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ORIGINAL RESEARCH

Association of the R67X and W303X non-sense polymorphisms in the protein Z-dependent protease inhibitor gene with idiopathic recurrent miscarriage

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ABSTRACT: Protein Z-dependent protease inhibitor (ZPI) is a 72 kDa single-chain serpin which inhibits the activated coagulation factors X and XI. Two non-sense polymorphisms of ZPI, R67X and W303X, were recently identified, and were linked with a prothrombotic state. Here, we investigated the association of the R67X (728C>T) and W303X (1438G>A) variants in the ZPI gene with recurrent spontaneous miscarriage (RSM). This was a case–control study involving a total of 288 women with a history of two consecutive or ≥3 non-consecutive pregnancy losses between 8 and 12th week of gestation, along with 304 age-matched and ethnically matched multiparous control women, with no personal or family history of pregnancy complications. The minor allele frequency of R67X ($P = 0.003$) and W303X ($P = 0.014$) were higher in RSM cases than in control women. Both single-nucleotide polymorphisms were significantly associated with RSM under the dominant genetic association model, and were in moderate linkage disequilibrium ($D' = 0.412$; $P < 0.001$). Taking the common ^{728}C/^{1438}G haplotype as reference, multivariate analysis confirmed the positive association of ^{728}T/^{1438}G [$P = 0.043$; odds ratio (OR) = 2.25; 95% confidence interval (CI) = 1.03–4.90], and ^{728}T/^{1438}A ($P = 0.022$; OR = 3.93; 95% CI = 1.23–12.59) haplotypes with increased RSM risk. These differences remained significant after controlling for some covariates. These results demonstrate that both *ZPI* R67X and W303X non-sense variants and specific *ZPI* haplotypes are significantly associated with RSM.

Key words: haplotype / protein Z-dependent protease inhibitor / recurrent miscarriage / genetic association

Introduction

The protein Z (PZ)-dependent protease inhibitor (ZPI) is a 72 kDa single chain anti-coagulant glycoprotein (Han *et al.*, 1999), belonging to the serpin superfamily of protease inhibitors (Han *et al.*, 1999, 2000; Rezaie *et al.*, 2008; Vass, 2011). ZPI is synthesized in the liver (Han *et al.*, 2000; Vass, 2011), and mediates its anti-coagulant effects by two distinct mechanisms (Vass, 2011). ZPI exerts its effects by binding the 62 kDa PZ cofactor, resulting in the formation of PZ/ZPI complex, which in turn inactivate coagulation factor Xa in a Ca^{2+}-and phospholipid-dependent manner (Corral *et al.*, 2007; Vass, 2011). The significance of the PZ-ZPI formation on factor Xa inhibition was highlighted by the findings that the absence of PZ reduces ZPI inhibitory effects by 1000 folds, resulting in a specific, but poor, factor Xa inhibition (Huang *et al.*, 2011). ZPI also directly inhibits coagulation factor XIa in a PZ-independent fashion, by binding heparin (Rezaie *et al.*, 2008; Huang *et al.*, 2011).

Successful pregnancy depends on maintaining a fine balance between pro- and anti-coagulant mechanisms (Isermann *et al.*, 2003; Zygmunt *et al.*, 2003), and the maintenance of early pregnancy is tightly linked with placental growth and differentiation (Demir *et al.*, 2007; Blois *et al.*, 2011). Defective maternal haemostatic responses, leading to thrombosis of the utero-placental circulation and subsequent fetal loss, was described as a major cause of recurrent spontaneous miscarriage (RSM), a common reproductive problem affecting 1–3% of pregnancies (Brown, 2008; Warren and Silver, 2008). While very early pregnancy loss was attributed to chromosomal abnormalities, later losses were attributed to altered vascular permeability of the placenta. In this regard, it was shown that pregnancy, delivery and post-partum are associated with differential changes in the levels

of coagulation factors (Szecsi *et al*., 2010) favoring a hypercoagulable state associated with increased clotting factors, decreased fibrinolytic activity and reduction in anti-coagulant levels (Brenner, 2004; D'Uva *et al*., 2008; Thornton and Douglas, 2010). This becomes more apparent in RSM (Rai, 2003; D'Uva *et al*., 2008), whereby a significant proportion of RSM women present with identifiable prothrombotic risk factors (Krause *et al*., 2005; Sottilotta *et al*., 2006), thereby posing greater risk state of miscarriage in future pregnancies.

Several variants in the *ZPI* gene were previously reported (van de Water *et al*., 2004; Vossen *et al*., 2004), of which the two non-sense polymorphisms, R67X and W303X, were associated with venous thromboembolism (VTE) in New Zealand (van de Water *et al*., 2004), likely due to altered ZPI levels given their location within structurally important sites within the *ZPI* gene, as was suggested (van de Water *et al*., 2004). Other studies reported lack of association of the *ZPI* variants with venous (Razzari *et al*., 2006; Fabbro *et al*., 2007; Dentali *et al*., 2008) or arterial (Refaai *et al*., 2006) thrombosis and an ethnic contribution of the *ZPI* variants (W303X) to venous thrombosis was proposed (Gonzalez-Conejero *et al*., 2005; Fabbro *et al*., 2007). While previous studies have documented a link between altered PZ levels in normal pregnancy (Quack Loetscher *et al*., 2005), and pregnancy complications (Erez *et al*., 2007; Kusanovic *et al*., 2007), no single study has investigated the association between changes in ZPI levels and the *ZPI* variants to the risk of RSM. Here, we investigate the association of the R67X and W303X *ZPI* non-sense variants to RSM in 288 women with confirmed RSM and 304 age-matched and ethnically matched control women.

Materials and Methods

Subjects

A total of 288 women with confirmed RSM (mean age 31.6 $\pm$ 5.2 years) were consecutively recruited from the outpatient OB/GYN clinics in Manama, Bahrain for assessment of idiopathic RSM, as per the Royal College of Obstetricians and Gynaecologists Guidelines (http://www.aetna.com/cpb/medical/data/300_399/0348.html), which were consistent with ACOG Guidelines. These included endometrial biopsies for evaluating luteal phase defect, pelvic ultrasound scan to assess ovarian morphology and the uterine cavity, hysterosalpingography, hysteroscopy or sonohysteroscopy to evaluate uterine anatomic abnormality (Fallopian tubes openness, any evidence of peritoneal cavity scarring); all cases (100%) had these procedures done. Data on lupus anti-coagulant and anti-cardiolipin antibodies and karyotyping of fetal products and peripheral blood of both partners were available for 195 (67.7%) and 115 (39.9%) cases, respectively. In total, 193 patients (67.0%) had early miscarriages (<10 weeks gestation), while the remaining 95 (33%) had later miscarriages (>10 weeks gestation).

The inclusion criteria were three or more consecutive pregnancy losses of unknown etiology during natural pregnancies with no underlying classical risk factors with the same partner, which occurred during the first trimester of gestation. Among RSM cases, 210 patients (72.9%) did not have any live birth, 59 (19.8%) had 1 live birth and the remaining 19 (6.6%) had 2 live births. Exclusion criteria included parental and fetal karyotype aberrations (if available), Rh blood group incompatibility (which can cause maternal alloimmunization and hemolytic disease of the fetus and newborn in later pregnancies), older age (>40 years or older at first miscarriage), preclinical miscarriages and/or biochemical pregnancy and pre-eclampsia elevated systolic and diastolic blood pressure (BP) >145/95 mmHg, or rise in systolic/diastolic BP >30/15 mmHg on at least two occasions). Patients were also excluded if they reported systemic autoimmune disease, diabetes mellitus and thyroid dysfunction, anatomical disorders, infections (toxoplasmosis, human cytomegalovirus, rubella, human immunodeficiency virus, Group B streptococci, *Chlamydia trachomatis*, hepatitis B and C and bacterial vaginosis), liver function abnormalities, anti-phospholipid syndrome and hyper-prolactinemia prior to luteal phase defects.

Controls were composed of 304 consecutively recruited multi-parous, age-matched and ethnically matched women who had at least two live births and no miscarriages (spontaneous or induced), and did not have a family history of RSM. Both patients and controls were Bahrain Arabs. Blood samples were taken from all participants in EDTA-containing tube, after signing a consent form before they were included in the study. The Research and Ethics Committee of the Arabian Gulf University approved the study protocol.

ZPI genotyping

Total genomic DNA was isolated from peripheral blood lymphocytes of study subjects by the Qiagen mini-spin column method. *ZPI* polymorphisms were analyzed by bidirectional allele-specific PCR (van de Water *et al*., 2004). For R67X, the following primers were used: [67M] 5′-TGC CTC ATG GAG ATC TTA CA-3′, [67N] 5′-CTT-CGA TTC AGC CTG ATG C-3′, [67P1] 5′-TGG AGC CCT CTC TTG ATG TG-3′ and [67P2] 5′-CCA GAC CAG CAG GGT TGT-3′. For W303X, the following primers were used: [303M] 5′-GAC CGC AGA CTT GGT GGA GAC CTG A-3′, [303N] 5′-CCT GGT TTT CAT GTT TCT AAG-C-3′, [303P1] 5′-GAC CCT GTC TTC ACC GAA GTC-3′ and [303P2] 5′-GTG ACA GAT GCT GGG GAT AGA GTG G-3′, Amplified products were detected by ethidium bromide-stained agarose gel.

Statistical analyses

Data were expressed as mean $\pm$ SD for continuous variables, or as percent of total for categorical variables. Intergroup significance was assessed by Student's *t*-test (continuous variables), and χ^2 test (categorical variables). Allele frequencies were calculated by the gene-counting method; each single-nucleotide polymorphisms (SNP) was tested for Hardy–Weinberg equilibrium using χ^2 goodness-of-fit test by HPlus 2.5 (http://cdsweb01.fhcrc.org/HPlus). The power was calculated for each SNP (http://pngu.mgh.harvard.edu/~purcell/gpc/cc2.html) At $\alpha = 0.05$, this sample size provided 88.28 and 73.32% power for R67X and W303X, respectively. Pairwise linkage disequilibrium (LD) values were calculated with SNPStats (http://bioinfo.iconcologia.net/snpstats); R67X and W303X were in moderate LD ($D' = 0.412$; $P < 0.001$). Haplotype estimation was done by the expectation maximization method with the HPlus 2.5. Logistic regression analysis was performed to calculate specific *P* values, odds ratios (ORs), 95% confidence intervals (CIs) after controlling for, age, BMI and menstrual history.

Results

Study subjects

The demographics and clinical characteristics of study subjects are reported in Table 1. Although age ($P = 0.571$) and smoking prevalence ($P = 0.776$) were comparable between RSM cases and control women, significant differences were seen with regard to BMI ($P = 0.003$) and obesity ($P = 0.005$), menarche ($P = 0.001$), irregular menstrual history ($P = 0.011$) and gravida ($P < 0.001$). Accordingly, the latter were the covariates that were controlled for in subsequent analysis.

Association studies

Table 2 illustrates the association between *ZPI* SNPs studied and RSM in cases and controls. Genotype distribution of R67X ($P = 1.00$) and W303X ($P = 1.00$) were in Hardy–Weinberg equilibrium among control women. The minor allele frequency (MAF) of R67X ($P = 0.003$; OR, 2.66; 95% CI, 1.40–4.87) and W303X ($P = 0.014$; OR, 2.44; 95% CI, 1.21–4.71) were significantly associated with RSM, and both SNPs were in moderate linkage dysequilibrium (LD) ($P < 0.001$; $D' = 0.412$; $r = 0.375$). This association remained significance after applying the Bonferroni correction for multiple testing.

Table 3 summarizes the association between R67X and W303X *ZPI* variants and RSM. In the virtual absence of homozygous carriers, these were analyzed under the dominant genetic model. Both *ZPI* SNPs showed a significant association with RSM under the model selected. This association remained significant after adjusting for BMI and menarche. Neither R67X (MAF: 0.065 versus 0.043; $P = 0.060$), nor W303X (MAF: 0.043 versus 0.063; $P = 0.084$) were associated with the status of miscarriage (primary/childless or secondary/at least one live birth). However, R67X (MAF: 0.106 versus 0.052; $P = 0.037$), more so than W303X (MAF: 0.083 versus 0.032; $P = 0.058$), was associated with fetal miscarriages (>10 weeks gestation).

Table I Clinical characteristic of patients and controls.

Characteristic	Controls (304)	Patients (288)	P[a]
Mean age (years)	31.8 ± 4.5	31.6 ± 5.2	0.571
Smoking[b]	29 (10.6)	27 (11.9)	0.776
Mean BMI (kg/m^2)	24.8 ± 3.9	26.1 ± 5.3	0.003
Obesity (BMI >30 kg/m^2)[b]	31 (10.2)	56 (19.4)	0.005
Menarche	12.7 ± 1.0	12.2 ± 1.0	0.001
Irregular menstrual history[b]	25 (8.2)	35 (12.2)	0.011
Gravida	3.5 ± 1.0	4.3 ± 1.1	<0.001
Live births	3.5 ± 1.0	0.95 ± 1.1	<0.001
Abortion	0.0 ± 0.0	3.7 ± 1.3	<0.001

[a]Student's *t*-test for continuous variables, Pearson's χ^2 test for categorical variables.
[b]Number of subjects (percent of total).

Haplotype analysis

Two-locus (R67X and W303X) ZPI haplotypes were constructed based on the prevalence of individual SNPs and LD between them. Taking the common ^{728}C/^{1438}G as reference (OR = 1.00), multivariate analysis confirmed the association of the ^{728}T-containing haplotypes ^{728}T/^{1438}G ($P = 0.043$; OR, 2.25; 95% CI, 1.03–4.90), and ^{728}T/^{1438}A ($P = 0.022$; OR, 3.93; 95% CI, 1.23–12.59) haplotypes with RSM, thus conferring disease susceptibility nature to these haplotypes (Table 4).

Discussion

In this case–control study, we investigated the association of the R67X and W303X non-sense *ZPI* variants as risk factors for RSM, and found strong association of R67X (OR = 2.66), and to a lower extent W303X (OR = 2.44), *ZPI* variants with RSM risk. Of

Table II Minor allele frequencies of the *ZPI* SNPs analyzed[a].

SNP	Gene position	HWE	Alleles	Controls[b]	Cases[b]	P-value[c]	OR (95% CI)
R67X	728C>T	1.00	C : T	14 (0.02)	34 (0.06)	0.003	2.66 (1.40–4.87)
W303X	1438G>A	1.00	G : A	12 (0.02)	27 (0.05)	0.014	2.44 (1.21–4.71)

HWE, Hardy–Weinberg equilibrium.
[a]Minor allele defined based on frequency in controls.
[b]A total of 288 RSM cases and 304 control women were genotyped. Values indicate number of alleles (frequency)
[c]Observed *P*-value.

Table III RSM association for ZPI SNPs adjusted for BMI and menarche[a].

SNP	Genotype	Controls	Cases	P-value	aOR (95% CI)[c]
R67X (728C>T)	CC	290 (0.95)[b]	255 (0.89)	0.005	1.00 (Reference)
	CT	14 (0.05)	32 (0.11)		2.68 (1.40–5.12); C/T-T/T versus C/C
	TT	0 (0.0)	1 (0.01)		
W303X (1438G>A)	GG	292 (0.96)	263 (0.91)	0.025	1.00 (Reference)
	GA	12 (0.04)	23 (0.08)		2.31 (1.14–4.70); G/A-A/A versus G/G
	AA	0 (0.00)	2 (0.007)		

[a]Analyzed by SNPstats software.
[b]Number of subjects (frequency).
[c]aOR, adjusted odds ratios, covariates controlled for included BMI and menarche. Analysis conducted under dominant genetic model.

Table IV Haplotype frequencies across the ZPI SNPs analyzed.

Haplotype[a]	Total[b]	Controls[b]	Cases[b]	*P*-value	aOR (95% CI)[c]
^{728}C/^{1438}G	0.941	0.964	0.918	—	1.00 (Reference)
^{728}T/^{1438}G	0.026	0.017	0.036	0.043	2.25 (1.03–4.90)
^{728}C/^{1438}A	0.018	0.013	0.023	0.210	1.71 (0.74–3.94)
^{728}T/^{1438}A	0.015	0.006	0.024	0.022	3.93 (1.23–12.59)

[a]ZPI haplotype construction was done using SNPstats software.
[b]Haplotype frequency.
[c]aOR, adjusted odds ratios, covariates controlled for included BMI and menarche.

significance was the finding that R67X, and to a lesser extent W303X, was associated with fetal, but not embryonic miscarriages. To the best of our knowledge, this is the first report investigating the contribution of these polymorphisms to increased RSM risk.

In contrast to pre-embryonic/embryonic pregnancy losses, which occur secondary to chromosomal abnormalities or intrauterine infections (Porter and Scott, 2005), later losses are due to altered maternal vascular permeability, resulting from constitutional defective homeostatic mechanisms (Paidas *et al*., 2005; Porter and Scott, 2005). In our study, which comprised 288 women with confirmed RSM diagnosis and 304 age-matched and ethnically matched control women, patients were excluded if they had systemic or autoimmune diseases and other maternal factors previously implicated in pregnancy losses in the fetal period (Davis and Olson, 2007; Metwally *et al*., 2010). We reasoned that since pregnancy is associated with increased levels of clotting factor (Szecsi *et al*., 2010), and reduced fibrinolysis and levels of a number of anti-coagulants (Brenner, 2004; D'Uva *et al*., 2008; Thornton and Douglas, 2010), which is more evident in RSM (Rai, 2003; D'Uva *et al*., 2008), the presence of inherited prothrombotic risk factors, including *ZPI* mutations, significantly increases the risk of miscarriage in future pregnancies.

It is noteworthy that the MAF of the R67X (2%) and W303X (2%) were generally comparable to prevalence rates established for Italians (Razzari *et al*., 2006), but were higher than reported rates of Spanish (Gonzalez-Conejero *et al*., 2005; Corral *et al*., 2006), Dutch (Al-Shanqeeti *et al*., 2005) and New Zealanders (van de Water *et al*., 2004). This difference in the distribution of the *ZPI* variants may be attributed to small sample size adopted by some of the studies (Fabbro *et al*., 2007), selection criteria of controls (blood donors versus volunteers), and to differences in ethnicity as was proposed (Gonzalez-Conejero *et al*., 2005; Fabbro *et al*., 2007). Additional studies involving larger sample size and diverse ethnic background are needed to test this notion.

Few studies have looked into a possible link between *ZPI* polymorphisms and VTE but with inconclusive findings, and none examined its association with recurrent miscarriage. Since ZPI/PZ complex was identified as an anti-coagulant system, deficiency in ZPI or its PZ was found to increase the risk of VTE, ischemic stroke and pregnancy complication according to some (Gris *et al*., 2002; van de Water *et al*., 2004; Corral *et al*., 2006; van Goor *et al*, 2008; Erez *et al*., 2007; Vass, 2011) but not all studies (Al-Shanqeeti *et al*., 2005; Corral *et al*., 2007). Functionally, ZPI reportedly acts by inhibiting factor Xa (Huang *et al*., 2011) and factor Xia (Rezaie *et al*., 2008; Huang *et al*., 2011). However, compared with endothelium-associated inhibitor of factor Xa, anti-thrombin, the phospholipid- and calcium-dependent ZPI activity appears to localize to the platelet membrane, suggesting that the roles of ZPI and anti-thrombin in controlling haemostasis are complementary.

In conclusion, our results clearly demonstrated for the first time, a link between R67X and W303X *ZPI* variants and RSM, suggesting that RSM is associated with constitutional and functional ZPI deficiency, brought about in part by the *ZPI* mutations. Our study has strengths, namely: (i) it was sufficiently powered to detect meaningful associations, (ii) cases and controls were ethnically matched, thereby minimizing the problems relating to different genetic background inherent in genetic association studies and (iii) potential covariates were controlled for throughout the analysis. However, our study has some limitations. We did not measure ZPI levels and/or activity, and as such could not establish the functional attributes of R67X and W303X variants. In addition, our study examined only two *ZPI* variants, thus prompting the speculation of other *ZPI* variants in RSM pathogenesis, and that it was limited to Bahraini Arabs, thereby necessitating follow-up studies in RSM cases from different ethnic groups. Furthermore, while we adjusted for a number of conventional RSM risk factors, we cannot exclude the possible contribution of other unmeasured risk factors, and given the modest OR associated with either SNP, this questions the potential for clinical utility of our findings. Large independent prospective population-based studies with different ethnicity are needed to confirm the contribution of *ZPI* polymorphisms to the risk of RSM.

Authors' roles

F.S.A. performed the genotyping assays and analysis of results and contributed toward the drafting of the manuscript. R.R.F. participated in patient careening and referral. A.W.A. contributed toward specimens preparation and performed genotyping assays. F.E.M. participated in patient screening and referral. W.Y.A. was the project leader and contributed toward data analysis and manuscript preparation.

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