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Relevance of *KISS1* gene polymorphisms in susceptibility to polycystic ovary syndrome and its associated endocrine and metabolic disturbances

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ABSTRACT

Background: Variations in *KISS1* may be an emerging factor in polycystic ovary syndrome (PCOS) We hypothesised links between *KISS1* polymorphisms in PCOS and its associated endocrine and metabolic disturbances.

Methods: The study included 104 PCOS women and 109 controls. Endocrine (kisspeptin, LH, FSH, LH-FSH ratio, oestradiol) and metabolic (cholesterol, triglycerides, HDL, LDL, insulin and glucose) parameters were measured. PCR and nucleotide sequencing were carried out to screen single nucleotide polymorphisms (SNPs) of *KISS1*. Endocrine and metabolic parameters of PCOS women were compared in the genotypes.

Results: Three novel SNPs (rs1213704663C>G, rs1481572212T>G and rs775770652G>A) were detected in *KISS1*. Of these SNPs, the genotype and allele frequencies of rs1213704663C>G were all significantly associated $p<0.001$ with PCOS. The LH and oestradiol hypersecretion, and increased LH-FSH ratio of PCOS women were significantly influenced by the GG genotype of rs1213704663, but, this SNP did not influence kisspeptin levels. The other two SNPs rs1481572212T>G and rs775770652G>A exhibited no clinical significance.

Conclusion: rs1213704663C>G variation in *KISS1* is linked to PCOS and its associated endocrine and metabolic disturbances (LH and oestradiol hypersecretion, and increased LH/FSH).

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Introduction

Polycystic ovary syndrome (PCOS) is a common gynaecological issue that occurs in 3–10% of women of childbearing age with menstrual irregularity, excessive hair growth, weight gain and infertility, resulting in depression, anxiety, and sexual dysfunction [1,2]. The pathological cause of this disease is controversial. The aetiology of PCOS is multifactorial with genetic, endocrine, metabolic and environmental factors [3]. Mutations in the hypothalamic-pituitary-gonadal (HPG) axis associated with *KISS1* are postulated as a major genetic factor linked to PCOS [4].

The female reproductive system mainly depends on the proper development and maturation of the HPG axis [5]. *KISS1* contributes significantly in regulating the HPG axis through involving a mechanism in which the *KISS1* encoded kisspeptin-GPR54 releases GnRH into the portal circulation, and stimulates the secretion of LH and FSH from the anterior pituitary. These act on gonads to direct the secretion of oestrogen, testosterone and progesterone [6,7].

In cases of genetic variations in the exonic region of *KISS1*, kisspeptin-GPR54 pathway is disturbed, leading to dysfunction in GnRH secretion. This causes excess formation of androgens by the abnormal stimulatory effects of LH, and encourages anovulatory hyperandrogenism associated PCOS [8]. Therefore, the involved mechanism of *KISS1* SNPs in the aetiology of PCOS may be associated with the disturbances in the endocrine system. Previous investigations have reported the influence of *KISS1* SNPs in the pathogenesis of PCOS and related endocrine disturbances, the results of these investigations being unclear (9, 10). Therefore, further studies are required to confirm the relevance of *KISS1* in PCOS and its associated endocrine and metabolic disturbances (11-13). The most frequent characterization of metabolic disturbances encountered in PCOS is mediated through insulin hypersecretion and insulin resistance, which appear to have direct effect in deregulating the mechanism of GnRH/LH secretion and to induce excess androgen production and so the development of PCOS (14). Women with PCOS also suffer from dyslipidemia, and related studies concerning the links in metabolic disturbances with PCOS

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are known (15, 17), but the relevance of *KISS1* SNPs in relation to metabolic disturbances of PCOS, is unknown. We therefore hypothesised links between *KISS1* SNPs in PCOS, and effects on hormonal and metabolic parameters.

Methods

We tested our hypothesis on 104 women with PCOS group and 109 healthy women, aged between 19 and 36 years of age. Selection of the PCOS group was based on the Rotterdam 2003 criteria, having at least two of oligomenorrhea or amenorrhea (menstrual period >35 days or menstrual period absent for six month), hyperandrogenism, or polycystic ovaries on ultrasound (18). Patients having other causes of hyperandrogenism or menstrual irregularity such as Cushing's syndrome, prolactinoma and congenital adrenal hyperplasia, were excluded. Pregnant women and the women with first postpartum year were also exculsed. Healthy women with no history of PCOS and having regular menstrual cycle of 21-35 days, menstrual period up to seven days, normal androgen levels and no symptoms of hirsutism, was recruited as controls. Study participants who had received any kind of lipid lowering therapy, or were on medicines known to affect hormonal concentration and glucose metabolism in previous three months of the study, were excluded. The study was approved by the ethical committee of the Institutional Review Board, Umm Al-Qura University, Makkah, Saudi Arabia (IRB No. 235), and informed consent was taken from each participant. Blood samples were drawn after an 8-10 hour overnight fasting state and processed for obtaining serum, plasma and buffy coat through centrifugation, and consequently stored at -80°C until analysis.

The plasma level of kisspeptin and serum levels of LH, FSH and estradiol (E_2) were measured by ELISA (Phoenix Pharmaceuticals, Belmond, CA, USA). Total cholesterol, HDL, LDL and triglycerides were measured by an enzymatic colorimetric method (Boehringer Mannheim, Germany). Plasma glucose level was determined by the glucose oxidase method (Beckman Analyzer, Fullerton, CA, USA). Serum insulin was determined by electro-chemiluminescence immunoassay (Elecsys 1010/2010 and Modular Analytics E170, Roche Diagnostic, Mannheim, Germany).

Genomic DNA was extracted from a buffy coat, utilizing Gentra Puregene Blood Kit (Qiagen, Hilden, Germany). The purity and concentration of the DNA was determined using a Nanodrop spectrophotometer. KISS1 was amplified by polymerase chain reaction (PCR) using the following amplification primers *KISS1*-F: 5′-ACCTGCCGAACTACAACTGG-3, *KISS1*-R: 5′-TGAAGGAACAGGCGGTTAGT-3′. The PCR amplification conditions consisted of initial denaturation step at 95°C for 15 minutes, followed by 34 cycles of denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute, with final extension at 72°C for 10 minutes. The PCR product was checked for purity and size, and subjected to nucleotide sequencing utilizing an ABI Big Dye Terminator protocol on an ABI 3100 Avant Genetic Analyzer.

Statistical analyses were conducted using SPSS version 20.0. The descriptive characteristics of the different continuous variables were expressed as mean [SD]. Student's t-test compared mean values of the continuous variables. Genotype and allele frequencies of *KISS1* SNPs were calculated and consequently tested for Hardy-Weinberg equilibrium using the link http://ihg.gsf.de/cgi-bin/hw/hwa1.pl. Chi-Square test analysed the difference between categorical variables. Odds ratio (OR) and its 95% confidence interval (CI) were obtained. The levels of endocrine and metabolic parameters in the women with PCOS were determined in different genotypes of *KISS1* SNPs and the significance of difference between the three genotypes was calculated by ANOVA test and post hoc with Pairwise t-test. Results were considered significant at $p<0.05$.

Results

The demographic characteristics and the serum and plasma endocrine and metabolic parameters, in the PCOS and control groups are presented in Table 1. The two study groups were matched in age and BMI, while the mean value of waist–hip ratio (WHR) showed significant difference between PCOS and control groups. No significant difference was found in the level of kisspeptin and FSH between the study groups.

Table 1. Demographic, endocrine and metabolic characteristics of the study groups.

Characteristics	PCOS group (n = 104)	Control group (n = 109)	*p*-value
Demographic			
Age (years)	25.1 ± 3.9	25.0 ± 5.6	0.886
BMI (kg/m^2)	29.4 ± 5.9	29.9 ± 7.3	0.633
WHR	0.84 ± 0.07	0.77 ± 0.06	0.0001
Endocrine			
Kisspeptin (ng/ml)	0.41 ± 0.16	0.39 ± 0.07	0.415
FSH (IU/L)	5.09 ± 1.54	4.83 ± 1.55	0.223
LH (IU/L)	14.4 ± 7.4	4.5 ± 1.3	0.0001
LH-FSH ratio	2.94 ± 1.07	0.98 ± 0.37	0.0001
Oestradiol (pmol/L)	203 ± 93	141 ± 71	0.0001
Metabolic			
Cholesterol (mmol/L)	4.4 ± 0.8	3.7 ± 0.6	0.0001
Triglycerides (mmol/L)	1.08 ± 0.39	0.93 ± 0.42	0.007
HDL (mmol/L)	1.14 ± 0.34	1.21 ± 0.33	0.105
LDL (mmol/ml)	2.4 ± 0.7	1.8 ± 0.6	0.0001
Fasting glucose (mmol/L)	5.1 ± 0.4	4.8 ± 0.5	0.0001
Fasting insulin (pmol/L)	97 ± 58	79 ± 39	0.008

WHR- waist–hip ratio, FSH – follicle stimulating hormone, LH – luteinizing hormone, HDL – high-density lipoprotein cholesterol, LDL – low-density lipoprotein cholesterol.

LH, oestradiol and LH-FSH ratio were increased in PCOS. In the control group, kisspeptin showed a weak but significant negative correlation with FSH ($r = -0.208$; $p = 0.03$), but not in the patients ($r = -0.186$; $p = 0.059$)

When both study groups were compared in regards to metabolic parameters, significantly higher levels of total cholesterol, triglycerides, LDL, fasting insulin and fasting glucose were observed in PCOS group compared to the controls. The level of HDL was not significantly different between the groups.

Direct nucleotide sequencing of *KISS1* gene displayed several SNPs and of these three SNPs were selected for this study (rs1213704663 C/G, rs1481572212 T/G and rs775770652 G/A). The genotype and allele frequencies were calculated for each SNP and compared between PCOS and control groups. Hardy–Weinberg equilibrium was maintained for these three SNPs, in the control group ($p > 0.05$).

A novel SNP rs1213704663 C/G was identified in the 3′-untranslated region. Both the homozygous (GG) and heterozygous (CG) genotypes of rs1213704663 occurred at a significantly higher frequency in the PCOS group as compared to controls (Table 2). The G allele frequenciy was significantly higher in PCOS (Table 3).

The other two SNPs rs1481572212 T/G and rs775770652 G/A were novel and were located in intron 1 and exon 2, respectively. Tables 2 and 3 present the genotype and allele frequencies of rs1481572212 and rs775770652 and show that these are not significantly different between the two groups.

To study the effects of these three SNPs in leading to endocrine and metabolic disturbances, women with PCOS were grouped on the bases of the genotypes of each SNP, and the values of demographic, endocrine and metabolic parameters were separately analysed and compared in different genotypes of *KISS1* polymorphisms. The results are presented in Table 4. When the significance of rs1213704663 polymorphism was seen in regards to demographic characteristics of PCOS women, the values of BMI and waist–hip ratio showed no significant difference among the genotypes of this SNP.

As presented in Table 1, the PCOS group showed the feature of endocrine disturbances in terms of LH and oestradiol hypersecretion, and increased value of LH-FSH ratio compared to controls., When the values of LH, LH-FSH ratio and oestradiol were compared in three genotypes of rs1213704663, a significant difference was obtained (LH: $p = 0.001$, LHFSH ratio: $p = 0.001$, oestradiol: $p = 0.006$) (Table 4). In post hoc pairwise analysis, the females with GG genotype have significantly ($p < 0.05$) higher level of LH, LH/FSH and oestradiol, as compared to CC and CG genotypes. Kisspeptin level showed no significant difference in the three genotypes of rs1213704663. Similarly, the levels of FSH and studied metabolic parameters showed no significant difference in the different genotypes.

Among the metabolic parameters, the post hoc pairwise analysis revealed that the levels of the other metabolic parameters (cholesterol, triglycerides, LDL, HDL and fasting glucose) showed no significant difference between the three genotypes of rs1213704663.

Table 2. Genotype frequencies of *KISS1* gene polymorphisms in PCOS and control groups.

		PCOS (104)	Control (109)
SNPs Genotypes	N (Frequency)	N (Frequency)	OR (95%CI)
rs1213704663			
CC	45 (0.43)	82 (0.75)	Reference
CG	45 (0.43)	23 (0.21)	3.56 (1.92–6.62)[a]
GG	14 (0.13)	4 (0.04)	6.37 (1.98–20.5)[b]
CG+GG	59 (0.57)	27 (0.25)	3.98 (2.22–7.13)[c]
rs1481572212			
T/T	87 (0.84)	86 (0.79)	Reference
TG	17 (0.16)	23 (0.21)	0.73 (0.36–1.46)[d]
rs775770652			
G/G	82 (0.79)	87 (0.79)	Reference
GA	18 (0.17)	18 (0.17)	1.06 (0.52–2.18)[e]
AA	4 (0.04)	4 (0.04)	1.06 (0.25–4.38)[f]
GA+AA	22 (0.21)	22 (0.20)	0.95 (0.23–3.91)[g]

OR – odds ratio, CI – confidence interval.; [a]p = 0.0004; [b]p -= 0.0006; [c]p = 0.0002; [d]p = 0.374; [e]p = 0.871; [f]p = 0.934; [g]p = 0.946.

Table 3. Allele frequencies of *KISS1* gene polymorphisms in PCOS and control groups.

	Frequency		
SNPs Alleles	PCOS Group (104)	Control group (109)	OR (95%CI)
rs1213704663 C/G			
C	0.65	0.86	Reference
G	0.35	0.14	3.26 (2.03–5.25)[a]
T	0.92	0.89	Reference
G	0.08	0.11	1.32 (0.68–2.55)[b]
rs775770652 G/A			
G	0.87	0.88	Reference
A	0.12	0.12	0.94 (0.53–1.69)[c]

**Highly significant at $p < 0.0001$, OR – odds ratio, CI – confidence interval.; [a]p < 0.001; [b]p -= 0.4; [c]p = 0.856.

Discussion

Poly-cystic ovarian syndrome is emerging as a major health issue in the female population of childbearing age, through exerting effects not only on the reproductive system but also on the entire female body [19]. It also influences endocrine and metabolic parameter levels and hence leads to several complications [20–22]. The genetic factors responsible for the etiopathogenesis of PCOS are not yet fully understood [23]. Recently, SNPs in *KISS1* have been identified as markers of central precocious puberty [24]. The present study intended to investigate *KISS1* SNPs, which could be used as markers of PCOS. Three

Table 4. Relevance of *KISS1* gene polymorphism (rs1213704663) in demographic, endocrine and metabolic characteristics of PCOS women (n = 104).

Parameters	Genotypes of rs1213704663			*p*-value (ANOVA)
	CC n = 45	CG n = 45	GG n = 14	
Demographic				
BMI (kg/m^2)	29.7 ± 6.1	29.5 ± 6.0	28.2 ± 5.8	0.428
WHR	0.84 ± 0.08	0.83 ± 0.07	0.86 ± 0.07	0.367
Endocrine				
Kisspeptin (ng/ml)	0.40 ± 0.18	0.41 ± 0.14	0.42 ± 0.13	0.466
LH (IU/L)	14.00 ± 7.06	14.37 ± 7.03	15.97 ± 10.2	0.001
FSH (IU/L)	5.00 ± 1.68	5.02 ± 1.49	5.57 ± 1.15	0.459
LH-FSH ratio	2.81 ± 0.94	2.94 ± 1.07	2.96 ± 1.12	0.001
Oestradiol (pmol/L)	179 ± 69	195 ± 70	230 ± 112	0.006
Metabolic				
Cholesterol (mmol/L)	4.49 ± 0.78	4.30 ± 0.91	4.45 ± 0.78	0.367
Triglycerides (mmol/L)	1.09 ± 0.35	1.05 ± 0.35	1.13 ± 0.59	0.601
HDL (mmol/L)	1.18 ± 0.38	1.11 ± 0.32	1.07 ± 0.27	0.572
LDL (mmol/L)	2.35 ± 0.81	2.48 ± 0.61	2.42 ± 0.69	0.536
Fasting insulin (pmol/L)	95 ± 66	99. ± 54	98 ± 42	0.481
Fasting glucose (mmol/L)	5.07 ± 0.37	5.04 ± 0.49	5.04 ± 0.46	0.933

BMI – body mass index, WHR – waist–hip ratio, FSH – follicle stimulating hormone, LH – luteinizing hormone, HDL – high-density lipoprotein cholesterol, LDL – low-density lipoprotein cholesterol.

novel SNPs (rs1213704663 C/G, rs1481572212 T/G and rs775770652 G/A) were identified and investigated.

For rs1213704663, the mutant G allele increased in PCOS over three times compared to the wild type C allele and the genotypes GG, increased over six times. The mode of inheritance of the G allele displayed a dominant model. This SNP is located in the 3′-untranslated region (3′UTR) of *KISS1*. The 3′ UTRs of mRNAs regulate mRNA-based processes, including mRNA stability, mRNA localization, and mRNA translation. It also regulates diverse protein features, such as protein complex formation, posttranslational modifications, and may alter protein conformations [25]. Hence, 3′ UTR-mediated information transfer can regulate protein features that are not encoded in the amino acid sequence. Our results show that the SNP rs1213704663 does not influence the level of kisspeptin as there was no difference in its level in the three different genotypes of rs1213704663, but may be effecting kisspeptin conformation or its binding to the receptor. Most of the previous studies have suggested a major role of kisspeptin in PCOS [26,27]. Tang and co-workers recently published a systematic literature search in which they compared the levels of kisspeptin in PCOS as reported in 12 different studies. They showed that 8 of the 12 studies investigated reported higher levels of kisspeptin in PCOS and hypothesized that 'an overactive KISS1 system leads to enhanced HPG-axis activity, thereby causing irregular menstrual cycles and excessive androgen release in PCOS women' [28]. This review also showed that there are contradictory findings and several studies do not report elevated kisspeptin level in PCOS. Our finding is in line with these reports and this may be explained on the basis of the type of mutations or polymorphisms in the KISS gene. One of the earlier findings from our group confirms this suggestion [9]. The altered behaviour of kisspeptin was suggested as a major cause to deregulate the mechanism of GnRH/LH secretion and to induce the condition of hyperandrogenism associated PCOS [28,29]. In contrast to this study, Umayal and co-workers reported two SNPs (rs4889 and rs5780218) in *KISS1* gene in relation to PCOS and found no association of kisspeptin with these two SNPs [30].

In some studies, the most evident endocrine disturbances in PCOS were reported as LH hypersecretion, increased value of LH-FSH ratio and low or normal level of FSH [31]. Some women with PCOS also experienced high level of oestradiol with dysfunctional uterine bleeding [32]. In our present study, the PCOS group revealed the features of endocrine disturbances through increased level of LH, oestradiol and LH-FSH ratio with normal level of FSH, as compared to controls. When the influence of genotypes of rs1213704663 was investigated on the levels of the endocrine and metabolic parameters, no effect was seen on the latter. However, the GG genotype showed significantly higher level of LH, oestradiol and LHFSH ratio as compared to CG and CC genotypes, while, FSH level showed no significant difference among the genotypes of rs1213704663. These results show that the variant GG rs1213704663, influences endocrine levels in PCOS.

The other two SNPs rs775770652 G/A, and rs1481572212 T/G were not significantly associated with the risk of PCOS. The former showed a significant influence on kisspeptin level, however, both did not influence the endocrine or metabolic parameters.

Thus, we postulate that the polymorphism rs1213704663 C/G in the 3′UTR of *KISS1* may plays a role in oestradiol hypersecretion, which in turn may be responsible for deregulating the mechanism of GnRH secretion and stimulation of hypersecretion of LH with increased LH-FSH ratio [33,34]. Subsequently,

the condition of PCOS may be induced with excess production of androgens by the direct effect of LH hypersecretion. The significant role of elevated level of oestradiol for inappropriate GnRH secretion associated PCOS pathogenesis is suggested by Granger et al. [35]. Our finding is supported by previous reports in which the oestradiol level positively correlates with LH level and GnRH pulse frequency [36,37]. Many recent studies have addressed the role of oestradiol in GnRH secretion with both inhibitory and stimulatory effects, depending upon the phases of the menstrual cycle [38,39]. In our study, the presence of LH hypersecretion in PCOS patients indicates increased GnRH secretion and this abnormality may be due to the direct stimulatory effect of high level of oestradiol which is ultimately highly influenced by the polymorphism rs1213704663 of *KISS1*.

Further studies with a higher sample size are needed in order to identify other SNPs in KISS1, and to reach a conclusive mechanism for the role of *KISS1* SNPs in PCOS related issues.

This work represents an advance in biomedical science because it shows that women having rs1213704663 C/G variation in *KISS1* are more likely to have PCOS, and points to a role for rs1213704663 in PCOS associated LH and oestrogen hypersecretion, and increased LH-FSH ratio. Interestingly, this novel and significant SNP showed no influence on kisspeptin level of PCOS women.

Summary table

What is known about this subject:
- SNPs in *KISS1* are postulated as a genetic factor influencing the risk of PCOS and its associated endocrine and metabolic disturbances.
- The relevance of *KISS1* polymorphisms in PCOS and associated endocrine disturbances are unclear.

What this paper adds:
- SNP rs1213704663 C/G of *KISS1* is altered in PCOS.
- This SNP showed no influence on kisspeptin level and metabolic disturbances.
- LH and oestradiol hypersecretion, and increased LH-FSH ratio of PCOS women were significantly influenced by this SNP.

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