Variations in the thrombomodulin and endothelial protein C receptor genes in couples with recurrent miscarriage

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BACKGROUND: Recurrent miscarriage (RM) has been suggested to be caused by mutations in genes coding for various coagulation factors resulting in thrombophilia. Mouse models indicate that genes involved in the protein C anticoagulant pathway are essential for normal embryonic development. Loss of function of two of these genes, thrombomodulin (TM) and endothelial protein C receptor (EPCR), causes embryonic lethality in mice. The aim of this study was to determine whether variations in the human TM or EPCR genes are associated with an increased risk for RM. METHODS: Forty-six RM patients and 191 controls were screened for mutations in TM and EPCR using denaturing high-performance liquid chromatography (DHPLC). The partners of 40 RM patients were also screened. RESULTS: One exonic and one intronic variation in TM and two exonic and two intronic sequences in EPCR were detected. Four variants were common in both patients and controls. A previously identified truncating mutation in EPCR, suggested to have a role in pregnancy complications, was identified in two patients and one control. A novel deletion in the 3’UTR region of TM was detected, but its significance remains unsolved. CONCLUSIONS: These data suggest that mutations in the TM or EPCR genes are not a major cause of RM, although they may exert a modifier effect in combination with other variants.

Key words: DHPLC/endothelial protein C receptor/miscarriage/recurrent spontaneous abortion/thrombomodulin

Introduction

Recurrent miscarriage (RM), defined as three or more consecutive pregnancy failures, is estimated to occur in 0.5–1% of all couples (Tulppala et al., 1993; Katz and Kuller, 1994). The cause, however, remains unknown in ∼50% of all cases (Plouffe et al., 1992; Clifford et al., 1994). Recently, several studies have shown that both hereditary and acquired thrombophilia increase the risk for adverse pregnancy outcomes, including miscarriages (Robertson et al., 2004). Thus, several studies have analysed the association of inherited forms of thrombophilia with miscarriage. Although an increased risk has been found for carriers of known predisposing mutations such as the Leiden mutation, other studies have been unable to confirm these results, and the role of each of these mutations in RM remains uncertain (reviewed by Kupferminc, 2003; Kutteh and Triplett, 2006).

In an attempt to find candidate genes for human RM, we have searched for animal models for this condition, that is, genes essential for normal embryonic development. These include two thrombophilia-associated genes, namely thrombomodulin (TM) and endothelial protein C receptor (EPCR). Loss of function of TM causes early post-implantation embryonic lethality before establishment of a functional cardiovascular system in the mouse embryo (Healy et al., 1995). TM expression in non-endothelial placental cells is required for proper function of the early placenta, whereas the absence of TM from blood vessel endothelium causes excessive activation of the embryonic blood coagulation system. In TM-deficient mice, embryogenesis is disrupted at two different developmental stages, indicating a crucial role for TM in both (Isermann et al., 2001).

Deletion of the EPCR gene in mice leads to embryonic lethality before embryonic day 10.5 (Gu et al., 2002). However, EPCR<−− embryos removed from extraembryonic membranes and tissues at day E7.5 and cultured in vitro developed beyond E10.5, suggesting a role for EPCR in the normal function of the placenta and/or at the maternal–embryonic interface (Gu et al., 2002). EPCR is normally detected in giant trophoblast cells, which are in direct contact with the maternal circulation and its clotting factors (Crawley et al., 2002). If EPCR is not expressed on the giant trophoblast cells, even an enhanced expression of EPCR in the embryo cannot rescue the embryo. Conversely, selective EPCR expression on the giant trophoblast cells rescues EPCR-deficient embryos (Li et al., 2005). Thrombosis is observed surrounding the trophoblast giant cells.
derived from EPCR<sup>−/−</sup> embryos, but not around those derived from EPCR<sup>+/−</sup> or EPCR<sup>+/+</sup> cells (Gu et al., 2002). These observations suggest that extraembryonic EPCR expression is essential for embryonic viability and plays a critical role in the control of blood coagulation at the fetomaternal interface.

TM and EPCR are glycoprotein receptors that both play key roles in the protein C anticoagulant pathway, the major regulatory mechanism that suppresses coagulation. TM is an endothelial cell surface receptor expressed mainly on the endothelial surfaces of blood vessels and in the placenta. TM forms a complex with thrombin, which then converts protein C to activated protein C (Maruyama et al., 1985; Van de Wouwer et al., 2004; Dahlbäck and Villoutreix, 2005). EPCR is a type 1 transmembrane receptor, expressed primarily on endothelial cells of large blood vessels and in the placenta and developing cardiovascular system in the fetus. EPCR functions in the protein C pathway by binding protein C and presenting it to the TMthrombin complex on the endothelium, thereby increasing the rate of protein C activation (Stearns-Kurosawa et al., 1996; Laszik et al., 1997; Crawley et al., 2002).

Considering the crucial role of TM and EPCR in coagulation and the embryonic lethality of mutations in these genes, we hypothesized that these genes would be good candidates for RM. This study was performed to determine whether there is an association between sequence variations in the TM and EPCR genes and RM. The study was performed by screening the TM and EPCR genes for mutations in patients with RM and in controls with no known history of RM and at least one uncomplicated successful pregnancy.

**Materials and methods**

**Subjects**

Patients with RM treated at the Department of Gynaecology and Obstetrics of the Helsinki University Hospital during 2001-04 were recruited in the study. The inclusion criteria for the study were women: (i) aged 18-40 years with (ii) previous history of RM, defined as three or more consecutive miscarriages. A total of 46 patients of Caucasian origin with unexplained RM were included. For 40 patients, the partners were screened, and for the remaining 6 women, blood samples from their partners could not be obtained. In 43 women, all miscarriages had taken place during the first trimester (<13 weeks). In addition to first trimester losses, two women had experienced second trimester (from 13 to 23 + 6 weeks) miscarriages and one woman had a third trimester (>24 weeks) intrauterine fetal death.

Uterine anomalies were excluded by ultrasonography or hysterosonogram. Maternal and paternal karyotypes were tested from peripheral blood lymphocyte cultures and shown to be normal in all patients.

The control group, which was recruited from the same hospital during the same time, consisted of 191 women who had at least one normal pregnancy and no history of miscarriage. The controls were of same age and racial origin (Finnish Caucasian) as the patients. Informed consent was obtained from all participants before enrolment. The ethics committee of the Department of Obstetrics and Gynaecology, Helsinki University Central Hospital, approved the study protocol.

**Polymerase chain reaction**

DNA was extracted from whole blood collected from patients and controls using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, USA). Polymerase chain reactions of TM and EPCR (non-coding exons included) were performed in 25 μl of reaction mixes containing the following reagents: 50–100 ng of genomic DNA, 1× PCR buffer (Applied Biosystems, Foster City, USA), 2 nmol each dNTP, 10 pmol forward primer, 10 pmol reverse primer and 0.1 U AmpliTaq Gold DNA-polymerase (Applied Biosystems). Additionally, DMSO [final concentration 5% (v/v)] was added to some of the amplification mixes to improve amplification. Thermocycling was performed in a PTC-225 DNA Engine Tetrad thermocycler (MJ Research, Waltham, USA). Initial denaturation at 95°C for 10 min was followed by 35 cycles of denaturation at 95°C for 30 s, annealing for 45 s (temperature depending on the amplicon) and extension at 72°C for 45 s. A final extension was performed at 72°C for 10 min. PCR conditions and primer sequences are summarized in Table I. The specificity of the amplification was confirmed by agarose gel electrophoresis before further analysis.

**Mutation analysis**

Denaturing high-performance liquid chromatography (DHPLC) analysis of the samples was carried out using a Transgenomic WAVE<sup>®</sup> Nucleic Acid Fragment Analysis System (Transgenomic, Omaha, USA) and the associated Navigator software as described by Kaare et al. (2006). Conditions used for DHPLC analysis are summarized in Table I. Following DHPLC screening, samples showing heterozygous changes were sequenced in order to determine the nature of the sequence change. Additionally, for each amplicon, 10 samples showing only a homoduplex peak were sequenced to confirm that no variation went.

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Annealing temperature (°C)</th>
<th>Percentage of DMSO</th>
<th>F primer (5' and 3')</th>
<th>R primer (5' and 3')</th>
<th>DHPLC analysis temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMprom</td>
<td>55</td>
<td>–</td>
<td>ACCAAGAGATGAAAGAGGG</td>
<td>AGCCCTCTCCTGTCCGTCCA</td>
<td>64.1</td>
</tr>
<tr>
<td>TM1</td>
<td>TD 64–54</td>
<td>5</td>
<td>CAGCCGCAAGAAGCTGTCT</td>
<td>TGGTGTTTGTTGCTCCCGTA</td>
<td>64.6</td>
</tr>
<tr>
<td>TM2</td>
<td>55</td>
<td>5</td>
<td>CCCAGTCATATTCTTGCTA</td>
<td>CAGAATGCTGCGAGGATC</td>
<td>64.5</td>
</tr>
<tr>
<td>TM3</td>
<td>53</td>
<td>5</td>
<td>CCCCTGGCTCATACGCTAT</td>
<td>TACTCCGAGCTGCTCCGTA</td>
<td>64.5</td>
</tr>
<tr>
<td>TM4</td>
<td>TD 62–52</td>
<td>5</td>
<td>CTTTGAGTGCCACTGCTACC</td>
<td>CGGAGGACTCAAGGTGAG</td>
<td>64.3</td>
</tr>
<tr>
<td>TM5</td>
<td>66</td>
<td></td>
<td>ATGGGAGCCGAGCTGACCT</td>
<td>AGGCTCATTCCCTCCTCCT</td>
<td>61.6</td>
</tr>
<tr>
<td>EPCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 1</td>
<td>60</td>
<td>–</td>
<td>TGCTCCTTGGCTACAAAACCTG</td>
<td>CCCATCCAGTCCCAATTAGA</td>
<td>57.4</td>
</tr>
<tr>
<td>Exon 2+3</td>
<td>60</td>
<td>–</td>
<td>GAATCTGGCCGCATGAC</td>
<td>TGGTCTTGGTTTGTGGTGTG</td>
<td>58.5</td>
</tr>
<tr>
<td>Exon 4</td>
<td>60</td>
<td>–</td>
<td>ATCTCTCCTTGGCACTGCT</td>
<td>GATTCTCCGACGACCTGCA</td>
<td>63.1</td>
</tr>
<tr>
<td>Exon 5</td>
<td>60</td>
<td>–</td>
<td>CAGGCCTCCCAAAGCTTCT</td>
<td>CCTACTCACGCGCAGGTC</td>
<td>64.5</td>
</tr>
<tr>
<td>Exon 6</td>
<td>62</td>
<td>–</td>
<td>GACCCCTCTCTGCAACAGTC</td>
<td>CACCTATTTGTCTGGAACC</td>
<td>62.1</td>
</tr>
<tr>
<td>Exon 7</td>
<td>57</td>
<td>–</td>
<td>TAAAAGGGTTCCTTCTCCT</td>
<td>CCTCCCTCTCTCAATCCTCT</td>
<td>61.6 and 63</td>
</tr>
<tr>
<td>UTR</td>
<td>57</td>
<td>–</td>
<td>GGTGTTGGAGTCAGCCTTCT</td>
<td>ATGGGTATAGTTTACTTTGCCAGA</td>
<td>55.7</td>
</tr>
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</table>
undetected. Direct sequencing was performed using BigDye version 3.1 sequencing chemistry and an ABI 3730 DNA Analyzer (Applied Biosystems).

By sequencing, a c.1418C>T variation in TM was detected. This variation was not detected by DHPLC. All samples were genotyped for this variation using the restriction enzyme Cac8I (New England Biolabs, Ipswich, USA). Homozygosity for EPCR variations c.655A>G and c.717+16G>C was also detected by restriction fragment length polymorphism (RFLP) using restriction enzymes AciI and DdeI, respectively. After digestion, the restriction pattern was visualized in agarose gels. Homozygosity of EPCR variation c.323–20T>C was genotyped using DHPLC. To detect homozygous variations, 5 μl of the genotyped samples was mixed with 5 μl of a reference sample with no variations and analysed on the WAVE System. Homozygosity of TM variation c.1728+23_+40del and EPCR c.323–9_336dup was genotyped using 3% agarose gels, on which an 18/23-bp difference in the fragment sizes can be detected.

The nature of variations predicted to change an amino acid was analysed by the SIFT (Sorting Intolerant From Tolerant) program (http://blocks.fhcrc.org/sift/SIFT.html).

Statistical analysis

The χ² and Fisher’s exact tests ([SISA 1997 programs] http://home.clara.net/sisa) were used for statistical analysis of the data. The allele frequencies were compared in patients and controls to determine whether any of the variations were more frequent in either group. Differences were considered as statistically significant for P-values of <0.05.

Results

Eighty-six patients (40 couples and 6 women) with a history of unexplained RM and 191 controls were screened with DHPLC for mutations in the TM and EPCR genes. The nature of the variations detected was studied by sequencing. In total, two sequence variations in TM and four in EPCR were found (Table II).

Variations in the TM gene

In the TM gene, one intronic deletion and one exonic variation were detected. The novel variation 1728+23_+40del, an 18-bp deletion downstream of the coding region of the gene, was detected in five patients and five controls. The c.1418C>T change, a previously reported, common variation, was detected in both patients and controls in heterozygous and homozygous states. The variation is a non-synonymous substitution, predicting an amino acid change at codon 455 from alanine to valine. In the SIFT analysis, any substitution of the amino acid at position 455 was predicted to be tolerated.

Variations in the EPCR gene

In the EPCR gene, two variations in the non-coding regions of the gene and two variations within the exons were detected. The c.323–20T>C substitution, located 20 bp upstream of exon 3, and the c.717+16G>C substitution, located in the 3'UTR, are predicted to be common polymorphisms. The 23-bp (TATC-CACAGTTCCCTGACCACCATC) c.323–9_336 duplication in exon 3 is predicted to code for five amino acids (Tyr, Pro, Gln, Phe and Leu) followed by a stop codon. The c.655A>G substitution in exon 4 is a non-synonymous change, predicting a Ser219Gly change. The variation is tolerated according to the SIFT analysis.

All variations were detected in both patients and controls. There were no significant differences in the allele or genotype frequencies between patients and controls for any of the sequence variations. The 40 couples included in this study were also analysed to determine whether the variations existed in both partners of a couple. The common variations c.323–20T>C, c.655A>G and c.717+16G>C in EPCR and c.1418C>T in TM were all detected in both partners of a couple enabling homozygous states in the fetus. Additionally, the newly identified 1728+23_+40 deletion was detected in a heterozygous state in both partners of one couple.

Discussion

It has been suggested that many cases of RM and other pregnancy complications are caused by defects in maternal haemostatic responses, leading to disturbances of the uteroplacental vasculature and, in some cases, subsequent fetal loss. Accordingly, recent studies have reported an increased prevalence of thrombophilic mutations such as the Leiden mutation, prothrombin G20210A and MTHFR C677T and deficiencies in protein S, protein C and antithrombin in women with RM (Younis et al., 2000; Kujovich, 2004; Krabbendam et al., 2005; Yamada et al., 2005; Kutteh and Triplett, 2006). These findings suggest that thrombophilia may have an important role in the pathogenesis of these complications. Here, we have studied two

<table>
<thead>
<tr>
<th>DNA variation (predicted amino acid change)</th>
<th>Location</th>
<th>Heterozygous patients (n = 86)</th>
<th>Homozygous patients (n = 86)</th>
<th>Heterozygous controls (n = 191)</th>
<th>Homozygous controls (n = 191)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM variations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1418C&gt;T (Ala455Val)</td>
<td>Exon 1</td>
<td>41 (48%)</td>
<td>5 (6%)</td>
<td>84 (44%)</td>
<td>8 (4%)</td>
</tr>
<tr>
<td>c.1728+23_+40del</td>
<td>3'UTR</td>
<td>5 (6%)</td>
<td>0</td>
<td>5 (3%)</td>
<td>0</td>
</tr>
<tr>
<td>EPCR variations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.323–20T&gt;C</td>
<td>intron 2</td>
<td>41 (48%)</td>
<td>15 (17%)</td>
<td>95 (50%)</td>
<td>33 (17%)</td>
</tr>
<tr>
<td>c.323–9_336dup (TyrProGlnPheLeuSTOP)</td>
<td>exon 3</td>
<td>2 (2%)</td>
<td>0</td>
<td>1 (0.5%)</td>
<td>0</td>
</tr>
<tr>
<td>c.655A&gt;G</td>
<td>exon 4</td>
<td>22 (26%)</td>
<td>1 (1%)</td>
<td>49 (26%)</td>
<td>2 (1%)</td>
</tr>
<tr>
<td>c.717+16G&gt;C</td>
<td>3'UTR</td>
<td>41 (48%)</td>
<td>15 (17%)</td>
<td>93 (49%)</td>
<td>34 (18%)</td>
</tr>
</tbody>
</table>

Numbering of the nucleotides is relative to the adenine in the ATG start codon in the reference sequence.

*Novel variation (not previously reported).

*Includes partners for 40 couples.
thrombophilia-related genes, TM and EPCR, for possible mutations in patients with RM. While mutations in TM or EPCR may cause thrombophilia in the mother, thereby constituting a risk factor, homozygous mutations in the fetus may cause miscarriage by other mechanisms (Isermann et al., 2001). In mice, an important role for the TM-protein C-EPCR system in placental development and maintenance of pregnancy is firmly established (Healy et al., 1995; Gu et al., 2002), but the relevance of these mechanisms for pregnancy-associated complications in humans remains unknown. The data on mouse models, the known sequence homology of murine and human TM and EPCR, and the similar type of placentation in both species (Dittman and Majerus, 1989; Cross et al., 1994), however, all suggested TM and EPCR as candidate genes for RM.

In this study, we have analysed the entire coding regions of the TM and EPCR genes including exon–intron boundaries and the 5′ and 3′ UTR for mutations in 46 patients with RM, partners of 40 RM patients and 191 controls. As a result of screening using DHPLC, one exon and one intronic sequence variation in the TM gene and two exonic and two intronic sequence variations in the EPCR gene were detected.

A novel 18-bp deletion in the 3′UTR of the TM gene, 1728+23_40del, was detected in five patients (6%) and five controls (3%). In one couple, both partners carried the deletion creating a 25% chance for every conceptus to be homozygous for the deletion. Unfortunately, no samples are available to study whether these variations may have existed in a homozygous state in the spontaneously aborted pregnancies. The variation is located 23 bp downstream of the exon–intron boundary and may affect splicing or alternatively have an effect on the stability of the mRNA. Further studies would, however, be required to confirm a phenotypic effect of this variation. The other variation detected in TM, c.1418C>T, is a frequent polymorphism resulting in an Ala to Val substitution at amino acid position 455. It is located in the sixth epidermal growth factor (EGF)-like domain of the gene, a region responsible for thrombin binding and protein C activation (Esmon, 1989). However, the variation is likely to be neutral, as it is tolerated according to the SIFT program, and was commonly detected in a homozygous state in both patients and controls in our study. We found no significant differences in the allele frequencies between patients and controls, and the variation has previously been shown to be neutral in respect to thrombophilia (van der Velden et al., 1991).

The intronic substitutions in EPCR, c.323–20T>C, located 20 bp upstream of exon 3 at base position 3997, and c.717+16G>C, located in the 3′UTR at base position 4678, have been previously reported as common polymorphisms. The c.717+16G>C polymorphism, however, is suggested to have a modifier effect on the risk of venous thromboembolism (VTE). Individuals homozygous for the C allele have elevated levels of activated protein C and a lower risk of VTE (Medina et al., 2004). Furthermore, the risk of VTE in Leiden mutation carriers is correlated with the CC genotype carrying a lower risk than GG or GC genotypes (Medina et al., 2005). In our series, the proportion of GC heterozygotes and CC homozygotes was similar in patients (48 and 17%) and controls (49 and 18%). The c.655A>G substitution in EPCR exon 4 at base position 4600 is a non-synonymous change, predicting a Ser219Gly change. It has been noted that individuals carrying the G allele have higher sEPCR levels than AA genotypes but do not have an increased risk of VTE (Medina et al., 2004; Ireland et al., 2005). The variation is also tolerated in the SIFT prediction. The proportion of different genotypes was similar in patients and controls also for this variant in our series, with 26% of both patients and controls being heterozygotes and altogether three individuals being GG homozygotes.

The 23-bp insertion, c.323–9_336dup, in EPCR exon 3, which duplicates the preceding 23 bases at position 4031, results in a premature STOP codon downstream from the insertion point. The mutation leads to the formation of a truncated receptor lacking the extracellular and transmembrane domains. Expression studies have shown that the truncated protein is not localized on the cell surface, cannot be secreted from the cells and does not bind activated protein C (Biguzzi et al., 2001). Franchi et al. (2001) reported that pathological examination revealed placental thrombosis in a woman with late fetal loss carrying the 23-bp insertion. The mutation seems to be rare, and its role in pregnancy complications is still unclear. We detected this variation in one female patient and one male partner and in one control woman. The female patient with the duplication has had three miscarriages and has no children. The couple where the male is a duplication carrier has had three miscarriages and a recent successful pregnancy with heparin treatment.

In screening TM and EPCR genes, a high frequency of some variants, namely c.1418C>T in TM and c.323–20T>C, c.655A>G and c.717+16G>C in EPCR, was identified in patients and controls. Whereas some of these may be true silent polymorphisms, others may have functional significance and modifier effects in combination with thrombophilic mutations or polymorphisms in other genes as has been shown for the c.717+16G>C variant and Leiden mutation (Medina et al., 2005). In a pregnancy, homozygous variants and mutations could also function through other mechanisms than the thrombophilic effect. However, further studies are needed to resolve this.

In this study, we detected two interesting variations, c.323–9_336dup in EPCR and 1728+23_40del in TM, which may have a role in miscarriages. The c.323–9_336dup is a truncating mutation in exon 3 of EPCR, previously identified in a patient with pregnancy loss. The 1728+23_40del is a newly identified variation in the 3′UTR region of TM. As these mutations were rare in our series, more patients/couples should be studied to determine their exact role in RM.

Excluding the common variants, the mutation rate in TM and EPCR was low. This may be because of the fact that mutations in these genes are rare in general or that they are a rare reason for RM or other pregnancy complications, as indicated by other studies (Franchi et al., 2001; Faioni et al., 2002). As TM and EPCR mutations seem to be rare and the aetiology of RM is genetically heterogenous, the number of patients used in this study may be too small and more couples would be needed to detect a difference in variation frequencies between patients and controls and to determine the role of these genes in RM.

Additionally, we may also have missed some mutations as one variant in TM c.1418C>T was detected by random sequencing
of samples showing only homozygous peaks in the DHPLC analysis. Although DHPLC has been shown to be a highly sensitive and specific method for mutation detection (sensitivity and specificity of DHPLC vary between 95 and 100%), some mutations may go undetected or can be detected only at a specific temperature (Xiao and Oefner, 2001).

Taken together, our study results from this small sample suggest that clear-cut mutations in the TM or EPCR genes are not a major cause of RM in Finnish patients. However, some mutations and variants may play a role as indicated by mouse models. Recently, Ghosh and co-workers have suggested that there is an urgent need for a multicentric trial with mutually agreed criteria to study thrombophilia and pregnancy loss (Ghosh et al., 2006). While this may be true, we also suggest that when planning these trials, collection of samples should be extended to include samples from the spontaneously aborted pregnancies as well as both partners of the couples experiencing RM.

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References


