RESEARCH ARTICLE

Study of Association of Forkhead Box P3 (*FOXP3*) Gene Polymorphisms with Unexplained Recurrent Spontaneous Abortions in Indian Population.

Shivani Mishra, Anukool Srivastava, Kausik Mandal, Shubha R Phadke*

Department of Medical Genetics, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, 220614, Uttar Pradesh, India.

**Correspondence to: Dr Shubha R Phadke, Professor, Department of Medical Genetics, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, 220614, Uttar Pradesh, India.*

Email ID: shubharaophadke@gmail.com ; Contact- +91-9839220199.

ABSTRACT

Purpose: Recurrent spontaneous abortions (RSA) is defined as 3 or more consecutive pregnancy losses before 20 weeks of gestation. Various causes of RSA have been identified, still 50% cases remains unexplained after evaluation. One of the causes of unexplained recurrent spontaneous abortions (URSA) is supposed to be the disruption of immunological tolerance at fetal-maternal interface. Regulatory T cells (Tregs) are responsible for development of immune-tolerant environment at fetal-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.

Methods: 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).

Results: −924A/G and +459T/C polymorphisms were found to be associated with URSA. −3279C/A and −20G/A polymorphism were not found 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.

Conclusion: 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

Forkhead box P3 (FOXP3) gene; Regulatory T cells (Tregs); Single-nucleotide polymorphism (SNP); Unexplained recurrent spontaneous abortion (URSA).

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 remains unexplained and are termed as unexplained recurrent spontaneous abortion (URSA) (Ford et al., 2009). Association of URSA with various immune factors which may cause disruption of immunological tolerance at fetal-maternal interface have been reported (Leber et al., 2011). Regulatory T cells (Tregs) are specialized T cells which are responsible for development of immune-tolerant environment at fetal-maternal interface and supports pregnancy (Zenclussen et al., 2006). In URSA, it has been 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 fetal-maternal interface (Mei et al., 2010).

Human *FOXP3* gene (Gene ID: 50943, MIM number: 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 affects 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 few studies in other population on association of *FOXP3* gene polymorphisms and URSA (Wu et al., 2012; Faezeh 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 counseling of recurrent spontaneous abortions. All patients had at least three pregnancy losses with unexplained etiology before 20th week of gestation. The control group consisted 100 ethnically matched women (reproductive age group, no history of autoimmune disorder) with at least one normal pregnancy and no spontaneous abortion, preterm labor, 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 EDTA (ethylene diamine-tetra acetic acid) 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 match the criteria for URSA: (1) Chromosomal abnormalities of recurrent abortions in couple were excluded by karyotype of couple. (2) Anatomic causes including intrauterine malformations, uterine fibroids and intrauterine adhesions (Asherman's Syndrome) were excluded by pelvic examination and ultrasound. (3) Hormonal causes such as hyperprolactinemia, luteal insufficiency and hyperandrogenemia were evaluated using blood measurements. (4) Autoimmune and thrombotic causes such as lupus and antiphospholipid antibody syndrome were excluded by evaluating lupus anticoagulant, anticardiolipin antibodies and anti-beta-2 glycoprotein. (5) Medical causes such as thyroid disorders, diabetes mellitus, polycystic ovarian syndrome and systemic lupus erythematosus were also excluded.

Genetic analysis of FOXP3 gene single-nucleotide polymorphisms

Genomic DNA was extracted from anticoagulated peripheral blood using a QIAGEN DNA extraction kit method as per 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, 1xTaq 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- Primers used in the genotyping of FOXP3 gene polymorphisms by PCR-RFLP.

SNP	Forward Primer (FP)/ Reverse Primer (RP)	Restriction Enzyme	PCR Product	Allele size (bp)
−3279C/A (rs3761548)	FP- CCTCTCCGTGCTCAGTGTAG RP- CCTCACCTAGCCCAGCTCTTG	PstI; 37°C	301 bp	C:159+142[a] A:301[b]
−924A/G (rs2232365)	FP- AGGAGAAGGAGTGGGCATTT RP- GAATACGGGGGTCTGGATCT	Tth111I; 65°C	221 bp	A:221[a] G:124+97[b]
−20G/A (rs2232368)	FP- GGGGCTCAGAGGAGAGAACT RP- TTTGCGCACTATCCCTATCC	NlaIII; 37°C	242 bp	G:153+89[a] A:242[b]
+459T/C (rs2280883)	FP- TAACTCCTTCCCCAGCCTTT RP- TTCAGGTTTGGGGTTAGGTG	HpyCH4III; 65°C	229 bp	T:229[a] C:110+119[b]

a-RFLP product for wild-type allele.

b-RFLP product for mutant allele.

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

Statistical methods

Genotype and allele frequency distribution among cases and controls were analyzed 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 have been taken into consideration. The Boneferroni adjustment was used to address the issue of false positive findings arising from multiple comparisons. GraphPad InStat software and SPSS version 20.0 software package was used for data analysis and $p < 0.05$ was considered significant.

RESULTS

As described in statistical methods we have calculated genotype and allele frequencies for all the four SNPs. The additive, dominant and recessive models of inheritance have been 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 2- Genotype frequency of SNPs among cases and controls.

SNP	Genotype	Patient (N=100)	Control (N=100)	Odds ratio (OR)	95% CI (Confidence Interval)	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 genotype taken together;

Dominant model: Mutant homozygous and heterozygous genotype taken together compared with wild homozygous genotype.

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.

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

SNP	Allele	Patient (N=200)	Control (N=200)	Odds ratio (OR)	95% CI (Confidence Interval)	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.

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.

−3279C/A polymorphism, an increased risk of fetal loss ranged from almost 3-fold-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 odds ratio (OR) for GG genotype in additive model was 5.3 (95%CI 1.7–16.7; p=0.027) and odds ratio (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 odds ratio (OR) for CC genotype in additive model was 22.1 (95%CI 2.8–174.0; p<0.001) and odds ratio (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 have been implicated in etiopathogenesis 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 fetal cells by developing immune-tolerant environment at the fetal–maternal interface in pregnancy (Mold JE et al., 2008). But, there are evidences which shows 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 causes 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 (OR= 3.6; 95% CI 2.1–6.1; p=0.001) (Faezeh et al., 2015).

In −3279C/A polymorphism, an increased risk of fetal loss ranged from almost 3-fold-2-fold respectively in additive, recessive and dominant models of inheritance has been 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 (Faezeh 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) have been reported (Gao et al., 2010).

To prevent rejection of fetus 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). Out 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 model, 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 (Faezeh et al., 2015). +459T/C polymorphism is also associated with severe psoriasis (Gao et al., 2010). Faezeh et al, has reported 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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CONFLICTS OF INTEREST

Shubha R Phadke has received a grant from the Indian Council of Medical Research, Government of India, New Delhi, India (Grant number 63/8/2010-BMS). The other authors declare that they have no conflict of interest.

ETHICAL APPROVAL

The study has been 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.

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