Association between *trefoil factor* 3 gene variants and idiopathic recurrent spontaneous abortion

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**Abstract**  *Trefoil factor* 3 (*TFF3*) gene is an inflammatory mediator expressed in human endometrium during the window of implantation. The aim of this study was to evaluate the possible genetic association of *TFF3* variants in recurrent spontaneous abortion. Women with a history of recurrent spontaneous abortion (*n* = 164) and healthy pregnant women (*n* = 143) were genotyped for five *TFF3* polymorphisms (rs225439 G/A, rs533093 C/T, rs225361 A/G, rs11701143 T/C and rs77436142 G/C). In addition, haplotypes formed within the gene were analysed. Within the recurrent spontaneous abortion group, women who at some point had given birth and childless women had 4.19 ± 1.75 and 5.34 ± 3.42 consecutive spontaneous abortions, respectively. Women who had experienced recurrent spontaneous abortions had a lower allele frequency of the rs11701143 promoter region minor C allele compared with fertile women (0.02 versus 0.05, *P* = 0.015). Patients with rs225361 AG genotype had significantly more successful pregnancies before spontaneous abortion than those with homozygous AA and GG genotypes (*P* = 0.014). No significant differences in haplotype frequencies...
between patients and controls were detected. Possible genetic risk factors identified that might contribute to the pathogenesis of idiopathic recurrent spontaneous abortion were TFF3 gene variants. © 2014 Published by Elsevier Ltd on behalf of Reproductive Healthcare Ltd.

KEYWORDS: haplotype, intestinal trefoil factor, polymorphism, recurrent miscarriage, trefoil factor

Introduction

Recurrent spontaneous abortion, defined as the occurrence of three or more consecutive pregnancy losses, affects 1–3% of women trying to conceive (Kolte et al., 2011; Teklenburg et al., 2010a), with a prevalence of 0.5–1.0% in fertile couples (Maybin et al., 2011), and an incidence that increases with rising maternal age (Brigham et al., 1999; Quenby and Farquharson, 1993). Recurrent spontaneous abortion is a heterogeneous condition of multifactorial causes (Kolte et al., 2011; Li et al., 2002a), including uterine abnormalities, endocrine disorders, paternal and fetal chromosomal abnormalities (Ljungner et al., 2011), autoantibodies, thrombophilia, as well as immunological and genetic disorders (Kolte et al., 2011; Li et al., 2002b). In up to 50% of recurrent spontaneous abortion cases, none of these factors are found in the couple, and is therefore considered idiopathic (Li et al., 2002c).

During the endometrial mid-secretory (receptive) phase, the limited time period of the implantation window ensures coordinated endometrial and embryonic development for successful implantation (Aghajanova et al., 2008; Altmäe et al., 2012). Increasing evidence shows that recurrent spontaneous abortion is a result of a selection failure in preventing ‘poor quality’ embryos from implanting, and being subsequently rejected as their development fails, leading to a later recurrent pregnancy loss (Quenby et al., 2002; Salker et al., 2010; Teklenburg et al., 2010b; Weimar et al., 2012). Furthermore, some endometrial factors can negatively affect the implantation process in women who have experienced recurrent spontaneous abortion (Coughlan et al., 2014; Maybin et al., 2011), causing alterations in factors essential for implantation and maintenance of full-term pregnancy (Lee et al., 2007). Recurrent spontaneous abortion might be a consequence of impaired decidualization, improper invasion or angiogenesis during the early establishment of pregnancy (Singh et al., 2012), thus being associated with aberrant expression of genes responsible for endometrial decidualization and inflammatory processes (Rull et al., 2012), ultimately leading to a prolonged endometrial receptive phase in women who have experienced recurrent spontaneous abortion (Salker et al., 2012).

Different association studies have searched for molecular markers for recurrent spontaneous abortion. Several polymorphisms within the genes involved in inflammation, thrombosis and cardiovascular system, detoxification system, immune response, hormonal regulation and placental function have been shown to be associated with recurrent spontaneous abortion, including tumour necrosis factor alpha gene, matrix metalloproteinase, tissue inhibitor of metalloproteinase, vascular endothelial growth factor, progestosterone receptor, endocrine gland-derived vascular endothelial growth factor, p53, endothelial nitric oxide synthase, phosphodiesterase 8B, transforming growth factor beta 1, chorionic gonadotropin beta, annexin A5 gene, among other genes (Almawi et al., 2013; Eller et al., 2011; Granfors et al., 2012; Hayashi et al., 2013; Lee et al., 2013; Magdoud et al., 2013; Pereza et al., 2013; Rull et al., 2008, 2013; Schweikert et al., 2004; Singh et al., 2012; Su et al., 2010a, 2010b, 2011).

Nevertheless, most of these findings are controversial, and have not been replicated in follow-up studies (Rull et al., 2012), so the search for molecular risk factors for recurrent spontaneous abortion continues.

In a previous genome expression analysis, we identified trefoil factor 3 (TFF3) as one factor that could have a role in the receptive endometrium at the time of embryo implantation (Altmäe et al., 2010). Trefoil factor 3, also known as intestinal trefoil factor, is a member of the trefoil factor mucin-associated peptides family that is produced in epithelial surfaces (Madsen et al., 2007; Samson et al., 2008), and is detected in the human endometrium (Borthwick et al., 2003; Kao et al., 2002). TFF3 possesses a mitogenic effect, which promotes epithelial cell migration during wound healing, and assists in the maintenance and restoration of the epithelial surface integrity (Thim, 1997; Williams and Wright, 1997). Furthermore, as TFF3 is expressed in almost all tissues containing mucin-secreting cells, an effect related to that of mucins is suggested (Kjellev, 2009; Wiede et al., 2001). It has previously been shown that women who have experienced spontaneous recurrent abortion have lower levels of endometrial mucins in the secretory phase compared with normal fertile women (Aplin et al., 1996). On the basis of these findings, the possible associations between recurrent spontaneous abortion susceptibility and genetic variation in the TFF3 gene were analysed. Haplotype pattern within the TFF3 gene and its association with recurrent spontaneous abortion were also analysed.

Materials and methods

Study participants

The present study was approved by the Ethical Review Boards at Uppsala University and Karolinska Institutet, Sweden on 1 May 2009 (International Review Board reference number: 2006/1545-31/4), and written informed consent was obtained from all participants. A total of 307 women participated in the study. Blood samples were collected from 164 patients with a mean age of 30.36 ± 5.71 years who had experienced three or more consecutive spontaneous abortions in the first or second trimester of pregnancy (5–21 weeks of gestation), and were recruited from the Departments of Obstetrics and Gynecology at the Uppsala University Hospital, Huddinge University Hospital, Karolinska University Hospital, and Danderyd University Hospital, Sweden (Table 1). Women with a history of spontaneous recurrent abortion diagnosed between the 1989 and 2009 were considered eligible, with the exclusion of patients with obvious risk factors...
causing their spontaneous recurrent abortion, such as thyroid disease, thyroperoxidase (TPO) antibodies, cardiolipin, lupus, paternal chromosomal abnormalities, thrombophilia, factor (II, V leiden, VIII, and XIII) deficiencies, von Willebrand disease, antithrombin III deficiency, homocysteine, protein S and protein C deficiency, plasminogen activator inhibitor-1 and -2, prothrombin complex (PK), activated partial thromboplastin time, uterine fibroids and uterine abnormalities.

All women in this group had conceived naturally. All participants were white and of European origin.

The control group consisted of 143 pregnant women (28.52 ± 4.84 years), who had conceived spontaneously and were in the second trimester of their pregnancy (16–18 weeks of gestation). The women were randomly chosen from Uppsala University biobank, and were healthy volunteers, with no history of spontaneous abortions, and at least one full-term pregnancy resulting in a live birth. Controls were matched for maternal age at the first attempted pregnancy, and for ethnicity as white of European origin.

Genotyping TFF3 polymorphisms

Genomic DNA was extracted from whole peripheral blood using QIAamp® DNA Blood Maxi protocol (QIAGEN, Venlo, Netherlands) according to manufacturer’s instructions. Real-time polymerase chain reaction was carried out in accordance with TaqMan® SNP Genotyping Assays manufacturers’ instruction kit and manual (Applied Biosystems, Foster City, CA, USA), using 1–20 ng of purified genomic DNA as a template. Amplification was carried out for 40 cycles with a denaturation temperature of 95°C and an annealing and extension temperature of 60°C. Primers and probes were custom made, except for two of the TFF3 polymorphisms, which were designed using the Primer Express® Software Version 3.0 (Applied Biosystems®, Foster City, CA, USA).

The five single nucleotide polymorphisms (SNP) analysed in the TFF3 gene included rs77436142 G/C (promoter region), rs11701143 T/C (promoter region), rs225361 A/G (intron 1), rs533093 C/T (intron 2), and rs225439 G/A (3’ UTR). All SNP were genotyped using TaqMan® SNP Genotyping Assays (Applied Biosystems). Polymorphism selections were based on previously published data, and NCBI (http://www.ncbi.nlm.nih.gov) and HapMap (http://www.hapmap.org) databases. All five SNP were validated; SNP rs225439, rs533093, rs225361, and rs11701143 had a minor allele frequency 0.04 or greater; and based on the haplotype pattern, the selected SNP covered the gene locus.

Haplotype analysis

The program Haploview was used to calculate allele frequencies and estimate pair-wise linkage disequilibrium between SNP. Genotypes’ deviations from Hardy-Weinberg equilibrium was also assessed. Haplotypes and haplotype blocks were generated using Haploview’s default option, which is based on confidence intervals. The association between phenotypes and haplotypes was tested using Haploview software (version 4.1) (Barrett et al., 2005).

Statistical analysis

Statistical analyses were carried out using the Statistical Package for Social Sciences software (SPSS version 19.0 for Macintosh; IBM Corp., Armonk, NY, USA). Data are given as mean ± SD (continuous variables), or percentages (categorical variables), unless otherwise indicated. Nominal variables, genotypes and alleles, were analysed using chi-squared test. Correlation between any two variables was evaluated using Pearson’s correlation analysis test. P < 0.05 was considered statistically significant. Continuous variables, being normally distributed, were analysed using analysis of variance. When comparing genotypes (three groups) in analysis of variance, Bonferroni correction was applied, so that groups would be considered significantly different when P < 0.017 (0.05/3 = 0.017).

Results

Study group characteristics

The number of women who experienced three and four or more consecutive pregnancy losses was 75 (45.73%) and...
89 (54.27%), respectively, with an average of 4.94 ± 1.50 and 7.56 ± 2.78 pregnancies, respectively. According to Pearson’s correlation test, with each additional pregnancy, the consecutive spontaneous abortion rate increased by 0.749 (P < 0.01). Women with no children after recurrent spontaneous abortion had an average of 5.34 ± 3.42 consecutive spontaneous abortions, whereas women with children had an average of 4.19 ± 1.75 consecutive spontaneous abortions. Women in the recurrent spontaneous abortion group had a total live birth outcome of 79.14% (having a full term pregnancy with at least one child successfully being born).

**TFF3 gene variation**

Genotype and allele frequencies among women who have experienced recurrent spontaneous abortion and controls are shown in Table 2. All genotype distributions within were in Hardy-Weinberg equilibrium. Furthermore, all genotype frequencies were in agreement with the 1000 Genomes database (browser.1000genomes.org). In the present study, women who had experienced recurrent spontaneous abortion presented a lower frequency of the rs11701143 minor C allele compared with fertile controls (0.02 versus 0.05 P = 0.015). A significant difference in the genotype frequencies of the rs11701143 polymorphism was found between the patient group and controls, with the TT genotype being significantly more frequent and TC genotype less frequent among women who had experienced recurrent spontaneous abortion (96.3% and 3.7% versus 90.2% and 9.8%; P = 0.027). The genotype and allele frequencies of the other SNP were distributed similarly among fertile women and women who had experienced recurrent spontaneous abortion.

**TFF3 haplotype pattern and frequency**

The five analysed polymorphisms in the TFF3 gene formed linkage disequilibrium blocks, and linkage-disequilibrium seemed to exist between three polymorphisms: two polymorphisms in the intron regions (rs533093 C/T and rs225361 A/G) and one polymorphism in the 3’ UTR region (rs225439 G/A) (Figure 1). Haplotype prevalence analysis within the studied population showed the presence of four haplotype combinations of the variant alleles of rs225439 G/A, rs533093 C/T, and rs225361 A/G (Table 3). The haplotype formed of the major alleles, rs225439 G – rs533093 C – rs225361 A, was the most frequent in the studied population (56.0%), whereas

<table>
<thead>
<tr>
<th>Allele</th>
<th>Genotype</th>
<th>Frequency</th>
<th>Chi-squared</th>
<th>Frequency</th>
<th>Chi-squared</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs225439</td>
<td>GG</td>
<td>53 (32.9)</td>
<td>0.18</td>
<td>40 (28.56)</td>
<td>0.06</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>81 (50.3)</td>
<td></td>
<td>71 (50.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>27 (16.8)</td>
<td></td>
<td>29 (20.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p(G)</td>
<td>0.58</td>
<td></td>
<td>0.54</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>q(A)</td>
<td>0.42</td>
<td></td>
<td>0.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs533093</td>
<td>CC</td>
<td>67 (40.9)</td>
<td>0.27</td>
<td>54 (37.8)</td>
<td>0.52</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>78 (47.6)</td>
<td></td>
<td>71 (49.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>19 (11.6)</td>
<td></td>
<td>18 (12.76)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p(C)</td>
<td>0.65</td>
<td></td>
<td>0.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>q(T)</td>
<td>0.35</td>
<td></td>
<td>0.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs225361</td>
<td>AA</td>
<td>60 (36.6)</td>
<td>0.006</td>
<td>44 (30.8)</td>
<td>0.004</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>78 (47.6)</td>
<td></td>
<td>71 (49.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>26 (15.9)</td>
<td></td>
<td>28 (19.6)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>p(A)</td>
<td>0.60</td>
<td></td>
<td>0.56</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>q(G)</td>
<td>0.40</td>
<td></td>
<td>0.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs11701143</td>
<td>TT</td>
<td>157 (96.3)</td>
<td>0.06</td>
<td>129 (90.2)</td>
<td>0.38</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>6 (3.7)</td>
<td></td>
<td>14 (9.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>0 (0)</td>
<td></td>
<td>0 (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p(T)</td>
<td>0.98</td>
<td></td>
<td>0.95</td>
<td></td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>q(C)</td>
<td>0.02</td>
<td></td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs77436142</td>
<td>GG</td>
<td>163 (100)</td>
<td>NA</td>
<td>143 (100)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>0 (0)</td>
<td></td>
<td>0 (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>0 (0)</td>
<td></td>
<td>0 (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p(G)</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>q(C)</td>
<td>0.00</td>
<td></td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NA, not applicable; NS, non-significant.
the other combinations ATG, ACG and ACA were less frequent (36.0%, 6.0% and 2.0%, respectively). No significant differences were found in haplotype block frequencies between fertile women and women who had experienced recurrent spontaneous abortion (Table 3).

### Table 3

<table>
<thead>
<tr>
<th>Haplotype block</th>
<th>Overall frequency</th>
<th>Patient frequencies</th>
<th>Control frequencies</th>
<th>Chi-squared</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCA</td>
<td>0.56</td>
<td>0.58</td>
<td>0.53</td>
<td>1.45</td>
</tr>
<tr>
<td>ATG</td>
<td>0.36</td>
<td>0.35</td>
<td>0.37</td>
<td>0.35</td>
</tr>
<tr>
<td>ACG</td>
<td>0.06</td>
<td>0.04</td>
<td>0.07</td>
<td>1.82</td>
</tr>
<tr>
<td>ACA</td>
<td>0.02</td>
<td>0.03</td>
<td>0.02</td>
<td>0.34</td>
</tr>
</tbody>
</table>

P-values showed no significant differences between the two groups.

### Association between TFF3 gene variants and recurrent spontaneous abortion

The strict multiple corrections were taken into account, and the only association between recurrent spontaneous abortion and TFF3 variants that remained significant was between rs225361 and having had children before the first spontaneous abortion. Patients with the rs225361 homozygous AA or GG genotypes had children less frequently before their first spontaneous abortion compared with women who had the heterozygous AG genotype (AA 15/60 [25.0%] versus AG 33/78 [42.31%] versus GG 4/26 [15.38%], respectively, \(P = 0.014\)) (Figure 2). No significant differences between the number of previous spontaneous abortion, age and TFF3 genotypes were detected.

### Discussion

In the present study, the potential association between the TFF3 gene polymorphisms and haplotypes in patients who had experienced recurrent spontaneous abortion were analysed. The main findings show an association between TFF3 rs11701143 promoter region with idiopathic recurrent spontaneous abortion, and intronic SNP rs225361 with live birth before spontaneous abortion.

Several studies have addressed TFF3 gene expression in association with different cancers (Balcer-Kubiczek et al., 2002; Bignotti et al., 2008; Kannan et al., 2010). As far as is known, this is the first study to analyse genetic variation and haplotype pattern of this gene in women who have experienced recurrent spontaneous abortion. The TFF3 rs11701143 minor allele frequency (C) was significantly lower in women who had experienced recurrent spontaneous abortion compared with healthy fertile controls among the study groups. The function of this SNP is so far not known, but as this allele variant is located within the regulatory region of the transcription factor-binding site (http://www.ensembl.org), polymorphisms in this promoter region might interfere with the gene expression regulation (Daher et al., 2012). The effect of this polymorphism on gene expression level, however, is so far unknown. In addition, previous studies have reported TFF3 gene expression to be cycle-dependent (Borthwick et al., 2003; Kao et al., 2002).

The intronic polymorphism rs225361 A/G within TFF3 was associated with live birth before spontaneous abortion in our study. This intron variant has been found to be located within a regulatory region (http://www.1000genomes.org).
Nevertheless, the mechanisms by which introns enhance gene expression are still poorly understood; however, introns seem to affect various steps of mRNA maturation, including splicing, transcription initiation, elongation, and termination, polyadenylation, nuclear export, and mRNA stability (Chorev and Carmel, 2012). Yet, the functional effect of the identified promoter and intronic variant on TFF3 gene expression need to be assessed in future studies.

Among the studied women, a relative increase in the number of consecutive spontaneous abortions with an increase in age was observed. The highest number of consecutive spontaneous abortions occurred in women aged between 35 and 44 years. Live birth rates were higher in women who had their first spontaneous abortion aged between 25 and 34 years, and women with children had significantly fewer spontaneous abortions. Therefore, a major factor that influenced subsequent live births among these women was the number of consecutive spontaneous abortions. This result is in line with previous studies, which have found a relationship between the history of previous spontaneous abortions and subsequent birth rates (Anokute, 1987; Li et al., 2002a).

It is also well known that increasing maternal age and the number of previous spontaneous abortions have negative effects on pregnancy outcome (Clifford et al., 1997; Li et al., 2002b; Quenby and Farquharson, 1993).

Furthermore, interactions of the TFF3 pathway with certain mucins have been recently identified (Haroun et al., 2013). Mucins are known to be important in the receptive endometrium, in which they are required for selection and implantation of high-quality embryos. Nevertheless, endometrial expression of certain mucins has previously been found to be significantly lower in women who have experienced recurrent spontaneous abortion compared with fertile women. This reduced expression may cause inappropriate endometrium receptivity, allowing defective embryos to implant (Aplin et al., 1996; Gipson et al., 2008; Li et al., 2002c; Quenby et al., 2002; Teklenburg et al., 2010a; Xu et al., 2012). Whether a similar effect might be caused by TFF3 gene variants, requires further investigation.

In conclusion, our study results indicate that individual and combined TFF3 genetic variations could be a contributing risk factor of recurrent spontaneous abortion. Further studies of the function of the polymorphisms, and the gene’s expression pattern in endometrial samples, are recommended to give a deeper understanding of the possible role of TFF3 in recurrent spontaneous abortion.

Acknowledgements

This study was supported by grants from Uppsala University; the Family planning foundation, Uppsala; Karolinska Institutet; Estonian Ministry of Education and Research (grant SF0180044s09); Enterprise Estonia (grant EU30020); EU-FP7 Eurostars program (grant NOTED, E6478); EU-FP7 IAPP project (grant SARM, 324509); and the Spanish Ministry of Education (grant no. SB2010-0025). We are grateful to all participants in the study, and to the staff who collected the study material at Uppsala University Hospital, Huddinge University Hospital, Karolinska University Hospital, and Danderyd University Hospital, Sweden. Special thanks go to Tiina Murto for her help in collecting the study material.

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Declaration: The authors report no financial or commercial conflicts of interest.

Received 12 February 2014; refereed 18 August 2014; accepted 19 August 2014.