Molecular thrombophilic profile in Mexican patients with idiopathic recurrent pregnancy loss

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ABSTRACT. Idiopathic recurrent pregnancy loss (IRPL) is defined by three or more consecutive miscarriages occurring before the twentieth week of gestation as a result of unidentified etiological factors. The results of previous studies have indicated that prothrombotic factors play a pathogenic role in early and late pregnancy. This study aimed to identify inherited prothrombotic and hypofibrinolytic risk factors

in Mexican-Mestizo patients with IRPL. Fifty-six women with IRPL and 50 control women with at least two full-term pregnancies and no history of RPL were included in this case-control study. Four prothrombotic (F5 G1691A, F2 G20210A, MTHFR C677T-A1298C) and one hypofibrinolytic (PAI1 4G/5G) restricted fragment length polymorphisms were subjected to molecular analysis. In the case of hypofibrinolytic ACE Ins/Del (I/D), identification was performed by direct PCR. The independent risk correlated with the presence of polymorphisms in IRPL patients was estimated using odds ratio (OR) with a 95% confidence interval (CI). *MTHFR* 677TT was the most frequent prothrombotic factor in the IRPL group (23%), followed by the compound-heterozygous *C677T*-A1298C (16%) and heterozygous *F2* 20210GA (3.6%). The heterozygous *ACE* I/D (62%) was the main hypofibrinolytic risk factor of IRPL, followed by the homozygote *PAI1* 4G/4G (18%). The *ACE* I/D polymorphism was the only significantly different factor among the cases and controls. The dominant genetic model D/D+I/D *vs* I/I showed an OR (95%CI) of 2.89 (1.22-6.89) and P = 0.019 in Mexican-Mestizo women. The results of this study support an association between the *ACE* I/D polymorphism and IRPL risk in a Mexican population.

Key words: *ACE I/D* polymorphism; Gene-disease association; Hypofibrinolysis; Recurrent pregnancy loss; Thrombophilia

INTRODUCTION

Recurrent pregnancy loss (RPL), initially defined as "three or more consecutive miscarriages before the 20th week of gestation", has been reported to affect 1-2% of reproductive-age females (Bashiri et al., 2012). Over the years, this definition has been updated to "two or more pregnancy losses"; consequently, there has been an increase in the frequency of reproductive-age women with RPL to 1-5% (Bashiri et al., 2012). RPL has a multifactorial etiology including a wide spectrum of abnormalities; causative risk factors of RPL include anatomic, immune, endocrine, infectious, chromosomal, and environmental factors, and inherited or acquired thrombophilia (Kiwi, 2006; Thomas and van der Spuy, 2010; Bennett et al., 2012). Idiopathic RPL (IRPL) is defined in ~50% of the couples seeking diagnosis who present none of the above etiological factors (Thomas and van der Spuy, 2010).

Evidence from previous control trials, cohort studies, and meta-analyses has supported that inherited prothrombotic factors play a pathogenic role in early and late pregnancy, in effect, causing a thrombogenic physiological state (Bennett et al., 2012).

Thrombosis, and its clinical manifestations, can be attributed to a large number of deaths worldwide. Thrombosis arises because of an imbalance between thrombogenic factors and antithrombotic mechanisms (including fibrinolysis) (Rosenberg and Aird, 1999). Rosendaal (1999) described this phenomenon as a multicausal disease. Thrombus formation can appear at various levels of vascular integrity, affecting veins and arteries, as well as the fetal-maternal circulatory system, resulting in miscarriage (Bennett et al., 2012). Arterial thrombosis is a major cause of death worldwide every year. Alternately, 1 in 1000 individuals

are afflicted with deep venous thrombosis (Rosendaal, 1999). Women with thrombophilia caused by individual (or a combination of) defects in antithrombin, protein C, or protein S production are at an increased risk of fetal loss, possibly leading to RPL (Preston et al., 1996).

Inherited thrombophilia induces abnormal coagulation in the utero-placental circulation during the early stages of pregnancy or an increased predisposition to placental infections, leading to late fetal loss, intrauterine growth retardation, and abruptio placentae (Kiwi, 2006).

In particular, approximately 15% of all women with RPL are afflicted with primary thrombophilia caused by the G1691A (FV Leiden or dbSNP: rs6025), G20210A (dbSNP: rs1799963), and C677T (dbSNP: rs1801133) combined with deficiency of natural anti-coagulant (antithrombin, C protein, and S protein) (Kiwi, 2006; Bennett et al., 2012). Several studies have reported an association between *F5* G1691A and *F2* G20210A polymorphisms and RPL, primarily during the first trimester, and late non-recurrent fetal loss (Bennett et al., 2012). According to various meta-analyses, mild hyperhomocysteinemia (HHC) is a risk factor for thrombosis in the presence of low plasma folate levels (Boushey et al., 1995; den Heijer et al., 1998). Likewise, HHC is strongly associated with recurrent early pregnancy loss, whereas the association between the C677T mutation related to HHC and RPL is controversial because of a dependent intake of folates and B vitamins (Nelen et al., 2000; Rey et al., 2003).

The association between the A1298C polymorphism (dbSNP: rs1801131) in *MTHFR* and RPL is also controversial. Nair et al. (2013) concluded that the presence of the A/C and C/C genotypes significantly increased the risk of RPL in an Indian population based on the results of their meta-analysis. However, another meta-analysis indicated that the A1298C polymorphism in *MTHFR* is not a risk factor for RPL (Rai, 2014).

Mutations in antifibrinolytic factors, as well as those in pro-coagulant factors, have been associated with RPL (Su et al., 2013). Normal fibrinolysis prevents excess fibrin deposition in the placental and fetal vasculature; moreover, fibrin polymerization stabilizes the placental basal plate (Su et al., 2013). The angiotensin I-converting enzyme Ins/Del (*ACE* I/D) and plasminogen activator inhibitor-1 (*PAI1*) 4G/5G polymorphisms, associated with low fibrinolysis, and their synergistic physiological effect on thrombosis, have also been extensively studied. ACE converts angiotensin I to angiotensin II, a potent vasoconstrictor that promotes vasoconstriction, inflammation, and thrombosis in the vascular endothelium and vessel walls (Su et al., 2013). Angiotensin II is a proatherogenic agent, which elevates the level of PAI-1, resulting in decreased fibrinolytic activity. Recent studies have indicated the important contribution of these hypofibrinolytic factors in the etiology of RPL (Su et al., 2013).

The *ACE* I/D polymorphism is involved in maternal uteroplacental and fetal circulation, thereby affecting the outcome of pregnancy (Mello et al., 2003). Previous meta-analyses have indicated that IRPL is strongly associated with the I/D polymorphism in *ACE* (dbSNP: rs4340). In fact, both the D/D and I/D genotypes have been shown to increase the risk of RPL by 29% (Su et al., 2013; Wang et al., 2013).

On the other hand, two previous meta-analyses have provided conflicting results regarding the association between the 4G/5G polymorphism in *PAI1* (dbSNP: rs587776796) and RPL (Sotiriadis et al., 2007; Su et al., 2013). RPL must be carefully studied in diverse ethnic populations to elucidate the role of the *PAI1* 4G/5G in preventing RPL (Su et al., 2013). Neither of these meta-analyses has included RPL patients from the Mexican-Mestizo population (Sotiriadis et al., 2007; Su et al., 2013).

The main objective of this study was to identify inherited thrombophilia markers (polymorphisms in prothrombotic and hypofibrinolytic risk factors) in patients with IRPL, and compare the results with those seen in a control group of women with normal pregnancy. This is the first study of this kind conducted in Mexico.

MATERIAL AND METHODS

Inclusion criteria for case and control groups

Fifty-six patients with IRPL (defined as three consecutive pregnancy losses prior to the 20th week of gestation), identified from a clinical database comprising 124 RPL Mexican-Mestizo patients, were included in this study. IRPL was diagnosed in the included patients after an exhaustive screening process to rule out RPL caused by secondary factors.

Patients with ectopic pregnancies or unfavorable anatomic factors, such as cervical insufficiency, intrauterine adhesion, uterine fibroids, and uterine polyps, were ruled out. The included patients tested negative for antiphospholipids, anti-DNA, and antinuclear and anticardiolipin antibodies. Lupus-like anticoagulants were used to rule out acquired thrombophilia due to lupus and antiphospholipid syndrome. Multiple-standard laboratory tests were performed to rule out endocrinal causes of RPL, including hyperthyroidism, hypothyroidism, hyperprolactinemia, luteal insufficiency, and diabetes. The patients were also tested for the TORCH panel, and subjected to vaginal screening (for bacteria and fungi), to rule out the presence of infectious diseases. Chromosomal abnormalities, including translocation, trisomy, and triploidy, were evaluated by cytogenetics experts. Fifty women with at least two normal full-term pregnancies and no more than one gestational loss were recruited as controls. The clinical, demographic, and hereditary information was obtained from all recruited individuals via a structured questionnaire. The included individuals were confirmed to be unrelated up to the previous two generations.

Molecular analysis

The thrombophilic marker profiles of the recruited IRPL patients and control subjects were constructed by analyzing the following RFLPs in these populations: *F5* G1691A, *F2* G20210A, *MTHFR* C677T-A1298C, *PAI1* 4G/5G. The *ACE* I/D marker was assessed in only 55 IRPL patients. Blood samples were collected from all patients and control subjects in EDTA-Na$_2$ tubes, and genomic DNA was purified from the peripheral blood leukocytes (10 mL) using a standard salting-out method (Miller et al., 1988). The selected markers were genotyped using previously described methods: *F5* G1691A, *F2* G20210A (Ripoll et al., 1997), *MTHFR* C677T (Frosst et al., 1995), *MTHFR* A1298C (van der Put et al., 1998), *ACE* I/D (Rigat et al., 1992), and *PAI1* 4G/5G (Isordia et al., 2009). The genotypes were identified by polyacrylamide gel electrophoresis (PAGE; 29:1, 6%) and stained by silver nitrate. The samples were genotyped at CIBO, IMSS (Guadalajara, Mexico).

Statistical analysis

Non-parametric statistical analyses, including the Fisher exact test and the Pearson chi-square exact test, were performed using SPSS v.22 for Windows (IBM, Armonk, NY,

USA). Independent risk of IRPL as a result of polymorphisms in various thrombotic factors was estimated by odds ratio (OR) and its 95% confidence interval (CI). The conformance of genetic markers in the control group with the Hardy-Weinberg equilibrium was also analyzed.

Ethical considerations

Written informed consent was obtained from all IRPL patients and control individuals prior to the study. All procedures and DNA sample collection were performed in accordance with the principles of the Helsinki Declaration, and were approved by the Institutional Scientific and Ethics Committee (registration number R-2010-1305-3).

RESULTS

The control subjects were age-matched with the patients with IRPL. The clinical and demographic characteristics of the controls and patients with IRPL are summarized in Table 1. The gravidity traits were also compared among the two groups (Table 1).

Table 1. Clinical and demographic characteristics of patients in the idiopathic recurrent pregnancy loss (IRPL) and control groups.

Clinical/demographic data	Mean (range)	
IRPL group (N = 55) Gravidity (cumulative total, N = 203)	4 (2-6)	
Control group (N = 50) Gravidity (cumulative total, N = 187)	3 (1-4)	
IRPL group (N = 47), age (years)	30 (18-41)	
Control group (N = 50), age (years)	30 (18-42)	
IRPL group (N = 49), BMI (kg/m^2)	27.6 (19-45)	
IRPL group (N = 55) GWMO (cumulative group total weeks, N = 1406)	9 (5-15)	
Miscarriage (M)/Parity (P)	M [N (%)]/P [N (%)]	P value[a]
IRPL group, total gravidity (N = 203) Miscarriage/Parity	161 (79)/42 (21)	<0.001
Control group, total gravidity (N = 187) Miscarriage/Parity	8 (4)/179 (96)	

[a]Fisher exact test. BMI = body mass index; GWMO = gestational week when miscarriage occurred.

The results of the molecular analyses (of the IRPL patients and the control subjects) are summarized in Table 2. The prothrombotic factor *MTHFR* 677TT was most frequently observed in IRPL patients (23%), followed by the compound-heterozygous *MTHFR* C677T-A1298C (16%) and the heterozygous *F2* 20210GA (3.6%) mutations (Table 2). The major hypofibrinolytic risk factor of IRPL was found to be the heterozygote genotype of *ACE* (I/D) (62%), followed by the homozygote genotype of *PAI1* (4G/4G; 18%) (Table 2).

We found no statistically significant differences in the genotype expression of the prothrombotic factors *F5* G1691A, *F2* G20210A, and *MTHFR* C677T-A1298C between the IRPL and control groups. However, the frequency of the heterozygous genotype of the I/D polymorphism in the hypofibrinolytic factor *ACE I/D* was significantly different (P = 0.031) between the IRPL patients and control subjects. Conversely, the genotype frequencies of the *PAI1* 4G/5G polymorphism did not differ significantly among the two groups (Table 2). The dominant genetic model D/D+I/D *vs* I/I (of *ACE* I/D) had an OR (95%CI) of 2.89 (1.22-6.89) (P = 0.019) in the analyzed Mexican-Mestizo population.

Table 2. Prothrombotic and hypofibrinolytic genotypes in idiopathic recurrent pregnancy loss (IRPL) patients and controls.

	IRPL group (N = 56)	Control group (N = 50)	P value
F5 G1691A [N (%)]			
A/G	1 (1.8)	1 (2)	>0.99[a]
G/G	55 (98.2)	49 (98)	
F2 G20210A [N (%)]			
A/G	2 (3.6)	2 (4)	>0.99[a]
G/G	54 (96.4)	48 (96)	
MTHFR C677T [N (%)]			
C/C	17 (30.4)	12 (24)	0.657[b]
C/T	26 (46.4)	23 (46)	
T/T	13 (23.2)	15 (30)	
MTHFR A1298C [N (%)]			
A/A	42 (75)	37 (74)	0.907[b]
A/C	13 (23)	13 (26)	
C/C	1 (2)	0 (0)	
MTHFR C677T-A1298C [N (%)]			
C/T + A/C	9 (16.1)	8 (16)	>0.99[b]
T/T + A/C	1 (1.8)	0 (0)	
C/C + A/A + C/C	46 (82.1)	42 (84)	
PAI1 4G/5G [N (%)]			
4G/4G	10 (17.9)	7 (14)	0.444[b]
4G/5G	25 (44.6)	18 (36)	
5G/5G	21 (37.5)	25 (50)	
ACE I/D [N (%)]	IRPL patients (N = 55)		
D/D	10 (18)	10 (20)	0.031[b]
D/I	34 (62)	19 (38)	
I/I	11 (20)	21 (42)	

[a]Fisher exact test. [b]Exact results of Pearson chi-square test.

DISCUSSION

RPL has a multifactorial etiology; in some cases, therefore, it is not possible to identify its cause. A large number of recurrent pregnancy losses are idiopathic. Forty-five percent of the analyzed cases (N = 124) presented IRPL, which was similar to a previously reported percentage of IRPL cases (50%) (Thomas and van der Spuy, 2010).

Pregnancy has been recognized as a hypercoagulable state; therefore, concomitant thrombophilic risk factors may enhance the thrombotic risk and impair fetal circulation, or cause thrombotic vasculopathy in the placenta, resulting in fetal loss (Bennett et al., 2012; Su et al., 2013). The low frequencies of prothrombotic factors *F5* G1691A and *F2* G20210A observed in the IRPL group (1.8 and 3.6%, respectively) were consistent with the reported frequencies in a review of diverse RPL populations (Bradley et al., 2012). The frequencies of these factors did not differ significantly between patients with IRPL and control individuals (women without IRPL). This result was similar to that reported in Mexican-Mestizo RPL patients by Quintero-Ramos et al. (2006), whose control group was comprised of normal women and men belonging to the same geographic region as included in our study. In contrast, Bradley et al. (2012) reported a significant association between the two groups in their review, wherein the OR was ~2 for both mutations in RPL patients (Bradley et al., 2012).

Elevated homocysteine levels due to mutations in *MTHFR* have been reported as risk factors for the pathogenesis of RPL; however, there has been no consensus regarding the association between *MTHFR* polymorphisms and IRPL (Cao et al., 2013). In this study, the 677TT and 1298CC genotypes were found at frequencies of 23 and 2% in the IRPL

patients, respectively. The 677TT genotype was the most frequent prothrombotic risk factor in women with IRPL; however, the genotype distributions of the *MTHFR* 677TT and 1298CC polymorphisms were not significantly different between the case and control groups. Cao et al. (2013) reported the highest OR (2.1) for the 677TT genotype in Asian women with IRPL; however, the *MTHFR* 1298CC polymorphism was not significant, consistent with previous meta-analyses (Cao et al., 2013; Rai, 2014). This result was also similar to that reported by Quintero-Ramos et al. (2006). However, the results reported by Rodríguez-Guillén et al. (2009) in a small group (23 cases) of Mexican women with spontaneous abortion and 74 normal controls were different; for example, these authors reported high ORs for the 677TT and 1298AC genotypes (5 and 5.5, respectively). Previous reports have suggested that compound heterozygous 677CT/1298AC individuals express 50% MTHFR activity, similar to those individuals with homozygous *MTHFR* 677TT (Weisberg et al., 1998). The frequency of the compound heterozygous genotype of *MTHFR* is reportedly very high in IRPL cases (16.1%), but did not differ significantly from the frequency observed in the control group (Zetterberg et al., 2002; Mtiraoui et al., 2006).

Early human trophoblastic invasion is regulated by the fibrinolytic system; plasmin promotes trophoblastic invasion by activating matrix metalloproteinases that directly degrade various components of the extracellular matrix of the decidua. Thus, hypofibrinolytic factors can lead to IRPL.

The renin-angiotensin system (RAS) is activated in normal pregnancy, and the levels of involved proteins, such as angiotensinogen, angiotensin II, and aldosterone, are markedly elevated. Moreover, angiotensin II levels are modulated by ACE (Su et al., 2013). Uteroplacental and fetal umbilical circulation are dependent on the RAS system; moreover, Mello et al. (2003) suggested that the I/D polymorphism in *ACE* induces abnormal hypertensive maternal uteroplacental and fetal umbilical flow.

The most common risk factor for IRPL in our population was the heterozygote genotype *ACE* I/D (62%); this frequency was significantly different from that seen in the control group (Table 2) under a dominant genetic model. The OR of the *ACE* D/D+I/D *vs* I/I model was 2.89 (95%CI = 1.22-6.89; P = 0.019). These data are concordant with the data obtained by Su et al. (2013) in a previous meta-analysis conducted in RPL patients (OR of a dominant genetic model = 1.29; 95%CI = 1.02-1.62), which indicated a significant association between the *ACE* I/D polymorphism and RPL; moreover, women carrying the D/D or I/D genotypes were found to be at a 29% higher risk of developing RPL, compared to those expressing the I/I genotype (Su et al., 2013). This supports our results, as well as those reported by Wang et al. (2013), who established a dominant genetic model of the *ACE* I/D polymorphic variants contributing to RPL susceptibility (summary OR = 1.44; 95%CI = 1.05-1.97). However, Quintero-Ramos et al. (2006) reported that the genotype distribution of the *ACE* I/D polymorphism did not differ significantly between IRPL patients (idiopathicity not exhaustively described) and normal Mexican-Mestizo controls. The differences between our results and those reported by Quintero-Ramos et al. (2006) could be attributed to the inclusion of both male and female individuals in their control group (N > 200), compared to our woman-only (expressing a successful pregnancy phenotype) control population.

Despite the role played by PAI-1 in inhibiting fibrinolysis, and the effect of the 4G/4G polymorphism in *PAI1* on the PAI-1 levels, polymorphisms in the marker have not been associated with RPL in previous meta-analyses (Sotiriadis et al., 2007; Su et al., 2013), similar to the results seen in our IRPL study population. The frequency of the homozygous 4G/4G

and heterozygous 4G/5G genotypes in *PAI1* was quite high in the IRPL population (17.9 and 44.6%, respectively); this was concordant with the results reported by Flores-Alatriste et al. (2014) in a Mexican population with RPL (homozygous 4G/4G and heterozygous 4G/5G frequencies = 22 and 63%, respectively).

The results of this study were similar to those of previous meta-analyses (Su et al., 2013; Wang et al., 2013) reporting an association between the *ACE* I/D polymorphism and IRPL; this could be attributed to the role of ACE in uteroplacental and fetal umbilical circulation. However, this polymorphism is only a partial contributor (29%) to RPL, which is a polygenic condition (Su et al., 2013). Therefore, related genetic components, such as those involved in angiogenesis (leading to the establishment of feto-placental circulation), must be studied further. Samli et al. (2012) reported a significant association between the 1154 G/A polymorphism in the vascular endothelial growth factor gene (*VEGF*) and RLP. Future studies into polymorphisms occurring in factors influencing thrombophilia and angiogenesis would help confirm the role of impaired utero-fetal circulation in IRPL.

In conclusion, this is the first case-control study of prothrombotic and hypofibrinolytic genetic markers of IRPL in a Mexican Mestizo population. We observed a significant association (P = 0.019) between the *ACE* I/D polymorphic variant and risk of idiopathic recurrent pregnancy loss. Further well-designed studies with adequate inclusion criteria and cohorts are required to identify various genetic haplogroups of prothrombotic and hypofibrinolytic risk factors, the genetic factors affecting uteroplacental and fetal umbilical flows, and the alterations in various environmental elements involved in trophoblast invasion, which are some of the major causative factors of IRPL.

Conflicts of interest

The authors declare no conflict of interest.

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