

The influence of endothelial cell protein C receptor gene 6936A/G, 1651C/G, 4678G/C polymorphisms and soluble endothelial protein C receptor levels on in vitro fertilization outcomes

Original
Article

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ABSTRACT

Hypercoagulability could be intrinsic or caused by the hormone treatment preceding the IVF procedure. Endothelial cell protein C receptor (EPCR) enhances the generation of activated protein C by the thrombin–thrombomodulin complex. Soluble EPCR plays a role in the maintenance of pregnancy evidenced by the findings that EPCR expression is critical for embryo development. Specific gene variants linked with altered soluble EPCR levels were associated with poor pregnancy outcome. The aim of this study was to assess the predictive value of EPCR gene polymorphisms (6936A/G, 1651C/G, and 4678G/C) and sEPCR level on the IVF outcome in Egyptian women with repeated IVF failure. They were compared to healthy control patients eligible for IVF. The present study was conducted on 45 women with repeated IVF failure, three or more previous IVF-embryo transfer cycles, and 45 healthy age-matched women eligible for IVF. PCR-RFLP for the EPCR polymorphisms (6936A/G, 1651C/G, 4678G/C) was done for cases and control groups. Plasma-soluble EPCR levels were measured with ELISA. As regards the mutant, EPCR (6936A/G) genotypes (AG, GG) were higher than the wild type (AA) ($P < 0.001$, OR 4.125, 95% CI 2.198–7.740). The homozygous mutant genotype (GG) was higher in comparison to the wild type (AA). The mutant allele (G) was higher than the wild allele (A) ($P < 0.001$, or 2.549, 95% CI 1.601–4.061). Higher frequencies of the (1651C/G) genotype and lower soluble EPCR levels were noted both in (C/C) ($P = 0.004$; $Z = -0.2867$) and (C/G) ($P = 0.006$; $Z = -0.2767$) genotype carriers. Regarding, EPCR polymorphism (4678G/C), the homozygous mutant genotype (CC) was significantly lower than the homozygous wild type (GG), ($P = 0.014$, OR 0.289, 95% CI 0.108–0.776). Our data suggest that the 6936A/G and 1651C/G EPCR gene variants coupled with procoagulant diminished levels of sEPCR may be associated with a higher tendency for repeated implantation failure.

Key Words: IVF, pregnancy, protein C receptor

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INTRODUCTION

In vitro fertilization (IVF) is a well-documented risk factor for thromboembolic complications^[1]. Hypercoagulability could be intrinsic or caused by the hormone treatment preceding the IVF procedure^[2]. Possible plasma factor compositional reasons for the link between IVF and thrombotic events include activated protein C resistance^[3].

Protein C anticoagulant pathway regulates blood coagulation by inhibiting the activities of factors Va and VIIIa, and hence thrombin generation. Several factors regulate activated protein C (APC) activity including endothelial cell activated protein C receptor (EPCR), a 46-kDa type I transmembrane glycoprotein localized

to 20 q11.2, spanning approximately 8 kbp of genomic DNA and consisting of four exons interrupted by three introns^[4]. EPCR can increase the activity of PC/APC via the thrombin-thrombomodulin complex by five to 20-fold leading to markedly elevated anticoagulation activity^[5]. EPCR is expressed by the vascular endothelium of large vessels^[6], as well as, trophoblast giant cells at the fetomaternal boundary^[7]. APC also binds EPCR and activate protease-activated receptor 1 (PAR-1), thus triggering anti-inflammatory and cytoprotective signaling events in endothelial cells^[8].

Soluble EPCR (sEPCR) is a soluble form of EPCR that is constituted by metalloprotease cleavage and present in normal human plasma^[9]. Soluble EPCR plays a role in the maintenance of pregnancy, evidenced by the findings that

EPCR expression is critical for embryo development^[10] and blockade of EPCR activity by anti-EPCR autoantibodies was associated with adverse pregnancy outcomes^[11].

Several mutants of EPCR have been identified with differential expression levels and function. Four haplotypes involved in altering soluble EPCR levels were reported among which the haplotype 3 (H3) is associated with increased soluble EPCR levels. This haplotype is defined by the G/C/A/G combination at positions 1651, 3610, 4216, and 6936, respectively^[12]. In addition, 4678G/C (rs 9574) is a single-nucleotide polymorphisms (SNP) representative of one of the four different haplotypes in the EPCR gene, haplotype 1 (H1)^[12]. Specific gene variants linked with altered soluble EPCR levels were associated with poor pregnancy outcome in some^[13] but not all^[14] studies.

The aim of this study was to assess the predictive value of EPCR gene polymorphisms (6936A/G, 1651C/G, and 4678C/G) and sEPCR level 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 45 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 January 2015 and January 2016. The evolution of the pregnancy was not recorded.

Forty-five 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) were included in the study.

Methodology:

This prospective controlled trial included 90 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 (45) and control group with no prior history of ICSI treatment (45)

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 10 ml of peripheral blood was collected from each individual. Five milliliters of blood was placed in EDTA tube for DNA extraction and 5 ml in Na citrate (110/ volume) tube from which plasma was isolated for sEPCR estimation. Both EDTA and plasma samples were stored at -20°C for later use.

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 PC, protein S, antithrombin III and lupus anticoagulant.

PCR-RFLP for the EPCR polymorphisms (6936A/G, 1651C/G, 4678G/C) was done for cases and control groups. Plasma-soluble EPCR levels were measured with ELISA.

Detection of EPCR polymorphisms by PCR-RFLP:

Purification of DNA from peripheral blood samples of patients was done using 'FavorPrep Genomic DNA Mini kit' produced by Favorgen Biotech Lab, Pingtung, Taiwan. For DNA amplification, Firepol Master Mix ready to load was produced by Solis BioDyne, Tartu, Estonia.

The sequence of the used primers (product of Jena Bioscience GmbH (Germany) was as follows: for EPCR 6936A/G genotype:

Forward: 5' CCTACTTCGCTGGTCC TGGGCGTCCTGGTCTGC 3' and Reverse 5' CAAGTACTTTGTCCACCTCTCC 3'.

For EPCR 1651 C/G genotype: Forward: 5' GCTGAAATTTGTATTCTGTCC-3' and Reverse 5'-CCAGTATAATGGCTACATTTTACC-3'.

For EPCR 4678G/C genotype: Forward: 5' GCTTCAGTCAGTTGGTAAAC 3' and Reverse: 5' TCTGGCTTCACAGTGAGCTG 3'.

All reactions were performed in a total volume of 25 µl using 1 µl of each primer.

PCR was performed using DNA thermal cycler (PTC-100 programmable thermal controller; MJ Research,

Watertown, Massachusetts, USA). The thermocycler was programmed for initial heat denaturation at 94°C for 5 min; amplification included 40 cycles with the following programme: denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min. Last cycle extension was prolonged to 5 min at 72°C.

As regards EPCR polymorphism 6936A/G, after amplification, the PCR product (290 bp) was digested with PstI (Thermoscientific, Runcorn, Cheshire, UK) restriction enzyme.

For EPCR polymorphism 1651 C/G, after amplification, the PCR product (293 bp) was digested with Eco91I (Fermentas, Lithuania) restriction enzyme.

For EPCR polymorphism 4678 G/C, after amplification, the PCR product (314 bp) was digested with DdeI (New England BioLabs, Hitchin, Hertfordshire, UK) restriction enzyme. The digested products were detected by capillary electrophoresis using QIAxcel instrument and QIAxcel DNA High-resolution Kit.

sEPCR levels were estimated using ELISA kit (WKEA, Med Supplies, China) according to the manufacturer's instructions.

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

Controls were age matched to patients ($P = 0.217$), and BMI ($P = 0.352$). Soluble EPCR levels were significantly lower in cases than in controls ($P = 0.008$).

Results of EPCR polymorphism 6936A/G genotyping:

As shown in Table 1 ; the wild 6936 genotype (AA) was lower than mutant 6936 genotype (AG, GG) ($P < 0.001$, OR 4.125, 95% CI 2.198–7.740). The homozygous wild 6936 genotype (AA) was lower than the homozygous mutant 6936 genotype (GG) ($P = 0.008$,

OR 9.000, 95% CI 1.777-45.586). Likewise, the homozygous wild genotype (AA) was lower

than the heterozygous mutant genotype (AG) ($P < 0.001$, OR 3.838, 95% CI 2.023–7.284).

Table 1: Frequencies of EPCR polymorphism in IVF cases in comparison to healthy controls

Polymorphism	Genotype	Cases		Controls		P
		No (%)	No (%)	No (%)	No (%)	
EPCR polymorphism (6936A/G)	AA	12 (26.7)	27 (60.0)	27 (60.0)	17 (37.8)	< 0.001
	AG	29 (64.4)	17 (37.8)	17 (37.8)	1 (2.2)	
	GG	4 (8.9)	1 (2.2)	1 (2.2)	1 (2.2)	
EPCR polymorphism (1651C/G)	GG	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	< 0.001
	CC	35 (77.8)	42 (93.3)	42 (93.3)	3 (6.7)	
	CG	10 (22.2)	3 (6.7)	3 (6.7)	3 (6.7)	
EPCR polymorphism (4678G/C)	GG	21 (46.7)	16 (35.6)	16 (35.6)	16 (35.6)	0.049
	GC	20 (44.4)	20 (44.4)	20 (44.4)	20 (44.4)	
	CC	4 (8.9)	9 (20.0)	9 (20.0)	9 (20.0)	

* $P < 0.001$ (highly significant), EPCR; endothelial protein C receptor

Results of EPCR polymorphism 1651C/G genotyping:

The 1651G (minor) allele frequency [$P < 0.001$; OR (95% CI) = 3.80 (2.30-6.12)] and the heterozygous 1651C/G genotype [$P < 0.001$; OR (95% CI) = 4.18 (2.51–6.95)] were higher in cases than in controls as presented in Table 2. None of the cases or

controls was a 1651G/G homozygote.

Results of EPCR polymorphism 4678G/C genotyping:

The homozygous wild 4678G/C genotype (GG) was higher than the mutant 4678G/C genotype (CC) ($P = 0.014$, OR 0.289, 95% CI 0.108–0.776).

Table 2: Regression analysis

Polymorphism	P	OR	(95% CI)
EPCR polymorphism (6936A/G)	< 0.001	2.549	1.601- 4.061
EPCR polymorphism (1651C/G)	0.011	3.130	1.290 -7.570
EPCR polymorphism (4678G/C)	0.021	0.600	0.388- 0.927
Soluble EPCR	0.067	1.010	1.000 - 1.100

* $P < 0.001$ (highly significant), CI; confidence interval, EPCR; endothelial protein C receptor, OR; odds ratio

Results of sEPCR assay by ELISA:

As shown in Table 3; significantly lower sEPCR levels were detected in cases in comparison to controls, as sEPCR in cases ranged from 20.0–151.3 ng/ml with a median value of 129.6, while in controls, sEPCR ranged from 38.7–1073.1 ng/ml with a median value of 157.4, ($P = 0.008$). Lower sEPCR levels were noted in 1651C/C in comparison to 1651C/G genotype carriers in both cases ($P = 0.0032$) and controls ($P < 0.001$). According

to the specific genotype, significantly lower sEPCR levels were detected in cases both in 1651C/C ($P = 0.0046$) and 1651C/G ($P = 0.0032$) genotype carriers.

A highly significant relation was noticed between EPCR polymorphisms (6936A/G, 1651C/G) and lower sEPCR levels. There was a relation between low sEPCR levels and EPCR polymorphism 4678G/C genotypes, though it was not significant.

Table 3: sEPCR level in patients compared to controls

sEPCR (ng/ml)	Cases	Control	P
Mean \pm SD	106.3 \pm 46.1	265.6 \pm 255.1	
Min–Max	20.0–151.3	38.7–1073.1	0.008
Median	129.6	157.4	

* $P < 0.05$ (significant), EPCR; endothelial protein C receptor.

DISCUSSION

Appropriate hemostatic balance is essential for maintenance of pregnancy. EPCR is abundant on the trophoblast giant cells at the feto-maternal boundary from embryonic day 7.5 (E7.5) suggesting a role in the hemostatic regulation of the maternal blood that irrigates these surfaces^[15]. EPCR expression is critical for embryo development, this is supported by the enhanced embryonic lethality seen in EPCR knockout mice before embryonic day 10 as fibrin deposition around trophoblast giant cells results in thrombosis at the maternal- embryonic interface^[16]. In addition, blockade of EPCR activity by anti-EPCR autoantibodies was associated with adverse pregnancy outcomes^[17].

Soluble EPCR is generated by two mechanisms: shedding and mRNA splicing. EPCR 6936A/G causes serine to glycine substitution at residue 219 in the transmembrane domain, thus it becomes adjacent to another Gly at residue 218. The Gly-Gly interaction induces exposure of the cleavage site. After cleavage by matrix metalloproteinases, increased shedding of membrane-attached EPCR occurs leading to increased levels of sEPCR in the circulation and subsequently reduced APC because sEPCR inhibits both APC activity and PC activation by competing for PC with membrane-associated EPCR^[18]. Meanwhile, the splicing mechanism results in a truncated mRNA, which lacks intracytoplasmic and trans-membrane domain sequences, and thus produces protein incapable of membrane attachment and is later secreted as sEPCR^[19].

In the present study, it was found that frequencies of the mutant EPCR (6936A/G) genotypes (AG, GG) were higher in cases (73.3%) than the control group (40.0%); it was higher than the wild type (AA) ($P < 0.001$, OR 4.125, 95% CI 2.198-7.740). The homozygous mutant genotype (GG) was higher in comparison to the wild type (AA). Also, the mutant allele (G) was higher than the wild allele (A) ($P < 0.001$, OR 2.549, 95% CI 1.601-4.061). Similarly, Yin *et al.* reported that frequencies of the EPCR gene 6936AG and 6936GG genotypes were higher in patients with venous thromboembolism than in healthy subjects^[20]. Moreover, Medina *et al.* reported that those with homozygous mutant EPCR (6936A/G) genotype have an increased risk for venous thrombosis and increased plasma sEPCR levels^[21].

Furthermore, the current results presented higher frequencies of the (1651C/G) genotype and lower soluble EPCR levels in cases than in controls. Soluble EPCR production was decreased in cases than in control patients, both in (C/C) ($P = 0.004$; $Z = -0.2867$) and (C/G) ($P = 0.006$; $Z = -0.2767$) genotype carriers. In contrast, another study reported that elevated soluble EPCR levels were linked with the (1651C/G) genotype^[22].

As regards EPCR polymorphism (4678G/C), the homozygous mutant genotype (CC) was significantly lower than the homozygous wild type (GG), ($P = 0.014$, OR 0.289, 95% CI 0.108–0.776). There was also a relation between low sEPCR levels and EPCR polymorphism (4678G/C) genotypes, though it was not significant. These results were in agreement with Medina *et al.* who

confirmed that individuals with EPCR polymorphism (4678G/C) have reduced risk for venous thrombosis, increased plasma activated PC levels and reduced plasma sEPCR levels [21].

EPCR genotype was reported that it could augment the condition of increased resistance to APC that is normally observed in the second and third trimesters, thus increasing the risk of thrombosis during pregnancy, puerperium and contraceptive treatment [23]. Variants in EPCR haplotypes were reported for their contribution to the development of early vascular pregnancy complications [14]. This was supported by a study which demonstrated that the 23-bp insertion variant of the EPCR gene was seen in a patient with three miscarriages and no children who eventually had a successful pregnancy with heparin treatment [24].

The present results showed that the 6936A/G polymorphism and the 1651G allele are associated with a decreased production of sEPCR, while 4678G/C polymorphism had no effect on sEPCR level. It could be speculated that there is an interaction between EPCR polymorphisms (6936A/G, 1651C/G) and downregulation of EPCR expression which in turn suppresses soluble EPCR production. It was reported that soluble EPCR exerts anticoagulant, antiapoptotic and anti-inflammatory effects [25]. Therefore, reduced soluble EPCR levels seen in the studied patients promotes a local pro-inflammatory and procoagulant state, which in turn propagates coagulation mechanisms, thus leading to implantation failure.

CONCLUSION

In conclusion, data suggested that the 6936A/G and 1651C/G EPCR gene variants coupled with procoagulant diminished levels of sEPCR may be associated with a higher tendency for implantation failure. Nevertheless, larger scale studies are recommended to support these results.

CONFLICT OF INTEREST

There are no conflict of interest

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