis as well. In addition, compared with subjects with rs2541012 common genotype TT, subjects with the variant genotype CC (OR [95%CI] = 2.230[1.237–4.020]) and the combined genotype TC/CC (OR [95%CI] = 1.647[1.007–2.694]) for rs2541012 were more likely to suffer from osteoporosis. However, the variant genotypes GA (OR [95%CI] = 0.447[0.243–0.861]) and the combined GA/AA variant genotype (OR [95%CI] = 2.134[1.344–3.390]) were more likely to suffer from osteoporosis, compared with GG genotype. Associations mentioned above remained significant after adjusting for age, BMI, smoking, alcohol, SHBG and FT, with one exception that the association between the combined genotype TC/CC for rs2541012 and presence of osteoporosis lost its statistical significance after adjusting for those covaraints (adjusted OR [95%CI] = 2.174[1.344–3.517]) and the combined GA/AA variant genotype (OR [95%CI] = 2.134[1.344–3.390]) were more likely to suffer from osteoporosis.

3.6. Haplotype analysis

Based on patterns of LD two haplotype blocks were found: Block 1 consisted of the polymorphisms rs11078701, rs1017163, rs9898876, rs62058936 and rs2541012 (pairwise D = 0.80–1.0, r² = 0.04–0.29) and block 2 consisted of rs858521, rs858518, rs254102 and rs727428 (pairwise D = 0.77–0.95, r² = 0.04–0.35) (Fig. 1b). In block 1, five haplotypes each having a frequency above 5% were found comprising 93.8% of all haplotypes. Similarly, in block 2, five common haplotypes were found comprising more
than 95% of all haplotypes. Block1 haplo5 CCGGT was associated with a significant risk effect for osteoporosis (OR[95%CI] = 1.987 [1.066–3.702], \( P = 0.029 \)) compared with the most common genotype CGGGC. Also, block2 haplo5 CGGT was associated with a significant risk effect for osteoporosis (OR[95%CI] = 1.799 [1.008–3.208], \( P = 0.045 \)), compared with the most common haplotype GAGC (Table 6). We did not find the other haplotypes that had a statistically significant association with osteoporosis.

### 4. Discussion

We identified 12 SNPs in SHBG gene and 4 SNPs in neighboring genes among 404 Chinese males. In the single locus analysis, significant associations were found between SHBG levels and four polymorphisms (rs11078701, rs9901675, rs8988876 and rs2541012) in age- and BMI-adjusted models. In addition, we observed statistically significant difference between osteoporosis group and non-osteoporosis group in genotype distributions of rs9898876, rs2541012, rs6259 and rs3853894. These four SNPs were also associated with BMD and presence of osteoporosis, with or without adjustment for covariants (age, BMI, SHBG and FT levels). In the haplotypes analysis, CCGGT (constituted by rs11078701C, rs1017163C, rs9898876G, rs62059836G and rs2541012T) and haplotype CGGT (constituted by rs858521C, rs858518G, rs6259G and rs727428T) was associated with a significant risk effect for osteoporosis.

This study was designed to cover an extensive range of loci in SHBG gene or in neighboring genes which have been reported to be responsible for the association with SHBG levels. In a genome-wide association study (GWAS) meta-analysis examined numerous candidate genetic loci, significant linkages for serum SHBG levels, suggesting that SHBG levels may be regulated by many genes rather than one gene. Nevertheless, in contrast to the GWAS meta-analysis, no significant association was observed of rs3853894, rs9303218, rs9901675, and rs8077824 located on SHBG and its neighboring gene were exhibited [16]. The result of our current study confirmed the association between rs9901675 and SHBG levels, suggesting that SHBG levels may be regulated by many genes rather than one gene. Nevertheless, in contrast to the GWAS meta-analysis, no significant association was observed of rs3853894, rs9303218, rs8077824 and SHBG levels in this study. The discrepancy of this may be due to the...
relative small sample size and lack of power in the present study, or maybe explained by selection of the population, demographic difference (namely age), and ethnic and racial difference. It's worth being mentioned that our results showed a clear association of another 3 SNPs (rs11078701, rs9898876 and rs2541012) of SHBG gene with SHBG levels in men, which had not been described in other studies, but it supported the notion that SHBG levels are genetic heritable [17,38].

Findings of the difference in SHBG levels resulting from polymorphisms of the gene and their association with hormone-related diseases have been reported but mainly concentrated on PCOS [19,20], breast [21,22] and prostate cancer [23,24]. Very little information is available on the effect of SHBG SNPs on bone. Results from the limited studies that have examined this relationship showed either no association or discordant results. The research conducted by Eriksson et al. [34] which was a large scale study of male population showed that those with genotypes associated with high SHBG levels had significantly higher BMD compared with those with lower SHBG. Meanwhile, data from Limer's [32] study indicated that the polymorphisms (rs1799941, rs6259) genotyped in SHBG did not seem to influence ultrasound BMD in white aged men. While in the study of Napoli et al. [31], they did not find any significant difference in the SHBG levels among genotypes of the rs179941 and rs6259 polymorphisms but significant difference in BMD in the femoral sites was noted among the variants. Similar to the findings of Napoli, our data showed that polymorphism of rs6259 was not related to SHBG levels but was associated with FN–TH-BMD even when adjusted for age, BMI, SHBG and FT levels. Similarly, we showed polymorphisms of rs3853894 were significantly associated with BMD but not SHBG levels. Interestingly, we found that polymorphisms of rs9898876 and rs2541012 were not only associated with SHBG levels, but also associated with BMD and the presence of osteoporosis. That is, carriers of genotypes related to high SHBG levels were associated with decreased BMD and presence of osteoporosis, and vice versa.

The potential relationship of haplotypes in SHBG gene and osteoporosis was further evaluated, and we found that haplotype CCGGT (constituted by rs11078701C, rs1017163C, rs9898876G, rs62059836G and rs2541012T) and haplotype CGGT (constituted by rs858521C, rs858518G, rs6259G and rs727428T) was associated with a significant risk effect for osteoporosis, suggesting that those SNPs in SHBG gene may jointly increased risk of osteoporosis.

Our data suggested that polymorphisms in the SHBG gene or the neighboring genes may influence BMD in men. Polymorphisms within the coding sequence and, potentially, in the regulatory sequence of the SHBG gene, altering either the production or the metabolism of the protein, could represent part of the genetic background of sex steroid hormone activity in humans. The G/A rs6259 polymorphism is located in exon 8 and causes a change in the amino acid sequence of SHBG (Asp > Asn). It has been suggested that the Asp > Asn modification affects a potential glycosylation site and increases the half-life of the protein coded for the less common allele [39]. It was assumed that the difference in BMD between carriers of different genotypes of rs6259 might be due to the variation in functional activity of the SHBG protein rather than in the amount of protein produced [31]. Thus, regardless of the obvious absence of biochemical change in SHBG levels, difference in BMD was observed among the variants. The mechanism for that rs3853893 associated with BMD but not SHBG levels is unknown to us. As it is located in an extronic region (exon 2) of ZBTB4 gene, which may have influence on gene expression and result in changes in the phenotype of diseases, further work is needed to elucidate its true functional significance on bone. In terms of polymorphisms (rs9898876 and rs2541012) associated with both SHBG levels and BMD, one might speculate that polymorphisms could result in biochemical alteration of SHBG concentrations and affected BMD consequently. Interestingly, the associations between these SNPs and BMD didn't seem to be mediated by serum levels of SHBG or FT, as indicated by the fact that the association between these SNPs and BMD persisted even after adjusting for age, BMI, SHBG and FT. Thus, the true mechanism underlining the association of SHBG polymorphisms and BMD remains undetermined and needs further examination.

As far as we know, it is the first study that captured the majority of the genetic variance within SHBG gene or the neighboring gene, and investigated the relationship of the SNPs and haplotypes with bone health in men. However, there are some limitations in our current study. First, the sample size is relatively small with skewed age distribution, which may make the study less powerful and less generalization. A larger population-based study would be valuable to assure the scientific reliability of our findings. Second, the hormone levels were measured by chemiluminescent analysis, where mass spectroscopy provides a more accurate value, especially for low TT levels as would be expected in this population and the measurements were based on single serum sample. The appropriate method for hormonal analysis should be taken into consideration in future work and draw a more reliable conclusion. Third, we did not assay estradiol (E2) and Vitamin D, which has been found to be of major importance for bone health in men as well as in women and may influence SHBG levels (and vice versa) [8,40–42]. However, the study of Kyvernitakis et al. [43] showed that age-dependent SHBG changes were associated with QUS values and did not support the hypothesis that E2 is a key regulator of bone turnover. Whether E2 might have an effect on bone in our population or whether the impact of SHBG on bone was independent from E2 needs to be clarified in future work.

In conclusion, our study showed that polymorphisms in SHBG or the neighboring genes were associated with SHBG levels and BMD and presence of osteoporosis, confirming the probability of another genetic contribution to bone health. These SNPs might help to identify individuals who were at risk of low bone density but might be the candidate for osteoporosis. The mechanism of SHBG genetic variants in influencing bone density and the presence of osteoporosis was not clearly understood and further studies are needed.

Declaration of interest

All authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Author contributions

Study conception and design: Xiao-Yun Zha, Yu Hu. Acquisition of data: Xiao-Yun Zha, Ji-Heng Zhu, Gui-Lin Chang, Li Li. Analysis and interpretation of data: Xiao-Yun Zha, Yu Hu, Xiao-Na Pang. Drafting of manuscript: Xiao-Yun Zha, Yu Hu. All authors give final approval of the version of this manuscript to be submitted.

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Supplementary material

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