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Association of FOXP3 rs3761548 polymorphism and its reduced expression with unexplained recurrent spontaneous abortions: A South Indian study

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Abstract

Problem: Fork Head Box Protein 3 (FOXP3) is an X-linked gene, codes for a master transcription regulatory protein that controls the development and function of immunosuppressive T regulatory (Treg) cells. They are crucial mediators of maternal foetal tolerance and successful pregnancy outcome. The aim of the study is to evaluate the association of FOXP3 rs3761548 functional polymorphism and to assess the serum concentrations of full-length FOXP3 protein in Unexplained Recurrent Spontaneous Abortions (URSA) patients of Southern India.

Method of study: The study included blood samples from 150 URSA patients and 150 healthy, pregnant parous women. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism was done for rs3761548 FOXP3 genotyping. Serum concentrations of full-length FOXP3 protein were estimated by enzyme-linked immunosorbent assay.

Results: The frequencies of mutant A allele, CA and AA genotypes of rs3761548 functional polymorphism were significantly elevated in patients compared to healthy, pregnant parous women and exhibited a two, three and twofold increased risk respectively towards URSA. Serum concentrations of full-length FOXP3 protein were high in controls compared to patients (10.14 ± .30 vs. 8.84 ± 1.73 ng/ml; $p < .05$).

Conclusion: Our results advocate an association of FOXP3 rs3761548 polymorphism and reduced expression of full-length FOXP3 protein with URSA.

KEYWORDS
FOXP3, polymorphism, regulatory T cells

1 | INTRODUCTION

Recurrent Pregnancy Loss (RPL) is a serious growing reproductive problem among the young couples, defined as three or more consecutive miscarriages before 20 weeks of gestational age,[1] while the American Society for Reproductive Medicine (ASRM) defines recurrent miscarriage as two previous losses.[2] Despite various clinical and experimental tests, yet there is not an accurate and efficient diagnostic method during the early stages of pregnancy in more than half of RPL patients.[3] The risk factors linked to its pathogenesis are

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genetic disorders such as foetal chromosomal abnormalities, maternal factors including anatomical deformities, placental anomalies, thrombophilia, endocrine disorders, immune dysfunction, infection, smoking, psychological trauma, stress and environmental factors.[4,5] Among these factors, immune function appears to play a significant role in protecting the pregnancy by preventing response to the semi allograft.[6] Functional variations in the genes coding for the expression and regulation of immune response may predispose the mother to recurrent abortions.[7–11]

The Fork Head Box Protein 3 (FOXP3) gene, a member of transcription factor winged helix family, located on chromosome Xp11.23, regulates the development and function of CD4+CD25+ regulatory T (Treg) cells.[12,13] In unexplained recurrent spontaneous abortions (URSA), it is found that Tregs (CD4+CD25+) are reduced in peripheral blood as well as in decidua of pregnant women.[14] In addition, in peripheral blood and decidua, decreased expression of FOXP3 gene, a marker of Treg cell, indicating that alteration in the Treg cell-dependent maintenance of feto-maternal tolerance contributes to pregnancy loss.[15,16]

Increased FOXP3 promoter methylation downregulates the expression of the FOXP3 protein associated with URSA.[17] Further, different single-nucleotide variants (SNVs) in the promoter region of FOXP3, which affect the expression of FOXP3 and impair the Treg differentiation and function, have been allied with occurrence of URSA in various populations.[7–11] The rs3761548 FOXP3 (−3279) C>A variant is located in the core of 'TG**C**AGGCCTC' sequence of the putative binding site for the transcription factor specificity protein 1 (Sp1), and the A allele is correlated with the reduction in FOXP3 expression,[18] which was extensively studied in several human diseases. Previously, we reported an association of this polymorphism with the patients of preeclampsia, vitiligo and breast cancer in South Indian patients.[19–21]

The aim of the current study is not only to evaluate the association of FOXP3 rs3761548 (−3279) C>A functional polymorphism with URSA, but also to assess the full-length FOXP3 serum protein levels in URSA patients of Southern India.

2 | MATERIALS AND METHODS

The study population consists a total of 300 subjects that include 150 URSA patients, who had a history of at least two successive miscarriages with unexplained aetiology before 12 weeks of gestation, and 150 age-matched healthy, pregnant parous women as controls with at least two live births and with no history of spontaneous abortions or any other known diseases (age: 18–40 years) were recruited based upon the information available from case sheets of patients, where (clinical pregnancies documented by ultrasound or histopathology) test reports of diabetes, thyroid, polycystic ovary syndrome, progesterone, oestrogen, cervical incompetence, chromosomal abnormality etc. were screened, diagnosed and investigated by the Department of Obstetrics & Gynaecology of Gandhi Medical and Niloufer Hospital, Hyderabad. Women with history of only one spontaneous abortion, with history of induced abortions and abortions with known reasons such as anatomical problems, hormonal imbalances etc. were excluded from the study. Clinical and demographical information such as age, Body Mass Index (BMI), Haemoglobin (Hb), age at menarche, age at first conception, number of abortions, consanguinity was collected from both patients and controls. Informed consent was obtained from all patients and controls. The study was approved by the institutional ethics committees, (Institute of Genetics and Hospital for Genetic Diseases, Osmania Medical College) Hyderabad. This manuscript refers URSA and RPL/RSA interchangeably.

2.1 | Selection of SNP—rs3761548 (−3279) C>A in FOXP3 gene region

For the present study, SNP databases, Ensembl (http://www.ensembl.org/index.html) and the International HapMap database (http://hapmap.ncbi.nlm.nih.gov/index.html.en) were used to select rs3761548 (−3279) C>A polymorphism in the FOXP3 gene region from chromosome X at nucleotide position 49,261,409 to 49,261,972. Further, published data show scientific evidence to illustrate potential role of rs3761548 in RPL[7–11] and auto-immune diseases.[22]

2.2 | Blood collection and DNA extraction

Blood samples (5 ml) from both URSA patients and healthy controls were collected in EDTA-coated and clot activator collection vials. Genomic DNA was extracted from whole blood using QIAamp DNA Blood Mini Kit (CAT No. /ID: 51104) according to the manufacturer's protocol.

2.3 | Genotyping of rs3761548 (−3279) C>A polymorphism

Genotypes of rs3761548 polymorphism were determined using the Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP), presented in Table 1. In short, PCR for amplification of 564 bp region in the FOXP3 promoter was performed in

TABLE 1 Primers used in the genotyping of −3279 C>A polymorphism by PCR-RFLP

Marker/method	Primer type	Primer sequence (5′–3′)	Annealing temperature	Product size	Restriction enzyme
(−3279)C/A PCR-RFLP	Forward	GACTTAACCAGACAGCGTAG	51°C	564 bp	Pst1
	Reverse	CTGGTGTGCCTTTGGTCT			

a volume of 25 µl, containing 2 µg/ml of genomic DNA, 10 pmol of each primer, 2.0 mM of dNTPs, 1.5 mM of MgCl2, 10× PCR buffer and 0.5 U Taq DNA polymerase (G Biosciences). The PCR conditions for amplification of rs3761548(−3279) C>A location were initial denaturation step at 95°C for 5 min, followed by 28 cycles of 94°C for 35 s, annealing temperature of 51°C for 40 s, extension at 72°C for 40 s, final extension at 72°C for 5 min and holds at 4°C. The amplified PCR product of 564 bp was digested with 0.5 µl of Pst1 (CAT No: R0140S) restriction enzyme at 37°C for an hour and then separated on a 2% agarose gel stained with ethidium bromide at 100 V for 20 min. and visualized under ultraviolet light with a 100-bp DNA ladder. Genotypes of rs3761548 C>A were identified by the presence of three different bands: C/C (374 bp, 190 bp), C/A (564 bp, 374 bp and 190 bp) and A/A (564 bp) in the ethidium bromide stained gel.

2.4 | Estimation of FOXP3 serum protein levels

The concentrations of full-length FOXP3 protein were measured before 12th week of pregnancy for all the patients and controls using enzyme-linked immunosorbent assay (ELISA) kit (KINESISDx, CAT No. K12-0693), as per the manufacturer's instructions.

TABLE 2 Clinical and demographic characteristics of the study group

Category	Controls (150) X ± SD	Patients (150) X ± SD	*p*-Value
Age (years)	24.64 ± 3.13	24.91 ± 4.16	.53
BMI (kg/m^2)	21.38 ± 4.38	21.91 ± 4.00	.27
Hb (g/dl)	10.23 ± 1.66	9.98 ± 1.31	.15
Age at menarche (years)	12.5 ± 1.35	12.84 ± 1.46	.03*
Age at first conception (years)	19.18 ± 2.80	20.24 ± 3.64	.005*
No. of abortions	NA	2.68 ± 1.01	NA
Mean gestational age (months)	4.36 ± 1.14	5.04 ± 1.32	<.0001*

Note: Values are represented as mean ± SEM.
*p < .05.

2.5 | Statistical analysis

Hardy–Weinberg equilibrium was tested for FOXP3 (−3279) C>A variant in URSA patients and healthy controls. Further deviation in the allele and genotype frequencies between patients and controls was tested for statistical significance by Fisher's exact test and the odds ratio at 95% Confidence Interval (C.I.) using Open EPI6 online statistical software (Open EPI v 2.3.1, Emory University). All the p values were two-sided, and the level of significance was considered at p < .05. t-test (Independent sample test) was used to test the difference between two sample means. ANOVA was done to test the significant difference among the groups using IBM SPSS statistics. Relationship between the risk factors for URSA with respect to genotypes was assessed through Multiple Logistic Regression (MLR).

3 | RESULTS

The clinical and demographic characteristics of the study group (N = 300) were presented in Table 2. A significant variation was observed between patients and controls with respect to mean age at menarche (p = .03) and mean age at first conception (p < .01).

Further, 7% of the patients showed irregular menstrual cycles in comparison with only 3% in controls. Eleven per cent of the patients experienced more than 3 pregnancy losses whereas 89% had ≤3 pregnancy losses. The percentage of consanguinity was 25% in patients and 20% in control group (p = .3).

3.1 | Genotype and Allele distribution among controls and patients

The genotype distribution of rs3761548 (−3279) C>A of FOXP3 gene in URSA patients and healthy controls is presented in Table 3. Individuals with CC genotype predominated in controls compared to URSA patients (OR 0.10, 95% C.I: 0.05–0.19, p = < .0001), while women with CA and AA genotypes were in higher frequency among the patients and exhibited an odds ratio of 3.18 and 2.23 (CA vs.

TABLE 3 Genotype and allele frequency distribution of FOXP3 (−3279) C>A polymorphism among the study group

SNP	Genotype	Controls (150) *n* (%)	Patients (150) *n* (%)	χ2 value	OR (95% CI)	*p* Value
rs3761548 C/A	CC	72 (48)	13 (9)	55.22	0.10 (0.05–0.19)	<.0001*
	CA	60 (40)	102 (68)	22.56	3.18 (1.98–5.11)	<.0001*
	AA	18 (12)	35 (23)	5.86	2.23 (1.19–4.15)	<.01*
	C	204 (68)	128 (43)	Reference		
	A	96 (32)	172 (57)	37.93	2.85 (2.04–3.98)	<.0001*
	HWE	Controls		0.98 (0.32)		
	X^2 (p-value)	Patients		22.8 (0.000002)		

Abbreviation: 95% C.I, Confidence Interval.
*p<.05.

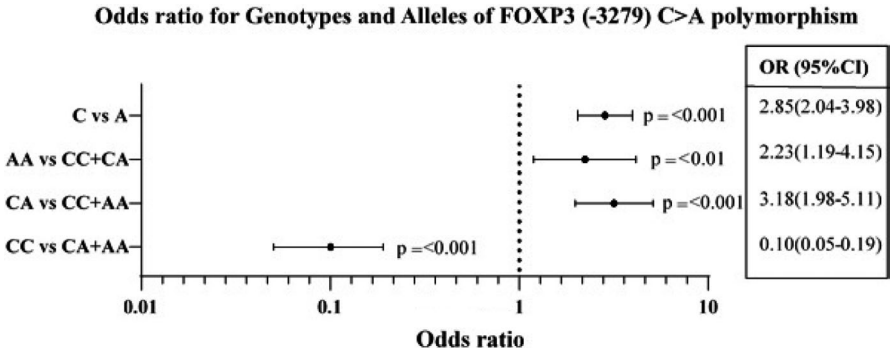

FIGURE 1 Forest plot representing the odds ratio for genotypes and alleles of FOXP3 (−3279) C>A polymorphism in URSA patients and controls

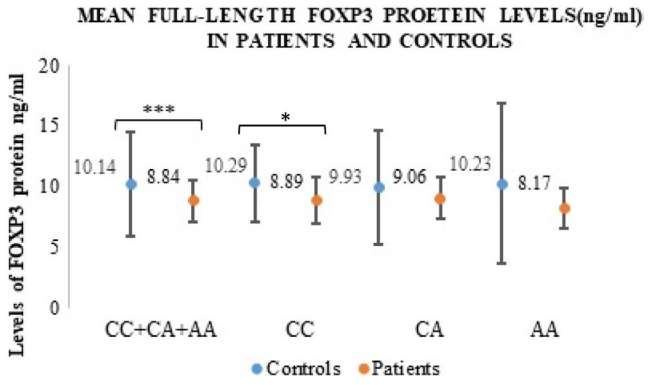

FIGURE 2 Full-length FOXP3 protein concentrations in patients and controls based on FOXP3 rs3761548 genotypes

CC+AA, OR 3.18, 95% C.I: 1.98–5.11, p = < .0001; AA vs. CC+CA, OR 2.23, 95% C.I: 1.19–4.15, p = < .01), respectively. Further, variant allele A was significantly elevated in case of patients compared with healthy counterparts (A vs. C, OR: 2.85, 95% C.I: 2.04–3.98, p = < .0001) shown in Figure 1.

3.2 | Circulating levels of full-length FOXP3 protein among the study group

Circulating levels of full-length FOXP3 protein were significantly higher in controls compared to patients (10.14 ± 4.30 vs. 8.84 ± 1.73 ng/ml) shown in Figure 2. Further, genotype-dependent variation with respect to the protein concentrations was noted within the patients but not in controls. However, in correspondence to CC genotype, there was a significant variation between patients and controls, presented in Table 4.

3.3 | Multiple logistic regression analysis in the URSA group

The multiple logistic regression analysis was performed to identify the independent risk factors for recurrent abortions among URSA patients, but none of the clinical and demographic characters showed the association (Data not shown).

TABLE 4 Serum levels of full-length FOXP3 protein with respect to rs3761548 genotypes in URSA patients and healthy controls

Protein (ng/ml) Genotypes	Controls X ± SD (*n*)	Patients X ± SD (*n*)	*p* Value
CC+CA+AA	10.14 ± 4.30 (150)	8.84 ± 1.73 (150)	.001***
CC	10.29 ± 3.20 (72)	8.89 ± 1.88 (13)	.03*
CA	9.93 ± 4.68 (60)	9.06 ± 1.69 (102)	.17
AA	10.23 ± 6.59 (18)	8.17 ± 1.67 (35)	.2
(CC,CA & AA) *p* value	0.88	0.03*	-

Note: Student *t*-test & ANOVA.
*p-Value .05.; ***p-Value < 0.01.

4 | DISCUSSION

Normal pregnancy progression is the result of timely regulated local shifts between pro- and anti-inflammatory immune responses allowing implantation and placentation in a pro-inflammatory environment, foetal growth in an anti-inflammatory environment and finally the induction of labour and delivery again under pro-inflammatory conditions.[23] RPL is the complex and challenging scenario in reproductive medicine, needs coordination in evaluation and management of patients include gynaecologists, geneticists, immunologists and reproductive specialist. During pregnancy, human chorionic gonadotropin actively recruit peripheral Treg cells into the foetal-maternal interface,[24] mediate local expansion of decidual Treg cells[25] and convert conventional T cells into Treg cells.[26,27] This suggests that Tregs have a pivotal role in the induction and maintenance of foetal-maternal immunologic tolerance. Growth and development of Treg cells require expression of FOXP3 protein,[28] a Treg cell-specific marker; its attenuated expression due to epigenetics/FOXP3 gene variants may result in reduced production of regulatory T cells.[29,30]

Based upon the importance of FOXP3 protein for Treg cell function in pregnancy, firstly, we evaluated the link between functional variant of FOXP3 rs3761548 C>A, a recognized polymorphism

associated with decreased expression of full-length FOXP3 protein and, secondly, to measure the serum concentrations of full-length FOXP3 protein in RPL patients from Southern India.

The findings of the present study exhibited protective role of high producing CC genotype against RPL; a threefold and twofold increased risk for women carrying CA and AA genotypes correspondingly towards RPL. Moreover, A allele was shown to be significantly associated with RPL susceptibility. There is an inconsistency in the literature regarding the association of this polymorphism with RPL in different populations. Individually, a marked difference of FOXP3 rs3761548 genotype frequencies among URSA patients and healthy controls in Han Chinese and North Indian population was reported.[7,9] Allele A and AA genotype were also reported to be higher in RPL women in Gaza strip representing that this polymorphism is a risk factor for RPL, whereas a significant 2.5-fold increased risk of AA genotype towards RPL in Palestine population[8,10] and a significant association of CA and AA genotypes and A allele of FOXP3 rs3761548 polymorphism with URSA in Egyptian population was identified.[11] In contrast, there was no association seen in Iranian and North Indian URSA women.[31–33]

The functional aspect of FOXP3 rs3761548 polymorphism was first reported, showing that substitution of C>A leads to loss of binding to the c-Myb and E47 transcription factors, with subsequent defect in the FOXP3 gene transcription, as this region has a binding site for the transcription factor Sp1 where the A allele affects the interaction of sp-1 protein with FOXP3 gene promoter region.[18] Therefore, patients with the AA genotype may have weaker suppressive function and are difficult to accommodate foetal tolerance.

Having observed the CA and AA as risk and CC as protective genotypes, we sought to assess the genotype-dependent variation with respect to full-length FOXP3 protein among patients and controls. Overall, there was a prominent increase in the concentration of full-length FOXP3 protein in controls compared to patients. Further, genotype-dependent variation was observed within patients but not in the control group; a significant difference in protein levels between patients and controls of women with homozygous wild type (CC) was noted, which is considered to be a high producing genotype.

There are several studies that support our observation of low levels of FOXP3 protein in RPL patients than in controls. A study from China reported that the expressing quantity of FOXP3 protein in the decidua of URSA patients is lower than that in normal pregnant women.[14] In the decidua, significantly reduced proportions of (CD4+CD25+) Treg cells and FOXP3 expression were found in URSA patients with early miscarriages compared with normal early pregnant women.[15] FOXP3 protein quantity in the decidua of the URSA group was lower than that in Recurrent Spontaneous Abortion (RSA) patients with an abnormal embryo and in normal pregnant women.[17] Decreased concentration of FOXP3 was reported in RPL patients than in healthy controls among Egyptian women.[34] The FOXP3 expression in trophoblasts in the term placenta was significantly decreased compared with that in the normal early pregnancy. Moreover, reduced expression of FOXP3 mRNA and protein was observed in women with RPL.[35] Our results are in agreement with above discussed reports, showing that circulating levels of full-length FOXP3 protein were significantly elevated in controls compared to patients.

In the light of existing literature and our observations, it may be surmised that high levels of FOXP3 in women during early pregnancy are crucial in regulating the immunological events through Treg cells at feto-maternal interphase; low levels may prove to be a risk in handling the dynamic events required in the continuation of the pregnancy, leading to adverse pregnancy outcome. This opinion needs to be substantiated by systematic cell-based studies.

Lack of genotype-dependent variation with respect to full-length FOXP3 protein levels within controls to certain extent can be contributed to other genetic factors like X-chromosomal inactivation [36,37] or FOXP3 isoform profile, as alternative splicing of FOXP3 appears to add another layer of complexity to Treg cell biology.[38]

5 | CONCLUSION

We conclude that A allele and AA/AC genotype of rs3761548 functional polymorphism of FOXP3 is associated with pregnancy loss in our ethnicity. Reduced concentrations of full-length FOXP3 protein linked with pregnancy loss appear to impact the Treg population at maternal-foetal interphase. Determination of genetic variants contributing to early miscarriages can be used for a better understanding of RPL's pathophysiology to improve molecular diagnosis. Large systematic studies in relation to genetic polymorphisms of FOXP3, its expression at various stages of gestation and Treg cells are warranted. Consequently, the information generated may open up novel Protein or cell-based medical interventions in the field of Unexplained Recurrent Pregnancy Loss which is crucial for species survival.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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