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Prevalence of coagulation factor XIII and plasminogen activator inhibitor-1 gene polymorphisms among Egyptian women suffering from unexplained primary recurrent miscarriage

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ABSTRACT

Recurrent miscarriage (RM) is an obstetric challenge. Polymorphisms of factor XIII (FXIII) and plasminogen activator inhibitor-1 (PAI-1) may cause an imbalance between coagulation and fibrinolysis that can end in RM. The aim of the work was to determine the prevalence of FXIII Val34Leu and PAI-1 4G/5G gene polymorphisms in Egyptian women presenting with unexplained primary first trimester RM. Genotyping of 120 unexplained primary first trimester RM patients and 130 healthy controls by polymerase chain reaction (PCR) amplification of target genes followed by the allele-specific restriction enzyme digestion (RFLP technique). Among the cases, 67.5% of individuals had wild-type FXIII; 21.7% were heterozygous and 10.8% were homozygous for the FXIII Val34Leu polymorphism. Among controls, the proportions were 89.2%, 8.5% and 2.3% respectively. In addition, comparison between the two groups regarding Leu and 4G allele frequencies showed statistically significant differences (P values = 0.0001 and 0.027 respectively). RM is more frequent in women with combined polymorphisms than in women with a single gene polymorphism (RR = 3.91; OR = 4.51; 95% CI = 1.79–11.38; P = 0.002). FXIII Val34Leu and PAI-1 4G/5G polymorphisms are prevalent in Egyptian women, with unexplained primary first trimester RM and combined polymorphisms statistically increasing the risk.

1. Introduction

Recurrent miscarriage (RM) refers to three or more consecutive pregnancy losses before viability (defined as less than 24 weeks' gestation in the UK) (Royal College of Obstetricians and Gynaecologists, 2011). Using this definition, RM affects about 1% of couples trying to have children (Royal College of Obstetricians and Gynaecologists, 2011).

There are various alleged causes of RM, some of which may be amenable to treatment. Among the identifiable risk factors of RM are genetic, anatomical, endocrine, epidemiological and immune factors, infectious agents, antiphospholipid syndrome (APS), and inherited thrombophilic defects (Royal College of Obstetricians and Gynaecologists, 2011).

Despite major advances in genetics, biology and immunology, RM still poses a challenging and frustrating

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condition for the patient and her obstetrician. Routine clinical investigations identify a possible "cause" of RM in less than 50% of cases (Li et al., 2002).

Some investigators have found that women with RM have different frequencies of polymorphisms of genes involved in coagulation and fibrinolysis than women with normal reproductive outcomes (Buchholz et al., 2003). This had led to the suggestion that related disturbances in the balance between coagulation and fibrinolysis or perturbations in proteolysis and remodeling of maternal tissue may have an adverse impact on trophoblast invasion (Buchholz et al., 2003).

Factor XIII (FXIII) is a transglutaminase, which plays an important role in placentation during the first trimester of pregnancy (Kappelmayer et al., 1994). A common G-to-T polymorphism in exon 2 causes a valine (Val) to leucine (Leu) change at position 34 (Val34Leu). Presence of the Val34Leu polymorphism in exon 2 of the A-chain FXIII gene could have an anti-fibrinolytic effect through the early cross-linking of fibrin with a tendency toward hemorrhage, a potential risk factor for RM (Buchholz et al., 2003).

In addition, for the successful implantation of the fertilized egg, invasion of the cytotrophoblast to the proper depth of the uterus is crucial. It provides anchorage for the conceptus and promotes the adaptation of the uteroplacental circulation (Kappelmayer et al., 1994). Plasminogen activator inhibitors are up-regulated during implantation to modify the invasion of the trophoblast cells and to prevent hemorrhage during placentation (Feng et al., 2000). PAI-1 also plays a vital role in hypofibrinolysis and thrombotic complications. PAI-1 gene expression is modulated by a 4G/5G polymorphism in the PAI-1 promoter, 675 bp upstream from the start site of transcription (Floridon et al., 2000).

This study aimed to investigate the prevalence of FXIII Val34Leu and PAI-1 4G/5G gene polymorphisms in unexplained primary first trimester RM in Egyptian women.

2. Subjects and methods

2.1. Study population

This comparative (case–control) observational study was conducted in the Departments of Obstetrics and Gynaecology and Clinical and Chemical Pathology, Kasr Al-Aini Hospital, Cairo, Egypt, during the period from January 9, 2011 to August 20, 2013. After obtaining ethics committee approval, we collected 120 serum samples from non-pregnant women with a history of unexplained, recurrent first trimester miscarriage and no prior live births (primary RM). Controls included 130 healthy women who had had no previous miscarriages and had completed at least two successive pregnancies without complications. All cases and controls were Egyptians, non-smokers, and were of similar age, weight, and BMI. All women who enrolled in the study gave informed consent.

All subjects underwent a history and physical examination. Cases had known or suspected "causes" of RM excluded via an evaluation that included normal findings for sonohysterography and parental karyotyping, as well as normal concentrations or negative results for fasting and 2-h post-prandial blood glucose (70–100 mg/dL and 90–130 mg/dL respectively), thyroid-stimulating hormone (0.4–4.5 μIU/mL), serum free triiodothyronine (2.3–4.2 pg/mL), serum free thyroxine (0.8–1.8 ng/L), mid-luteal phase progesterone (4.5–25.2 ng/mL), early follicular serum follicle-stimulating hormone (less than 9 IU/L), early follicular serum luteinizing hormone (2.4–12.6), anticardiolipin IgG and IgM, anti-β2 glycoprotein-1 IgG and IgM, lupus anticoagulant screen, protein C activity, protein S activity, antithrombin III activity, Toxoplasma IgM (negative), cytomegalovirus IgM (negative), rubella IgM (negative), and Herpes simplex type 2 IgM (negative). Eligible candidates (cases and controls) were subjected to the genotyping of the −675 4G/5G polymorphism in the promoter region of the PAI-1 gene and the Val34Leu polymorphism of the A-chain factor XIII gene.

2.2. Exclusion criteria

Women with unexplained secondary RM and cases with second trimester RM were excluded from this study. All subjects were at least 3 months post-delivery or miscarriage.

2.3. Sample collection for genotyping

Five milliliters of venous blood was collected from eligible candidates in ethylenediaminetetraacetic acid (EDTA) by sterile venipuncture using a sterile vacutainer tube. Samples were stored in the same vacutainer at −20 °C for genomic DNA extraction and subsequent PAI-1 and FXIII genotyping. DNA extraction was carried out using Gene JET™ Genomic DNA Purification Kit-Fermentas.

Genotyping of the −675 4G/5G polymorphism in the promoter region of the PAI-1 gene and the FXIII Val34Leu polymorphism was performed by the PCR technique using primers (shown in Table 1) purchased from BIONEER, EU.

The reaction was obtained using Dream Taq™ Green PCR Master Mix supplied by Fermentas. All reactions were performed in a total volume of 25 μL, 12.5 μL of Ready-to-use PCR Master Mix, 1 μL of forward primer, 1 μL of reverse primers, 5 μL of genomic extracted DNA and completed by 5.5 μL of water nuclease-free. Initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. Final extension was conducted at 72 °C for 5 min. The amplification products were analyzed by gel electrophoresis (3%). After generation of the PCR product, BseRI (catalog number R0581S, Biolabs) and DdeI (catalog number FD1884, Fermentas, EU), restriction enzymes were added and incubated at 37 °C for 15 min for the identification of the −675 4G/5G polymorphisms in the promoter region of the PAI-1 gene, and FXIII Val34Leu polymorphism respectively, followed by detection using agarose gel electrophoresis and ultra-violet light transillumination.

2.4. Interpretation of genetic analysis

A PCR product of the FXIII Val34Leu polymorphism had a length of 192 bp with no restriction site for the DdeI

Table 1
Primers of the −675 4G/5G polymorphism in the promoter region of the PAI-1 gene and FXIII Val34Leu polymorphism.

Polymorphism	Primer	PCR product	Restriction enzyme
PAI-1 −675 4G/5G	5′-CAC AGA GAG AGT CTG GAC ACG TGA-3′[a] 5′-TGC AGC CAG CCA CGT GAT TGT CTA-3′[b]	148 bp	BseRI
Factor XIII Val34Leu	5′-CAT GCC TTT TCT GTT GTC TTC-3′[a] 5′-TAC CTT GCA GGT TGA CGC CCC GGG GCA CTA-3′[b]	192 bp	DdeI

[a] The forward primer.
[b] The reversed primer.

enzyme. The mutation in FXIII created a restriction site and produced two fragments (161 bp and 31 bp) after enzyme digestion. The original 148-bp PCR product of the PAI-1 gene has no restriction site for the BseRI enzyme, but the combination of nucleotide substitution and genetic abnormality creates the enzyme cleavage site and cleaves the amplicon into two fragments (110 bp and 38 bp).

2.5. Statistical methods

Data were statistically described in terms of mean ± standard deviation (±SD), median (range), or frequencies (number of cases) and percentages where appropriate. Comparison of numerical variables between the study groups was carried out using Student's *t*-test for independent samples. Stratified, the Mantel–Haenszel method (2 × 2 tables) was used to calculate the relative risks, odds ratios, and 95% CI for each gene mutation in the cases and controls. To compare the categorical data, the Chi-squared (χ^2) test was performed. Fisher's exact test was used instead when the expected frequency was less than 5. *P* values less than 0.05 were considered statistically significant. All statistical calculations were performed using SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

2.6. Sample size calculation

Before this study, sample sizes were determined using an a priori estimate of the prevalence of FXIII Val34Leu and 4G/5G PAI-1-gene polymorphisms and a significance level of 0.05 and a power of 95% (G*Power 3.1.3; Franz Faul, Christian-Albrechts-Universität, Kiel, Germany). We found that 113 subjects were required in each arm. We corrected the sample size by adding 24 samples (∼10%) to compensate for discontinuations (expected to be more in the control group); thus, we recruited 120 samples from cases and 130 samples from the control group.

3. Results

Pertinent clinical and demographic features of the cases and controls are shown in Table 2. The mean ages (±SD) of the cases and controls were 28.50 (±4.61) and 29.10 (±3.12); these were not statistically different. The median (range) weight and BMI were 60.6 (45.1–88.6) and 24.5 (18.1–36.7) for the cases and 61.1 (46.2–87.3) and 23.9 (17.8–37.4) for the controls. The differences were not statistically significant.

Among the RM cases, 26 women (21.7%) were heterozygous for the FXIII Val34Leu polymorphism and 13 (10.8%) were homozygous, while among the controls, 11 (8.5%) were heterozygous and 3 (2.3%) were homozygous for this polymorphism. These differences were statistically significant ($P = 0.0151$ and 0.0197 respectively; Table 3). The prevalence of the PAI-1 4G/5G polymorphism among cases was 37 (30.8%) for the heterozygous state and 8 (6.7%) for the homozygous state vs 28 (21.5%) and 3 (2.3%) respectively, for the controls. Neither of these differences was statistically significant ($P = 0.2$ and 0.12 respectively; Table 3).

With regard to the risk of RM, we found that women with RM are more often carriers of the heterozygous and homozygous factor XIII polymorphism than controls. This suggests that carriers of the heterozygous and homozygous factor XIII polymorphism might be at an increased risk of RM. In addition, a clear relation to the risk of RM was established with the homozygous FXIII Val34Leu polymorphism (RR = 5.49; Table 4).

Regarding the PAI-1 4G/5G polymorphism, we found that women with RM were more likely to carry the 4G allele ($P = 0.027$), while the frequency of the heterozygous and homozygous polymorphisms did not reach statistical significance between the cases and controls.

In order to extend our results we further subdivided the study population into combined (FXIII Val34Leu and PAI-1 4G/5G) and single gene polymorphism subgroups to study the frequency of RM in each subgroup. We observed

Table 2
Clinical data of the groups studied.

	Cases ($n = 120$)	Controls ($n = 130$)	*P* value
Age in years (SD)	28.50 ± 4.61	29.10 ± 3.12	0.226
No. of RM	4.0 ± 1.82	–	–
Gestational age at miscarriage (weeks)	8.76 ± 0.76	–	–
No. of smokers (%)	0	0	0.968
Median weight in kg (range)	60.6 (45.1–88.6)	61.1 (46.2–87.3)	0.119
Median BMI in kg/m^2 (range)	24.5 (18.1–36.7)	23.9 (17.8–37.4)	0.263

Table 3
The FXIII Val34Leu polymorphism and PAI-1 4G/5G polymorphism of the groups studied.

FXIII Val34Leu polymorphism			
Wild-type	81/120 (67.5%)	116/130 (89.2%)	0.1502
Heterozygous	26/120 (21.7%)	11/130 (8.5%)	0.0151*
Homozygous	13/120 (10.8%)	3/130 (2.3%)	0.0197*
Val allele frequency	188 (78.3%)	243 (93.5%)	0.1832
Leu allele frequency	52 (21.7%)	17 (6.5%)	0.0001**
PAI-1 4G/5G polymorphism			
Wild-type	75/120 (62.5%)	99/130 (76.2%)	0.320
Heterozygous	37/120 (30.8%)	28/130 (21.5%)	0.201
Homozygous	8/120 (6.7%)	3/130 (2.3%)	0.124
4G allele frequency	53 (22.1%)	34 (13.1%)	0.027*
5G allele frequency	187 (77.9%)	226 (86.9%)	0.412
Combined FXIII Val34Leu and PAI-1 4G/5G polymorphisms	25/120	6/130	0.002*
Both heterozygous	14/120	3/130	0.014*
34Leu + 4G/5G	6/120	1/130	0.088
Val34Leu + 4G/4G	3/120	1/130	0.312
Both homozygous	2/120	1/130	0.530

* Significant.
** Highly significant.

Table 4
Relative risks of RM in the PAI-1 4G/5G and FXIII Val34Leu polymorphisms.

	OR	95% CI	*P* value
4G/5G vs 5G/5G	1.74	0.98–3.10	0.058
4G/4G vs 4G/5G	2.02	0.49–8.30	0.331
4G/4G vs 5G/5G	3.52	0.90–13.72	0.070
Val34Leu vs 34Val	3.38	1.58–7.24	0.002*
34Leu vs Val34Leu	1.83	0.43–7.73	0.409
34Leu vs 34Val	6.21	1.71–22.48	0.005*
Combined mutations	4.51	1.79–11.38	0.002*

* Significant.

that RM is more frequent in the women with combined FXIII Val34Leu and PAI-1 4G/5G polymorphisms than in the women with single gene polymorphism (RR = 3.91; OR = 4.51; 95% CI = 1.79–11.38; $P = 0.002$), as shown in Tables 3 and 4.

4. Discussion

In this case–control study, we demonstrated that heterozygous and homozygous FXIII Val34Leu polymorphisms and Leu allele frequency were significantly more common in women with RM than in controls. In addition, this study demonstrated that the prevalence of homozygous and heterozygous PAI-1 4G/5G polymorphism did not differ significantly between cases and controls, although the 4G allele frequencies was more frequent in cases. Moreover, our findings do suggest that RM might be more common in women with combined FXIII Val34Leu and PAI-1 4G/5G polymorphisms than in women with a single gene polymorphism.

Similar results were reported by Goodman et al. (2006), who found that the FXIII Val34Leu genotype was statistically more frequent in women with RM than in controls, and by Yenicesu et al. (2010), who reported that the heterozygous mutations of FXIII Val/Leu were significantly more frequent in RM women than in control women. Earlier studies by Barbosa et al. (2004), Sotiriadis et al. (2007), and Bagheri et al. (2011), in contrast, did not reveal significant differences between cases and controls with regard to the FXIII Val/Leu polymorphism.

The differences between these studies might be attributable to the different ethnicities of the populations studied. The gene frequencies of North African populations, including Egypt, are intermediate between those of the Near East, the Horn of Africa, southern Europe and Sub-Saharan Africa (Cavalli-Sforza et al., 1994).

Regarding the PAI-1 4G/5G polymorphism, we also observed that women with primary RM were more likely to carry the 4G allele, although the prevalence of the heterozygous and homozygous polymorphisms did not differ significantly between the cases and controls. Similar results were obtained by Buchholz et al. (2003), Dossenbach-Glaninger et al. (2003), and Al Sallout and Sharif (2010), none of whom found significant differences between women with RM and controls. In contrast, Aarabi et al. (2011) and Torabi et al. (2012) found a significantly higher prevalence of the 4G/4G genotype in Iranian women with RM than in controls.

To our knowledge, ours is the first study to associate the FXIII Val/Leu PAI-1 4G/5G polymorphisms with RM in the Egyptian population. We also demonstrate that the RM is more prevalent among women with combined FXIII Val/Leu and PAI-1 4G/5G polymorphisms than among women with a single gene polymorphism (RR = 3.91; OR = 4.51; 95% CI = 1.79–11.38; $P = 0.002$). These findings are comparable with those of a previous study by Dossenbach-Glaninger et al. (2003), who concluded that the risk of early pregnancy loss was significantly increased in women with homozygosity of either FXIII Val/Leu or PAI-1 4G/5G or with a compound carrier status of both mutations.

In conclusion, our results demonstrate that the FXIII Val34Leu polymorphism and combined FXIII Val/Leu and PAI-1 4G/5G polymorphisms are statistically more common in Egyptian women with unexplained primary first-trimester RM than in controls. In vitro mechanistic studies may identify potential therapeutic targets. We recommend larger studies of well-characterized women with

RM to confirm our findings, as well as longitudinal studies of RM patients with or without these polymorphisms to determine whether or not the polymorphisms are of prognostic significance.

Conflict of interest

The authors declare that they have no conflict of interest.

Source of support

None.

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