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ORIGINAL ARTICLE

A replication study of a candidate locus for follicle-stimulating hormone levels and association analysis for semen quality traits in Japanese men

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In men, follicle-stimulating hormone (FSH) acts on the seminiferous tubules and enhances spermatogenesis. Recently, a candidate locus (rs2414095) for FSH levels was identified by a genome-wide association study (GWAS) in Chinese men. The rs2414095 single-nucleotide polymorphism (SNP) is found on the third intron of the *cytochrome P450, family 19, subfamily A, peptide 1* (*CYP19A1*) gene encoding an aromatase. In the present study, we performed a replication study in 1687 Japanese men (901 from cohort 1 and 786 from cohort 2) to assess whether this SNP is associated with circulating FSH levels. Furthermore, we investigated whether the rs2414095 SNP is correlated with semen quality traits in 2015 Japanese men (1224 from cohort 1 and 791 from cohort 2). The rs2414095 SNP was significantly associated with circulating FSH levels ($\beta_{STD} = 0.15$, $P = 9.7 \times 10^{-5}$), sperm concentration ($\beta_{STD} = 0.073$, $P = 0.032$) and total sperm number (TSN) ($\beta_{STD} = 0.074$, $P = 0.027$) in a combined analysis of the two Japanese male cohorts. We successfully replicated, in Japanese men, the results of the previous GWAS for the rs2414095 SNP in Chinese men, and found that the rs2414095 SNP was related with sperm production.
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INTRODUCTION

The gonadotropin-releasing hormone secreted by the hypothalamus stimulates the production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the pituitary gland. LH acts on Leydig cells in the testes and induces testosterone secretion.[1,2] The main action of testosterone is the development of sexual characteristics and genitalia. Sex hormone-binding globulin (SHBG) binds to 50%–60% of the testosterone in circulation, and decreases the concentration of biologically active testosterone in circulation.[3] Inhibin B is a peptide hormone, the synthesis and secretion of which is promoted by FSH. Secreted inhibin B in the circulation directly acts on adenohypophysis by a feedback mechanism, and specifically inhibits FSH secretion.[4] In men, FSH stimulates Sertoli cells and increases sperm production.[5,6] Therefore, low levels of FSH decrease spermatogenesis.

It has been suggested that sex hormone levels are heritable.[7,8] However, little is known about the genetic determinants of sex hormone levels. Recently, a genome-wide association study (GWAS) in 3495 healthy Chinese men revealed that the rs2414095 single-nucleotide polymorphism (SNP) in *cytochrome P450, family 19, subfamily A, polypeptide 1* (*CYP19A1*) at 15q21.2 was significantly associated with estradiol levels ($P = 6.5 \times 10^{-31}$) and FSH levels ($P = 1.6 \times 10^{-16}$).[9] Here, we conducted a replication study to assess whether the rs2414095 SNP is associated with circulating FSH levels or other hormone levels (including testosterone, SHBG, LH, inhibin B and calculated free testosterone) in Japanese men. Furthermore, we conducted an association analysis for semen quality traits (including sperm concentration, semen volume, sperm motility, total sperm number (TSN) and/or total motile sperm number) to establish whether this SNP influences spermatogenesis.

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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.

Two Japanese cohort samples

Two Japanese cohorts, 1224 young men from the general Japanese population (cohort 1: aged 20.8 ± 1.7 years; mean ± s.d.) and 791 Japanese men of proven fertility (cohort 2: aged 31.2 ± 4.8 years; mean ± s.d.), were included in this study. Among these subjects, the hormone levels of 901 men from cohort 1 and of 786 men from cohort 2 were measured and used for the replication study for FSH levels, and samples from all subjects were used for the association analysis for semen quality. Some of the subjects in this study have been described in previous reports.[10–19] Briefly, cohort 1 subjects consisted of male university students from four study centers based in the urology departments of university hospitals in four Japanese cities (Kawasaki, Kanazawa, Nagasaki and Sapporo). Cohort 2 subjects consisted of the partners of pregnant women who attended obstetric clinics in four Japanese cities (Sapporo, Kanazawa, Osaka and Fukuoka).

Measurement of clinical traits

Age, body weight and height were self-reported. Body mass index was calculated from body weight and height. Blood samples were obtained and analyzed as previously described.[11,12] Briefly, blood samples were drawn from the cubital vein of each subject usually in the mornings to reduce the effect of diurnal variations in hormone levels. Testosterone, SHBG, FSH and LH levels were determined using a time-resolved immunofluorometric assay (Delfia, Wallac, Turku, Finland), and inhibin B was measured by a specific two-sided enzyme immunometric assay (Serotec, Kidlington, UK) at the Department of Growth and Reproduction, Rigshospitalet, in Copenhagen, Denmark. Calculated free testosterone was calculated from a value of 1×10^9 mol l^{-1} for the association constant of SHBG to testosterone, a value of 3.6×10^4 mol l^{-1} for the association constant of albumin to testosterone and a fixed plasma albumin concentration of 43 g l^{-1} using Vermeulen's formula.[20] Semen samples were obtained and analyzed by using standardized protocols as previously described.[11,12] Briefly, semen samples were obtained by masturbation after sexual abstinence for at least 48 h and were ejaculated into clean, wide-necked, sterile and nontoxic collection containers. The samples were protected from temperature extremes and were liquefied at 37 °C before testing. The sperm concentration of each sample was assessed using a Bürker–Türk hemocytometer (Erma, Tokyo, Japan) after fixation with sodium bicarbonate–formaldehyde solution. Only sperm with tails were counted. Semen volume was measured with a graduated 5 ml syringe (Terumo, Tokyo, Japan). Sperm motility was assessed from 10 μl of well-mixed semen placed on a clean glass slide, covered and then examined microscopically at 400× magnification at 37 °C on a microscope stage. The motility assessment was repeated twice, and the average value from two samples was calculated. The sperm were assessed using the World Health Organization motility classes A, B, C and D.[21] In this study, sperm categorized as class A or B were considered motile.

Genotyping

Genomic DNA was extracted from the peripheral blood samples of subjects using a QIAamp DNA blood kit (Qiagen, Tokyo, Japan). The rs2414095 SNP was genotyped using TaqMan probes (C_15798408_20; Applied Biosystems, Tokyo, Japan) with the ABI 7900HT real-time PCR system (Applied Biosystems).

Statistical analysis

Hardy–Weinberg equilibrium was assessed in the two cohorts using Pearson's χ^2 test for genotypes.

To minimize deviation from a normal distribution, the analyses for testosterone, SHBG, FSH, LH, inhibin B and calculated free testosterone were processed using natural log-transformed variables, and sperm concentration, semen volume, TSN and total motile sperm number were processed using square-root transformed values. The associations between the rs2414095 SNP and sex hormone values were assessed using a standardized multiple linear regression test under an additive genetic model with adjustments for age and body mass index, and the associations between the rs2414095 SNP and semen quality traits were assessed using a standardized multiple linear regression test under an additive genetic model with adjustments for age, body mass index and ejaculation abstinence. Ejaculation abstinence and time from masturbation to test were additionally adjusted for in determining sperm motility and total motile sperm number.

The results from the two cohorts were combined in a meta-analysis using the meta package for R, version 3.1.2 (http://www.R-project.org/). The extent of heterogeneity among studies was quantified by the I^2 statistic[22] 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 >0.1, a fixed-effects model using the inverse variance method was used. Otherwise, the random-effects model using the DerSimonian and Laird method was used.

All statistical analyses were performed using R, version 3.1.2, and statistical significance was considered at *P*-values of <0.05.

RESULTS

The characteristics of circulating sex hormones obtained from the two Japanese male cohorts are presented in Supplementary Table S1 as previously reported.[12,17] To investigate the associations between the rs2414095 SNP and circulating FSH levels, we genotyped the SNP in a total of 1687 men (901 from cohort 1 and 786 from cohort 2). The allele frequencies of the SNP analyzed in each cohort are shown in Table 1. The missing genotyping rate of the rs2414095 SNP was 0.1%. The genotypes of the SNP were in Hardy–Weinberg equilibrium in the two cohorts. Multiple linear regression analyses under the additive genetic model revealed that the rs2414095 SNP was significantly correlated with circulating FSH levels in both cohorts ($\beta_{STD} = 0.17$, $P = 1.2 \times 10^{-3}$ in cohort 1; $\beta_{STD} = 0.12$, $P = 0.026$ in cohort 2) (Table 1). In the meta-analysis of the two Japanese cohorts, we found that the rs2414095 SNP was significantly associated with circulating FSH levels ($\beta_{STD} = 0.15$, $P = 9.7 \times 10^{-5}$). The rs2414095 SNP contributed to 0.9% of variance in FSH levels.

Table 1 Association analysis between the rs2414095 SNP and circulating FSH levels in two Japanese male cohorts

						Cohort 1 (N = 901)			*Cohort 2 (N = 786)*			*Combined*			*Heterogeneity*	
SNP	*Chr*	*Position*	*Gene*	*Location*	*Effect/other*	*EAF*	β_{STD} *(s.e.)*	P-*value*	*EAF*	β_{STD} *(s.e.)*	P-*value*	β_{STD} *(s.e.)*	P_{meta}	*Var (%)*[a]	P_{hetero}	I^2 *(%)*
rs2414095	15	51524292	*CYP19A1*	Intron	A/G	0.280	0.17 (0.052)	**1.2×10^{-3}**	0.299	0.12 (0.055)	**0.026**	0.15 (0.038)	**9.7×10^{-5}**	0.9	0.54	0.0

Abbreviations: β_{STD}, standardized regression coefficient; Chr, chromosome; EAF, effect allele frequency; FSH, follicle-stimulating hormone; P_{hetero}, *P*-value for heterogeneity; SNP, single-nucleotide polymorphism.
Data are shown as the estimated standardized liner regression statistic β_{STD}, s.e. and *P*-value with adjustments for age and body mass index (BMI). FSH was processed using natural log-transformed variables.
The β-coefficient and its s.e. were summarized using an inverse variance-weighted meta-analysis under fixed-effects model.
Bold numbers indicate $P<0.05$.
[a]Percentage of phenotypic variance (Var) explained by SNP.

We next investigated the associations between the rs2414095 SNP and other circulating hormone levels (including testosterone, SHBG, LH, inhibin B and calculated free testosterone) in the two Japanese male cohorts. We found that the rs2414095 SNP was significantly associated with circulating inhibin B levels in cohort 1 ($\beta_{STD} = 0.11$, $P = 0.030$), but not in cohort 2 (Table 2). In the meta-analysis of the two Japanese cohorts, the rs2414095 SNP was significantly associated with circulating inhibin B levels ($\beta_{STD} = 0.076$, $P = 0.042$).

We hypothesized that the SNP associated with FSH levels might influence sperm production, because FSH contributes to spermatogenesis.[5,6] To verify this, we performed an association study between the rs2414095 SNP and semen quality traits. The characteristics of semen parameters obtained from the two Japanese cohorts are presented in Supplementary Table S2 as previously reported.[11,12,15,18] The SNP was genotyped in a total of 2015 men (1224 from cohort 1 and 791 from cohort 2). The effect allele frequencies of this SNP was 0.29 in cohort 1 and 0.30 in cohort 2, and the genotypes of the SNP were in Hardy–Weinberg equilibrium in the two cohorts. Multiple linear regression analyses under the additive genetic model revealed that the rs2414095 SNP was significantly associated with sperm concentration ($\beta_{STD} = 0.11$, $P = 0.016$), TSN ($\beta_{STD} = 0.11$, $P = 0.012$) and total motile sperm number ($\beta_{STD} = 0.098$, $P = 0.024$) in cohort 1 (Table 3). However, this SNP did not display associations with semen parameters in cohort 2. In the meta-analysis of the two Japanese cohorts, we found that the rs2414095 SNP was significantly associated with sperm concentration ($\beta_{STD} = 0.073$, $P = 0.032$) and TSN ($\beta_{STD} = 0.074$, $P = 0.027$).

DISCUSSION

A recent GWAS reported that the rs2414095 SNP was significantly associated with FSH levels in Chinese men.[9] In this replication study, the rs2414095 SNP was found to significantly associate with FSH levels in a combined analysis of two cohorts in Japanese men. Therefore, we successfully validated the results of the association of the rs2414095 SNP from the previous GWAS.

The rs2414095 SNP is located on the third intron of the *CYP19A1* gene that encodes aromatase. This enzyme is induced by FSH and LH secretion, and converts testosterone to estradiol (E2).[23–25] Aromatase deficiency in men and women suppressed the concentration of estradiol, and elevated the concentrations of FSH and LH in circulation.[26] The treatment of an aromatase inhibitor elevated serum FSH levels in nonobstructive azoospermia,[27] in male breast cancer patients[28] and in boys with delayed puberty.[29] Insertion[30] or missense[31–35] mutations in the coding region of the *CYP19A1* gene can cause aromatase deficiency. Mutations in the splicing site were also found in aromatase-deficient patients.[35–37] Aromatase deficiency with mutations in the *CYP19A1* gene resulted in markedly elevated FSH concentrations.[32,34,38–41] In this study, the A allele of the rs2414095 SNP was positively correlated with circulating FSH levels. The previous GWAS study reported that the G allele of the rs2414095 SNP was

Table 2 Association analysis between the rs2414095 SNP and other circulating sex hormone levels in two Japanese male cohorts

		Cohort 1 (N = *901*)		*Cohort 2* (N = *786*)		*Combined*		*Heterogeneity*	
SNP (effect allele)	*Trait*	β_{STD} *(s.e.)*	P-*value*	β_{STD} *(s.e.)*	P-*value*	β_{STD} *(s.e.) (model)*[a]	P_{meta}	P_{hetero}	I^2 *(%)*
rs2414095 (A)	Testosterone	–0.019 (0.052)	0.71	–0.035 (0.052)	0.50	–0.027 (0.037) (F)	0.46	0.83	0.0
	SHBG	–0.090 (0.050)	0.072	0.011 (0.051)	0.82	–0.040 (0.051) (R)	0.43	0.15	50.6
	LH	–0.017 (0.052)	0.74	0.0035 (0.056)	0.95	–0.0076 (0.038) (F)	0.84	0.79	0.0
	Inhibin B	**0.11 (0.052)**	**0.030**	0.036 (0.054)	0.51	**0.076 (0.038) (F)**	**0.042**	0.31	3.3
	cFT	–0.032 (0.052)	0.54	0.050 (0.054)	0.35	0.0081 (0.037) (F)	0.83	0.28	15.9

Abbreviations: β_{STD}, standardized regression coefficient; cFT, calculated free testosterone; LH, luteinizing hormone; P_{hetero}, *P*-value for heterogeneity; SHBG, sex hormone-binding globulin; SNP, single-nucleotide polymorphism.
Data are shown as the estimated standardized liner regression statistic β_{STD}, s.e. and *P*-value with adjustments for age and body mass index (BMI). Testosterone, SHBG, LH, inhibin B and cFT were processed using natural log-transformed variables.
Bold numbers indicate $P < 0.05$.
[a]The β-coefficient and its s.e. 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).

Table 3 Association analysis between the rs2414095 SNP and semen parameters in two Japanese male cohorts

		Cohort 1 (N = *1224*)		*Cohort 2* (N = *786*)		*Combined*		*Heterogeneity*	
SNP (effect allele[a]*)*	*Trait*	β_{STD} *(s.e.)*	P-*value*	β_{STD} *(s.e.)*	P-*value*	β_{STD} *(s.e.) (model)*[b]	P_{meta}	P_{hetero}	I^2 *(%)*
rs2414095 (A)	Conc.	**0.11 (0.043)**	**0.016**	0.022 (0.055)	0.69	**0.073 (0.034) (F)**	**0.032**	0.24	28.1
	Vol.	0.026 (0.043)	0.55	0.017 (0.055)	0.76	0.022 (0.034) (F)	0.51	0.90	0.0
	TSN	**0.11 (0.042)**	**0.012**	0.018 (0.055)	0.74	**0.074 (0.033) (F)**	**0.027**	0.20	38.0
	TMSN	**0.098 (0.043)**	**0.024**	–0.012 (0.056)	0.83	0.048 (0.055) (R)	0.38	0.12	59.1
	Motility (%)	0.010 (0.044)	0.82	–0.039 (0.056)	0.48	–0.0089 (0.034) (F)	0.80	0.49	0.0

Abbreviations: β_{STD}, standardized regression coefficient; Conc., sperm concentration; P_{hetero}, *P* value for heterogeneity; SNP, single-nucleotide polymorphism; TMSN, total motile sperm number; TSN, total sperm number; Vol., semen volume.
Data are shown as the estimated standardized liner regression statistic β_{STD}, s.e and *P*-value with adjustments for age, body mass index (BMI) and ejaculation abstinence. Motility and total motile sperm number were additionally adjusted for time from masturbation to test. The sperm concentration, semen volume, total sperm number and total motile sperm number were processed using square-root-transformed values. Bold numbers indicate $P < 0.05$.
[a]Effect allele indicates the allele that showed positive association with follicle-stimulating hormone (FSH) levels.
[b]The β-coefficient and its s.e. 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).

positively correlated with estradiol levels,[9] indicating that the A allele of this SNP was negatively correlated with estradiol levels. Although the relationship between the rs24154095 SNP and aromatase expression and activity is unknown, it is suggested that the positive correlation between the A allele of this SNP and circulating FSH levels might depend on a positive feedback mechanism through decrease in the circulating estradiol levels. The rs2414095 SNP was also related with circulating inhibin B levels. Because the synthesis and secretion of inhibin B is promoted by FSH,[4–6] it is suggested that the relationship between the rs2414095 SNP and inhibin B levels is an indirect connection through FSH levels. However, further studies are needed to elucidate the molecular mechanisms through which the rs24154095 SNP affects the respective circulating levels of FSH, E2 and inhibin B.

In this study, we found that the A allele of the rs2414095 SNP was positively correlated with sperm concentration and TSN. FSH activates Sertoli cells and induces spermatogenesis in the presence of testosterone.[42] Therefore, the relationship between the rs2414095 SNP and sperm production may also be indirectly affected by FSH levels. Further replication studies using larger samples with different circulating FSH levels and low sperm concentrations such as azoospermic and/or oligozoospermic patients will be needed to provide compelling evidence for this association.

In summary, we replicated the association between the rs2414095 SNP and circulating FSH levels in Japanese men. In addition, we found that the rs2414095 SNP was associated with sperm production. It is suggested that the rs2414095 SNP has an indirect influence on spermatogenesis through regulating FSH levels.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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