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## Genome-wide association study identifies HMGN3 locus for spine bone size variation in Chinese

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## Abstract

Bone size (BS) is one of the major risk factors for osteoporotic fractures. BS variation is genetically determined to a substantial degree with heritability over 50%, but specific genes underlying variation of BS are still largely unknown. To identify specific genes for BS in Chinese, initial genome-wide association scan (GWAS) study and follow-up replication study were performed. In initial GWAS study, a group of 12 contiguous single-nucleotide polymorphism (SNP)s, which span a region of ~ 25 kb and locate at the upstream of HMGN3 gene (high-mobility group nucleosomal binding domain 3), achieved moderate association signals for spine BS, with  $P$  values ranging from  $6.2E-05$  to  $1.8E-06$ . In the follow-up replication study, eight of the 12 SNPs were detected suggestive replicate associations with BS in 1,728 unrelated female Caucasians, which have well-known differences from Chinese in ethnic genetic background. The SNPs in the region of HMGN3 gene formed a tightly combined haplotype block in both Chinese and Caucasians. The results suggest that the genomic region containing HMGN3 gene may be associated with spine BS in Chinese.

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## Introduction

Osteoporosis is a skeletal disorder characterized by excessive skeletal fragility leading to an increased risk of fracture. Bone size (BS) is one of the major parameters in determining bone quality and strength (Ahlborg et al. 2003; Augat et al. 1996; Fox et al. 1998; McCreadie and Goldstein 2000). Abnormal BS significantly contributes to the pathology of osteoporotic fracture (Deng et al. 2002c; Seeman et al. 2001), the most serious clinical complication of osteoporosis. BS variation is genetically determined to a substantial degree (Deng et al. 2002a; Tan et al. 2008) with heritability over 50%, but specific genes underlying variation of BS are still largely unknown.

Genetic studies via candidate gene association analyses were performed to identify genes contributing to variation of BS (Fang et al. 2007; Lei et al. 2005a, b, c, 2005; Malkin et al. 2005; van der Sluis et al. 2003; Zmuda et al. 2001). However, the number of candidate

genes tested in previous candidate gene association analyses is very limited, partly because the selection of candidate genes was based on known significance in bone biology.

Recently developing genome-wide association scan (GWAS) studies are powerful tools to rapidly and systematically identify genes for complex traits/diseases. In Caucasian, our group performed the first GWAS study for BS by interrogating ~380,000 SNPs on the Affymetrix platform in 1,000 homogeneous unrelated Caucasian subjects, and identified that *PLCL1* (phospholipase c-like 1) is a novel gene associated with variation of BS (Liu et al. 2008). However, in Chinese, which is the largest population in the world, has well-known difference from Caucasian in ethnic genetic background, no such GWAS study on BS has been performed to dissect the genetic basis of BS at the genome-wide scale.

Here, we performed an initial GWAS study in 1,627 unrelated Chinese subjects using highly dense Affymetrix Genome-Wide Human SNP Arrays that examine ~909,622 SNPs and a subsequent replication study to identify genes important to BS.

## Materials and methods

The study was approved by the necessary Institutional Review Board or Research Administration of the involved institutions. Signed informed consent documents were obtained from all study participants before entering the study. For the initial GWAS and replicate samples, areal BS values of lumbar spine (L1–L4) were measured using dual energy X-ray absorptiometry (DXA) machines (Hologic Inc., Bedford, MA, USA) that were calibrated daily.

For the initial GWAS sample, a total of 1,627 Han adult unrelated subjects (age  $34.5 \pm 13.2$  years) including 802 males and 825 females were recruited from two cities (Xi'an and Changsha) and their surrounding areas. All study subjects belong to Chinese Han ethnic group. Strict exclusion criteria (Deng et al. 2002b) were adopted to minimize any known and potential confounding effects on variation of bone phenotype. Briefly, patients with chronic diseases/conditions that may potentially affect bone mass or bone metabolism were excluded. These diseases/conditions included chronic disorders involving vital organs (heart, lung, liver, kidney, and brain), serious metabolic diseases (diabetes, hypo- or hyperparathyroidism, and hyperthyroidism), other skeletal diseases (Paget's disease, osteogenesis imperfecta, and rheumatoid arthritis), chronic use of drugs affecting bone metabolism (corticosteroid therapy, and anticonvulsant drugs), and malnutrition conditions (chronic diarrhea and chronic ulcerative colitis). For the replication study sample, a total of 1,728 random samples (age  $51.6 \pm 12.9$  years) were recruited in Midwestern US in Kansas City, Missouri and Omaha, Nebraska. All identified subjects were American Caucasians. Basic characteristics of study subjects were summarized in Table 1.

## Genotyping

Genomic DNA was extracted from whole human blood using a commercial isolation kit (Gentra systems, Minneapolis, MN, USA) following the protocol of the kit. For the initial GWAS sample, genotyping with the Affymetrix Genome-Wide Human SNP Array 6.0 was performed using the standard protocol recommended by the manufacturer. Fluorescence

intensities were quantified using an Affymetrix array scanner 3000 7G. Data management and analyses were performed using the Affymetrix® Gene-Chip® Command Console® Software (AGCC). Contrast QC threshold, which measures how well experiments resolve SNP signals into three genotype clusters, was set at the default value of >0.4 for quality control. Subjects with contrast QC of <0.4 were subject to re-genotyping. The final average contrast QC across the entire sample reached a high level of 2.62. The average missing call rate for all SNPs is 1.67%. Out of the initial full-set of 909,622 SNPs, there are 17,888 SNPs with allele frequencies extremely deviating from Hardy–Weinberg equilibrium ( $P < 0.01$ ), 202,984 SNPs with minor allele frequency (MAF) <1% and 618 with both. Therefore, the final SNP set maintained in the subsequent analyses contained 689,368 SNPs, yielding an average marker spacing of ~4 kb throughout the human genome. For the replication study sample, genotyping of SNPs was performed with the same Affymetrix Genome-Wide Human SNP Array 6.0 using the standard protocol. The final average contrast QC across the entire sample also reached a high level of 2.32. The average missing call rate for all SNPs is 1.27%.

### Statistical analysis

**GWAS analysis**—Gender and age, two significant covariates, were used to adjust the raw spine BS values for subsequent analyses by using a linear regression method. Spine BS values and gender & age were used as dependent and independent variables, respectively, and the output residuals of spine BS were used as phenotype value in association analysis. PLINK (Purcell et al. 2007) was used to perform genotypic association analyses with the command “–assoc”. Genotypic association analyses were used to compare the difference of mean BS values among three genotypic groups for each SNP. The adjusted spine BS values were in agreement with the asymptotic multivariate normal distribution.

**Haplotype analysis**—The linkage disequilibrium (LD) [standardized  $D'(D/D_{\max})$ ] patterns for the detected SNPs were analyzed and plotted using the Haploview program (Barrett et al. 2005) (<http://www.broad.mit.edu/mpg/haploview/>). Haplotype-specific association analysis in a quantitative trait association model was performed with the command “–hap-assoc” in the PLINK.

**Population stratification**—EIGENSTRAT was employed to perform principal component analysis to correct for stratification in GWAS studies. We used ~700,000 SNPs to calculate the principal components and the ten default main eigenvectors were used as covariates to adjust BS.

**Meta-analysis**—Meta-analysis for initial discovery and replication studies for BS was performed with the command “–meta-analysis” in the PLINK. The PLINK calculated four parameters for each SNP including  $P(F)$ : fixed-effects meta-analysis  $P$  value;  $P(R)$ : random-effects meta-analysis  $P$  value;  $Q$ :  $P$  value for Cochran’s  $Q$  statistics to test presence ( $P < 0.05$ ) versus absence of heterogeneity;  $I$ :  $I^2$  heterogeneity index to test the extent of heterogeneity (0–100).

## Results

### Initial GWAS study

As shown in a quantile–quantile (Q–Q) plot (Fig. 1) that demonstrate the distribution of  $P$  values involving 689,368 eligible SNPs in the initial sample, the observed  $P$  values matched the expected  $P$  values over the range of  $1 < -\log_{10}(P) < 4.5$ , but little departed at the extreme tail ( $-\log_{10}(P) > 4.5$ ), probably suggesting that the suggestive associations identified are likely due to true variants rather than potential biases such as genotyping error, potential population stratification, and cryptic family relatedness.

Figure 2 shows the distribution of  $P$  values in association analyses across the genome. We detected no significant associations according to the conservative genome-wide threshold of Bonferroni correction ( $P < 7.3E-08$ ) ( $0.05/689,368$ ). However, we detected 75 SNPs with moderate association signals ( $P < 1.0E-04$ ). The most significant association was detected at rs17743190 ( $P = 1.8E-06$ ), which is located at the upstream of high-mobility group nucleosomal binding domain 3 (HMGN3 gene). Moreover, at the HMGN3 locus, another 11 contiguous SNPs (Table 2), which span a region of ~25 kb, also achieved moderate association signals for spine BS, with  $P$  values ranging from  $6.2E-05$  to  $6.1E-06$ . In the follow-up replication study, eight of the 12 SNPs were detected suggestive replicate association signals with BS in 1,728 unrelated female Caucasians, which have well-known difference from Chinese in ethnic genetic background. We also identified other two distinct groups of contiguous SNPs with moderate consistent association signals to BS, one in the upstream region of ALCAM gene (activated leukocyte cell adhesion molecule) and the other in the intron of COMMD7 gene (COMM domain containing 7) (Supplementary Table 1), but no significant or suggestive replicate association signals were detected for these two distinct groups in the 1,728 unrelated female Caucasians. Due to high phenotypic correlation between BS and height, we also performed association analysis between the 75 SNPs and height. Among the 75 SNPs, 48 SNPs were associated with height ( $P < 0.05$ ) with the strongest association at rs16878913 ( $P = 9.5E-07$ ). After we adjusted BS value with height, most of the association  $P$  values for the 75 SNPs were much weaker than those without adjustment of height (data not shown). These results taken together suggest that some of the detected SNPs have evident pleiotropic effects on both BS and height.

As shown in Table 2 and Supplementary Table 1, the fixed-effects meta-analysis  $P$  values ( $P(F)$ ) for most of the 75 SNPs are little weaker than in initial study, but much stronger than in replicate study. However, for the 75 SNPs except for five SNPs (rs2955948, rs16960741, rs16999576, rs16999576 and rs926598),  $P$  values for Cochran's  $Q$  statistic are significant and  $I^2$  heterogeneity indexes are high, suggesting high heterogeneity in effect of SNP across Chinese and Caucasians. In this situation, random-effects meta-analysis  $P$  values ( $P(R)$ ) are more appropriate to evaluate the combined effects than  $P(F)$ . The  $P(R)$  values for most of the 75 SNPs are much weaker than in initial study, but little stronger than in replicate study.

Figure 3 shows the LD pattern and the haplotype block structure for the SNP group of HMGN3 locus in initial Chinese sample. The SNPs in the region of HMGN3 gene formed a tightly combined haplotype block in initial Chinese sample (Fig. 3) and also in replicate Caucasian sample (data not shown). As shown in Supplementary Table 2, the PLINK

detected two haplotypes for the 12 SNPs in the initial Chinese sample, and the associations for the two haplotypes are significant ( $P = 6.8E-05$  at HAPL-1 and  $P = 9.3E-06$  at HAPL-1). However, in the replicate Caucasian sample, six haplotypes were detected, but only one haplotype (HAPL-6) is significant.

Previous genetic studies on BS are relatively few. Together, about seven genes (ANKH, PLCL1, COL1A1, COL1A2, ESR1, VDR, and CYP17) (Lei et al. 2005a; Liu et al. 2008; Long et al. 2004; Malkin et al. 2005; van der Sluis et al. 2003; Zmuda et al. 2001) were studied and found significant associations with BS at various skeletal sites (e.g., spine, femoral neck, hip, and hand). We selected 353 SNPs, which were located in the seven genes above and genotyped with Affymetrix Human Genome SNP 6.0, for association analysis with spine BS in initial Chinese sample. Only six SNPs from two genes (estrogen receptor-alpha and vitamin receptor) have  $P$  values of  $<0.05$ , but these  $P$  values will be not significant if adapting multiple testing corrections (FDR\_BH).

## Discussion

In Caucasian, a lot of genetic studies using genome-wide linkage scan (GWLS) (Shen et al. 2006; Xu et al. 2004) or candidate gene association analyses (Long et al. 2004; van der Sluis et al. 2003) identified quite a few chromosomal regions potentially harboring genes underlying variation of spine BS, or some variants in candidate genes associated with spine BS variation. Recently developing GWAS studies are powerful tools to rapidly and systematically identify genes for complex traits/diseases. Our group performed the first GWAS study of BS in 1,000 homogeneous unrelated Caucasian subjects (Liu et al. 2008). No such GWAS study on BS was performed to dissect the genetic basis of BS at the genome-wide scale in Chinese, which has well-known difference in genetic background from Caucasian. Therefore, it is necessary to perform independent genetic studies to rapidly and systematically identify genes for BS variation in Chinese. This study represents the first GWAS study for BS in Chinese.

HMG3 is a nuclear protein that binds to nucleosomes, promotes chromatin unfolding, enhances access to nucleosomes, and modulates transcription from chromatin templates. This gene is a new, but important regulator of glucose homeostasis and demonstrates the relevance to glucose-stimulated insulin secretion (Ueda et al. 2009). Insulin acts directly on osteoclast activity, and thiazolidinediones (TZD) may have a negative effect by switching mesenchymal progenitor cells to adipose rather than bone tissue (Montagnani et al. 2011). The data from expression database demonstrated that this gene is expressed in tissues of bone and bone marrow (<http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?UGID=140116&TAXID=9606&SEARCH=HMG3>). The current detected association between the HMG3 gene and spine BS added further evidence on supporting the link between HMG3 locus and bone. Such association results should be considered exploratory and will require more replications in larger cohorts to better understand their role on bone. Focused molecular functional studies deserve to be pursued to clarify the regulating mechanisms possibly from the aspect of insulin signaling and glucose metabolism.

In a previous GWAS study of BS in Caucasian subjects, we identified that PLCL1 (phospholipase c-like 1) is a novel gene associated with variation of BS at hip in Caucasian (Liu et al. 2008). However, we have not found any replicate association evidence in the current study at spine in Chinese, possibly suggesting the differences at skeletal sites in genetic determination of BS.

Some bone phenotypes are clearly under ethnic-specific genetic determination (Dvornyk et al. 2005, 2003; Lei et al. 2006, 2003). For example, five candidate genes (Dvornyk et al. 2005) were tested for their contribution of ethnicity to bone mineral density variation in Caucasian and Chinese. The frequencies and distribution patterns of SNPs of some prominent bone candidate genes were different between Caucasian and Chinese (Lei et al. 2003). In this study, all association signals for the 75 detected SNPs (Table 2 and Supplementary Table 1) in the initial study are stronger than in replication study, and some of them even have reverse direction of effect ( $\beta$ ). Moreover, all the studied SNPs except for five SNPs (rs2955948, rs16960741, rs16999576, rs16999576, and rs926598),  $P$  values for Cochran's  $Q$  statistic are significant and  $I^2$  heterogeneity indexes are high, suggesting high heterogeneity in effect of SNP across Chinese and Caucasians. The above observations combined together suggest evident differences in genetic regulators of BS in Chinese versus Caucasians.

This study has several limitations. First, the age between two cohorts are quite different (34.5 in Chinese versus 51.6 in Caucasian). Second, the sex composition is different, with both men and women in Chinese, but only women in Caucasian. These two above-mentioned differences may be the two important factors underlying discrepancy of the findings between Chinese and Caucasian. Second, the statistical power is relatively low. Calculated with a GWA-Power (Feng et al. 2011), the current sample size ( $n = 1,627$ ) in initial study has only over 11% of power to detect the studied SNPs with strong LD ( $D' = 0.9$ ) with QTLs that explain over 1% of spine BS variation according to the conservative genome-wide threshold of Bonferroni correction ( $P < 7.3E-08$ ) (0.05/689,368).

In conclusion, this study reported the first GWAS for BS in Chinese population. The results of this study suggest that HMGN3 locus may be associated with spine BS.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

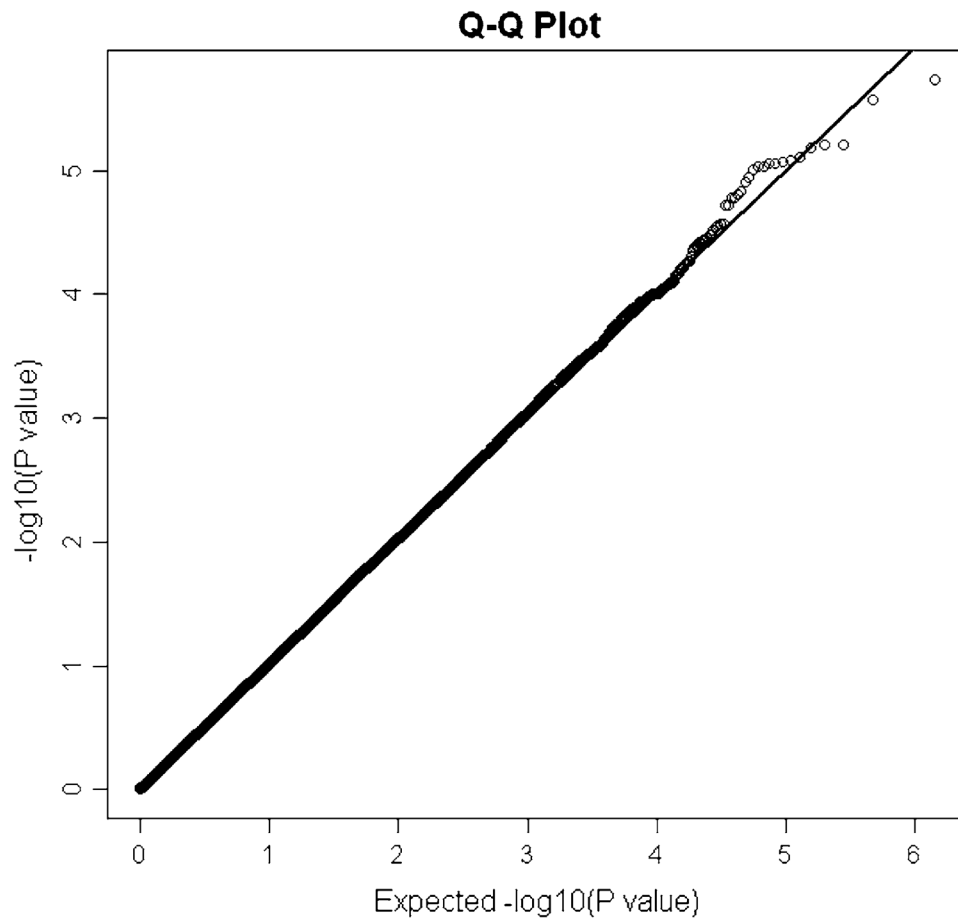
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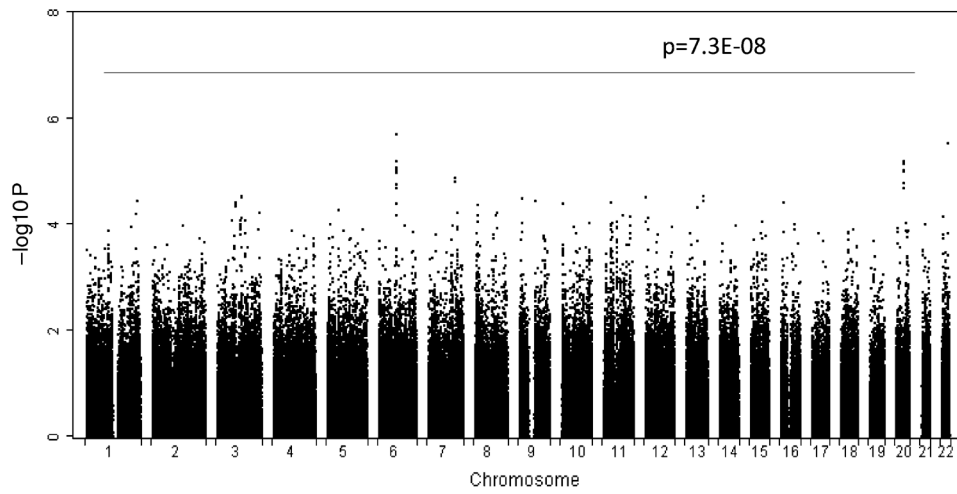
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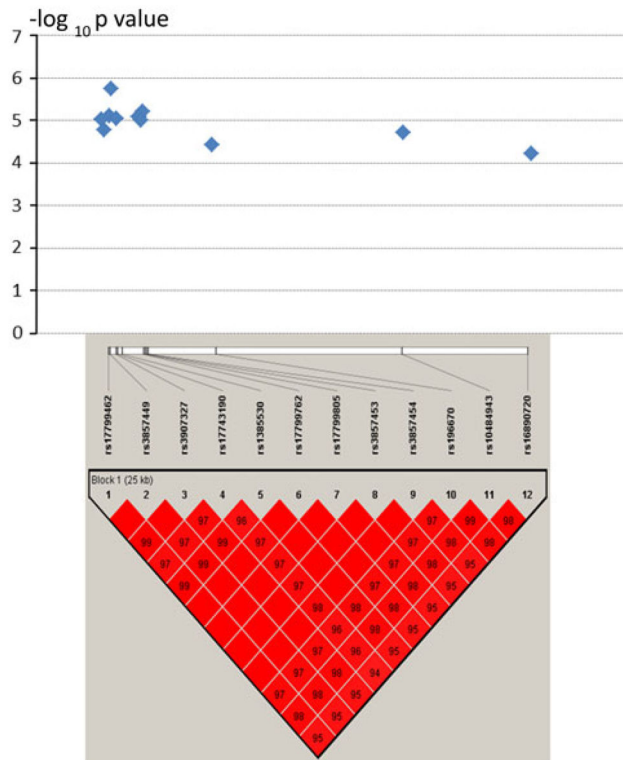
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**Fig. 1.** Quantile–quantile (Q–Q) plot for BS in Chinese. From the Q–Q plot, the observed  $P$  values for BS match the expected  $P$  values under the null distributions over the range of  $(1 < -\log_{10}(P) < 4.5)$



**Fig. 2.** Distribution of  $P$  values across the genome in association analyses. The  $-\log_{10} P$  values from 689,368 SNPs in 1,627 Chinese are plotted according to its physical position on successive chromosomes. “ $P = 7.3E-08$ ” is the conservative genome-wide threshold of Bonferroni correction in this study



**Fig. 3.** Haplotype block for 12 SNPs detected in HMGN3 gene locus in Chinese. The detected SNPs are located at the upstream of HMGN3 (~200 kb). The LD patterns for the 12 SNPs were analyzed and plotted using the Haploview program

**Table 1**

## Basic characteristics of study subjects

<b>Trait</b>	<b>Initial discovery</b>	<b>Replication</b>
Sample size	1,627	1,728
Age (years)	34.5 (13.2)	51.6 (12.9)
Height (m)	1.64 (0.08)	1.63 (0.06)
Weight (kg)	60.1 (10.5)	71.4 (16.0)
Spine BS (cm <sup>2</sup> )	60.4 (7.0)	58.6 (5.7)

Mean (SD)

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**Table 2**

Associations of 12 SNPs in HMGN3 locus with BS and height

SNP ID	Position	Allele <sup>a</sup>	BS		Initial discovery		Replication		Height		Meta-analysis <sup>d</sup>			
			MAF	$\beta$	MAF	$\beta$	MAF	$\beta$	$P$	$\beta$	$P$	$P(F)$	$P(R)$	$Q$
rs17743190	80164398	A/G	0.09	1.83	1.8E-06	0.09	0.40	0.27	0.61	0.11	4.2E-05	0.12	6.6E-03	86.5
rs3857454	80166252	A/G	0.11	1.57	6.1E-06	0.12	0.56	6.2E-02	0.70	4.2E-02	1.1E-05	3.6E-02	2.9E-02	79.2
rs3907327	80164301	C/A	0.11	1.54	7.8E-06	0.12	0.52	8.5E-02	0.68	4.8E-02	2.2E-05	4.7E-02	2.5E-02	80.2
rs17799762	80165971	C/T	0.11	1.53	8.3E-06	0.12	0.51	8.5E-02	0.67	5.1E-02	2.4E-05	4.9E-02	2.4E-02	80.5
rs17799805	80166050	C/A	0.11	1.53	8.4E-06	0.12	0.55	6.7E-02	0.67	5.0E-02	1.6E-05	3.7E-02	3.0E-02	78.7
rs1385530	80164684	G/A	0.11	1.53	8.8E-06	0.12	0.55	6.4E-02	0.63	6.8E-02	1.5E-05	3.6E-02	3.2E-02	78.4
rs17799462	80163839	C/T	0.09	1.52	9.3E-06	0.09	0.17	0.60	0.68	5.0E-02	5.2E-04	0.21	4.7E-03	87.5
rs3857453	80166130	G/C	0.11	1.52	9.9E-06	0.12	0.53	7.8E-02	0.68	5.0E-02	2.2E-05	4.3E-02	2.9E-02	79.2
rs3857449	80163957	T/C	0.11	1.48	1.7E-05	0.12	0.56	6.3E-02	0.67	5.2E-02	2.2E-05	3.0E-02	4.2E-02	75.7
rs10484943	80181477	T/C	0.11	1.50	1.9E-05	0.12	0.52	8.5E-02	0.61	8.6E-02	4.1E-05	4.4E-02	3.2E-02	78.2
rs196670	80170314	A/G	0.19	1.43	3.8E-05	0.19	0.13	0.59	0.53	0.13	4.7E-03	0.24	2.2E-03	89.4
rs16890720	80189011	A/G	0.15	1.40	6.2E-05	0.16	0.22	0.40	0.59	9.1E-02	2.0E-03	0.18	6.9E-03	86.3

HMGN3 gene is located at 6q14

<sup>a</sup>The first allele represents the minor allele of each SNP

<sup>b</sup> $P$  values after correction of population stratification: EIGENSTRAT was employed to calculate the principal components and the ten default main eigenvectors were used as covariate to adjust BS

<sup>c</sup>The direction of effect indicated by  $\beta$  is calculated for minor allele

<sup>d</sup>Meta-analysis for initial discovery and replication studies for BS was performed with the command “-meta-analysis” in PLINK

$P(F)$  fixed-effects meta-analysis  $P$  value,  $P(R)$  random-effects meta-analysis  $P$  value,  $Q$   $P$  value for Cochran’s  $Q$  statistic,  $I^2$  heterogeneity index