

# An independent validation study of three single nucleotide polymorphisms at the sex hormone-binding globulin locus for testosterone levels identified by genome-wide association studies

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**STUDY QUESTION:** Are the single nucleotide polymorphisms (SNPs) rs2075230, rs6259 and rs727428 at the sex hormone-binding globulin (*SHBG*) locus, which were identified by genome-wide association studies (GWASs) for testosterone levels, associated with testosterone levels in Japanese men?

**SUMMARY ANSWER:** The SNP rs2075230, but not rs6259 and rs727428, is significantly associated with testosterone levels in Japanese men.

**WHAT IS ALREADY KNOWN:** Previous GWASs have revealed that rs2075230 is associated with serum testosterone levels in 3495 Chinese men and rs6259 and rs727428 are associated with serum testosterone levels in 3225 men of European ancestry.

**STUDY DESIGN, SIZE, AND DURATION:** This is an independent validation study of 1687 Japanese men (901 in Cohort 1 and 786 in Cohort 2).

**PARTICIPANTS/MATERIALS, SETTING AND METHOD:** Cohort 1 (20.7 ± 1.7 years old, mean ± SD) and Cohort 2 (31.2 ± 4.8 years) included samples obtained from university students and partners of pregnant women, respectively. The three SNPs were genotyped using either TaqMan probes or restriction fragment length polymorphism PCR. Blood samples were drawn from the cubital vein of the study

participants in the morning, and total testosterone and SHBG levels were measured using a time-resolved immunofluorometric assay. Association between each SNP and testosterone levels was evaluated by meta-analysis of the two Japanese male cohorts.

**MAIN RESULTS AND THE ROLE OF CHANCE:** The age of the two cohorts was significantly different ( $P < 0.0001$ ). We found that rs2075230 was significantly associated with serum testosterone levels ( $\beta_{\text{STD}} = 0.15$ ,  $P = 7.2 \times 10^{-6}$ ); however, rs6259 and rs727428 were not ( $\beta_{\text{STD}} = 0.17$ ,  $P = 0.071$ ;  $\beta_{\text{STD}} = 0.082$ ,  $P = 0.017$ , respectively), after adjusting for multiple testing in a combined analysis of two Japanese male cohorts. Moreover, rs2075230, rs6259 and rs727428 were significantly associated with high SHBG levels ( $\beta_{\text{STD}} = 0.22$ ,  $P = 3.4 \times 10^{-12}$ ;  $\beta_{\text{STD}} = 0.23$ ,  $P = 6.5 \times 10^{-6}$  and  $\beta_{\text{STD}} = 0.21$ ,  $P = 3.4 \times 10^{-10}$ , respectively).

**LARGE SCALE DATA:** Not applicable.

**LIMITATIONS, REASONS FOR CAUTION:** This study had differences in the age and background parameters of participants compared to those observed in previous GWASs. In addition, the average age of participants in the two cohorts in our study also differed from one another. Therefore, the average testosterone levels, which decrease with age, between studies or the two cohorts were different.

**WIDER IMPLICATIONS OF THE FINDINGS:** The three SNPs have a considerable effect on SHBG levels and hence may indirectly affect testosterone levels.

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**Key words:** independent validation study / testosterone / Japanese men / single nucleotide polymorphism / sex hormone-binding globulin / genome-wide association studies

## WHAT DOES THIS MEAN FOR PATIENTS?

Previous studies have indicated that there may be a hereditary factor associated with men's testosterone levels. One particular DNA variation has been linked with the testosterone levels of Chinese men and two others have been linked with the testosterone levels of European men.

This research was carried out on two groups of Japanese men aimed to confirm the previous results. The DNA variation which was linked to testosterone levels in Chinese men had similar links to the testosterone levels of the men in this study. There was also a link with levels of a protein present in the blood which carries testosterone around the body. The two other DNA variations which had been linked with testosterone of European men were not significant for the Japanese men. However, the researchers did find that the levels of the protein were associated with all three variations.

This study backs up research which has found a link between men's DNA and their testosterone levels. As levels of testosterone as well as the protein can affect men's fertility and their general health, this study demonstrates that particular DNA variations can play a role in this in different groups of men.

## Introduction

Testosterone, secreted by the testes, is one of the major androgens. It contributes to the development of sexual characteristics and genitalia and to the maturation of sperm (Kaufman and Vermeulen, 2005). In addition, differing testosterone levels have been observed to affect health adversely causing diseases, including metabolic syndromes (Kupelian et al., 2006; Haring et al., 2009), type two diabetes (Vikan et al., 2010), cardiovascular diseases (Vikan et al., 2009; Araujo et al., 2011) and carcinogenesis (Sharifi et al., 2005). Approximately 50–60% of the testosterone in circulation is bound to sex hormone-binding globulin (SHBG) and 40–50% is bound to albumin. Unbound testosterone (1–2%), which is termed free testosterone, and albumin-bound testosterone act as biologically active hormones (Kaufman and Vermeulen, 2005).

Twin studies have shown that the heritability of sex hormone levels, including those of testosterone and SHBG, ranges from 56% to 81% (Ring et al., 2005; Kuijper et al., 2007). However, the genetic determinants of sex hormone levels remain largely unknown. To

date, there have been six genome-wide association studies (GWASs) regarding sex hormone levels, including those of testosterone, dihydrotestosterone, SHBG, dehydroepiandrosterone sulfate and FSH. Of these, the results of one GWAS of 3495 Chinese men indicated the association of the SHBG locus at 17p13 with testosterone ( $P = 1.1 \times 10^{-8}$  for single nucleotide polymorphism (SNP) rs2075230) and SHBG levels ( $P = 4.8 \times 10^{-19}$  for SNP rs2075230) (Chen et al., 2013). A GWAS of 3225 men of European descent has shown that the SHBG locus is associated with serum testosterone ( $P = 1.3 \times 10^{-12}$  for SNP rs727428;  $P = 5.8 \times 10^{-8}$  for SNP rs72829446;  $P = 3.3 \times 10^{-7}$  for SNP rs6259) and dihydrotestosterone levels ( $P = 1.5 \times 10^{-11}$  for rs727428;  $P = 9.5 \times 10^{-10}$  for rs72829446;  $P = 4.04 \times 10^{-9}$  for rs6259) (Jin et al., 2012). SNPs rs72829446 and rs6259 were found to be in strong linkage disequilibrium (LD) ( $r^2: 0.88$ ) (Jin et al., 2012).

This independent validation study was conducted to assess whether the three SNPs (rs2075230, rs6259 and rs727428) of the SHBG locus

were associated with testosterone levels in two Japanese male cohorts. The three specific SNPs have been previously reported as strongly associated with testosterone levels with minor allele frequencies  $>0.05$  in the HapMap-JPT population of male subjects. Pairwise  $r^2$  of the three SNPs measured by HapMap JPT (Phase II + III data set) are as follows: 0.129 (rs2075230–rs6259); 0.415 (rs2075230–rs727428) and 0.129 (rs6259–rs727428). Therefore, these three SNPs are in incomplete LD and not highly correlated with each other, although pairwise  $ID'1$  values among the three SNPs are 1.

In addition, to provide evidence for the biological association between the *SHBG* locus and testosterone levels, we conducted association studies between the three SNPs and SHBG and calculated free testosterone (cFT) levels. Furthermore, we investigated associations between the three SNPs and serum total testosterone levels, assuming covariates for SHBG levels.

## Materials and Methods

This study was approved by the ethics committees of the University of Tokushima and St. Marianna Medical University. All participants provided written informed consent.

### Samples from two Japanese cohorts

Two Japanese cohorts consisting of 901 young men from the general Japanese population ( $20.7 \pm 1.7$  years old, mean  $\pm$  SD: Cohort 1) and 786 Japanese men of proven fertility ( $31.2 \pm 4.8$  years old, mean  $\pm$  SD: Cohort 2) were included in the independent validation study. The subjects in this study have been described in previous reports (Nakahori *et al.*, 2012; Iwamoto *et al.*, 2013a,b; Sato *et al.*, 2013a,b, 2014a,b, 2015a,b,c). Briefly, Cohort 1 samples were recruited from the university students in the urology departments of university hospitals in four Japanese cities (Kawasaki, Kanazawa, Nagasaki and Sapporo). Cohort 2 samples were recruited from the partners of pregnant women who attended obstetric clinics in four Japanese cities (Sapporo, Kanazawa, Osaka and Fukuoka).

### Measurement of clinical characteristics

Physical characteristics and hormone levels of the study participants have been analyzed in a previous study (Iwamoto *et al.*, 2013a,b). Briefly, age, body weight and height were self-reported. BMI ( $\text{kg}/\text{m}^2$ ) was calculated from body weight and height. Blood was drawn from the cubital vein of each participant usually in the morning to reduce the effect of diurnal variation in hormone levels. Serum total testosterone and SHBG levels were determined using a time-resolved immunofluorometric assay (Delfia, Wallac, Turku, Finland). It has been reported that cFT calculated using Vermeulen's formula (Vermeulen *et al.*, 1999) is related to measured FT in the Japanese population (Okamura *et al.*, 2005; Iwamoto *et al.*, 2009). Further, cFT calculated using Vermeulen's formula in the Japanese population has been used in some other reports (Yoshinaga *et al.* 2014; Tanabe *et al.* 2015), including two reports on our cohorts (Iwamoto *et al.*, 2013a,b). Therefore, the values of cFT, calculated from testosterone and SHBG levels by using Vermeulen's formula, were used in this study. Briefly, a value of  $1 \times 10^9$  mol/l for the association constant of SHBG for testosterone, a value of  $3.6 \times 10^4$  mol/l for the association constant of albumin for testosterone, and a fixed plasma albumin concentration of 43 g/l were used to calculate the free testosterone (Vermeulen *et al.*, 1999).

### Genotyping and LD structure

Genomic DNA was extracted from the peripheral blood samples of subjects using a QIAamp DNA blood kit (Qiagen; Tokyo, Japan), as previously

described (Nakahori *et al.*, 2012; Sato *et al.*, 2013a,b, 2014a,b, 2015a,b,c). The rs2075230 and rs6259 SNPs were genotyped using TaqMan probes rs2075230 (C\_16165982\_10; Applied Biosystems; Tokyo, Japan) and rs6259 (C\_11955739\_10; Applied Biosystems) in the ABI 7900HT real-time PCR system (Applied Biosystems). The rs727428 SNP was detected by restriction fragment length polymorphism PCR using the following primer sets: 5'-AAGTGGACCAAGACTAGGAG-3' (forward) and 5'-GAAGCTACTCCC TTTGAGAC-3' (reverse). DNA from each subject was amplified using Taq DNA polymerase (Promega, Tokyo, Japan) under the following PCR cycling parameters: initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min; and final extension for 3 min at 72°C. The resulting PCR products were then digested using the *Hinf*I restriction enzyme (New England Biolabs Japan Inc., Tokyo, Japan). The digested products were separated by electrophoresis on a 2.5% agarose gel. The following fragment sizes were used for allele identification on gels: 274 bp (A-allele) and 195 + 79 bp (G-allele). Genotyping was performed once, and the call rates of the three SNPs were 100%.

Pairwise  $r^2$  and  $ID'1$  values among SNPs were measured by HapMap-JPT data set (Phase II + III). The LD plots were obtained with Haploview software version 4.2 (Broad Institute, Cambridge, MA, USA; online at <https://www.broadinstitute.org/haploview/haploview>) (Barrett *et al.*, 2005), using the HapMap-JPT and CEU database (Phase III) as per the definition by Gabriel *et al.* (2002).

### Statistical analysis

Hardy–Weinberg equilibrium (HWE) was assessed in the two cohorts by using Pearson chi-square test for genotypes. The genotype distributions for the three SNPs were in HWE in the two cohorts ( $P > 0.05$ ).

In a previous GWAS report, testosterone values were not transformed (Chen *et al.*, 2013). On the other hand, in another GWAS report by Jin *et al.*, the testosterone value underwent logarithmic (log) transformation in the analysis (Jin *et al.*, 2012). In our study, testosterone values were not normally distributed. Previously, Iwamoto *et al.* analyzed the same samples that were used in our study using natural log-transformed testosterone values (Iwamoto *et al.*, 2013a,b). When we performed the Shapiro–Wilks normality test to confirm whether natural log-transformed testosterone is normally distributed, the results showed significant normality in Cohort 1 ( $P > 0.05$ ) and none in Cohort 2 ( $P = 0.02$ ). However, there was a reduction in the skewness of distribution of the natural log-transformed testosterone in Cohort 2. Therefore, we decided to use the natural log-transformed testosterone values for analysis in the present study. For the same reason, SHBG and cFT also were processed using natural log-transformed variables to minimize deviation from a normal distribution. The associations between SNPs and sex hormone values were assessed using standardized multiple linear regressions under an additive genetic model, with adjustments for age and BMI. In a separate analysis, rs6259 and rs727428 were additionally adjusted for rs2075230.

The results obtained from the two cohorts were combined in a meta-analysis, using the meta-package for the R version 3.1.2 statistical environment (The R Project for Statistical Computing; online at <http://www.R-project.org/>). The extent of heterogeneity among studies was quantified by the  $I^2$  statistic (Higgins *et al.*, 2003) and statistically assessed by Cochran's  $Q$  test. If there was no heterogeneity, as determined by an  $I^2$  statistic  $<50\%$  or a  $P$  value more than 0.1, a fixed-effects model using the inverse variance method was used. Otherwise, the random-effects model using the DerSimonian–Laird method was employed.

All statistical analyses were performed using R version 3.1.2 (<http://www.R-project.org/>), and statistical significance was considered at  $P$  values  $< 0.0083$  (0.05/6 tests [= 2 studies  $\times$  1 trait  $\times$  3 SNPs]) for the independent validation study and at  $P$  values  $< 0.0042$  (0.05/12 tests [= 2 studies  $\times$

2 traits  $\times$  3 SNPs]) for other hormone parameters, after adjusting for multiple testing.

## Results

The sex hormone concentrations in blood samples obtained from the two Japanese cohorts are presented in the [Supplementary Table S1](#). In concurrence with previous reports ([Iwamoto et al., 2013b](#); [Sato et al., 2015a](#)), sex hormone levels significantly differed between Cohorts 1 and 2.

Multiple linear regression analysis under the additive genetic model revealed that rs2075230 and rs6259 were significantly correlated with testosterone levels in Cohort 1 (standardized  $\beta$  ( $\beta_{STD}$ ) = 0.18,  $P = 1.3 \times 10^{-4}$  in Cohort 1) and Cohort 2 ( $\beta_{STD} = 0.26$ ,  $P = 3.8 \times 10^{-4}$  in Cohort 2), respectively; however, rs727428 did not display a correlation with testosterone levels in both cohorts, after adjusting for multiple testing (Table I). The combined analysis of the two cohorts revealed that only rs2075230 was significantly associated with testosterone levels ( $\beta_{STD} = 0.15$ ,  $P = 7.2 \times 10^{-6}$ ), after adjusting for multiple testing.

Next, we investigated the association of the three SNPs with SHBG and cFT levels in the two Japanese male cohorts. We found that the three SNPs were significantly associated with SHBG levels in both cohorts (rs2075230,  $\beta_{STD} = 0.20$ ,  $P = 6.5 \times 10^{-6}$  in Cohort 1;  $\beta_{STD} = 0.25$ ,  $P = 1.3 \times 10^{-7}$  in Cohort 2/rs6259,  $\beta_{STD} = 0.20$ ,  $P = 6.4 \times 10^{-3}$  in Cohort 1;  $\beta_{STD} = 0.25$ ,  $P = 3.0 \times 10^{-4}$  in Cohort 2/rs727428,  $\beta_{STD} = 0.18$ ,  $P = 8.1 \times 10^{-5}$  in Cohort 1;  $\beta_{STD} = 0.23$ ,  $P = 9.8 \times 10^{-7}$  in Cohort 2). The combined analysis of the two cohorts also revealed that the three SNPs were significantly linked with SHBG levels after adjusting for multiple testing (rs2075230,  $\beta_{STD} = 0.22$ ,  $P = 3.4 \times 10^{-12}$ ; rs6259,  $\beta_{STD} = 0.23$ ,  $P = 6.5 \times 10^{-6}$ ; rs727428,  $\beta_{STD} = 0.21$ ,  $P = 3.4 \times 10^{-10}$ ). However, none of the three SNPs were significantly associated with cFT levels after being corrected for multiple testing (Table II).

Testosterone levels strongly correlate with SHBG levels in both cohorts ([Supplementary Tables S2 and S3](#)). Therefore, it was suggested that the observed associations between these SNPs and testosterone levels could be affected by inter-individual differences in circulating SHBG levels. To ascertain this, we conducted association analysis of the three SNPs with the testosterone levels adjusted for SHBG levels. The associations between the three SNPs and testosterone levels were very weak, and non-significant (Table III).

The rs2075230, rs6259 and rs72748 SNPs associated with SHBG levels are located near or on the *SHBG* gene. Therefore, we performed conditional logistic regression analysis additionally adjusted with rs2075230, which had the most significant associations with SHBG levels, to investigate whether rs6259 and rs727428 affected SHBG levels, independently. After adjusting for the effect of rs2075230, the strength of associations of rs6259 and rs727428 with SHBG levels was reduced; however, the two SNPs still showed statistically significant associations with SHBG levels (rs6259,  $\beta_{STD} = 0.14$ ,  $P = 8.9 \times 10^{-3}$ ; rs727428,  $\beta_{STD} = 0.10$ ,  $P = 0.014$ ) ([Supplementary Table S4](#)).

## Discussion

Recent GWASs reported that rs2075230 was significantly associated with testosterone and SHBG levels in 3495 Chinese men ([Chen et al., 2013](#)), and rs6259 and rs727428 were significantly associated with

**Table I** An association analysis of the three SNPs with serum testosterone levels in two Japanese male cohorts.

SNP	Chr	Position	Gene	Location	Effect/ other	Cohort 1 (N = 901)			Cohort 2 (N = 786)			Combined			Heterogeneity	
						EAF	$\beta_{STD}$ (SE)	P	EAF	$\beta_{STD}$ (SE)	P	$\beta_{STD}$ (SE) [model] <sup>a</sup>	P <sub>meta</sub>	Var (%) <sup>b</sup>	P <sub>hetero</sub>	I <sup>2</sup> (%)
Testosterone																
rs2075230	17	7487108	SHBG	Upst.	A/G	0.565	<b>0.18 (0.046)</b>	<b><math>1.3 \times 10^{-4}</math></b>	0.556	0.12 (0.049)	$1.4 \times 10^{-2}$	<b>0.15 (0.033) [F]</b>	<b><math>7.2 \times 10^{-6}</math></b>	1.1	0.39	0.0
rs6259	17	7536527	SHBG	Exon	A/G	0.107	0.073 (0.075)	0.33	0.116	<b>0.26 (0.072)</b>	<b><math>3.8 \times 10^{-4}</math></b>	0.17 (0.092) [R]	0.071	0.5	0.076	68.1
rs727428	17	7537792	SHBG	Dwnst.	G/A	0.396	0.11 (0.048)	0.019	0.366	0.050 (0.049)	0.31	0.082 (0.034) [F]	0.017	0.3	0.36	0.0

Data are shown as the estimated standardized linear regression statistic  $\beta_{STD}$ , SE and P value with adjustments for age and BMI. Testosterone and sex hormone-binding globulin (SHBG) were processed using natural log-transformed variables. Bold numbers indicate significance ( $P$  value  $< 0.0083$ ) after adjusting for multiple testing.

SNP, single nucleotide polymorphisms; Chr, chromosome; EAF, effect allele frequency;  $\beta_{STD}$ , standardized regression coefficient; P<sub>hetero</sub>, P value for heterogeneity; Upst., upstream; Dwnst., downstream.

<sup>a</sup>The  $\beta$ -coefficient and its SE were summarized using an inverse variance-weighted meta-analysis, under fixed-effects model [F] or the DerSimonian and Laird method under random-effects model [R].

<sup>b</sup>Percentage of phenotypic variance (log-transformed) explained by SNP.

**Table II** An association analysis between the three SNPs and other sex hormone levels in two Japanese male cohorts.

SNP (effect allele)	Trait	Cohort 1		Cohort 2		Combined		Heterogeneity	
		$\beta_{STD}$ (SE)	P	$\beta_{STD}$ (SE)	P	$\beta_{STD}$ (SE) [model] <sup>a</sup>	P <sub>meta</sub>	P <sub>hetero</sub>	I <sup>2</sup> (%)
rs2075230 (A)	SHBG	<b>0.20 (0.044)</b>	<b>6.5 × 10<sup>-6</sup></b>	<b>0.25 (0.047)</b>	<b>1.3 × 10<sup>-7</sup></b>	<b>0.22 (0.032) [F]</b>	<b>3.4 × 10<sup>-12</sup></b>	0.46	0.0
	cFT	-0.097 (0.047)	0.039	0.034 (0.050)	0.50	-0.033 (0.065) [R]	0.62	0.058	72.3
rs6259 (A)	SHBG	0.20 (0.072)	6.4 × 10 <sup>-3</sup>	<b>0.25 (0.070)</b>	<b>3.0 × 10<sup>-4</sup></b>	<b>0.23 (0.050) [F]</b>	<b>6.5 × 10<sup>-6</sup></b>	0.57	0.0
	cFT	0.021 (0.075)	0.78	-0.12 (0.074)	0.11	-0.049 (0.053) [F]	0.35	0.19	42.1
rs727428 (G)	SHBG	<b>0.18 (0.046)</b>	<b>8.1 × 10<sup>-5</sup></b>	<b>0.23 (0.047)</b>	<b>9.8 × 10<sup>-7</sup></b>	<b>0.21 (0.033) [F]</b>	<b>3.4 × 10<sup>-10</sup></b>	0.43	0.0
	cFT	-0.030 (0.048)	0.54	0.11 (0.050)	0.031	0.039 (0.069) [R]	0.57	0.046	74.8

Data are shown as the estimated standard linear regression statistic  $\beta_{STD}$ , SE and P value with adjustments for age and BMI. SHBG and calculated free testosterone (cFT) were processed using natural log-transformed variables. Bold numbers indicate significance (P value < 0.0042) after adjusting for multiple testing.

<sup>a</sup>The  $\beta$ -coefficient and its SE were summarized using an inverse variance-weighted meta-analysis, under fixed-effects model [F] or the DerSimonian and Laird method under random-effects model [R].

testosterone levels in 3225 men of European descent (Jin *et al.*, 2012). In this independent validation study, rs2075230 showed significant association with testosterone and SHBG levels in a combined analysis of two cohorts of Japanese men. Therefore, we could successfully validate the results of rs2075230 obtained in the previous GWAS. However, rs6259 and rs727428 were not associated with testosterone levels in our study, after adjustment for multiple testing in Japanese men. The previous GWAS was conducted using 3225 samples, whereas ours was conducted using 1687 samples, being approximately half the sample size. Sample sizes have a potent influence on the results of statistical analysis. Studies with larger sample sizes could yield highly significant associations of low-effect SNPs. On the other hand, studies with smaller sample sizes may not reach that level of significance even if the effects of SNPs are high. Since the phenotypic variances explained by rs6259 and rs727428 were low (0.5% and 0.3%, respectively) in our study, and  $\beta_{STD}$  results of rs727428 displayed the opposite direction compared with that of previous GWASs, it is suggested that the non-significant associations displayed by the two SNPs for testosterone levels cannot just be explained by the difference in sample sizes. Regarding the characteristics of subjects, the previous GWAS recruited men ( $62.76 \pm 6.00$  years old, mean  $\pm$  SD) from the Reduction by Dutasteride of Prostate Cancer Events/REDUCE study, which was designed to evaluate the effect of dutasteride on prostate cancer risk (Andriole *et al.*, 2004, 2010). On the other hand, our independent validation study recruited men from the general population ( $20.7 \pm 1.7$  years old, mean  $\pm$  SD) and from a population of proven fertility ( $31.2 \pm 4.8$  years old, mean  $\pm$  SD), who were generally healthy. Testosterone levels in men peak in the second decade of life and decrease later with age (Iwamoto *et al.*, 2009). In fact, in our study, the testosterone levels were observed to be lower in Cohort 2 than in Cohort 1 patients (Supplementary Table S1), and testosterone levels of previous GWAS subjects were observed to be lower than those observed for our subjects. Although there is no association between testosterone levels and prostate cancer (Endogenous Hormones and Prostate Cancer Collaborative Group *et al.*, 2008; Sawada *et al.*, 2010), the difference in the average age of subjects may be one of the reasons for the lack of association of rs6259 or rs727428 with testosterone values. Additionally, the differences in genetic background based on ethnicity may also be another reason for this lack of association, since the LD structure around these SNPs in HapMap JPT was slightly different from that in HapMap CEU (Supplementary Fig. S1).

On the other hand, we found that rs6259 and rs727428 were significantly associated with SHBG levels in two Japanese male cohorts, who were relatively young. It has been previously reported that the variant allele of rs6259 is significantly associated with higher levels of circulating SHBG in post-menopausal women (Cousin *et al.*, 2004; Dunning *et al.*, 2004; Haiman *et al.*, 2005; Thompson *et al.*, 2008). In addition, Ding *et al.* (2009), using the Women's Health Study cohort ( $60.3 \pm 6.1$  years old, mean  $\pm$  SD) and Physicians' Health Study II cohort of men ( $63.7 \pm 7.6$  years old, mean  $\pm$  SD), have reported that carriers of an rs6259 variant allele had significantly higher SHBG levels, suggesting that the variant allele of rs6259 may be associated with higher SHBG levels in spite of the difference in sex, age and population. The rs727428 SNP has also been previously reported to be associated with SHBG levels (Thompson *et al.*, 2008; Wickham *et al.*, 2011; Prescott *et al.*, 2012). However, there are no reports, except for a previous GWAS (Chen *et al.*, 2013), that rs2075230 is associated with SHBG levels. Our study is the first to replicate the association between rs2075230 and SHBG



**Table III** An association analysis of the three SNPs with serum testosterone levels, after adjusting for SHBG levels in the two Japanese male cohorts.

SNP	Cohort 1 (N = 901)		Cohort 2 (N = 786)		Combined			Heterogeneity	
	$\beta_{STD}$ (SE)	P	$\beta_{STD}$ (SE)	P	$\beta_{STD}$ (SE) [model] <sup>a</sup>	P <sub>meta</sub>	Var (%) <sup>b</sup>	P <sub>hetero</sub>	I <sup>2</sup> (%)
Testosterone									
rs2075230	0.076 (0.041)	0.63	-0.024 (0.041)	0.57	0.026 (0.050) [R]	0.60	0.03	0.086	66.2
rs6259	-0.028 (0.065)	0.66	0.11 (0.060)	0.063	0.044 (0.070) [R]	0.53	0.04	0.11	60.0
rs727428	0.019 (0.042)	0.64	<b>-0.086 (0.041)</b>	<b>0.037</b>	-0.034 (0.053) [R]	0.52	0.05	0.07	69.0

Data are shown as the estimated standardized linear regression statistic  $\beta_{STD}$ , SE and P value with adjustments for age, BMI and SHBG. Testosterone and SHBG were processed using natural log-transformed variables. Bold numbers indicate significance (P value < 0.05).

<sup>a</sup>The  $\beta$ -coefficient and its SE were summarized using an inverse variance-weighted meta-analysis, under fixed-effects model [F] or the DerSimonian and Laird method under random-effects model [R].

<sup>b</sup>Percentage of phenotypic variance explained by SNP.

levels. In this study, we also reported that after adjusting for SHBG levels, the associations between the three SNPs and testosterone levels were extremely reduced. In addition, there were no associations between the three SNPs and cFT. Therefore, we suggested that the three SNPs have a considerable effect on SHBG levels rather than on testosterone levels.

The values of pairwise  $r^2$  among the three SNPs (rs2075230, rs6259 and rs727428) are modest (maximum  $r^2 = 0.415$ , between rs2075230 and rs727428); however,  $ID^1$  values are 1, and these SNPs are located in the same LD block according to HapMap-JPT data (Supplementary Fig. S1). Therefore, the three SNPs are considered to be in LD. In fact, the significant associations between rs6259 or rs727428 with SHBG and testosterone were attenuated by adjustment for the effect of rs2075230. Hence, it is suggested that the haplotype (AAG) consisting of the effector alleles of rs2075230, rs6259 and rs727428 is possibly associated with higher SHBG levels. The rs6259 is a non-synonymous SNP in Exon 8 of *SHBG*, which leads to the substitution of asparagine with aspartic acid in codon 356 (D356N, also known as D327N) (Cui et al., 2005). The rs727428 is located in the downstream region of *SHBG*, whereas rs2075230 is located in the upstream region of *SHBG*. In general, non-synonymous SNPs in genes could exert effects on the functions of proteins rather than on gene expression, and SNPs located in the upstream regions of genes may influence gene expression. In this study, rs2075230 SNP located in the upstream region of *SHBG* displayed a significant association with SHBG levels. We identified the most significant SNP rs2075230 in an SPI transcription factor binding site using a GENETYX software program version 12 (Genetyx Co., Tokyo, Japan). Therefore, it is suggested that the variant allele of rs2075230 may influence the SHBG levels. To assess if more than one haplotype within the *SHBG* locus have independent effects on circulating SHBG levels, fine-scale genetic mapping of this locus and functional analyses is necessary.

In summary, we could replicate the association of rs2075230 with testosterone levels, but not the associations of rs6259 or rs727428 with testosterone levels. However, we found that the three SNPs (rs2075230, rs6259 and rs727428) in the *SHBG* locus were significantly associated with SHBG levels.

## Supplementary data

Supplementary data are available at *Human Reproduction Open* online.

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## Authors' roles

Y.S. and A.Ta.: study design and data analysis; Y.S. and M.K.: genotyping; S.N., M.Y., E.K., J.K., M.N., K.M., A.Ts., K.K., N.I., J.E. and T.I.: cohort collection and characterization; Y.S., A.Ta., M.K., S.N., M.Y., E.K., J.K., M.N., K.M., A.Ts., K.K., N.I., J.E., I.I., A.Y. and T.I.: preparation and approval of the final version of the manuscript.

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## Conflict of interest

None declared.

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**Supplementary Table S1** Serum sex hormone levels measured in the two Japanese male cohorts.

	Cohort 1 (N = 901)	Cohort 2 (N = 786)	P
Age (years)	20.7 ± 1.7	31.2 ± 4.8	<0.0001
BMI (kg/m <sup>2</sup> )	21.5 ± 2.5	23.3 ± 3.0	<0.0001
Testosterone (nmol/l)	23.2 ± 7.7	19.5 ± 6.6	<0.0001
SHBG (nmol/l)	26.6 ± 10.4	33.3 ± 14.6	<0.0001
cFT (pmol/l)	585.3 ± 192.7	423.3 ± 146.5	<0.0001

Data are presented as mean ± SD. *P* values were obtained with Student's unpaired *t*-test.  
SHBG, sex hormone-binding globulin; cFT, calculated free testosterone.

**Supplementary Table SII Correlation between age, BMI and sex hormone parameters in Cohort I of Japanese men.**

	<b>Age</b>	<b>BMI</b>	<b>Testosterone</b>	<b>SHBG</b>	<b>cFT</b>
Age	1.000				
BMI	0.074*	1.000			
Testosterone	-0.019	-0.131***	1.000		
SHBG	-0.001	-0.251***	0.525***	1.000	
cFT	-0.024	-0.017	0.871***	0.054	1.000

Pearson's correlation coefficient is shown. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

**Supplementary Table SIII Correlation between age, BMI and sex hormone parameters in Cohort 2 of Japanese men.**

	<b>Age</b>	<b>BMI</b>	<b>Testosterone</b>	<b>SHBG</b>	<b>cFT</b>
Age	1.000				
BMI	0.172***	1.000			
Testosterone	-0.197***	-0.291***	1.000		
SHBG	0.022	-0.337**	0.573***	1.000	
cFT	-0.246***	-0.090*	0.780***	-0.040	1.000

Pearson's correlation coefficient is shown. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

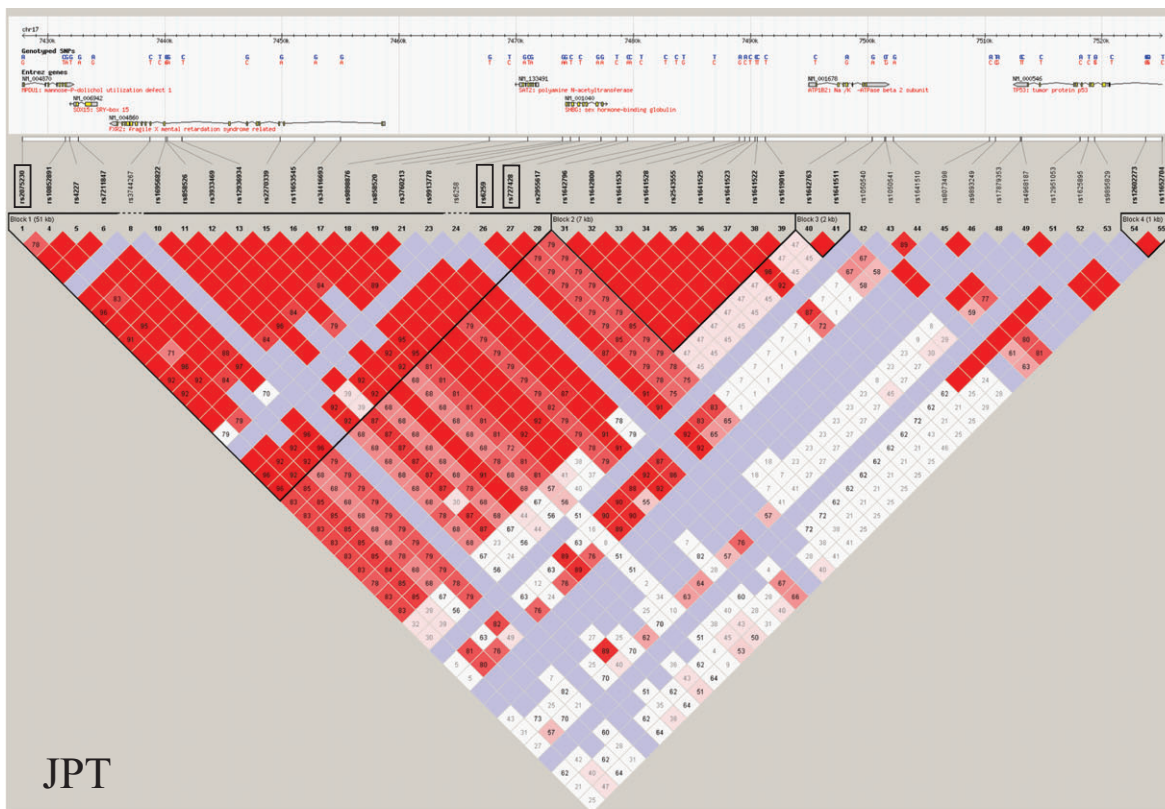
**Supplementary Table SIV** An association analysis of two SNPs with serum testosterone and SHBG levels, after adjusting for rs2075230 in two Japanese male cohorts.

SNP	Cohort 1 (N = 901)		Cohort 2 (N = 786)		Combined			Heterogeneity	
	$\beta_{STD}$ (SE)	P	$\beta_{STD}$ (SE)	P	$\beta_{STD}$ (SE) [model] <sup>a</sup>	P <sub>meta</sub>	Var (%) <sup>b</sup>	P <sub>hetero</sub>	I <sup>2</sup> (%)
Testosterone									
rs6259	-0.0058 (0.077)	0.94	<b>0.22 (0.075)</b>	<b>3.3 × 10<sup>-3</sup></b>	0.11 (0.11) [R]	0.34	0.2	0.035	77.6
rs727428	-0.00058 (0.060)	0.99	-0.047 (0.064)	0.46	-0.023 (0.044) [F]	0.61	0.02	0.60	0.0
SHBG									
rs6259	0.12 (0.074)	0.12	<b>0.15 (0.073)</b>	<b>0.035</b>	<b>0.14 (0.052) [F]</b>	<b>8.9 × 10<sup>-3</sup></b>	0.4	0.72	0.0
rs727428	0.086 (0.058)	0.14	<b>0.12 (0.061)</b>	<b>0.047</b>	<b>0.10 (0.042) [F]</b>	<b>0.014</b>	0.5	0.67	0.0

Data are shown as the estimated standardized linear regression statistic  $\beta_{STD}$ , SE and P value with adjustments for age, BMI and rs2075230. Testosterone and SHBG were processed using natural log-transformed variables. Bold numbers indicate significance (P value < 0.05).

<sup>a</sup>The  $\beta$ -coefficient and its SE were summarized using an inverse variance-weighted meta-analysis, under fixed-effects model [F] or the DerSimonian and Laird method under random-effects model [R].

<sup>b</sup>Percentage of phenotypic variance explained by SNP.



JPT



CEU

**Supplementary Figure S1** Linkage disequilibrium (LD) plot around the human sex hormone-binding globulin (*SHBG*) gene including the single nucleotide polymorphisms (SNPs) rs2075230, rs6259 and rs727428 according to HapMap Phase III JPT (upper) and CEU (lower) data.