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RESEARCH ARTICLE

Study of the association of forkhead box P3 (*FOXP3*) gene polymorphisms with unexplained recurrent spontaneous abortions in Indian population

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Abstract. Recurrent spontaneous abortions (RSA) is defined as three or more consecutive pregnancy losses before 20 weeks of gestation. Various causes of RSA have been identified, still 50% cases remain unexplained after evaluation. One of the causes of unexplained recurrent spontaneous abortions (URSA) is supposed to be the disruption of immunological tolerance at foeta–maternal interface. Regulatory T cells (Tregs) are responsible for the development of immune-tolerant environment at foetal–maternal interface and supports pregnancy. Forkhead/winged helix transcription factor (*FOXP3*) gene plays an important role in the development and function of Tregs. In URSA, Tregs (CD4+CD25+) are reduced in peripheral blood and decidua of pregnant women. This reduction of Tregs (CD4+CD25+) is associated with decreased expression of *FOXP3* gene. This study evaluated the association between single-nucleotide polymorphisms (SNPs) in *FOXP3* gene and URSA in Indian population. In this study, 100 patients with a history of URSA and 100 healthy ethnically matched women with at least one normal pregnancy and no abortion were included as case and control groups, respectively. Four SNPs of *FOXP3* gene, two in the promoter region: −924A/G and −3279C/A, and two intronic, −20G/A and +459T/C, were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). −924A/G and +459T/C polymorphisms were found to be associated with URSA. −3279C/A and −20G/A polymorphism were not found to be associated with URSA. The odds ratio (OR) of mutant allele G for −924A/G polymorphism was 2.5 (95% CI 1.7–3.8; $P < 0.001$) and mutant allele C for +459T/C polymorphism was 1.7 (95% CI 1.1–2.6; $P = 0.01$). For −20G/A polymorphism, only GG genotype was found in both URSA and controls. These results suggest that −924A/G and +459T/C polymorphisms of the *FOXP3* gene might be associated with URSA and −20G/A polymorphism is likely to be rare in Indian population and might not be associated with URSA.

Keywords. *FOXP3* gene; regulatory T cells; single-nucleotide polymorphism; unexplained recurrent spontaneous abortion.

Introduction

Recurrent spontaneous abortions (RSA) or recurrent pregnancy loss (RPL) is defined as three or more consecutive pregnancy losses before 20 weeks of gestation. According to Practice Committee of the American Society for Reproductive Medicine (ASRM), RSA is redefined as two or more consecutive pregnancy losses and needs proper evaluation. About 1% couples are affected by RSA (Li *et al.* 2002). Various causes have been reported in the pathogenesis of RSA including anatomical, thrombophilic, immunological, infectious, hormonal and genetic. But, still 50% cases of RSA remain unexplained and are termed as unexplained recurrent spontaneous abortion (URSA) (Ford *et al.* 2009). Associations of URSA with various immune factors, which may cause disruption of immunological tolerance at foetal–maternal interface have been reported (Leber *et al.* 2011). Regulatory T cells (Tregs) are specialized T cells which are responsible for the development of immune-tolerant environment at foetal–maternal interface and supports pregnancy (Zenclussen *et al.* 2006). In URSA, it is found that Tregs (CD4+CD25+) are reduced in peripheral blood and decidua of pregnant women (Yang *et al.* 2008). In URSA, this reduction of Tregs (CD4+CD25+) is associated with decreased expression of forkhead box P3 (*FOXP3*) gene, which may result in disruption of immune barrier at foetal–maternal interface (Mei *et al.* 2010).

Human *FOXP3* gene (gene ID: 50943, OMIM: 300292) is a transcriptional regulator that belongs to the forkhead/winged-helix family and is located on chromosome X (Xp11.23). *FOXP3* gene is a main transcription factor for the development and function of Tregs (CD4+CD25+) (Wu *et al.* 2006). *FOXP3* gene polymorphisms in the promoter region affect transcription initiation and thus, gene expression. Intronic polymorphism results in alternative splice site formation and affects RNA processing.

FOXP3 gene polymorphisms are also found associated with autoimmune diseases such as systemic lupus erythematosus (SLE) (Andre *et al.* 2011), allergic rhinitis (Zhang *et al.* 2009), autoimmune thyroid diseases (AITDs) (Inoue *et al.* 2010), and type I diabetes (TID) (Bassuny *et al.* 2003). There are a few studies in other population on association of *FOXP3* gene polymorphisms and URSA (Wu *et al.* 2012; Naderi-Mahabadi *et al.* 2015). In this study, we aimed to identify the association of *FOXP3* gene polymorphisms with URSA in Indian population. We studied the following four SNPs of the *FOXP3* gene: −924A/G (rs2232365) and −3279C/A (rs3761548) in the promoter, −20A/G (rs2232368) located in intron 1 and +459T/C (rs2280883) in intron 9 regions of the gene.

Materials and methods

This study consisted of 100 Indian women of reproductive-age group diagnosed with URSA, who attended as outpatients in Department of Medical Genetics in Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, for genetic evaluation and counselling of recurrent spontaneous abortions. All patients had at least three pregnancy losses with unexplained aetiology before 20th week of gestation. The control group consisted of 100 ethnically matched women (reproductive age group, no history of autoimmune disorder) with at least one normal pregnancy and no spontaneous abortion, preterm labour, or pre-eclampsia. Objectives of the study were explained to the couple and written informed consent was obtained from each individual for collection of clinical information and peripheral blood samples in ethylenediamine tetraacetic acid (EDTA) tubes.

Diagnostic criteria of URSA

In woman of reproductive-age group, who had at least three pregnancy losses before 20th week of gestation, the diagnosis of URSA was made when they matched the criteria for URSA: (i) chromosomal abnormalities of recurrent abortions in couple were excluded by karyotype of couple. (ii) Anatomic causes including intrauterine malformations, uterine fibroids and intrauterine adhesions (Asherman's syndrome) were excluded by pelvic examination and ultrasound. (iii) Hormonal causes such as hyperprolactinaemia, luteal insufficiency and hyperandrogenaemia were evaluated using blood measurements. (iv) Autoimmune and thrombotic causes such as lupus and antiphospholipid antibody syndrome were excluded by evaluating lupus anticoagulant, anticardiolipin antibodies and anti-beta-2 glycoprotein. (v) Medical causes such as thyroid disorders, diabetes mellitus, polycystic ovarian syndrome and systemic lupus erythematosus were also excluded.

Genetic analysis of FOXP3 gene SNPs

Genomic DNA was extracted from anticoagulated peripheral blood using a QIAGEN DNA extraction kit method as per the instructions provided. Genetic analysis of *FOXP3* gene polymorphisms was performed by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) in isolated genomic DNA template. PCR reaction mixture contained 200 ng of DNA, 1×Taq DNA polymerase master mix (Fermentas) and 10 pmol of each specific primer. The PCR products were digested by the restriction enzymes followed by agarose gel electrophoresis (table 1).

Validation of obtained results was done by Sanger sequencing of 10 samples of each polymorphism.

Statistical methods

Genotype and allele frequency distribution among cases and controls were analysed using the Fisher's exact test. The homozygous and heterozygous *FOXP3* gene SNPs were grouped accordingly, and risk of URSA in association with the presence of each of the polymorphisms is calculated by odds ratio (OR). The additive, dominant and recessive models of inheritance were taken into consideration. The Boneferroni adjustment was used to address the issue of false positive findings arising from multiple comparisons. GraphPadInStat software and SPSS ver. 20.0 software package were used for data analysis and $P < 0.05$ was considered significant.

The study was approved by the Institute Ethics Committee. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional committee.

Results

As described in statistical methods, we have calculated genotype and allele frequencies of all the four SNPs. The additive, dominant and recessive models of inheritance were taken into consideration. Genotype and allele frequencies of the −3279C/A, −924A/G, −20G/A and +459T/C *FOXP3* gene polymorphisms in the URSA women and controls are shown in tables 2 and 3.

Table 1. Primers used in the genotyping of *FOXP3* gene polymorphisms by PCR-RFLP.

SNP	Forward/reverse primers	Restriction enzyme	PCR product (bp)	Allele size (bp)
−3279C/A (rs3761548)	F: CCTCTCCGTGCTCAGTGTAG R: CCTCACCTAGCCCAGCTCTTG	*Pst*I; 37°C	301	C:159 + 142[a]A:301[b]
−924A/G (rs2232365)	F: AGGAGAAGGAGTGGGCATTT R: GAATACGGGGGTCTGGATCT	*Tth*111I; 65°C	221	A:221[a]G:124 + 97[b]
−20G/A (rs2232368)	F: GGGGCTCAGAGGAGAGAACT R: TTTGCGCACTATCCCTATCC	*Nla*III; 37°C	242	G:153 + 89[a]A:242[b]
+459T/C (rs2280883)	F: TAACTCCTTCCCCAGCCTTT R: TTCAGGTTTGGGGTTAGGTG	*Hpy*CH4III; 65°C	229	T:229[a] C:110 + 119[b]

[a]RFLP product for wild-type allele.
[b]RFLP product for mutant allele.

−3279C/A polymorphism, an increased risk of foetal loss ranged from almost 3-fold to 2-fold, respectively, in additive, recessive and dominant models. But, the association of −3279C/A polymorphism with URSA was not found significant. The frequency of the mutant allele A in URSA women was not found significantly higher than that in the controls (OR = 1.5; 95% CI 0.99–2.3; $P = 0.074$).

−924A/G polymorphism was found significantly associated with URSA. The OR for GG genotype in additive model was 5.3 (95% CI 1.7–16.7; $P = 0.027$) and OR in recessive model was 10.2 (95% CI 4.7–21.8; $P < 0.001$) for URSA. The frequency of the mutant allele G in URSA women was significantly higher than that in the controls (OR = 2.5; 95% CI 1.7–3.8; $P < 0.001$).

+459T/C polymorphism was also found associated with increased risk for URSA. The OR for CC genotype in additive model was 22.1 (95% CI 2.8–174.0; $P < 0.001$) and OR in recessive model was 21.7 (95% CI 2.8–166.4; $P < 0.001$) for URSA. The frequency of the mutant allele C in URSA women was significantly higher than that in the controls (OR= 1.7; 95% CI 1.1–2.6; $P = 0.012$).

In −20G/A polymorphism, only GG genotype was found in both URSA and controls. Confirmation of results was done by Sanger sequencing in few cases and controls.

Discussion

Various causes were implicated in aetiopathogenesis of recurrent abortions and association of many genetic polymorphisms with URSA are being investigated, immunological factors being one of the important factor. Deficient activity of *FOXP3* gene can result in decreased suppressive function of Tregs, which are crucial to support pregnancy (CD4+CD25+) (Williams and Rudensky 2007). They suppress maternal allo-reactive immune responses against paternal antigens in foetal cells by developing immune-tolerant environment at the foetal–maternal interface in pregnancy (Mold *et al.* 2008). But, there are evidences which show decreased number of Tregs (CD4+CD25+) in both peripheral blood and decidua of URSA women and its association with decreased expression of *FOXP3* gene in these women (Mei *et al.* 2010). This data and our findings suggest that *FOXP3* gene polymorphisms cause increased risk to URSA.

We have evaluated two *FOXP3* gene SNPs, −924A/G (rs2232365) and −3279C/A (rs3761548), in the promoter region. Genetic distribution of −924A/G polymorphism was found to be significantly different between the URSA and control groups. The risk of URSA in the women with the mutant G allele was 2.5 times higher than that in the women carrying the wild A allele. −924A/G polymorphism was found significantly associated with URSA. These results are similar to that obtained in URSA women in Chinese Han population (OR = 1.7; 95% CI 1.1–2.3; $P = 0.010$) (Wu *et al.* 2012) and in Iranian population

Table 2. Genotype frequency of SNPs among cases and controls.

SNP	Genotype	Patient ($n = 100$) (%)	Control ($n = 100$) (%)	OR	95% CI	P value[a]
−3279 C/A rs3761548	CC	35 (35)	47 (47)	1.00 (reference)		
	CA (additive model)	52 (52)	46 (46)	1.52	0.84–2.74	0.180
	AA (additive model)	13 (13)	07 (07)	2.49	0.90–6.90	0.085
	AA+CA vs CC (dominant model)			1.65	0.93–2.91	0.114
	AA vs CA+CC (recessive model)			1.99	0.76–5.21	0.238
−924A/G rs2232365	AA	09 (09)	09 (09)	1.00 (reference)		
	AG (additive model)	38 (38)	81 (81)	0.47	0.17–1.28	0.182
	GG (additive model)	53 (53)	10 (10)	**5.30**	1.69–16.65	**0.027**[b]
	GG+AG vs AA (dominant model)			1.00	0.38–2.64	1.00
	GG vs AG+AA (recessive model)			**10.15**	4.74–21.75	< **0.001**[b]
+459T/C rs2280883	TT	35 (35)	43 (43)	1.00 (reference)		
	TC (additive model)	47 (47)	56 (56)	1.03	0.57–1.86	1.000
	CC (additive model)	18 (18)	01 (01)	**22.11**	2.81–174.03	< **0.001**[b]
	CC+TC vs TT (dominant model)			1.40	0.79–2.48	0.310
	CC vs TC+TT (recessive model)			**21.73**	2.84–166.35	< **0.001**[b]
−20G/A rs2232368[c]	GG	100 (100)	100 (100)			

Additive model: comparing mutant homozygous and heterozygous genotypes individually with wild homozygous genotypes; recessive model: comparing mutant homozygous genotype with wild homozygous and heterozygous genotypes taken together; dominant model: mutant homozygous and heterozygous genotype taken together compared with wild homozygous genotype. The results that are statistically significant are in bold.

[a]Analysis by Fisher's exact test with Bonferroni correction.

[b]Statistically risk associated genotypes for unexplained recurrent spontaneous abortion.

[c]In −20G/A polymorphism, only GG genotype was found in both URSA and controls. OR, odds ratio; CI, confidence interval.

Table 3. Allele frequency of SNPs among cases and controls.

SNP	Allele	Patient ($n = 200$) (%)	Control ($n = 200$)	OR	95% CI	P value[a]
–3279C/A rs3761548	C	122 (61)	140 (70)	1.00 (reference)		
	A	78 (39)	60 (30)	1.49	0.99–2.26	0.074
–924A/G rs2232365	A	56 (28)	99 (49.5)	1.00 (reference)		
	G	144 (72)	101 (50.5)	**2.52**	1.66–3.82	< **0.001**[b]
+459T/C rs2280883	T	117 (58.5)	142 (71)	1.00 (reference)		
	C	83 (41.5)	58 (29)	**1.74**	1.15–2.63	**0.012**[b]
–20G/A rs2232368[c]	G	200 (200)	200 (200)			
	A	00	00			

[a]Analysis by Fisher's exact test with Bonferroni correction. The results that are statistically significant are in bold.
[b]Statistically risk associated alleles for unexplained recurrent spontaneous abortion.
[c]In −20G/A polymorphism only GG genotype was found in both URSA and controls.

(OR = 3.6; 95% CI 2.1–6.1; $P = 0.001$) (Naderi-Mahabadi *et al.* 2015).

In −3279C/A polymorphism, an increased risk of foetal loss ranged from almost 3-fold to 2-fold, respectively, in additive, recessive and dominant models of inheritance were found (table 3). However, the association of −3279C/A polymorphism with URSA was not found statistically significant. This finding is consistent with previous studies on endometriosis and infertility (Andre *et al.* 2011) in addition to URSA (Naderi-Mahabadi *et al.* 2015), but is in contrast to the finding in URSA in the Chinese Han population where −3279C/A polymorphism was found associated with URSA (OR = 1.7; 95% CI 1.2–2.5; $P = 0.003$) (Wu *et al.* 2012). Association of −3279C/A polymorphism with psoriasis, allergic rhinitis (AR), Grave's disease and systemic lupus erythematosus (SLE) were reported (Gao *et al.* 2010).

To prevent rejection of foetus in maternal uterus, Th1/Th2 cytokine balance with Th2 polarized condition is required. −924A/G SNP is located in putative-binding site for GATA-3, a transcription factor, which prompts *FOXP3*-mediated development of regulatory T cells, which are then advanced to Th2 conversion (Wang *et al.* 2010). This mechanism is disturbed in presence of high frequencies of G allele and GG genotype in URSA patients. −3279C/A SNP is located in the core of 'GGGCGG' sequences of putative DNA-binding site for the transcription factor specificity protein-1 (sp-1). It may be suggested that −3279C/A SNP variant may affect the interaction of sp-1 protein with *FOXP3* gene promoter region, which may confer increased risk of URSA.

We have also investigated two SNPs of the *FOXP3* gene in the intronic region as they can affect mRNA levels through regulatory mechanisms such as alternative splicing of mRNA (Moyer *et al.* 2011) or demethylation of CpG residues in intronic regions of the *FOXP3* gene (Floess *et al.* 2007). Of the two investigated SNPs in the intronic regions, one is −20A/G (rs2232368) located in intron 1 and the other is +459T/C (rs2280883) located in intron 9. +459T/C and −20G/A SNPs of *FOXP3* gene were found associated with idiopathic infertility (Andre *et al.* 2011). In the present study, genetic distribution of +459T/C polymorphism was found to be significantly different between the URSA and control groups and odds ratio were 22.1 (95% CI 2.8–174.0; $P < 0.001$) and 21.7 (95% CI 2.8–166.4; $P < 0.001$) for additive and recessive models, respectively. The frequency of the mutant allele C in URSA women was significantly higher than that in the controls. Thus, +459T/C polymorphism was found significantly associated with URSA. The result is in contrast to the finding in URSA in the Iranian population where +459T/C polymorphism was not found associated with URSA (Naderi-Mahabadi *et al.* 2015). +459T/C polymorphism is also associated with severe psoriasis (Gao *et al.* 2010). Naderi-Mahabadi *et al.* (2015) have reported that the association of −20G/A polymorphism with URSA in Iranian population. Thus, from the present study, we can conclude that −20G/A polymorphism is likely to be rare in Indian population and not associated with URSA in them as only GG genotype was found in both URSA and controls.

Conclusion

In this study, we have found high odds ratio (OR) of mutant allele G for −924A/G polymorphism, i.e. 2.5 (95% CI 1.7–3.8; $P < 0.001$) and mutant allele C for +459T/C polymorphism, i.e. 1.7 (95% CI 1.1–2.6; $P = 0.01$), which may indicate strong association of these *FOXP3* gene polymorphisms with URSA in a group of Indian patients. −3279C/A and −20G/A polymorphism were not found associated with URSA. In −20G/A polymorphism, only GG genotype was found in both URSA and controls. Thus, we can conclude that −20G/A polymorphism is likely to be rare in Indian population and might not be associated with URSA in them.

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