**Forkhead box p3 (Foxp3) gene polymorphisms and risk of unexplained recurrent spontaneous abortion among Egyptian women**

Research Article

Dr Hanan Mohamed Farhan1*, Dr Maha Hassan Katta 2
1 Department of Clinical and Chemical Pathology, Faculty of Medicine, Beni-Suef University
2 Department of Gynaecology and Obstetrics, Faculty of Medicine, Beni-Suef University

*Correspondence: Dr Hanan Mohamed Farhan, Department of Clinical and Chemical Pathology, Faculty of Medicine, Beni-Suef University
Email: hananmohamedfarhan@gmail.com

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Abbreviations: Transcription factor forkhead box p3 (Foxp3), unexplained recurrent spontaneous abortion (URSA).

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Summary

Transcription factor forkhead box p3 (Foxp3) gene plays crucial role in T regulatory (Treg) cells development and function. Tregs are involved in mediating maternal tolerance to the foetus and avoiding immunological rejection of the foetus. Our study aimed to determine whether there is a relationship between genetic polymorphisms of Foxp3 and susceptibility to unexplained recurrent spontaneous abortion (URSA).

Materials and Methods:
Using polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) assay, we examined the frequency of Foxp3, rs3761548AC and rs2294021TC, gene polymorphisms in Egyptian URSA patients (n = 40) and control individuals (n = 40).

Results:
Foxp3 rs3761548AC (p = 0.012) and rs2294021TC (p<0.001) genotype polymorphisms were significantly more frequent in women with URSA than in controls with odds ratio (OR) values 6.3 (95%CI 1.3-31.1) and 9.3 (95%CI 3.3-26.1), respectively. There were differences in the distribution of A allele of rs3761548AC (OR=1.5, 95%CI=0.8-2.9, p=0.197) and T allele of rs2294021TC (OR=4, 95%CI=1.9-8, p<0.001) between URSA and controls; where only T allele reached statistical significance.

Conclusion:
Foxp3, rs3761548AC and rs2294021TC, gene polymorphisms may be considered as genetic risk factors in development of URSA in Egyptian patients.

I. Introduction

Recurrent spontaneous abortion (RSA) is defined as two or more consecutive pregnancy losses before the 20th week of gestation (Beaman et al., 2012; Jahaninejad et al., 2013; Mei et al., 2010). Complex aetiological factors including; chromosomal, anatomic, endocrine, autoimmune abnormalities and infections of the reproductive tract could be reported as causes of RSA. But still the factors inducing abortion in at least 35-44% of patients remain unclear; known as unexplained recurrent spontaneous abortion (URSA) (Christiansen et al., 2004; Kwak-Kim et al., 2010; Wilczyński et al., 2012). During pregnancy, the foetus is allowed to grow within the maternal uterus due to multiple mechanisms of immune tolerance in spite of being semi-allogeneic foreign tissue which would immediately lead to a strong immune response directed to destroy foetus and placenta (Leber et al., 2011). This adaptation of the maternal immune response is necessary for pregnancy success, and any maternal tolerance disturbances could result in infertility and reproductive pathologies (Guerin et al., 2009). URSA has been proposed to be failure of foetalmaternal immunologic tolerance that belongs to an alloimmune disease (Leber et al., 2011; Wilczyński, 2006).

A subset of T-lymphocytes known as T regulatory (Treg) cells are cells having potent suppressive activity minimizing destructive immune responses and preventing autoimmune disease (Guerin et al., 2009). Tregs are essential for normal immune homeostasis through regulation of T cell activation (Oda et al., 2013). Immune suppression is a key function of Treg cells (Park et al., 2005; Yang et al., 2010). During pregnancy, Tregs play a critical role for foetal-maternal immunological tolerance at the fetal-maternal interface (Zenclussen et al., 2006). Treg cells have been considered to have reduced suppressive capacity in URSA (Jiang et al., 2009).

Forkhead box p3 (Foxp3) gene encodes a transcription factor important for the development and function of Treg cells (Oda et al., 2013). Foxp3 is a transcriptional regulator that belongs to the forkhead/winged-helix family on chromosome X (Xp11.23), 1296 bp in size and consists of 11 different exons (Coffer and Burgering 2004; Fontenot et
al., 2005). Up regulation of Foxp3 expression results in the conversion of naïve T cells to Tregs (Jahan et al., 2013). Foxp3 protein is expressed transiently in CD4+CD25+ effector T cells upon T-cell receptor (TCR) stimulation (Gavin et al., 2006; Wang et al., 2007). CD4+ CD25+ Foxp3+ subset of regulatory T cells can suppress the activity of autoreactive T cells that have escaped deletion in the thymus enabling tolerance to self antigens (Campbell and Ziegler, 2007). CD4+ CD25+ Treg cells play critical role in peripheral tolerance, transplantation tolerance and maternal tolerance to the foetus through immunosuppression by different mechanism. First, by inhibiting the conventional T cells immunostimulation through cell-to-cell contact. Second, through immunosuppressive cytokines such as interleukin 10 and transforming growth factor-beta. Third, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on CD4+CD25+ regulatory T cells up-regulate indoleamine 2,3-dioxygenase (IDO) expression in dendritic cells which play important roles for immunosuppression (Saito et al., 2005). Deficiency of Foxp3 gene impairs the suppressive function of Treg cells (Okumura et al., 2008). Dysfunction of Foxp3 gene product could lead to chronically activated CD4+T cells because of lack of Treg cells. This causes increased levels of several activation markers and cytokines, resulting in some autoimmune diseases (Oda et al., 2013). Association has been reported between Foxp3 gene polymorphisms and autoimmune diseases, such as systemic lupus erythematosus (SLE) (Lin et al., 2011), autoimmune thyroid diseases (AITDs) (Inoue et al., 2010), type 1 diabetes (TID) (Holm et al., 2006), and allergic rhinitis (Zhang et al., 2009). Previous studies suggest that women with URSA had remarkable deficiency and/or functional deficiency of CD4+CD25+ Treg cells in peripheral blood as well as in decidua (Mei et al., 2010; Sasaki et al., 2004; Yang et al., 2008). The reduction of Treg cells in URSA patients is closely related to the decreased expression of Foxp3 (Mei et al., 2010). In our study, we analyzed Foxp3 gene polymorphisms at the rs3761548AC and rs2294021TC loci among Egyptian women with URSA.

II. Materials & Methods

II.A. Study subjects

A total of 40 unrelated URSA patients (age 29.3 ± 4.5 years) were selected from those attending the Gynaecology and Obstetrics Clinic of Beni-Suef University Hospital during the period between October 2014 and April 2015. All patients had histories of at least two successive miscarriages with unexplained aetiology. Patients were subjected to the following: [1] Pelvic examination; [2] Transvaginal ultrasound; [3] Hysterosalpingography and diagnostic hysteroscopy; [4] Vaginal swab; [5] Serum progesterone and prolactin assays; [5] Antinuclear antibodies (ANA), lupus anticoagulant (LA), and anticardiolipin antibodies (ACL) assays; [6] Semen analysis for all male partners.

The control group of 40 women (age 27 ± 3 years) with at least one live birth was derived from volunteers with no history of spontaneous abortion or preterm labour. The subjects in both patients group and control group were examined on the endocrine and immune factors to exclude individuals with diabetes, hyper- or hypo- thyroidism, SLE and APS. The study design was approved by the Scientific Research Committee, Faculty of Medicine, Beni-Suef University. Data confidentiality was preserved according to the Revised Helsinki Declaration of Bioethics (2008) (World Medical Association, 2008). Informed consent was obtained from all participants in this study.

II.B. DNA extraction

Peripheral blood samples of the patients were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) for DNA isolation. The genomic DNA was extracted from each blood sample using QIAamp DNA mini Blood kit (cat. no.51304) (Qiagen, Germany) according to the manufacturer's instructions.

II.C. Genotyping of Foxp3 rs3761548AC and rs2294021TC Polymorphisms

Foxp3 rs3761548AC and rs2294021TC Polymorphisms were determined using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay (Wu et al., 2012). The PCR primers are presented in Table 1.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3761548A/C</td>
<td>5′-GCCCTTTGCTA CATCCACGCCTCT-3′</td>
<td>5′-CAGCCTTGCCTA ATACAGAGCC-3′</td>
</tr>
<tr>
<td>rs2294021T/C</td>
<td>5′-CACACACAAATC CATCCAGTCACC-3′</td>
<td>5′-ATCTCCATGCC CTAAGAACCCACC-3′</td>
</tr>
</tbody>
</table>

PCR assay was performed for each sample in a final reaction volume of 25 μL using 5 μL DNA, 12.5 μL universal master mix, 1 μL forward primer, 1 μL reversed primer, together with 5.5 μL distilled water (DW).

The PCR conditions were as follows: an initial denaturing step at 98°C for 1 min, followed by 35 cycles of 98°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min, and a final extension at 72°C for 7 min (Wu et al., 2012). All reactions were done using the thermal cycler Applied Biosystems (Perkin-Elmer 9600, USA).

After amplification, the PCR products were treated at 37°C for 1 h, followed by 35 cycles of 98°C for 20 sec, annealing at 55°C for 20 sec, and extension at 72°C for 1 min. The PCR products were then resolved on 2% agarose gel containing ethidium bromide, then visualized using UV transilluminator. DNA molecular weight marker (Cat No. # SM0373, Lot/ 244669 Fermentas Life Sciences, USA) and HaeIII (Cat No. # FD0154, Fermentas Life Sciences, USA) were used for size estimation of PCR-RFLP products. Genotypes of rs3761548AC were defined by the presence of different bands: AA (487 bp), AC (487 bp, 329 bp and 158 bp), and CC (329 bp, 158 bp). For rs2294021TC, genotypes were defined by the presence of distinct patterns of bands: TT (322 bp, 87 bp, and 20 bp), TC (322 bp, 216 bp, 106 bp, 87 bp, and 20 bp), CC (216 bp, 106 bp, 87 bp, and 20 bp).
The 20 bp band was running out of the gel. The representative gels of typical patterns of bands for Foxp3 rs3761548AC and rs2294021TC genes are presented in figures 1 and 2, respectively.

### II.D. Statistical methods

Data were analyzed using IBM SPSS advanced statistics version 22 (SPSS Inc., Chicago, IL). 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 was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann–Whitney test (non-parametric t-test). Odds ratio (OR) with its 95% confidence interval (CI) was used for risk estimation. A p-value < 0.05 was considered significant (Dawson and Trapp, 2001).

### III. Results

All patients were diagnosed unexplained abortion by: absence of uterine and cervical abnormalities excluded by pelvic examination and transvaginal ultrasound in all cases while hysterosalpingography and diagnostic hysteroscopy were done when needed; absence of Chlamydia and Ureaplasma diagnosed by Vaginal swab; luteal phase defect and hyperprolactinemia were excluded by serum progesterone and prolactin assays; autoimmune factors associated with systemic lupus erythematosus (SLE) and the antiphospholipid syndrome (APS) were excluded through testing for antinuclear antibodies (ANA), lupus anticoagulant (LA), and anticardiolipin antibodies (ACL); those with endocrine diseases including diabetes, hyperthyroidism and hypothyroidism were excluded; and all male partners had normal semen status.

As regards rs3761548AC genotypic distribution, the frequency of the combined genotypes AC/AA in the URSA group (95%) was significantly higher than that in the control group (75%) (OR=6.3, 95% CI=1.3-31.1, p=0.012); shown in Table 2 and Figure 3. The frequency of A allele in the URSA group (65%) was higher than that in the control group (55%), yet it did not reach statistical significance (OR=1.5, 95% CI=0.8-2.9, p=0.197); shown in Table 3 and Figure 4.

The genotypic distribution of genotypes TC/TT at the locus rs2294021TC was also significantly higher in the URSA group (80%) than control groups (30%) (OR=9.3, 95% CI=3.3-26.1, p=0.001); shown in Table 4 and Figure 5. The frequency of T allele in the URSA group (50%) was statistically higher than that in the control group (20%) (OR=4.0, 95% CI=1.9-8.9, p<0.001); shown in Table 5 and Figure 6.

### Table 2: Genotype frequency of Foxp3 rs3761548 polymorphism in controls and URSA patients

<table>
<thead>
<tr>
<th>Marker</th>
<th>Genotyp e</th>
<th>URSA N (%)</th>
<th>Control N (%)</th>
<th>P-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3761548</td>
<td>C/C</td>
<td>2(5%)</td>
<td>10(25%)</td>
<td>-</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>A/C</td>
<td>24(60%)</td>
<td>16(40%)</td>
<td>-</td>
<td>7.5(1.5-38.9)</td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>14(35%)</td>
<td>14(35%)</td>
<td>-</td>
<td>0.06(0.9-27.1)</td>
</tr>
<tr>
<td></td>
<td>C/C + A/C</td>
<td>14(35%)</td>
<td>14(35%)</td>
<td>-</td>
<td>1(0.4-2.5)</td>
</tr>
<tr>
<td></td>
<td>A/C</td>
<td>26(65%)</td>
<td>26(65%)</td>
<td>-</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>C/C + A/A</td>
<td>2(5%)</td>
<td>10(25%)</td>
<td>-</td>
<td>6.3(1.3-31.1)</td>
</tr>
</tbody>
</table>
Figure 3: Genotype frequency of Foxp3 rs3761548 polymorphism in controls and URSA patients

Table 3: Allele frequency of Foxp3 rs3761548 polymorphism in controls and URSA patients

<table>
<thead>
<tr>
<th>Marker</th>
<th>Allele</th>
<th>URSA N (%)</th>
<th>Control N (%)</th>
<th>P-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3761548</td>
<td>A</td>
<td>52(65%)</td>
<td>44(55%)</td>
<td>0.197</td>
<td>1.5(0.8-2.9)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>28(35%)</td>
<td>36(45%)</td>
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</tr>
</tbody>
</table>

Figure 4: Allele frequency of Foxp3 rs3761548 polymorphism in controls and URSA patient

Table 4: Genotype frequency of Foxp3 rs2294021 polymorphism in controls and URSA patients

<table>
<thead>
<tr>
<th>Marker</th>
<th>Genotype</th>
<th>URSA N (%)</th>
<th>Control N (%)</th>
<th>P-value</th>
<th>OR (95% CI)</th>
</tr>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marker</td>
<td>Allele</td>
<td>URSA N (%)</td>
<td>Control N (%)</td>
<td>P-value</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>---------</td>
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<td>-------------</td>
</tr>
<tr>
<td>rs2294021</td>
<td>C</td>
<td>40(50%)</td>
<td>64(80%)</td>
<td>&lt;0.001</td>
<td>4(1.9-8)</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>40(50%)</td>
<td>16(20%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 5:** Allele frequency of *Foxp3* rs2294021 polymorphism in controls and URSA patients

**Figure 5:** Genotype frequency of *Foxp3* rs2294021 polymorphism in controls and URSA patients
IV. Discussion

URSA has been suggested to be associated with the failure of foetal–maternal immunological tolerance in which Tregs play a crucial role (Naderi-Mahabadi et al., 2015). Foxp3 is an essential transcription factor for the induction and development of Tregs (Saxena et al., 2015). Foxp3 protein remains the best and most specific marker of Treg cells (Ziegler, 2006). Foxp3 expression serves as an on-and-off switch to regulate positively the physiology of Tregs (Wan and Flavell, 2007). Foxp3 mutations cause developmental or functional failure of natural Treg cells (Fontenot et al., 2003). Our study evaluated the association between Foxp3 rs3761548AC and rs2294021TC gene polymorphisms with URSA in an Egyptian population.

In our results we found that Foxp3 rs3761548AC and rs2294021TC gene polymorphisms were associated with URSA, Foxp3 rs3761548AC genotypic AC/AA and allelic A frequencies were higher in the URSA group than control group. Foxp3 rs2294021TC genotypic TC/TT and allelic T frequencies were higher in the URSA group than control group. Wu et al., 2012 found that genotypic frequency of Foxp3 rs3761548AA was significantly different between the URSA group and control while genotypic and allelic frequencies of rs2294021TC polymorphism showed no statistical difference. Saxena et al., 2015 reported that variants of Foxp3 SNPs namely; rs3761548 and rs2294021 may be associated with idiopathic recurrent miscarriages. Hadinedoushan et al., 2015 reported that Foxp3 rs3761548 promoter polymorphism is not a risk factor for recurrent spontaneous abortion. Naderi-Mahabadi et al., 2015 found no association for Foxp3 rs3761548 polymorphism with URSA. Shen et al., 2010 reported that individuals with a genotype of rs3761548AA have the lowest production of Foxp3 among the three genotypes of this polymorphism. Therefore, URSA patients with the AA genotype may have fewer Treg cells and/or weaker suppressive function and are difficult to accommodate foetal tolerance. These results are in agreement with other reports, showing a decrease in the proportion of Foxp3 Treg cells in URSA patients (Mei et al., 2010; Yang et al., 2008). Foxp3 gene; playing a crucial role in the development and function of Tregs; its polymorphism may cause a functional deficiency in Tregs (Wan and Flavell, 2007; Williams and Rudensky, 2007). Tregs increase during the first and second trimesters of pregnancy in peripheral blood and decidual tissue and decline before delivery to near prepregnancy levels (Guerin et al., 2009; Saito et al., 2005). Women with reduced frequency of Treg cells; in both decidua and peripheral blood; suffer reduced immunosuppressive competence and susceptibility to URSA (Yang et al., 2008). Decreased numbers of circulating Tregs, influenced by expression of Foxp3, have been observed in women with pregnancy complications, including recurrent pregnancy loss (Wu et al., 2012). These reports support our findings that Foxp3 gene polymorphisms were statistically significant in URSA women.

In conclusion, both Foxp3 rs3761548AC and rs2294021TC gene polymorphisms were related to the occurrence of URSA. Our results suggest the importance of Foxp3 in maintaining normal pregnancy. Yet, larger number of samples is recommended to gain more reliable conclusions. Future larger studies from different populations have to investigate both Foxp3 expression and Treg cells frequency among different Foxp3 genotypes in URSA patients, which may have clinical importance in modulating susceptibility to pregnancy loss.

Conflicts of interest: The authors have no conflicts of interest to declare. The authors alone are responsible for the content and writing of the paper. The authors did not receive any funds from any source.

Ethical considerations: All patients and healthy controls included in this study gave their informed consent and approval upon participating in the study. Neither patients' names nor photos were included in this study.
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