Research Article

Endothelial cell protein c receptor gene 6936a/G, 1651c/G, 4678g/C polymorphisms and soluble endothelial protein c receptor levels impact on in vitro fertilization outcomes

Abstract

Hypercoagulability could be intrinsic or may be induced by the hormonal treatment preceding in vitro fertilization (IVF) procedure. Endothelial cell protein C receptor (EPCR) enhances the generation of activated protein C by the thrombin–thrombomodulin complex. EPCR expression is critical for embryo development hence soluble EPCR plays a role in maintenance of pregnancy. Poor pregnancy outcome was associated with specific gene variants and altered soluble EPCR levels. The aim of this work was to evaluate the predictive value of EPCR gene polymorphisms (6936A/G, 1651C/G, 4678C/G) and sEPCR level on the IVF outcome. The study was conducted on 45 women with repeated IVF failure, three or more previous IVF-embryo transfer cycles, compared to 45 healthy age-matched women eligible for IVF with positive β-HCG were selected two weeks after embryo transfer as a control group. Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) for the EPCR polymorphisms (6936A/G, 1651C/G, 4678G/C) was done for both cases and controls. Soluble EPCR levels were measured with ELISA. As regards the mutant EPCR (6936A/G) genotypes (AG, GG) were higher than the wild type (AA). 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). Higher frequencies of the (1651C/G) genotype and lower soluble EPCR levels were noted, both in (C/C) and (C/G) genotype carriers. Regarding, EPCR polymorphism (4678G/C), the homozygous mutant genotype (CC) was significantly lower than the homozygous wild type (GG). The present data suggest that the 6936A/G and 1651C/G EPCR gene variants coupled with procoagulant diminished levels of sEPCR could be associated with a higher tendency for repeated implantation failure.

Keywords: endothelial protein C receptor, gene, polymorphisms, repeated implantation failure, in vitro fertilization

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Abbreviations: IVF, in vitro fertilization; sEPCR, soluble endothelial protein C receptor; PCR-RFLP, polymerase chain reaction restriction fragment length polymorphism; ELISA, enzyme linked immunosorbent assay

Introduction

In vitro fertilization (IVF) is a well-documented risk factor for thromboembolic complications.[1] Hypercoagulability could be either intrinsic or may be induced by the hormonal 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 regulates blood coagulation by inhibiting the activities of factors Va and VIIIa, and hence thrombin generation.[4] Activated protein C (APC) activity is regulated by several factors, including endothelial cell activated protein C receptor (EPCR). EPCR is a 46-kDa type I transmembrane glycoprotein spanning approximately 8kbp of genomic DNA, localized to 20q11.2, and consists of four exons interrupted by three introns.[5] EPCR can increase the activity of PC/APC via the thrombin-thrombomodulin complex by five to 20-fold, leading to markedly elevated anticoagulation activity.[6] EPCR is expressed by both the vascular endothelium of large vessels[7] and the trophoblast giant cells at the feto-maternal boundary.[8] When APC binds EPCR, it activates protease-activated receptor 1 (PAR-1), thus triggering both anti-inflammatory and cytoprotective signaling events in endothelial cells.[9] Soluble EPCR (sEPCR) is a soluble form of EPCR that is constituted by metalloprotease cleavage and present in normal human plasma.[10] Soluble EPCR plays a role in maintenance of pregnancy. That is confirmed by the findings that EPCR expression is critical for embryo development.[11] In addition, blocking of EPCR activity by anti-EPCR autoantibodies was associated with adverse pregnancy outcomes.[12] Several mutants of EPCR have been identified with differential expression levels and function. Four haplotypes involved in alteration of soluble EPCR levels were demonstrated, among which, the haplotype 3 (H3) is linked with increased soluble EPCR levels. This haplotype is identified by the G/C/A/G combination at positions 1651, 3610, 4216, and 6936, respectively.[13] Moreover, 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).[13] Poor pregnancy outcome was associated with specific gene variants and altered soluble EPCR levels in some[14] but not all[15] studies. The aim of this study was to assess the predictive value of EPCR gene polymorphisms (6936A/G, 1651C/G, 4678C/G)

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and sEPCR level on the IVF outcome in Egyptian women with repeated IVF failure. They were compared to healthy control patients eligible for IVF with positive β-HCG were selected two weeks after embryo transfer as a control group. Achievement of biochemical pregnancy was the end-point of the study.

Materials and methods

The present study was conducted between January 2015 and January 2016 on 45 women indicated for in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) treatment. They were selected from the IVF unit, department of Obstetrics and Gynaecology at Shatby University Hospital, Alexandria University. Fortyfive healthy, age-matched women eligible for IVF with positive β-HCG were selected two weeks after embryo transfer as a control group. They had no personal history of miscarriage or any thrombotic or bleeding disorder and without any history of known hereditary or acquired thrombophilic alteration. The study protocol 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 present study.

Exclusion criteria

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

Methodology

This prospective controlled study included 90 women indicated for IVF/ICSI treatment treated in Shatby IVF unit. All couples had a standard infertility evaluation that included a baseline hormonal profile that included FSH, LH, TSH, and PRL in the early follicular phase, evaluation of tubal patency either by hysterosalpingography (HSG) or laparoscopy, a baseline trans-vaginal ultrasonography, and a semen analysis using WHO criteria. Patients who have agreed to continue with ICSI were subdivided into two groups; 45 with history of recurrent ICSI failure and 45 with no prior history of ICSI treatment as control group.

Controlled ovarian stimulation was done using either agonist or antagonist protocols together with ovarian stimulation using recombinant human follicular stimulating hormone (FSH), in addition to human menopausal gonadotropins (HMG) at doses ranging from 75IU to 450IU per day depending on age, body mass index (BMI), antral follicle count, size and number of follicles and estradiol levels (E2). All participants were monitored by repeated vaginal ultrasound examinations and the dose of FSH /HMG was tailored according to their response and was continued till the day of HCG administration. A single dose of HCG injection (10000 IU) was administered when 3 follicles at least have reached 17mm in diameter. After 34 to 36hours of HCG injection, oocyte retrieval was scheduled and performed under vaginal ultrasound guidance. Then a semen sample was requested from the male partner and assessed for sperm concentration and motility. The most motile spermatozoa were selected by performing a sperm gradient technique with double sperm wash or a swim-up procedure.

Two or three embryos were transferred 72hours after oocyte retrieval into the uterine cavity under abdominal ultrasound guidance. Daily vaginal progesterone administration in a dose of 400mg twice daily was used for luteal phase support from the day of oocyte retrieval until the day of pregnancy test (Prontogest 400mg suppositories, IBSA, Cairo, Egypt). A pregnancy test was done 14days after oocyte retrieval and when the serum beta-HCG level was over 5 IU/l, it was considered positive. An early trans-vaginal scan was performed 4weeks after embryo transfer for women with a positive result. A clinical pregnancy was identified by the presence of a viable intrauterine gestational sac at 6-8weeks with a pulsating heart.

Blood sampling

Ten ml of peripheral blood was collected from each participant. Five ml of blood was placed in EDTA tube for DNA extraction and 5ml in Na citrate (1/10volume) tube from which plasma was isolated for sEPCR estimation. Patients were subjected to routine investigations, including complete blood picture, prothrombin time, international normalization ratio (INR) and activated partial thromboplastin time (aPTT)] and assay of PC, protein S, anti-thrombin III as well as 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. For 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 produced by Solis BioDyne, Tartu, Estonia was used.

The sequence of the used primers (product of Jena Bioscience GmbH (Germany) was as follows: for EPCR 6936A/G genotype: Forward: 5' CCTACACTTCGCTGGTCCTGGGCGTCCTGGTCTGC 3' and Reverse 5' CAAGTACTTTGTCCACCTCTCC 3'. For EPCR 1651 C/G genotype: Forward: 5′ GCTGAAATTTTGTATTCTGTCC-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 volume of 25µl using 1µl of each primer. PCR was performed using DNA thermocycler (PTC-100 programmable thermal controller; MJ Research, Watertown, Massachusetts, USA). The thermocycler was programmed for initial heat denaturation at 94°C for 5min; amplification included 40 cycles with the following programme: denaturation at 94°C for 1min, annealing at 60°C for 1min, extension at 72°C for 1min. Last cycle extension was prolonged to 5min at 72°C. As regards EPCR polymorphism 6936A/G, after amplification, the PCR product (290bp) was digested with PstI (Thermoscientific, Runcorn, Cheshire, UK) restriction enzyme. For EPCR polymorphism 1651C/G, after amplification, the PCR product (293bp) was digested with Eco91I (Fermentas, Lithuania) restriction enzyme. For EPCR polymorphism 4678G/C, after amplification, the PCR product (314bp) was digested with DdeI (New England BioLabs, Hitchin, Hertfordshire, UK) restriction enzyme. The digested products were detected by capillary electrophoresis using QIAxcel instrument

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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 was analysed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, Illinois, USA). Numerical data were expressed either as mean and standard deviation or as median and range. Qualitative data were expressed as frequency and percentage. To examine the relation between qualitative variables, Chi-square test or Fisher's exact test was used. For quantitative normally distributed data, comparison between two groups was done using Student's t-test and 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. P value less than 0.05 was considered significant.

Results and discussion

The control and patient groups were age and BMI matched (P=0.217, 0.352 respectively) but without any statistical significance. Soluble EPCR levels were significantly lower in cases than in controls (P=0.008).

Results of EPCR polymorphism 6936A/G genotyping

The wild 6936 genotype (AA) was lower than the 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) as shown in Table 1 & 2.

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

Polymorphism	Cases		Controls	
	Genotype	No (%)	No (%)	P
EPCR polymorphism (6936A/G)	AA	12(26.7)	27(60.0)	
	AG	29(64.4)	17(37.8)	<0.001
	GG	4(8.9)	1(2.2)	
EPCR polymorphism (1651C/G)	GG	0(0.0)	0(0.0)	
	CC	35(77.8)	42(93.3)	<0.001
	CG	10(22.2)	3(6.7)	
EPCR polymorphism (4678G/C)	GG	21(46.7)	16(35.6)	
	GC	20(44.4)	20(44.4)	0.049
	CC	4(8.9)	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 3.80, 95% CI 2.30-6.12) and the heterozygous 1651C/G genotype (P=0.001; OR 4.18, 95% CI 2.51–6.95) were higher in cases than in controls. None of the cases or controls was a 1651G/G homozygote as presented in Table 1 & 2.

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) as shown in Table 1 & 2.

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	3.13	1.290-7.570
EPCR polymorphism(4678G/C)	0.021	0.6	0.388-0.927
Soluble EPCR	0.067	1.01	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

Significantly lower sEPCR levels were detected in cases in comparison to controls, as sEPCR in cases ranged from 20.0-151.3ng/ml with a median value of 129.6, while in controls, sEPCR ranged from 38.7-1073.1ng/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 and controls as presented in Table 3. 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±SD	106.3±46.1	265.6±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

An appropriate hemostatic balance is crucial for maintenance of pregnancy. The presence of abundant EPCR on the trophoblast giant cells at the feto-maternal boundary from embryonic day 7.5 (E7.5), suggests its role in the hemostatic regulation of the maternal blood that irrigates these surfaces.[16] EPCR expression is essential for embryo development, this is supported by the accelerated embryonic lethality observed in EPCR knockout mice before embryonic day 10. This is owing to the fact that fibrin deposition around trophoblast giant cells results in thrombosis at the maternal- embryonic interface.[11] In addition, EPCR activity blockade by anti-EPCR autoantibodies was associated with adverse pregnancy outcomes.[12]

Soluble EPCR generation occurs 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

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EPCR occurs leading to increased levels of sEPCR in the circulation and subsequently reduced APC. This occurs because sEPCR inhibits both APC activity and PC activation by competing for PC with membrane-associated EPCR.[17] Meanwhile, the splicing mechanism results in a truncated mRNA, which lacks both intra-cytoplasmic and trans-membrane domain sequences, thus producing a protein incapable of membrane attachment and is later secreted as sEPCR.[10]

In the present study, it was found that frequencies of the mutant EPCR (6936A/G) genotypes (AG, GG) were higher in cases than the control group and it was higher than the wild type (AA). 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). 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.[18] 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.[19]

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) and (C/G) genotype carriers. In contrast, another study reported that higher soluble EPCR levels were linked with the (1651C/G) genotype.[20]

As regards EPCR polymorphism (4678G/C), the homozygous mutant genotype (CC) was significantly lower than the homozygous wild type (GG). 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 reported that individuals with EPCR polymorphism (4678G/C) have reduced risk for venous thrombosis, elevated plasma activated PC levels and reduced plasma sEPCR levels.[19]

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.[21] Variants in EPCR haplotypes were evidenced for their contribution to the development of early vascular pregnancy complications.[15] This was supported by a study which demonstrated that the 23-bp insertion variant of the EPCR gene was detected in a patient with three miscarriages and no children, then eventually had a successful pregnancy with heparin treatment.[22]

The present results show that the 6936A/G polymorphism and the 1651G allele are associated with a lower 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 has anticoagulant, antiapoptotic, and anti-inflammatory effects.[23] Therefore, reduced soluble EPCR levels seen in the studied patients initiates a local pro-inflammatory and procoagulant state, which consequently propagates coagulation mechanisms, thus resulting in implantation failure.[15]

Conclusion

The present 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 implantation failure.

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