Original Article

Polymorphisms of plasminogen activator iInhibitor-1 4G/5G, coagulation factor XIII Val34 Leu and angiotensin converting enzyme I/D impact on recurrent implantation failure

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ABSTRACT

Repeated implantation failure (RIF) is recognized when transferred embryos fail to implant following repeated in vitro fertilization (IVF) cycles. A functional balance of fibrinolysis and coagulation may secure adequate trophoblast invasion, which is crucial for a regular implantation. During cytotrophoblast invasion, plasminogen activator inhibitor 1 (PAI-1) appears to be involved in controlling proteolysis and remodeling of maternal tissue. Coagulation factor XIII (FXIII) may have an impact on the ability of the trophoblast to invade into the endometrium and to stabilize attachment with fibrin cross-linking. Angiotensin converting enzyme (ACE) participates in the regulation of vascular tone and changes in vascular metabolites affect the functions of the fetoplacental complex and may induce abnormalities of blood circulation in the placenta. The aim of this study was to assess the predictive value of PAI-1 4G/5G, FXIII Val34Leu and ACE I/D polymorphisms on the IVF outcome in Egyptian women with repeated IVF failure. The present study was conducted on 60 women with repeated IVF failure, three or more previous IVF-embryo transfer cycles and 60 healthy age-matched women eligible for IVF. PCR-RFLP for the PAI-1 4G/5G, FXIII Val34Leu and ACE I/D polymorphisms was done for cases and control groups. Cases with RIF showed higher prevalence of the 4G allele of the PAI-1 -675 4G/5G polymorphism (*P = 0.029*). Higher frequencies of the heterozygous and homozygous FXIII polymorphism were noted in cases (*P = 0.016, 0.020*, respectively) together with higher risk to carry the Leu allele (*P = 0.001*). The heterozygous ACE I/D polymorphism was predominant in the studied cases (*P = 0.011*). The data point to the importance of the PAI-1 4G/5G and the FXIII Val34Leu and to a lesser extent the ACE I/D, polymorphisms as possible biomarkers to select populations at risk of implantation failure prior to attempting pregnancy. Nevertheless, larger scale studies are recommended to support the results.

Key Words: IVF, Polymorphisms, pregnancy, recurrent implantation failure

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INTRODUCTION

Repeated implantation failure (RIF) is recognized when transferred embryos fail to implant following repeated in vitro fertilization (IVF) cycles [1]. RIF is commonly defined as the failure of implantation after three or more consecutive IVF attempts, with which 1-2 high-grade embryos were transferred in each cycle [2]. Invasion of the cytotrophoblast to the proper depth of the uterus provides anchorage for the conceptus which is needed for successful implantation [3]. A functional balance of fibrinolysis and coagulation may secure adequate trophoblast invasion which is crucial for a regular implantation [4].

During cytotrophoblast invasion, plasminogen activator inhibitor 1 (PAI-1) appears to be involved in controlling proteolysis and remodeling of maternal tissue[5]. PAI-1 inhibits tissue- and urokinase-type plasminogen activators, thus decreasing plasmin production and the dissolution of fibrin clots. The PAI-1 gene is located on the long arm of chromosome 7 (7q21.3-q22). Plasma PAI-1 levels are related to a common guanosine insertion/deletion gene polymorphism, 4G/5G, 675 bp upstream from the start site of translation and higher concentrations have been associated with homozygosity for the deletion genotype (4G/4G) than those associated with the insertion genotype (5G/5G), and hence with impaired fibrinolytic activity[6]. PAI-1, on one hand, inhibits urokinase plasminogen activator (uPA)-urokinase plasminogen activator receptor (uPAR) leading to inhibition of trophoblast invasion[7] and, on the other, it may initiate or intensify the trophoblast invasion process[8]. However, an

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association has been found between PAI-1 -675 4G/5G polymorphism and recurrent pregnancy loss (RPL) [9] and to a lesser extent RIF [10].

Coagulation factor XIII (FXIII) covalently cross-links fibrin and affects fibrinolysis, thus it may have an impact on the ability of the trophoblast to invade into the endometrium and to stabilize attachment with fibrin cross-linking. FXIII Val34Leu polymorphism in exon 2 of the FXIII gene is associated with earlier cross-linking, formation of a finer fibrin meshwork and reduced susceptibility to fibrinolysis[11]. This polymorphism has been reported to carry an elevated overall risk for RPL in women homozygous for the FXIII 34Leu [12].

Angiotensin converting enzyme (ACE) is a dipeptidyl carboxypeptidase which is encoded by the ACE gene located on chromosome 17q23 and contains 26 exons and 25 introns[13]. The ACE insertion (I)/deletion (D) polymorphism was strongly associated with the level of the circulating enzyme, where the D allele was associated with an elevated level [14]. The ACE participates in the regulation of vascular tone and changes in vascular metabolites were found to affect the functions of the fetoplacental complex and may induce abnormalities of blood circulation in the placenta resulting in RPL [15].

The aim of this study was to assess the predictive value of the polymorphisms of PAI-1 4G/5G, FXIII Val34Leu and ACE I/D on the IVF outcome in Egyptian women with repeated IVF failure. They were compared to healthy control patients eligible for IVF. Biochemical diagnosis of pregnancy was the end-point of the study.

PATIENTS AND METHODS

The present study was conducted on 60 women with repeated IVF failure. They were selected from the IVF unit, Department of Obstetrics and Gynaecology at El-Shatby University Hospital, Alexandria University between October 2014 and June 2016. The evolution of pregnancy was not recorded.

Sixty healthy age-matched women eligible for IVF were selected as a control group without any known hereditary or acquired thrombophilic alteration or personal history of thrombotic or bleeding disorder and without any personal history of miscarriage. The protocol of the study was in accordance to the commitment of the Helsinki declaration and was approved by the institutional ethics committee. All subjects provided informed written consent before inclusion in the study.

EXCLUSION CRITERIA

Women younger than 20 years or older than 37 years, weight less than 50 kg or more than 100 kg, with a personal or family history of venous thromboembolism (VTE) or hereditary or acquired thrombophilia, active anticoagulant or antiplatelet treatment or use of these agents during the last 30 days before inclusion, abnormal full blood count or platelet count and ongoing cardiovascular, renal or liver disease, malignancy, or arterial hypertension, known systematic or chronic disease (autoimmune syndrome, heart disease, severe or uncontrolled thyroid disease or HIV infection), treatment with non-steroid anti-inflammatory drugs within the last 10 days before inclusion, ovarian insufficiency (FSH > 9 IU/ml and/or number of antral follicles <8) or polycystic ovary syndrome (defined according to the Rotterdam criteria).

Methodology:

This prospective controlled trial included 120 women indicated for IVF/ICSI treatment treated in our IVF unit. All couples had a standard infertility evaluation that included a semen analysis using WHO criteria, evaluation of tubal patency either by hysterosalpingography (HSG) or laparoscopy, a baseline trans-vaginal ultrasonography and a baseline hormonal profile that included FSH, LH, TSH, and PRL in the early follicular phase.

All participants were counseled about the risks and benefits of enrollment in the study and an informed consent was obtained. The study was approved by the ethics committee of the Alexandria Faculty of Medicine. Patients who have agreed to continue with ICSI were divided into two groups, one with history of recurrent ICSI failure (60) and control group with no prior history of ICSI treatment (60)

All women in both groups had full blood count, platelet count, prothrombin time, activated partial thromboplastin time, fibrinogen, renal and liver function within the normal range.

Controlled ovarian stimulation was done using either agonist or antagonist protocols combined with ovarian stimulation with recombinant human follicular stimulating hormone (FSH) in addition to human menopausal gonadotropins (HMG) at doses ranging from 75 IU to 450 IU per day depending on age, body mass index (BMI), antral follicle count, size and number of follicles and estradiol levels (E2). In both groups, patients were monitored by repeated vaginal ultrasound examinations and the dose of FSH /HMG was tailored according to their response and continued until the day of HCG administration. A single injection of HCG (10000 IU) was administered when at least 3 follicles reached 17 mm in diameter. Oocyte retrieval was scheduled 34 to 36 hours after the HCG injection and performed under vaginal ultrasound guidance.

A semen sample was then requested from the male partner and assessed for sperm concentration and

motility. The most motile spermatozoa were selected by performing a swim-up procedure or a sperm gradient technique with double sperm wash. Embryo transfer was performed 72 hours after oocyte retrieval. Two or three embryos were transferred into the uterine cavity under abdominal ultrasound guidance. Daily vaginal progesterone administration was used for luteal phase support from the day of oocyte retrieval in a dose of 400 mg twice daily until the day of pregnancy test (Prontogest 400mg suppositories, IBSA, Cairo, Egypt). A pregnancy test was performed 14 days after oocyte retrieval and was considered positive when the serum beta-HCG level was over 5 IU/l. All women with a positive result were offered an early trans-vaginal scan 4 weeks after embryo transfer. A clinical pregnancy was defined as the presence of a viable intrauterine gestational sac at 68- weeks with a pulsating heart.

Blood sampling:

A total of 5 ml of venous blood was collected from each individual. Blood was stored in sterile EDTA vacutainer tube at - 20°C for genomic DNA extraction.

Patients were subjected to routine investigations including complete blood picture, coagulation profile [prothrombin time, international normalization ratio (INR) and activated partial thromboplastin time (aPTT)] and assay of protein C, protein S, antithrombin III and lupus anticoagulant.

PCR-RFLP for the PAI-1 4G/5G, FXIII Val34Leu and ACE I/D polymorphisms was done for cases and control groups.

Detection of PAI-1, FXIII and ACE polymorphisms by PCR-RFLP:

Genomic DNA was isolated from peripheral blood leucocytes using Wizard genomic DNA purification kit (Promega, Madison, WI, USA), according to the manufacturer's protocol.

The PAI-1 −675 4G/5G polymorphism was detected using forward (5′-CCA ACA GAG GAC TCT TGG TC-3′) and reverse (5′-CAC AGA GAG AGT CTG GCC ACG-3′) primers.

The FXIII Val34Leu polymorphism was detected using forward (5′-CAT GCC TTT TCT GTT GTC TTC-3′) and reverse (5′-TAC CTT GCA GGT TGA CGC CCC GGG GCA CTA-3′) primers.

The ACE I/D polymorphism was detected using forward (5′-CTG GAG ACC ACT CCC ATC CTT TCT-3′) and reverse (5′-GAT GTG GCC ATC ACA TTC GTC AGA T-3′) primers.

PCR was performed using DNA thermal cycler (PTC-100 programmable thermal controller; MJ Research, Watertown, Massachusetts, USA). The PCR mixture consisted of 1.5 mM MgCl2, 200 µM dNTPs, 0.4 µM of each primer, 1 U Taq DNA polymerase and 25 ng genomic extracted DNA in a total reaction volume of 25 µL. The DNA was denatured at 94°C for 5 min followed by amplification for 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. Final extension was conducted at 72°C for 5 min. DNA fragments were separated by 2% agarose gel electrophoresis and identified by ethidium bromide staining. After generation of the PCR product, restriction enzymes were added and incubated at 37°C for 15 min. for the detection of the PAI-1 4G/5G, FXIII Val34Leu and ACE I/D polymorphisms using agarose gel electrophoresis and ultra-violet light illumination.

STATISTICAL ANALYSIS

Data were analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, Illinois, USA). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test or Fisher's exact test was used to examine the relation between qualitative variables. For quantitative normally distributed data, comparison between two groups was done using Student's t-test. Comparison between three groups was done using Kruskal-Wallis test [nonparametric analysis of variance (ANOVA)]. Odds ratio (OR) with 95% confidence interval (CI) was used for risk estimation. All tests were two-tailed. P value less than 0.05 was considered significant.

RESULTS

Patients and controls matched in their age and BMI ($p>0.05$). Age at examination ($P = 0.290$) and mean BMI ($P = 0.353$) were comparable between cases and controls with no statistical significance.

Results of PAI-1 -675 4G/5G polymorphism:

The prevalence of PAI-1 -675 4G/5G polymorphism in total study population was (30%) for the heterozygous group and (8.3%) for the homozygous group, respectively. Meanwhile, the 4G/5G polymorphism among the control population was (20%) for the heterozygous group and (5%) for the homozygous group, respectively. Cases with RIF were more prone to carry the 4G allele (P= 0.029), whereas there was no statistical significance regarding the heterozygous and homozygous polymorphisms between cases and controls ($P = 0.2$ and 0.1, respectively) as shown in Table (1).

Table 1: PAI-1 4G/5G polymorphism in RIF patients and controls (60 each)

	Wild-type	Heterozygous	Homozygous	4G allele frequency	5G allele frequency
Cases	37(61.7%)	18 (30%)	5 (8.3%)	15 (21.4%)	55 (78.6%)
Controls	45 (75%)	12 (20%)	3 (5%)	17 (12.9%)	113 (87.1%)
P value	0.311	0.212	0.120	0.029*	0.421

Results of FXIII Val34Leu polymorphism:

As regards cases with FXIII Val34 Leu polymorphism (21.6%) were heterozygous and (11.7%) were homozygous, whereas (10%) of the controls were heterozygous and (1.7%) were homozygous. Cases were more likely to be carriers of the heterozygous and homozygous FXIII polymorphism than the control group (*P = 0.016, 0.020*, respectively) as shown in Table (2). This raises the potential that the heterozygous and homozygous FXIII polymorphism carriers are at higher risk to develop RIF. Cases were also found to be at higher risk to carry the Leu allele (*P = 0.001*).

Table 2: FXIII Val34Leu polymorphism in RIF patients and controls (60 each)

	Wild-type	Heterozygous	Homozygous	4G allele frequency	5G allele frequency
Cases	40 (66.7%)	13 (21.6%)	7 (11.7%)	54 (77.1%)	16 (22.9%)
Controls	53 (88.3%)	6 (10%)	1 (1.7%)	121 (93.1%)	9 (6.9%)
P value	0.151	0.016*	0.020*	0.187	0.001**

Results of ACE I/D polymorphism:

The results concerning the ACE I/D polymorphism are presented in Table (3). Cases were (16.7%) heterozygous for this polymorphism and (20%) were homozygous, while (6.7%) of the control group were heterozygous and (11.6%) were homozygous. Cases with RIF were more liable to carry the heterozygous ACE polymorphism than the control group (*P = 0.011*). Cases with RIF were more likely to carry the D allele than the control group but there was no statistical significance (*P = 0.322*).

Table 3 : ACE I/D polymorphisms in RIF patients and controls (60 each)

	Wild-type	Heterozygous	Homozygous	4G allele frequency	5G allele frequency
Cases	38 (63.3%)	10 (16.7%)	12 (20%)	20 (28.6%)	50 (71.4%)
Controls	49 (81.7%)	4 (6.7%)	7 (11.6%)	39 (30%)	91 (70 %)
P value	0.201	0.011*	0.102	0.143	0.322

DISCUSSION

Fine-tuned endometrial vascular remodeling with adequate fibrinolysis is essential for a sufficient trophoblast invasion into maternal spiral arteries and thus successful implantation and development of a low-resistance uteroplacental circulation [16]. As PAI-1 4G/4G, factor XIII V34L and ACE I/D polymorphisms interfere with fibrin cross-linking and regulation of fibrinolysis, they may be expected to contribute to RIF.

In the present study, there was no significant difference between cases and controls as regards both the heterozygous and homozygous PAI-1 -675 4G/5G polymorphisms. However, it was reported that women with a history of implantation failure have displayed

a significantly higher prevalence of PAI-1 4G/5G mutations than controls[17]. Meanwhile, the studied patients were more prone to carry the 4G allele than the control group. This result agrees with another study which has detected an increased incidence of the 4G allele and/or 4G/4G genotype among the patients with RPL[10]. Moreover, the prevalence of homozygous PAI-1 4G/4G polymorphism was demonstrated to be higher in patients with RIF and RPL[18]. It was also reported that low molecular weight heparin and metformin have been suggested to ameliorate pregnancy complications and improve implantation process in patients with 4G/4G genotype because of the hypofibrinolytic impact of overexpressed PAI-1 on implantation [19].

The heterozygous and homozygous FXIII Val34Leu polymorphisms as well as the Leu allele frequency were significantly higher in the studied patients than the control group. This coincides with the results of a recent study which pointed out that heterozygosity and, to a slightly larger extent, homozygosity for FXIII Val34Leu polymorphism might be considered as risk factors for RPL[20]. In addition, FXIII Val34Leu polymorphism has been reported to carry an elevated overall risk for RPL in women homozygous for the FXIII 34Leu as well as in compound carriers of the FXIII 34Leu [12].

In the current study, the prevalence of the heterozygous ACE I/D polymorphism among patients with RIF were significantly more common than the control group. Cases were also more likely to carry the D allele than controls but the difference was not statistically significant. The D allele was reported to induce an elevated expression of PAI-1 which is hypofibrinolytic [21]. Hence, the D allele may be predisposing to excess fibrin accumulations in spiral arteries and within the intervillous spaces that may impede perfusion and prevent implantation[22]. Furthermore, another study demonstrated an association between ACE I/D polymorphism and risk of RPL[23]. The ACE polymorphic D allele was also implicated as an increased risk factor for RPL[24].

CONCLUSION

In conclusion, the data pointed to the importance of the PAI-1 4G/5G and the FXIII Val34Leu, and to a lesser extent to the ACE I/D, polymorphisms as possible biomarkers to select populations at risk of implantation failure prior to attempting pregnancy. Nevertheless, larger scale studies are recommended to support our results.

CONFLICT OF INTEREST

There are no conflict of interest .

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