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Common genetic variants in the sex hormone-binding globulin (SHBG) gene in idiopathic recurrent pregnancy loss: a case control study

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Abstract

Background: A role for sex hormone-binding globulin (SHBG) in determining the pregnancy outcome was evidenced by the rise in SHBG levels during pregnancy linked with favorable pregnancy, while reduction in SHBG levels and hyperandrogenemia were linked with poor pregnancy outcome. Since SHBG production is genetically determined, this study investigated the association of *SHBG* polymorphisms with the susceptibility to recurrent pregnancy loss (RPL).

Methods: Retrospective case-control study, involving 308 women with RPL, and 310 control women RPL, defined as ≥3 consecutive miscarriages, and with the same partner, was the main outcome measure. *SHBG* genotyping was done by allelic exclusion method (real-time PCR).

Results: Of the seven tested *SHBG* SNP, lower MAF of rs6257 was seen in RPL cases than in control women, which was linked with lower risk of RPL, after controlling for key covariates. At the genotype level, significantly higher frequencies of heterozygous rs858521 and rs6259, and homozygous rs858521 genotype carriers, and reduced frequency of heterozygous rs6257 and homozygous rs6257 and rs6259 genotype carriers were seen in RPL cases vs. control women, respectively. Univariate regression analysis confirmed the positive association of rs858521 and rs6259 with RPL. Multivariate regression analysis confirmed the positive association of rs858521 heterozygote and homozygote genotypes with RPL; only heterozygous rs6259 remained associated with RPL. Haploview analysis demonstrated marked linkage disequilibrium among 6 of the 7 tested *SHBG* SNP. Of the possible 6-locus haplotypes, 12 were common, and were included in subsequent analysis. Within these haplotypes, only increased frequency of CCGTGA haplotypes was seen in RPL cases, thus conferring RPL susceptibility.

Conclusions: Specific *SHBG* variants, and *SHBG* haplotypes are associated with altered risk of RPL, suggesting role for *SHBG* as RPL candidate gene.

Keywords: Haplotypes, Hyperandrogenism, Polycystic ovary syndrome, Recurrent pregnancy loss, Sex hormone binding globulin

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Background

Recurrent pregnancy loss (RPL), defined as two or more clinically failed pregnancies, is a significant pregnancy complication which affects 2–3% of otherwise healthy women, with poor etiology [1–3]. RPL is associated with metabolic and hormonal disorders, including hyperprolactinemia [3, 4], hypothyroidism [4, 5], hyperinsulinemia and hyperandrogenemia [2, 3, 6]. Hormonal imbalances, particularly during early stages of pregnancy, were linked with RPL, as inadequate progesterone levels in early pregnancy was linked with termination of pregnancy [1, 7], and as elevated FSH/LH in early pregnancy were linked with pregnancy complications, including RPL [2, 8].

Several studies documented the association of hyperandrogenemia with RPL [2, 9], often with inconclusive findings. This was attributed to the wide variation in androgen levels, the time of sampling (i.e. within 7 days of the cycle), and the testosterone pool examined. The latter was attributed to the lipophilic nature of steroid sex hormones, in which circulating androgens and estrogens bind albumin and sex hormone binding globulin (SHBG), resulting in limited amounts of non-bound sex hormones, and hence reduced bioavailability [9, 10]. Accurate assessment of biochemical hyperandrogenemia requires measurement of either free testosterone, or free androgen index, which require determination of the serum levels SHBG, a 373-amino acid glycoprotein produced mainly by the liver, and binding the testosterone, dihydrotestosterone, and estradiol [11, 12], thus limiting their target tissue availability [12].

A role for SHBG in determining the pregnancy outcome was suggested. A rise in SHBG levels during pregnancy was proposed as protective for the fetus and mother from excessive androgen exposure [13], and reduction in SHBG levels and thus hyperandrogenemia were linked with pregnancy complications [11, 14]. *SHBG* gene is located on the short arm of chromosome 17 (17p13–17p12) [15], and several genetic variants in *SHBG* gene were identified, and were associated with varied levels of SHBG [16, 17], and with altered risk of RPL [18].

We hypothesize that genetic variants in *SHBG* gene associated with reduced SHBG expression, induce hyperandrogenemia, and hence increased risk of RPL. We tested this notion by examining the association of common *SHBG* gene variants among women with confirmed RPL diagnosis, and age- and ethnically-matched control women. This is the first study to examine this association in a Middle Eastern population.

Methods

Study subjects

This retrospective case-control study was performed at outpatient OB/GYN clinics in Manama, and Rifaa (Bahrain). Study subjects comprised 308 consecutively-recruited women with confirmed RPL (mean age 31.6 ± 5.4 years), and 310 age-matched control women. RPL diagnosis was based on Royal College of Obstetricians and Gynecologists guidelines (www.rcog.org.uk/guidelines), which were consistent with American College of Obstetricians and Gynecologists guidelines. These included screening of anti-phospholipid antibodies (lupus anticoagulant and anti-cardiolipin antibodies), karyotyping of both partners, pelvic ultrasound scan for evaluation of uterine anatomy, and screening of inherited thrombophilia (Factor V-Leiden, prothrombin/factor II G20210A).

These procedures were performed on all women with RPL. The inclusion criteria were three or more idiopathic (unknown etiology) miscarriages, which occurred during the 1st trimester of gestation with the same partner. Exclusion criteria were 40 years or older at first pregnancy, incompatibility in Rh blood groups, history of preeclampsia, which was defined as rise in systolic and diastolic blood pressure (BP) above 145/95 mmHg, and/or elevation in systolic/diastolic BP above 30/15 mmHg on two or more occasions, biochemical pregnancy and/or preclinical miscarriages. In addition, systemic autoimmunity, diabetes, and thyroid dysfunction, anatomical disorders, infections, and liver function abnormalities constituted added exclusion criteria. Due to personal, cultural and religious concerns, karyotyping of the products of conception was not routinely done.

Controls consisted of 310 multiparous women, with two or more full-term live pregnancies, no miscarriages (spontaneous or induced), and negative family history of miscarriages. Control women comprised ethnically-matched hospital and university students and employees, and volunteers from the community, and were recruited after a routine check-up after uncomplicated pregnancies. All participants had normo-ovulatory cycles, and none had evidence of polycystic ovary syndrome. Research and Ethics Committee of the Arabian Gulf University approved the study protocol, which was done in accordance with the Helsinki Declaration principle; all participants were asked to sign a consent form prior to inclusion in the study.

SHBG genotyping

Blood samples were taken from all participants in EDTA-containing tube for total genomic DNA extraction, which was done by the Qiagen minispin column method, according to the instructions of the manufacturer (Qiagen, Hilden, Germany). We selected polymorphisms in *SHBG* gene with a minor allele frequency (MAF) of > 5% in Caucasians, using SNPbrowser software (version 4.0, Applied Biosystems, Foster City, CA, USA). Genotyping was performed in 6-μl volume by the allelic discrimination method on StepOne Plus real-time

PCR system, according to manufacturer's instructions (Applied Biosystems). Assay-on-demand TaqMan primer pairs for the following SNPs: rs9898876, rs13894, rs858521, rs1799941, rs6257, rs6259, and rs727428 were ordered from Applied Biosystems. A typical genotyping reaction consisted of 2.2 μl DNA template added to 4. 0 μl TaqMan genotype master mix (TaqMan 2X mix, 1. 875 μl nuclease free water, and 0.125 μl 40X SNP primer mix) (Applied Biosystem). Pre-PCR (hold step; 30 s at 60 °C and 10 min at 95 °C) stage was followed by 35 cycles of denaturation (92 °C for 15 s), annealing and extension (60 °C for 1 min), followed by post-PCR stage at 60 °C for 30 s. Replicate blinded quality control samples were included to assess reproducibility of the genotyping procedure; concordance was > 99%.

Statistical analysis

Statistical analysis was performed on SPSS version 24.0 (IBM, Armonk, **NY**). Continuous variables were expressed as mean ±SD, while categorical data were represented as frequency (percentage of total). Student's *t*-test was used to determine differences in means, and Pearson χ^2 or Fisher's exact test was used to assess inter–group significance. Genotypes were tested for departures from Hardy–Weinberg equilibrium (HWE) using Haploview version 4.2 (www.broad.mit.edu/mpg/haploview). All analyses were conducted under additive genetic effect, using SNPStats software (www.bioinfo.iconcologia.net/snpstats/). CaTS Power Calculator (www.sph.umich.edu/csg/abecasis/cats) was used in calculating the power for detecting an association between *SHBG* variants and RPL. The parameters used were 308 RPL cases and 310 control women, genotypic relative risk for heterozygous and minor allele homozygous, and MAF of the tested SNPs for RPL cases and controls, and assuming a 2.5% RPL prevalence rate (unpublished Bahrain Ministry of Health statistics). Assuming these parameters, the overall power (81.0%) was calculated as the average power of the seven tested SNPs. Linkage disequilibrium (LD) analysis was performed using Haploview 4.2, and haplotype reconstruction was performed by the expectation maximization method (Haploview 4.2). Logistic regression analysis was used to examine the association between *SHBG* SNP and RPL, presented as odds ratio (OR) with 95% confidence intervals (CI), after controlling for the following potential confounders: BMI, systolic and diastolic blood pressure, number of pregnancies, and age at menarche; statistical significance being set at $P < 0.05$.

Results

Demographic and clinical characteristics of RPL cases and control women

The demographic and clinical characteristics of RPL cases and control women are shown in Table 1. Mean age at entry of study, serum glucose, gravida, and prevalence of smoking were comparable between cases and controls. Mean BMI ($P = 0.004$), menarche ($P < 0.001$), and systolic and diastolic blood pressure ($P < 0.001$) were different between RPL cases and control women. These were the main covariates selected that were controlled for in subsequent analysis.

Table 1 Demographics & Clinical Characteristics of Cases and Controls

	Cases[a]	Controls[a]	P[b]
Age at inclusion in study [c]	31.6 ± 5.4	31.6 ± 4.9	0.94
Body-mass index (kg/m^2) [c]	26.3 ± 5.4	25.2 ± 4.3	0.004
Obesity [n (%)] [d]	58 (19.6)	37 (12.1)	0.02
Smokers [n (%)] [d]	30 (10.1)	32 (10.8)	0.69
Systolic blood pressure (mmHg) [c]	114.2 ± 11.9	120.2 ± 17.0	< 0.001
Diastolic blood pressure (mmHg) [c]	72.0 ± 8.4	75.8 ± 9.1	< 0.001
Glucose (mmol/L) [c]	5.1 ± 0.9	5.2 ± 0.7	0.55
Menarche (years)[c]	12.2 ± 1.1	12.8 ± 1.0	< 0.001
Number of pregnancies [c]	4.2 ± 1.5	4.0 ± 1.1	0.11
Number of Children [c]	0.8 ± 1.1	4.0 ± 1.1	< 0.001
Miscarriages [c]	3.6 ± 1.0	0.0 ± 0.1	< 0.001
Serum IL-10 (pg/ml) [c]	5.3 ± 1.8	6.1 ± 1.2	0.002

[a]A total of 308 RPL cases and 310 control women were included
[b]Student's *t*-test (continuous variables), Pearson's χ^2 test (categorical variables)
[c]Mean ± SD
[d]Percent of total within each group/subgroup

Association between *SHBG* SNP and the risk of RPL

Genotype distributions of the tested *SHBG* variants were in Hardy-Weinberg equilibrium among study subjects Table 2. summarizes the association between *SHBG* SNP and RPL in cases and control subjects. At the allele level, lower MAF of rs6257 ($P = 0.001$) was seen in RPL cases than in control women, which translated into lower risk of RPL, after controlling for BMI, menarche, systolic and diastolic blood pressure [aOR (95% CI) = 0.59 (0.43–0.81)]. MAF of the remaining *SHBG* SNPs were not significantly different between RPL cases and control women.

The distribution of *SHBG* genotypes between RPL cases and control women are shown in Table 3. Significantly higher frequencies of heterozygous rs858521 (0. 51 vs. 0.44) and rs6259 (0.07 vs. 0.03), and homozygous rs858521 (0.28 vs. 0.26) genotype carriers, and reduced frequency of heterozygous rs6257 (0.23 vs. 0.32) and homozygous rs6257 (0.05 vs. 0.08) and rs6259 (0.003 vs 0.03) genotype carriers were seen in RPL cases vs. control women, respectively. The distribution of the remaining genotypes was comparable between RPL cases and control women.

Table 2 *SHBG* SNPs analyzed in RPL cases and control women

#	SNP	Position	HWE *P*	Alleles	Cases [a]	Controls [a]	χ^2	*P*	aOR [b] (95% CI)	Power
1	rs9898876	7,623,644	0.154	G:T	0.121	0.089	2.453	0.117		90
2	rs13894	7,626,584	0.149	C:T	0.345	0.370	0.751	0.386		63
3	rs858521	7,626,829	0.461	C:G	0.536	0.494	1.832	0.176		100
4	rs1799941	7,630,105	0.686	G:A	0.175	0.206	1.603	0.206		83
5	rs6257	7,630,399	0.084	T:C	0.163	0.249	10.82	0.001	0.59 (0.43–0.81)	100
6	rs6259	7,633,209	0.050	G:A	0.041	0.039	0.011	0.917		89
7	rs727428	7,634,474	0.783	G:A	0.411	0.433	0.262	0.609		42

[a]MAF frequency
[b]aOR = adjusted OR; variables that were controlled for were BMI, menarche, systolic and diastolic blood pressure

Risk of RPL associated with *SHBG* genotypes

The risk of RPL imparted by the tested *SHBG* variants was evaluated by logistic regression analysis, which was performed first at the univariate, and later at the multivariate levels, taking control status as an independent variable and the SHBG SNPs as dependent variables, after controlling for BMI, menarche, and systolic and diastolic blood pressure. Univariate regression analysis confirmed the positive association of rs858521 [$P = 0.01$; OR (95% CI) = 1.72 (1.13–2.60)] and rs6259 [$P = 0.03$; OR (95% CI) = 15.75 (1.29–192.46)] with RPL (Table 4). Multivariate regression analysis confirmed the positive association of rs858521 heterozygote [$P = 0.007$; aOR (95% CI) =1.80 (1.17–2.77)] and homozygote [$P = 0.05$; aOR (95% CI) = 1.60 (1.00–2.58)] genotypes with RPL. On the other hand, only heterozygous rs6259 remained associated with RPL after controlling for the confounders [$P = 0.03$; aOR (95% CI) = 16.08(1.28–202.08)] (Table 3).

Identification of *SHBG* haplotypes associated with RPL

We evaluated the interaction between the six tested *SHBG*SNPs and their mode of inheritance in RPL cases and control women. The *SHBG* SNPs were aligned according to their chromosomal locations (www.ncbi.nlm.nih.gov/snp), and the interaction between any all possible pair of SNPs was visualized by Haploview (Fig. 1). Haploview analysis demonstrated marked LD among *SHBG* SNPs (Fig. 1). Each haplotype contains the next six loci: rs13894- rs858521- rs1799941- rs6257- rs6259- rs727428 and only 12 haplotypes were captured. Of the theoretical 64 haplotypes, only 12 were found to be common, capturing 91.8% of all haplotype pool, and were included in subsequent analysis. Within these haplotypes, only increased frequency of CCGTGA (*P* = 6.5×10^{-3}) haplotypes was seen in RPL cases, thus conferring disease susceptibility (OR = 1.66 (1.16–2.38) (Table 5).

Discussion

Overview of the association of *SHBG* SNPs with RPL

Mounting evidence suggests that hyperandrogenemia in RPL is associated with abnormal LH levels and altered sex hormone production by ovaries or adrenal glands [2, 9], increased peripheral aromatase activity, or decreased testosterone clearance [19, 20]. Since SHBG is key to hyperandrogenemia, and as SHBG production is genetically determined [17], we investigated the association of *SHBG* variants with RPL. *SHBG* gene is highly polymorphic, and

Table 3 *SHBG* Genotype Frequencies

	1/1 [a]		1/2 [a]		2/2 [a]		
SNP	Cases	Controls	Cases	Controls	Cases	Controls	*P* [b]
rs9898876	247 (0.80) [c]	260 (0.84)	49 (0.16)	43 (0.14)	12 (0.04)	7 (0.02)	0.425
rs13894	136 (0.44)	133 (0.43)	131 (0.43)	131 (0.42)	41 (0.13)	46 (0.15)	0.872
rs858521	64 (0.21)	94 (0.30)	158 (0.51)	135 (0.44)	86 (0.28)	81 (0.26)	0.035
rs1799941	204 (0.66)	202 (0.65)	96 (0.31)	94 (0.30)	8 (0.03)	14 (0.05)	0.398
rs6257	222 (0.72)	187 (0.60)	70 (0.23)	98 (0.32)	16 (0.05)	25 (0.08)	0.019
rs6259	284 (0.92)	293 (0.95)	23 (0.07)	10 (0.03)	1 (0.003)	7 (0.02)	0.042
rs727428	115 (0.37)	107 (0.35)	143 (0.46)	142 (0.46)	50 (0.16)	61 (0.20)	0.877

[a]Genotypes were coded as per "1" = major allele, "2" = minor allele
[b]2-way ANOVA
[c]Number of subjects (frequency)

Table 4 Univariate and multivariate analysis of *SHBG* genotypes association with RPL[a]

	Univariate				Multivariate			
	1/2 [b]		2/2		1/2		2/2	
SNP	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	aOR [c] (95% CI)	*P*	aOR (95% CI)
rs9898876	0.46	1.56 (0.49–5.02)	0.26	1.87 (0.63–5.58)	0.34	1.78 (0.54–5.87)	0.20	2.07 (0.69–6.23)
rs13894	0.65	0.89 (0.54–1.48)	0.61	0.88 (0.53–1.45)	0.84	0.95 (0.57–1.59)	0.77	0.93 (0.56–1.55)
rs858521	0.01	1.72 (1.13–2.60)	0.07	1.53 (0.96–2.44)	0.007	1.80 (1.17–2.77)	0.05	1.60 (1.00–2.58)
rs1799941	0.19	1.94 (0.72–5.25)	0.19	1.92 (0.73–5.08)	0.16	2.15 (0.75–6.16)	0.19	1.99 (0.71–5.57)
rs6257	0.85	0.93 (0.44–1.99)	0.11	0.56 (0.27–1.14)	0.68	0.84 (0.38–1.89)	0.07	0.50 (0.23–1.06)
rs6259	0.03	15.75 (1.29–192.46)	0.11	6.42 (0.66–62.33)	0.03	16.08 (1.28–202.08)	0.11	6.42 (0.65–63.70)
rs727428	0.70	0.83 (0.32–2.17)	0.61	0.77 (0.28–1.10)	0.67	0.81 (0.31–2.13)	0.59	0.76 (0.28–2.08)

[a]Homozygous major allele genotypes were taken as reference (OR = 1.00)
[b]Genotypes were coded as per "1" = major allele, "2" = minor allele
[c]aOR = adjusted odds ratios; BMI and age were the main covariates that were controlled for

902 variants were identified in NCBI database (www.ncbi.nlm.nih.gov/gene/6462), some of which modulate circulating SHBG concentrations.

Significance of the findings

While many *SHBG* gene variants were reported, the novel finding here was the strong association of three of the seven tested variants (rs6257, rs6259, rs858521) with RPL. Compared to previous studies, this study is distinct in the relatively large sample size, new *SHBG* variants investigated, and the population investigated. This extends the list of *SHBG* gene variants implicated in RPL pathogenesis, thereby supporting a key role for SHBG in RPL, presumably through controlling hyperandrogenemia.

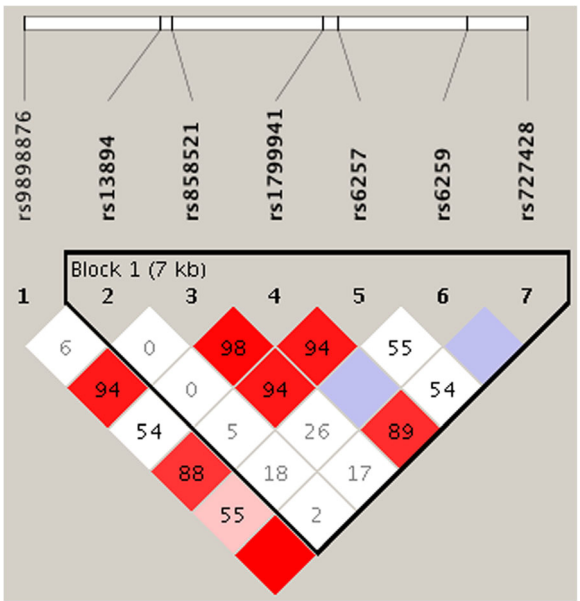

Fig. 1 Haploview graph of *SHBG* SNPs analyzed. The positions of the seven SNPs used (Build 37.3) are indicated along with the basic gene structure, and displayed above the Haploview output. The relative LD between specific pair of *SHBG* SNPs is indicated by the color scheme, which represents the LD relationships. This is based on D′ values (normalized linkage disequilibrium measure or D) multiplied by 100; D′ is calculated as D divided by the theoretical maximum for the observed allele frequencies. Values approaching zero indicate absence of LD, and those approaching 100 indicate complete LD. The square colored red represent varying degrees of LD < 1 and LOD (logarithm of odds) > 2 scores; darker shades indicating stronger LD

Main findings

The main finding here was the strong association of rs6257 with reduced risk of RPL at the allele level, while both rs858521 and rs6259 were positively associated with RPL at the genotype level. Of these, only heterozygous rs6259 remained associated with RPL after controlling for mean BMI, menarche, and blood pressure, since RPL cases were not matched to controls. Six-locus (rs13894-rs858521-rs1799941-rs6257-rs6259-rs727428) *SHBG* haplotype identified CCGTGA haplotype to be positively associated with increased risk of RPL. Significant differences were noted with respect to mean BMI, menarche, and systolic and diastolic blood pressure readings were different between RPL cases and control women. Although they did not constitute strong risk factors of RPL, they were selected as the covariates that were controlled for in subsequent analysis.

MAF of the tested *SHBG* variants among control women reflect the ethnic diversity of present-day Tunisians, which results from admixture of the ethnicities who sequentially invaded and populated Tunisia throughout history [21]. MAF of rs858521, rs1799941, and rs727428 was comparable between Bahraini and Caucasians (HapMap-CEU), and Africans. On the other hand, MAF of rs13894 and rs6257 were the highest recorded for any ethnic group, while MAF of rs6259 (0.039) was intermediate between those recorded for Caucasians (0.124) and Africans (0.009). Furthermore, MAF of rs9898876 recorded for Bahraini (0.089) was lower than that established for Caucasians (0.222) and Africans (0.132). This underscores the need for evaluation

Table 5 Haplotype frequencies across 7 *SHBG* SNPs analyzed [a]

Haplotype[b]	Frequency	Case:Control frequencies	χ^2	*P*	aOR (95% CI)
C C G T G A	0.138	0.165; 0.108	7.405	6.5×10^{-3}	1.66 (1.16–2.38)
C G G T G G	0.128	0.132; 0.124	0.163	0.687	
C C A T G G	0.115	0.106; 0.125	1.001	0.317	
T G G T G G	0.087	0.092; 0.081	0.420	0.517	
C G G C G G	0.069	0.061; 0.078	1.158	0.282	
T C G T G A	0.066	0.064; 0.067	0.030	0.863	
C G G C G A	0.060	0.047; 0.074	3.422	0.064	
T G G C G A	0.055	0.045; 0.067	2.411	0.121	
C C G T G G	0.054	0.062; 0.045	1.626	0.202	
T C A T G G	0.052	0.052; 0.052	0.001	0.986	
T C G T G G	0.047	0.044; 0.051	0.329	0.567	
C G G T G A	0.047	0.048; 0.045	0.055	0.814	

[a] *SHBG* haplotypes: rs13894- rs858521- rs1799941- rs6257- rs6259- rs727428
[b] Underlined indicates minor allele

of differences in ethnic/racial background in genetic association studies.

Interpretation of results

Progressive increases in maternal SHBG levels are seen throughout pregnancy, ranging from 1.61% in first trimester to almost 6% during mid-to-late pregnancy [6, 11, 22], which were suggested to protect mothers from androgen derived from the fetus [6, 23]. In contrast, minimal fluctuations in total testosterone (1.21–2.15%) and free testosterone (3.02–4.30%) levels were noted during pregnancy, suggesting that additional mechanisms operate in minimizing pregnancy-induced hyperandrogenemia [22, 23]. A role for SHBG in maintaining pregnancy was highlighted by the findings that reduction in SHBG levels were seen in miscarrying women compared to controls [14]. Insofar as genetic factors contribute to variation in *SHBG* levels, and thus and thus to the pathogenesis of gynecological diseases, low plasma SHBG levels, resulting from genetic variations in *SHBG* gene were associated with pregnancy complications, including RPL [24]. In this context, the present study evaluated the implication of seven SNPs in *SHBG* on RPL.

Based on its allele and genotypes distribution in RPL cases and controls, our results suggest that *SHBG* rs6257 (Intron 1) gene variant may modulate the risk of RPL. While this variant was previously associated with OB/GYN complications [25, 26] including PCOS [16, 27], this is the first report to document its association with RPL. Despite its intronic location, rs6257 appears to have functional capacity, as it maps to a potential binding site for Hepatocyte Nuclear Factor 3/Fox transcription factor, which was shown to influence SHBG levels [28]. While not tested here, carriage of rs6257 minor allele alters testosterone binding to SHBG, and hence testosterone bioavailability and action at target tissue site, including reproductive organs and tissues [29].

On the other hand, both rs6259 (D327N) and rs858521 were positively associated with RPL. While *SHBG* rs6259 is the most investigated of all reported *SHBG* variants, this is the first report describing its association with RPL. Mixed association of this variant with obstetric complications. For example, rs6259 was not associated with PCOS in Bahraini [16], Turkish [30], and Spanish [27] subjects. Moreover, this polymorphism was also studied in hormone-sensitive cancers and the results revealed that rs6259 minor allele was associated with reduced risk of endometrial cancer in postmenopausal, but not premenopausal women [31]. Reduced frequency of rs6259 minor allele was also reported in ovarian cancer [32], and breast cancer [33], presumably by increasing serum SHBG levels, particularly among postmenopausal women. Asn327 allele was associated with increased circulating SHBG levels, and with reduced estradiol-to-SHBG ratio, suggesting that rs6259 contributes the bioavailability of estrogens [34]. The functionality of D327N resides in the capacity of the (minor) Asn327 allele to decreases clearance rate of SHBG, resulting in increased half-life of SHBG and hence increased circulating SHBG levels [35].

Study strengths and shortcomings

Our study has strengths. RPL cases and control women were ethnically matched, hence minimizing the problems of differences in genetic background inherent in genetic association studies. It was also sufficiently powered, and key covariates were controlled for in single *SHBG* variant and haplotype analysis. However, our

study had several limitations. We did not measure circulating SHBG levels, and thus could not address the cause-effect relationship, as well as determine free testosterone levels and free androgen index (hallmarks of hyperandrogenemia). Furthermore, matching for ethnic origin was dependent on self-declared Arab vs. Non-Arab Tunisian origin, thus prompting the speculation of genetic population stratification bias. Lastly, the study examined the association of RPL with seven common *SHBG* gene variants distributed between intronic, exonic, and 5′ and 3′ untranslated regions, thus questioning of the potential association of other (untested) *SHBG* variants with RPL.

Conclusion

In conclusion, this is the first study that demonstrated that *SHBG* rs858521 and rs6259, and to a lesser extent rs6257 variants, are associated with RPL. As mounting evidence suggest that some *SHBG* genetic variants influence SHBG levels, the mechanism by which *SHBG* influence the risk of RPL will expand on the role of SHBG in infertility. Follow up studies on additional *SHBG* variants, and populations of related and distant ethnic origin are needed to fully elucidate the association of altered SHBG production stemming from the presence of specific *SHBG* variants, and consequently hyperandrogenemia with the risk of RPL.

Abbreviations

BP: blood pressure; LD: Linkage disequilibrium; MAF: minor allele frequency; RPL: Recurrent pregnancy loss; SHBG: sex hormone binding globulin; SNP: single nucleotide polymorphism

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MD Sample processing, drafting of manuscript. RRF Patient screening and referral. MM Genotyping assays. WYA Project leader, finalizing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by Arabian Gulf University Research & Ethics Committee (IRB approval: 35-PI-01/15, granted on 17 October 2014), and was done according to Helsinki II Declaration. All patients provided informed written consent before blood sampling.

Competing interests

The authors declare that they have no competing interests to declare.

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