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Effect of Forkhead Box Protein 3 Gene Polymorphisms in Recurrent Pregnancy Loss: A meta-analysis

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Abstract

Treg cells play an important role in development of tolerance in maternal immune system against the semi-allogenic embryo. Human forkhead box protein 3 (FOXP3) gene, is the major transcription factor responsible for the regulation of Treg function during pregnancy. Single nucleotide polymorphisms (SNPs) of FOXP3 gene have been reported as a risk factor for Recurrent Pregnancy Loss (RPL), however, results from previous studies are inconsistent. In this meta-analysis, we collected data from different studies to investigate the overall association of FOXP3 SNPs with risk of RPL. PubMed, Google Scholar, Elsevier, and Cochrane databases were searched to identify eligible studies. Odds Ratio (OR) and 95% Confidence Interval (CI), calculated via fixed effect or random effect models, were used to evaluate strength of association. This meta-analysis included 11 studies (1383 RPL cases and 1413 controls) of 6 SNPs: rs3761548 A/C, rs2232365 A/G, rs2294021 T/C, 2280883 T/C, rs5902434del/ATT and rs141704699 C/T, with ≥ 2 studies per SNPs and at least 1 significant result. We observed that FOXP3 polymorphism was predominantly present in Asian women with history of RPL. rs2232365 A/G, rs3761548 A/C, rs2294021 T/C, rs2280883 T/C and rs5902434del/ATT polymorphisms were significantly associated with risk of RPL in Indian population. Further, among the most commonly seen polymorphism, rs3761548 A/C was significantly associated with risk of RPL in women from Kazakhstan, China and Gaza, Palestine; rs2232365 A/G in populations of Kazakhstan, Egypt, Iran and Gaza, Palestine. Results of this study indicates that FOXP3 polymorphism is significantly associated with risk of RPL, especially in Asians.

Keywords: Recurrent Pregnancy Loss, SNP, FOXP3 polymorphism, Treg, meta-analysis, RSA

Introduction

Recurrent pregnancy loss (RPL), also referred to as Recurrent Spontaneous Abortion (RSA), is defined as two or more clinical loss of pregnancy before completing gestational age of 20-24 weeks [1]. The prevalence of RPL varies between 0.4-1.8% of clinical pregnancies [2] and affects 2-5% of couples [3,4]. The cause is multifactorial; genetic, endocrine abnormality, uterine factors, metabolic and immunological factors, however, in 50% of cases the etiology cannot be identified [5]. In such cases of unexplained RSA, dysregulated immune response at has been postulated as an important mechanism to understand the underlying etiology [2,6-8]. It has been

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postulated that regulatory T-cells (Tregs) play a crucial role in maintenance of immune tolerance at feto-maternal interface and thus supports pregnancy [9].

Treg cells play an important role in development of tolerance in maternal immune system against the semi-allogenic embryo. The levels of Treg increases at the initial stage of pregnancy, reaching a peak at the mid-stage and gradually decreases at the term stage of pregnancy [10]. However, in women with RPL, this inhibitory effect of Treg cell decreases. It has been observed that level of Treg (CD4+CD25+) cells is decreased in peripheral blood and decidua of women with unexplained RPL as compared to healthy pregnancies [11-13]. Human forkhead box protein 3 (FOXP3) gene, is a major transcription factor responsible for the regulation of Treg function. Decrease in Treg subset leads to decrease in expression of FOXP3 gene, which in turn will affect the development and function of CD4+CD25+ Tregs [14].

Polymorphism in the promoter region of FOXP3 gene affects initiation of transcription, by changing the kinetics and affects the binding efficiency of transcription factor which leads to altered gene expression. While mutation in the intronic region can create splice site which affects RNA processing [15] (Fig 1). Therefore, polymorphism in FOXP3 gene can result in inappropriate Tregs which fails to maintain immune tolerance and results in spontaneous abortions.

Due the major effect caused by FOXP3 gene polymorphism in RPL patients, many studies were conducted in different populations, however, the results are inconsistent and inconclusive, which could be due to discrepant study designs, diversity in population, smaller sample size and variation in statistical analysis used to compute the results.

Therefore, the present meta-analysis was conducted with an aim to provide comprehensive and more reliable conclusions for the association of polymorphism in FOXP3 gene with risk of RPL with database from eligible case-control studies around the globe. To the best of our knowledge, this is the first meta-analysis conducted on FOXP3 gene polymorphism in unexplained RPL patients.

Material and Methods

Search Strategy

An extensive literature search was conducted to identify relevant studies published from 2010 till March 2022. PubMed, Google Scholar, Elsevier, and Cochrane database were searched using following keywords: “forkhead box P3” OR “FOXP3”) AND (“recurrent pregnancy loss” OR “recurrent spontaneous abortion”) AND “polymorphism”. All relevant studies published in the English language were retrieved for further analysis. Additionally, references cited in review articles were manually searched for additional eligible studies. Flow chart for selection of studies is shown in Fig 2, as per the PRISMA 2020 guidelines.

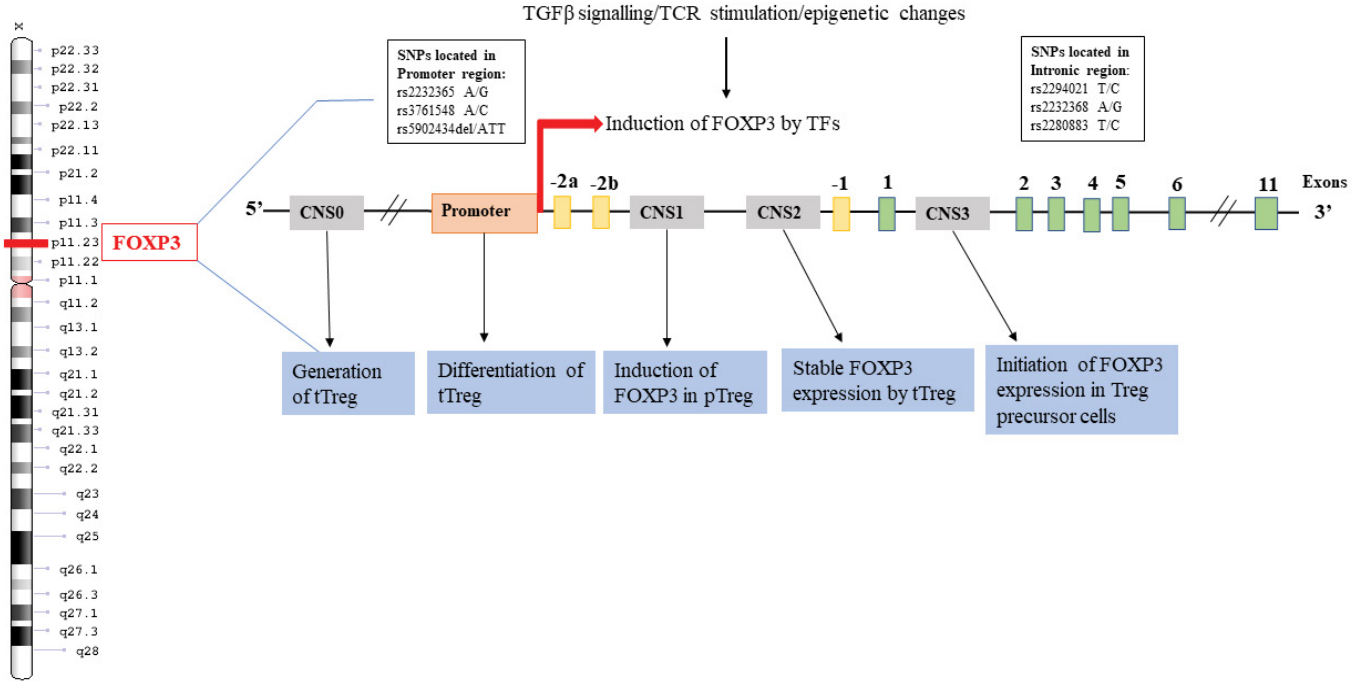

Figure 1: Schematic diagram of FOXP3 gene location on chromosome X (Xp11.12) and its transcriptional regulation. The FOXP3 gene locus has a promoter, four conserved non-coding sequences (CNSs), and 11 exons. The immune cells in trophoblast and decidua of pregnant women release cytokines that induce binding of various transcription factors to regulatory regions of FOXP3 gene. Activation of FOXP3 in turn regulates Treg expression.

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Selection Criteria

Based on following explicit inclusion criteria, studies were selected for the meta-analysis: (1) FOXP3 polymorphism association study on human subjects with RPL (2) Independent case-control studies (3) Complete genotyping data for both RPL cases and non-RPL controls (4) SNPs with atleast one significant association.

Studies were excluded based on following criteria: (1) SNP with less than 2 studies (2) unavailability of genotype frequency data (3) studies without data on healthy control population (4) non-compliance with the criteria for RPL (5) duplicate studies (6) commentaries, reviews, letter, abstract, conference editorials, unpublished data.

Data mining and Quality Assessment

Two independent researchers extracted the following data from each study: first author's name, year of publication, SNPs studied, country of origin and ethnicity of subjects, total sample size (cases and controls), allele and genotyping frequency in cases and controls, genomic genotyping method used and type of association. For quality assessment of included studies and to reduce the bias, Newcastle-Ottawa scale (NOS) score was used (Suppl. Data). Studies with NOS quality score less than 5, were excluded from the analysis.

Statistical Analysis

All the statistical analysis was done by using MetaGenyo web tool (http://bioinfo.genyo.es/metagenyo/). Two-tailed P-values with a significance at ≤0.05 level was used. The strength of association between FOXP3 gene polymorphism and RPL, in terms of Odds Ratio (OR) and 95% Confidence Interval (CI) were calculated by fixed-effects or random effects model. Based on the amount of heterogeneity, different models were selected for OR calculation. Cochran's Q-statistic (P-value< 0.10) was used to estimate the presence of heterogeneity and to report quantitative heterogeneity I2 test was used. Random effect model was selected if -significant

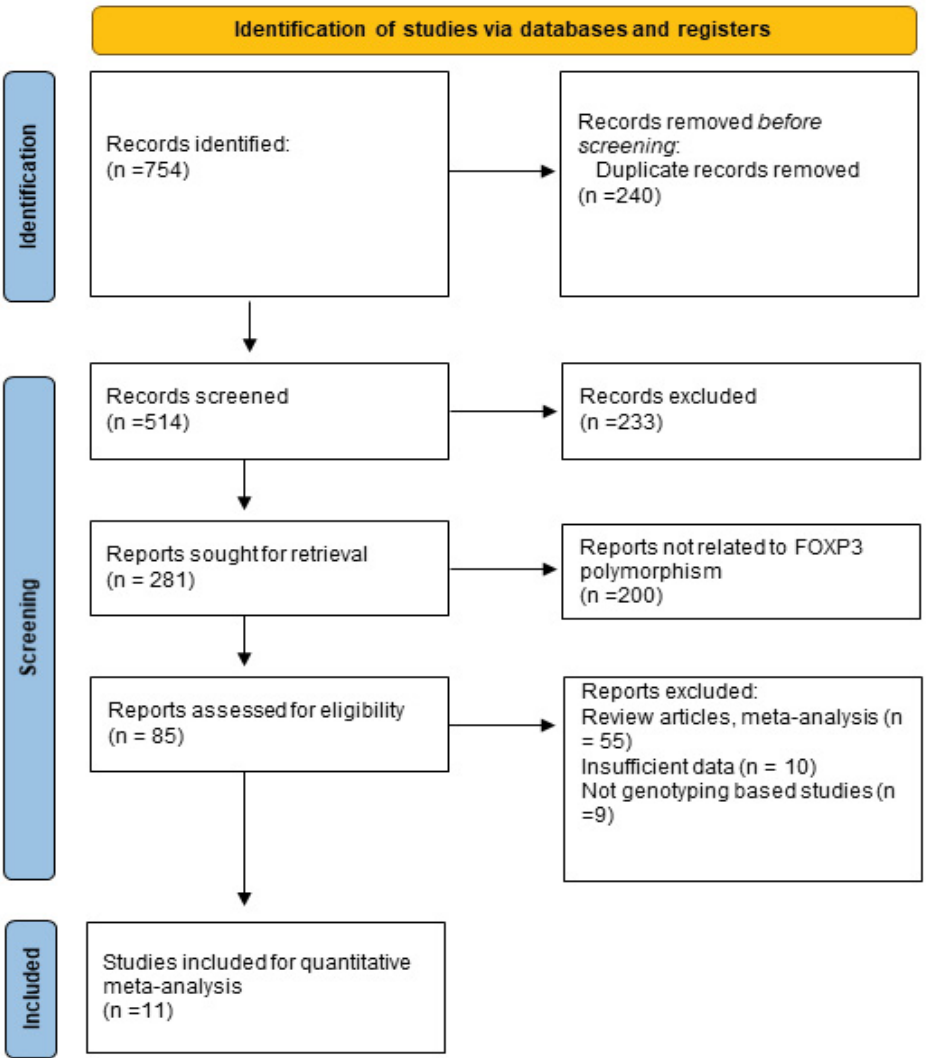

Figure 2: Flow chart for selection of studies (PRISMA 2020) [26].

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heterogeneity; I2 >50% and P-value <0.10, otherwise, fixed effect model was used when- no heterogeneity, I2 <50% and P-value >0.10. Meta-analysis was performed for different FOXP3 gene SNPs and their association with RPL under the allelic model, homozygous model, heterozygous model, dominant model and recessive model. To identify low-quality studies, Hardy Weinberg package was used to compute a P-value for each study in the control population. Funnel plot and Egger's tests were used for the diagnosis of potential publication bias. Furthermore, sub-group analysis based on countries/ethnicity was conducted to explore reasons for heterogeneity among studies. For all analysis, P-value <0.05 was considered statistically significant.

Results

Characteristics of selected study

The distribution of the FOXP3 genotypes in RPL cases and in control women are shown in Table 1. Overall, 754 studies were identified using different search engines. Using PRISMA checklist (Page et al., 2020), duplicate studies, review articles, meta-analysis and studies not following the defined inclusion-exclusion criteria were excluded. Finally, 11 studies were selected for meta-analysis, however, for further statistical analysis, SNPs with >2 studies were included. Among the studies included, all were from Asia (India, China, Iran, Kazakhstan and Gaza, Palestine) except one study which was from Africa (Egypt).

Selected SNPs

The selected 11 studies included 7 SNPs: rs3761548 A/C, rs2232365 A/G, rs2294021 T/C, rs2232368 A/G, 2280883 T/C, rs5902434del/ATT and rs141704699 C/T. For one of the FOXP3 SNP- rs141704699 C/T, only single study was found, therefore, it was removed from statistical analysis. Among the SNPs included for meta-analysis, three were located in the promoter region (rs2232365 A/G, rs3761548 A/C and rs5902434del/ATT) and remaining three were from the intronic region (rs2294021 T/C, rs2232368 A/G and rs2280883 T/C) of FOXP3 gene.

As summarized in Table 1, eleven studies including 1383 unexplained RPL cases and 1413 controls, were included for final analysis. Among the selected studies, most of the genotype distribution was consistent with Hardy–Weinberg equilibrium (HWE) but few studies didn't comply HWE (Table 2).

Further analysis was performed on 6 SNPs: rs3761548 A/C with 9 studies including 1134 cases/1103 controls, rs2232365 A/G with 8 studies (1053/1083), rs2294021 T/C with 3 studies (409/472), rs2232368 A/G with 2 studies (295/201), rs2280883 T/C with 2 studies (295/201) and rs5902434del/ATT with 2 studies (346/412). Genotype distributions in controls were in agreement with HWE in almost all included studies except for some studies in rs2232365 A/G [17,22,23]; rs3761548 A/C [15,19], rs2294021 T/C [25] and rs2280883 T/C [15,22].

FOXP3 variant rs2232368 A/G was excluded from further pooled analysis. The genotype frequencies among cases and controls and their p-values are shown in Table 2.

Quantitative Analysis

The pooled results of strength of association between different variants of FOXP3 gene polymorphism and their risk for RPL is shown in Table 2. Different genetic models were used for each variant and after pooling all the studies, we found that rs3761548 A/C, rs2232365 A/G, rs2280883 T/C and rs5902434del/ATT were significantly associated with risk of RPL. For each polymorphism forest plots in homogeneous model are shown in Figure 3.

Table 1: Baseline characteristics of studies included for meta-analysis

S. No	Study/First Author	Year	Country	Ethnicity	Genotyping Method	Case/Control	References
1	Wu Z et al	2012	China	Asian	PCR-SSP and PCR-RFLP	146/112	[16]
2	Jaber MO et al	2014	Gaza, Palestine	Asian	AS-PCR and PCR-RFLP	100/100	[17]
3	Naderi-Mahabadi F et al	2015	Iran	Asian	PCR-RFLP	195/101	[15]
4	Saxena D et al	2015	India	Asian	PCR-RFLP	200/300	[18]
5	Hadinedoushan H et al	2015	Iran	Asian	PCR-RFLP	80/80	[19]
6	Sharif FA et al	2016	Gaza, Palestine	Asian	PCR-RFLP	100/100	[20]
7	Zidan HE et al	2018	Egypt	African	PCR-RFLP	142/123	[21]
8	Mishra S et al	2018	India	Asian	PCR-RFLP	100/100	[22]
9	Gu Y et al	2018	China	Asian	Direct sequencing, Sequenom Mass ARRAY system.	107/187	[23]
10	Dirsipam K et al	2021	India	Asian	PCR-RFLP	150/150	[24]
11	Abdukassimova M et al	2021	Kazakhstan	Asian	RT-PCR	63/60	[25]

wherein, PCR- Polymerase Chain Reaction, AS- Allele-specific, RFLP-Restriction Fragment Length Polymorphism, SSP- sequence-specific primers,

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Table 2: Genotypic frequencies of different FOXP3 gene SNPs and their Hardy–Weinberg equilibrium adjusted P-values

Study			**Cases genotypes**			**Controls genotypes**				
rs2232365 A/G										
	Cases	**Controls**	**AA**	**AG**	**GG**	**AA**	**AG**	**GG**	**P-value**	**HW-adjusted P-value**
Jaber MO et al, 2014	100	100	20	49	31	25	63	12	**0.005**	**0.0163**
Abdukassimova M et al, 2021	63	60	28	26	8	36	22	2	0.076	0.8208
Naderi-Mahabadi F et al, 2015	195	101	106	77	12	84	16	1	**<0.0001**	0.8208
Mishra S et al, 2018	100	100	9	38	53	9	81	10	0.027	**0**
Wu Z et al, 2012	146	112	8	70	68	17	56	39	0.031	0.8208
Saxena D et al, 2015	200	300	77	85	38	149	126	25	**<0.001**	0.8208
Gu Y et al, 2018	107	187	35	34	22	63	66	41	0.968	**0.0163**
Zidan HE et al, 2018	142	123	35	62	45	65	46	12	**<0.001**	0.7322
rs3761548 A/C										
			AA	**AC**	**CC**	**AA**	**AC**	**CC**		
Jaber MO et al	100	100	33	37	30	44	42	14	**0.005**	0.497
Abdukassimova M et al	63	60	41	13	8	29	23	8	0.088	0.497
Naderi-Mahabadi F et al	195	101	89	47	59	38	30	33	0.387	**0**
Sharif FA et al	100	100	33	37	30	44	42	14	**0.011**	0.497
Wu Z et al	146	112	15	56	75	25	45	42	0.193	0.1809
Mishra S et al	100	100	35	52	13	47	46	7	0.18	0.497
Hadinedoushan H et al	80	80	47	20	13	42	17	21	0.30	**0**
Saxena D et al	200	300	68	87	45	139	128	33	**<0.001**	0.6658
Dirsipam K et al	150	150	13	102	35	72	60	18	**<0.001**	0.497
rs2294021 T/C										
			TT	**TC**	**CC**	**TT**	**TC**	**CC**		
Abdukassimova M et al, 2021	63	60	44	7	1	36	11	7	**0.002**	**0.0051**
Wu Z et al, 2012	146	112	65	70	11	40	57	15	0.18	0.6764
Saxena D et al, 2015	200	300	58	91	51	121	137	42	**<0.001**	0.7472
rs2232368 A/G										
			AA	**AG**	**GG**	**AA**	**AG**	**GG**		
Naderi-Mahabadi F et al, 2015	195	101	113	80	2	75	23	3	**0.008**	0.9148
Mishra S et al, 2018	100	100	100	0	0	100	0	0	**<0.001**	1.0
rs2280883 T/C										
			TT	**TC**	**CC**	**TT**	**TC**	**CC**		
Naderi-Mahabadi F et al, 2015	195	101	105	40	27	54	7	9	0.383	**0.0**
Mishra S et al, 2018	100	100	35	47	18	43	56	1	**<0.001**	**0.0003**
rs5902434del/ATT										
			del/del	del/ATT	ATT/ ATT	del/ del	del/ ATT	ATT/ ATT		
Wu Z et al, 2012	146	112	11	62	73	14	56	42	0.10	0.4805
Saxena D et al, 2015	200	300	63	89	48	114	147	39	**0.003**	0.4805

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rs3761548 A/C and RPL Risk

The SNP rs3761548 A/C was the most common among various Asian countries and Egypt. The results of combined analysis of all studies showed that rs3761548 A/C polymorphism was significantly associated with risk of RPL except for the recessive model (AA vs. AC+CC: OR=0.65, 95% CI [0.3971-1.0802], P=0.097). Further, substantial heterogeneity was present in all models except heterozygous comparison (AA vs AC: I2 <50%, Fig 1A). Subgroup analysis based on countries showed significant protective association for allele contrast model (A vs C) and dominant model (AA+AC vs. CC) in populations of China (OR=0.5669, 95% CI [0.3934-0.8170], P=0.002 and OR=0.568, 95% CI [0.3439-0.9380], P=0.0271; respectively), Gaza, Palestine (OR: 0.5718, 95% CI [0.3826-0.8544], P=0.006 and OR=0.3798, 95% CI [0.1870-0.7715], P=0.007; respectively) and India (OR=0.5183, 95% CI [0.3528-0.7614], P=0.0008 and OR=0.4431, 95% CI [0.3099-0.6337], P=0.00, respectively).

Similarly, recessive model (AA vs. AC+CC) was associated with protective OR in studies from China (OR:0.3985, 95% CI [0.1988-0.7985], P=0.009); Gaza, Palestine (OR=0.6269, 95% CI [0.4177-0.9408], P=0.0241); India (OR=0.3422, 95% CI [0.1234-0.9491], P=0.0393) and with significantly increased risk of RPL in population of Kazakhstan (OR=2.0870, 95% CI [1.0057-4.3311], P=0.0482). Both homozygous (AA vs CC) and heterozygous (AA vs AC) models were associated significantly with protective OR for populations of Gaza, Palestine and India, while for Chinese population homozygous model was significantly associated with RPL.

rs2232365 A/G and RPL Risk

The combined result of all eligible studies pooled into meta-analysis indicated that rs2232365 A/G polymorphism is significantly associated with risk of RPL for all genotyping/allelic models: Allele contrast (A vs. G: OR =0.5263, 95% CI [0.3997-0.6930], P <0.001), recessive model (AA vs. AG+GG: OR= 0.5265, 95% CI [0.3553-0.7800], P = 0.0013), dominant model (AA+AG vs. GG: OR= 0.3306, 95% CI [0.1920-0.5692], P<0.001), homozygous comparison (AA vs. GG: OR = 0.2900, 95% CI [0.1691-0.4972], P<0.001) and heterozygous model (AG vs GG: OR= 0.3836, 95% CI [0.2217-0.6635], P<0.001). Substantially significant heterogeneity was found in all models (I2 >50%, P <0.01, Fig 3B) Stratification based on geographic area showed that allele contrast model (A vs G) and homozygous comparison (AA vs GG) were significantly associated with risk of RPL in all Asian countries (Gaza-Palestine, India, Iran, Kazakhstan) except China and African country (Egypt).

rs2294021 T/C and RPL Risk

rs2294021 T/C polymorphism did not show significant associations with overall RPL risk (Table 3) in any of the genetic models. Substantial heterogeneity was found in all the models (Fig 3C).

Moreover, heterogeneity was removed on doing the subgroup analysis for stratification by country. Higher risk was observed in population of China and Kazakhstan, while significantly protective OR was observed for Indian population (Table 3).

rs2280883 T/C and RPL Risk

The pooled analysis of selected studies showed that rs2280883 T/C showed a significant strength of association with RPL for allelic and recessive models (P=0.0006 and P=0.0147, respectively). Substantial heterogeneity was also found for the above models (I2 <50%, Fig 3D).

While doing subgroup analysis based on countries, heterogeneity was removed. The results of subgroup analysis indicated that allele contrast model was significantly associated with risk of RPL in both Indian (OR=0.5758, 95% CI [0.3801- 0.8721], P=0.009) and Iranian (OR=0.5782, 95% CI [0.3531-0.9468], P=0.029) population, while homozygous, heterozygous and dominant models showed significant association in Indian population and recessive model in Iranian population.

rs5902434del/ATT and RPL Risk

All the genetic models except recessive model, showed protective ORs with significant P-values (Table 2). The results were as follows: for allele contrast model (OR=0.6893, 95% CI [0.5582-0.8514], P=0.0005), for dominant model (OR=0.5284, 95% CI [0.3754-0.7438], P=0.0002), for homozygous comparison (OR= 0.4498, 95% CI [0.2871-0.7047], P=0.0004) and for heterozygous model (OR=0.5561, 95% CI [0.3876-0.7978], P=0.0014). Substantial heterogeneity was observed for all models (I2 <50%, Fig 3E).

Heterogeneity was removed while doing subgroup analysis. Both allele contrast and dominant model showed significantly protective ORs in Indian (OR=0.6973, 95% CI [0.5394-0.9015], P=0.005) and Chinese (OR=0.6731, 95% CI [0.4646-0.9751], P=0.036) populations, while no significant association was found for recessive model in any of these populations. Further, in Indian population, both heterozygous and homozygous models showed significant strength of association with RPL (OR=0.449, 95% CI [0.2662-0.7573], P=0.002 and OR=0.4919, 95% CI [0.2990-0.8093], P=0.005; respectively).

Sensitivity Analysis and Publication Bias

evaluate the stability of overall results of the study, sensitivity analysis was done. Individual studies were

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sequentially omitted and it was observed that results did not change exactly. This indicates that no individual study influenced the overall results qualitatively, demonstrating that our results are statistically robust (data not shown).

To assess the publication bias in included studies funnel plot and Egger's test were used. The funnel plot analysis showed symmetrically shaped funnel for all the FOXP3 variants SNPs studied (Fig4). Furthermore, Egger's test showed no statistically significant publication bias for any of the polymorphism studied (Table 3).

Discussion

In cases of unexplained RPL, failure of immunologic tolerance at maternal-fetal interface has been suggested as an important cause for miscarriages [2,6-8]. The frequency of Tregs decrease remarkably in peripheral blood and decidua of women with unexplained RPL, which in turn decease the expression of FOXP3 gene [9,11,12]. Previous studies have shown that polymorphism in FOXP3 gene is associated with risk of unexplained RPL, however, its exact role in etiology of RPL is still poorly understood.

This meta-analysis is a step forward in the direction to understand the effect of different polymorphisms of FOXP3 gene among diverse ethnic groups around the globe. The pooled results of all the studies included in quantitative analysis showed that all the genotyping models for rs2232365 A/G polymorphism had significant protective association with RPL. While SNP rs3761548 A/C and rs5902434del/ATT were significantly associated with risk of RPL for all genotyping method except recessive model. rs2280883 T/C polymorphism showed significant association for allelic and recessive model, whereas rs2294021 T/C polymorphism showed no statistically significant association with RPL for any of the genotyping model.

The overall results of the subgroup analysis based on countries showed that FOXP3 gene SNPs rs2232365 A/G, rs3761548 A/C and rs2294021 T/C were significantly associated with risk of RPL in Indian population for all genotyping models. Further, rs2280883 T/C and rs5902434del/ATT polymorphism were also associated with significant susceptibility for RPL in Indian population for all genotyping models except for recessive model. This indicates that SNPs in either promoter or intronic region of FOXP3 gene are significantly present in women with unexplained RPL in India. The results are in corroboration with study conducted by Saxena D et al, wherein through single marker analysis they found that presence of rs2232365, rs3761548 and rs2294021 SNPs increase the risk of RPL by 2-3 folds in patients with unexplained RPL [18]. While, Multi Dimensionality Reduction (MDR) analysis revealed a 6-fold increase in risk of RPL for rs2232365, rs3761548 and rs2294021 SNPs in Indian women with RPL. On the contrary, Mishra et al, reported FOXP3 polymorphism rs2232365 as likely to be rare in Indian population [22].

In present study we found that among populations from Kazakhstan, polymorphism in FOXP3 gene variant rs3761548

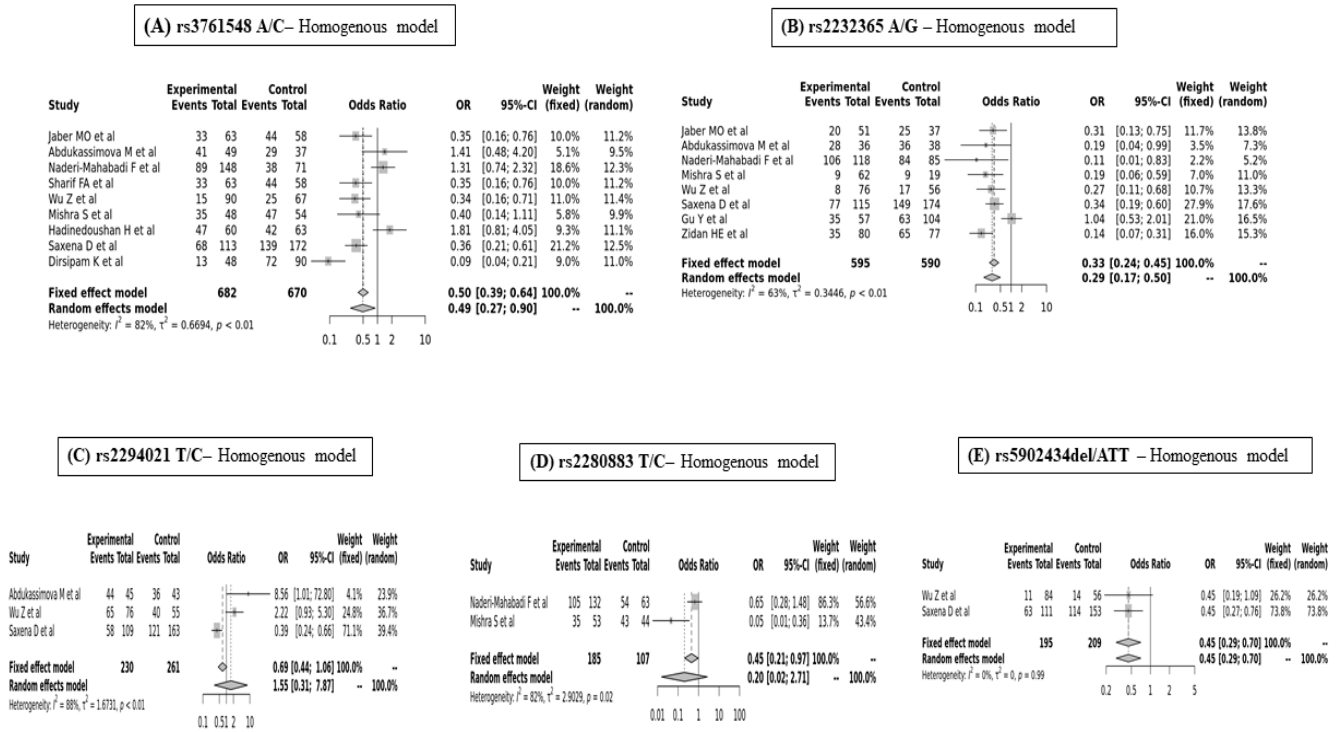

Figure 3: Forest plot for selected SNPs of FOXP3 (homogenous model)

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Table 3: Results of meta-analysis for the selected SNPs with RPL risk, heterogeneity test, and publication bias

Model	Number of studies	Test of association			Test of Heterogeneity			Publication bias
		OR	95% CI	p-val	Model	p-val	I^2	p-val (Egger's test)
rs3761548 A/C								
Allele contrast (A vs. C)	9	0.7316	[0.5360-0.9986]	**0.0489**	Random	0	83.56%	0.1884
Recessive model (AA vs. AC+CC)		0.655	[0.3971-1.0802]	0.097	Random	0	85.82%	0.8808
Dominant model (AA+AC vs. CC)		0.6194	[0.4341-0.8839]	**0.008**	Random	0.008	61.38%	0.7133
AA vs. CC		0.4929	[0.2713-0.8953]	**0.020**	Random	0	82.12%	0.8821
AC vs. CC		0.6585	[0.5204-0.8331]	**0.0005**	Fixed	0.2379	23.09%	0.7374
rs2232365 A/G								
Allele contrast (A vs. G)	8	0.5263	[0.3997-0.6930]	**<0.001**	Random	0.00	75.00%	0.3504
Recessive model (AA vs. AG+GG)		0.5265	[0.3553- 0.7800]	**0.0013**	Random	0.00	70.00%	0.8438
Dominant model (AA+AG vs. GG)		0.3306	[0.1920-0.5692]	**<0.001**	Random	0.00	76.00%	0.2518
AA vs. GG		0.2900	[0.1691-0.4972]	**<0.001**	Random	0.01	63.00%	0.2439
AG vs. GG		0.3836	[0.2217-0.6635]	**<0.001**	Random	0.00	73.00%	0.4483
rs2294021 T/C								
Allele contrast (T vs. C)	3	1.2942	[0.5839-2.8689]	0.525	Random	0	90.97%	0.293
Recessive model (TT vs. TC+CC)		1.2354	[0.5408-2.8219]	0.615	Random	0.0016	84.46%	0.3128
Dominant model (TT+TC vs. CC)		1.4332	[0.3662-5.6087]	0.605	Random	0.0013	84.88%	0.2741
TT vs. CC		1.5520	[0.3060-7.8701]	0.595	Random	0.0002	88.03%	0.3118
TC vs. CC		1.1589	[0.3973-3.3802]	0.787	Random	0.0257	72.67%	0.3199
rs2280883 T/C								
Allele contrast (A vs. G)	2	0.5768	[0.4198-0.7924]	**0.0006**	Fixed	0.989	0.0%	NaN
Recessive model (AA vs. AG+GG)		0.5893	[0.3853-0.9013]	**0.0147**	Fixed	0.324	0.0%	NaN
Dominant model (AA+AG vs. GG)		0.2239	[0.0140-3.5787]	0.289	Random	0.010	84.58%	NaN
AA vs. GG		0.2039	[0.0153-2.7091]	0.228	Random	0.018	81.89%	NaN
AG vs. GG		0.331	[0.0088-12.4807]	0.550	Random	0.001	89.75%	NaN
rs5902434del/ATT								
Allele contrast (del vs. ATT)	2	0.6893	[0.5582-0.8514]	**0.0005**	Fixed	0.8779	0.0%	NaN
Recessive model (del/del vs. del/ATT+ATT/ATT)		0.7158	[0.5071-1.0102]	0.0571	Fixed	0.5564	0.0%	NaN
Dominant model (del/del +del/ATT vs. ATT/ATT)		0.5284	[0.3754-0.7438]	**0.0002**	Fixed	0.4973	0.0%	NaN
del/del vs. ATT/ATT		0.4498	[0.2871-0.7047]	**0.0004**	Fixed	0.9897	0.0%	NaN
Del/ATT vs. ATT/ATT		0.5561	[0.3876-0.7978]	**0.0014**	Fixed	0.4835	0.0%	NaN

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rs3761548 A/C									
Allele contrast (A vs. C)	China	1	0.5669	[0.3934-0.8170]	**0.002**	Fixed	NA	NA	NA
	Gaza, Palestine	2	0.5718	[0.4304-0.7596]	**0.0001**	Fixed	1	0	NA
	India	3	0.5183	[0.3528-0.7614]	**0.0008**	Random	0.0179	0.7514	0.9538
	Iran	2	1.3048	[0.9900-1.7198]	0.0589	Fixed	0.5916	0	NA
	Kazakhstan	1	1.5773	[0.8968-2.7741]	0.113	Fixed	NA	NA	NA
Recessive model (AA vs. AC+CC)	China	1	0.3985	[0.1988-0.7985]	**0.009**	Fixed	NA	NA	NA
	Gaza, Palestine	2	0.6269	[0.4177-0.9408]	**0.0241**	Fixed	1	0	NA
	India	3	0.3422	[0.1234-0.9491]	**0.0393**	Random	0	0.9126	0.4886
	Iran	2	1.3516	[0.9185-1.9889]	0.126	Fixed	0.8491	0	NA
	Kazakhstan	1	2.0870	[1.0057-4.3311]	**0.0482**	Fixed	NA	NA	NA
Dominant model (AA+AC vs. CC)	China	1	0.568	[0.3439-0.9380]	**0.0271**	Fixed	NA	NA	NA
	Gaza, Palestine	2	0.3798	[0.2302-0.6269]	**0.0001**	Fixed	1	0	NA
	India	3	0.4431	[0.3099-0.6337]	**0.00**	Fixed	0.9538	0	0.0259
	Iran	2	1.3021	[0.8474-2.0008]	0.228	Fixed	0.2979	0.0773	NA
	Kazakhstan	1	1.0385	[0.3629-2.9716]	0.943	Fixed	NA	NA	NA
AA vs. CC	China	1	0.336	[0.1598-0.7065]	**0.004**	Fixed	NA	NA	NA
	Gaza, Palestine	2	0.35	[0.2018-0.6070]	**0.000**	Fixed	1	0	NA
	India	3	0.238	[0.0963-0.5878]	**0.001**	Random	0.017	0.7546	0.771
	Iran	2	1.4584	[0.9151-2.3242]	0.112	Fixed	0.5232	0	NA
	Kazakhstan	1	1.4138	[0.4757-4.2020]	0.533	Fixed	NA	NA	NA
AC vs. CC	China	1	0.6969	[0.4042-1.2014]	0.193	Fixed	NA	NA	NA
	Gaza, Palestine	2	0.4111	[0.2380-0.7102]	**0.001**	Fixed	1	0	NA
	India	3	0.62	[0.4246-0.9053]	**0.013**	Fixed	0.4205	0	0.7855
	Iran	2	1.1087	[0.6579-1.8684]	0.698	Fixed	0.1812	0.4407	NA
	Kazakhstan	1	0.5652	[0.1715-1.8632]	0.348	Fixed	NA	NA	NA
rs2232365 A/G									
Allele contrast (A vs. G)	China	2	0.7998	[0.4886-1.3092]	0.374	Random	0.0564	0.7254	NA
	Egypt	1	0.3454	[0.2405-0.4961]	**0.00**	Fixed	NA	NA	NA
	Gaza, Palestine	1	0.6173	[0.4159-0.9162]	**0.016**	Fixed	NA	NA	NA
	India	2	0.5095	[0.3323-0.7812]	**0.001**	Random	0.08	0.6738	NA
	Iran	1	0.2799	[0.1640-0.4777]	**0.00**	Fixed	NA	NA	NA
	Kazakhstan	1	0.54	[0.3049-0.9566]	**0.034**	Fixed	NA	NA	NA
Recessive model (AA vs. AG+GG)	China	2	0.6194	[0.1945-1.9722]	0.417	Random	0.0232	0.806	NA
	Egypt	1	0.2919	[0.1734-0.4912]	**0.00**	Fixed	NA	NA	NA
	Gaza, Palestine	1	0.75	[0.3849-1.4614]	0.397	Fixed	NA	NA	NA
	India	2	0.6711	[0.4774-0.9434]	**0.021**	Fixed	0.3887	0	NA
	Iran	1	0.241	[0.1333-0.4359]	**0.00**	Fixed	NA	NA	NA
	Kazakhstan	1	0.549	[0.2675-1.1268]	0.102	Fixed	NA	NA	NA
Dominant model (AA+AG vs. GG)	China	2	0.752	[0.5113-1.1060]	0.147	Fixed	0.2224	0.3285	NA
	Egypt	1	0.233	[0.1166-0.4658]	**0.00**	Fixed	NA	NA	NA
	Gaza, Palestine	1	0.3035	[0.1452-0.6343]	**0.001**	Fixed	NA	NA	NA
	India	2	0.2009	[0.0525-0.7678]	**0.018**	Random	0.0041	0.8788	NA
	Iran	1	0.1525	[0.0195-1.1900]	0.072	Fixed	NA	NA	NA
	Kazakhstan	1	0.2328	[0.0473-1.1450]	0.072	Fixed	NA	NA	NA
AA vs. GG	China	2	0.5507	[0.1478-2.0513]	0.373	Random	0.0208	0.8128	NA
	Egypt	1	0.1436	[0.0673-0.3064]	**0.00**	Fixed	NA	NA	NA
	Gaza, Palestine	1	0.3097	[0.1273-0.7531]	**0.009**	Fixed	NA	NA	NA
	India	2	0.302	[0.1807-0.5047]	**0.00**	Fixed	0.3676	0	NA

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	Iran	1	0.1052	[0.0134-0.8251]	**0.032**	Fixed	NA	NA	NA
	Kazakhstan	1	0.1944	[0.0382-0.9887]	**0.048**	Fixed	NA	NA	NA
AG vs. GG	China	2	0.8029	[0.5313-1.2133]	0.297	Fixed	0.4993	0	NA
	Egypt	1	0.3594	[0.1711-0.7550]	**0.006**	Fixed	NA	NA	NA
	Gaza, Palestine	1	0.3011	[0.1403-0.6462]	**0.002**	Fixed	NA	NA	NA
	India	2	0.2026	[0.0418-0.9832]	**0.047**	Random	0.0011	0.9063	NA
	Iran	1	0.401	[0.0486-3.3075]	0.396	Fixed	NA	NA	NA
	Kazakhstan	1	0.2955	[0.0567-1.5388]	0.147	Fixed	NA	NA	NA
rs2294021 T/C									
Allele contrast (T vs. C)	China	1	1.3805	[0.9584-1.9885]	0.083	Fixed	NA	NA	NA
	India	1	0.6254	[0.4837-0.8086]	**0.0003**	Fixed	NA	NA	NA
	Kazakhstan	1	3.1794	[1.4048-7.1959]	**0.005**	Fixed	NA	NA	NA
Recessive model (TT vs. TC+CC)	China	1	1.4444	[0.8710-2.3955]	0.154	Fixed	NA	NA	NA
	India	1	0.6042	[0.4121-0.8860]	**0.009**	Fixed	NA	NA	NA
	Kazakhstan	1	2.7500	[1.0719-7.0550]	**0.035**	Fixed	NA	NA	NA
Dominant model (TT+TC vs. CC)	China	1	1.8978	[0.8354-4.3116]	0.125	Fixed	NA	NA	NA
	India	1	0.4756	[0.3016-0.7500]	**0.001**	Fixed	NA	NA	NA
	Kazakhstan	1	7.5957	[0.9005-64.0732]	0.062	Fixed	NA	NA	NA
TT vs. CC	China	1	2.2159	[0.9265-5.3000]	0.073	Fixed	NA	NA	NA
	India	1	0.3947	[0.2360-0.6604]	**0.0003**	Fixed	NA	NA	NA
	Kazakhstan	1	8.5556	[1.0055-72.7965]	**0.049**	Fixed	NA	NA	NA
TC vs. CC	China	1	1.6746	[0.7136- 3.9298]	0.236	Fixed	NA	NA	NA
	India	1	0.547	[0.3362- 0.8901]	**0.015**	Fixed	NA	NA	NA
	Kazakhstan	1	4.4545	[0.4468-44.4135]	0.202	Fixed	NA	NA	NA
rs2280883 T/C									
Allele contrast (T vs. C)	India	1	0.5758	[0.3801- 0.8721]	**0.009**	Fixed	NA	NA	NA
	Iran	1	0.5782	[0.3531-0.9468]	**0.029**	Fixed	NA	NA	NA
Recessive model (TT vs. TC+CC)	India	1	0.7138	[0.4034-1.2629]	0.246	Fixed	NA	NA	NA
	Iran	1	0.4643	[0.2457-0.8776]	**0.018**	Fixed	NA	NA	NA
Dominant model (TT+TC vs. CC)	India	1	0.046	[0.0060-0.3521]	**0.003**	Fixed	NA	NA	NA
	Iran	1	0.7923	[0.3519-1.7838]	0.574	Fixed	NA	NA	NA
TT vs. CC	India	1	0.0452	[0.0057-0.3557]	**0.003**	Fixed	NA	NA	NA
	Iran	1	0.6481	[0.2847-1.4756]	0.301	Fixed	NA	NA	NA
TC vs. CC	India	1	0.0466	[0.0060-0.3624]	**0.003**	Fixed	NA	NA	NA
	Iran	1	1.9048	[0.6329-5.7324]	0.251	Fixed	NA	NA	NA
rs5902434del/ATT									
Allele contrast (del vs. ATT)	China	1	0.6731	[0.4646-0.9751]	**0.036**	Fixed	NA	NA	NA
	India	1	0.6973	[0.5394-0.9015]	**0.005**	Fixed	NA	NA	NA
Recessive model (del/del vs. del/ATT+ATT/ ATT)	China	1	0.5704	[0.2484-1.3099]	0.185	Fixed	NA	NA	NA
	India	1	0.7503	[0.5138-1.0956]	0.136	Fixed	NA	NA	NA
Dominant model (del/del +del/ATT vs. ATT/ ATT)	China	1	0.6	[0.3633-0.9908]	**0.045**	Fixed	NA	NA	NA
	India	1	0.4732	[0.2965-0.7552]	**0.001**	Fixed	NA	NA	NA
del/del vs. ATT/ATT	China	1	0.4521	[0.1882-1.0857]	0.075	Fixed	NA	NA	NA
	India	1	0.449	[0.2662-0.7573]	**0.002**	Fixed	NA	NA	NA
del/ATT vs. ATT/ATT	China	1	0.637	[0.3772-1.0758]	0.091	Fixed	NA	NA	NA
	India	1	0.4919	[0.2990-0.8093]	**0.005**	Fixed	NA	NA	NA

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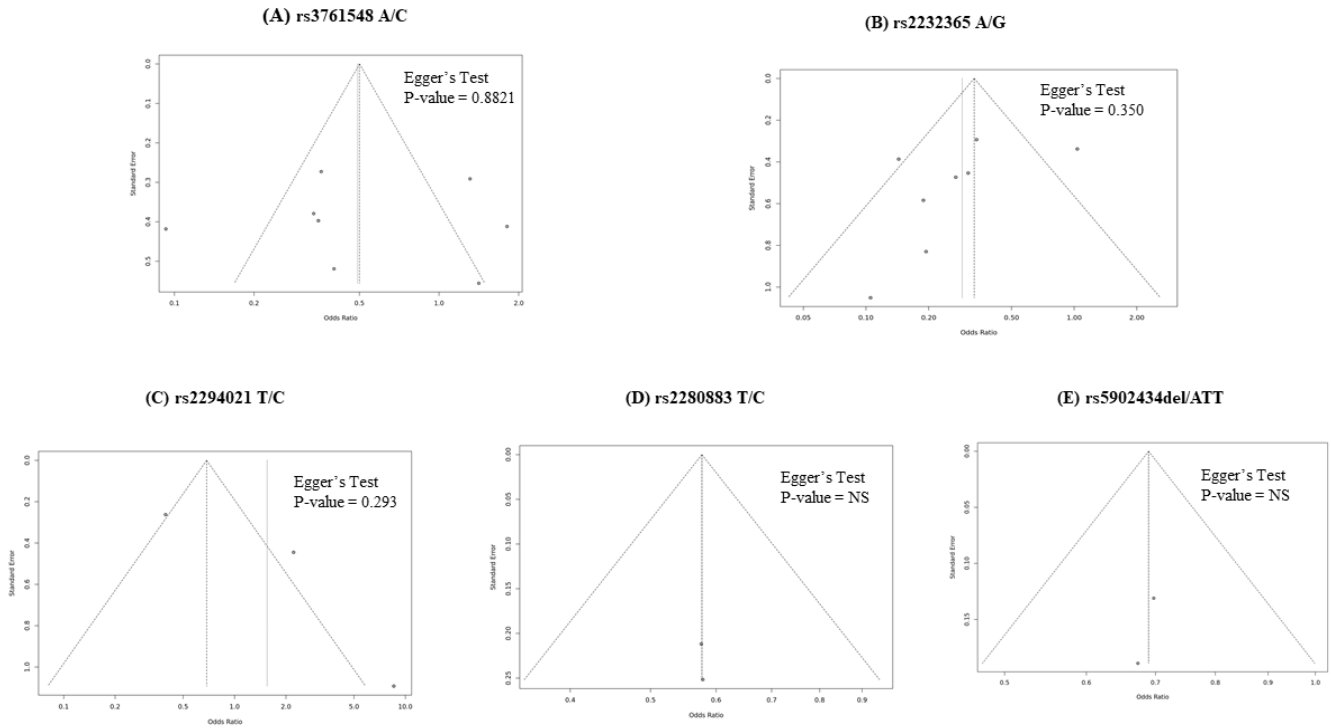

Figure 4: Funnel Plot for FOXP3 SNPs to analyse risk of bias

A/C (recessive model) was significantly associated with elevated risk of RPL (OR > 1). Further, our results showed a significantly protective association between rs2232365 A/G and RPL for allele contrast and homozygous model. While for rs2294021 T/C variant, allele contrast and recessive model were significantly associated with risk of RPL among women from Kazakhstan. However, contradictory results were reported by Abdukassimova M et al, in their study, wherein they reported minor allele frequency (MAF) of rs2294021 was significantly associated with protective OR, while rs2232365 MAF was significantly associated with RPL susceptibility [25].

The analysis also included studies from China, wherein the overall analysis indicated that rs3761548 A/C polymorphism shows significant association with RPL for all genotyping methods except for heterogenous comparison. While for rs5902434del/ATT, only allele contrast and dominant model showed significantly protective association with OR < 1. These results are in line with study by Wu Z et al, in Han Chinese population. They found that rs3761548A/C and rs2232365A/G polymorphisms to be significantly associated with unexplained RPL cases [16]. Further, difference in allelic distribution of rs5902434 del/ATT was observed in RPL women as compared to control, with haplotype del-A-G as a significant risk factor for RPL.

While in populations of Egypt, significantly protective OR was found for FOXP3 gene variant rs2232365 A/G for all genotyping models. Zidan HE et al, also reported that risk of RPL was significantly higher in Egyptian women for SNP rs2232365 [21]. Further, the risk was significantly higher in women carrying G allele as compared to allele A. Since rs2232365 polymorphism is located in the promoter region, increased frequency of G allele reduces Th2 response in RPL patients, thus disturbing the Th1/Th2 balance [16,21]. In addition, Zidan et al, also found that serum FOXP3 levels were significantly lower in women with GG and AG genotypes than AA genotype [21].

In populations of Gaza, Palestine, all genotyping models for rs3761548 A/C and rs2232365 A/G (except recessive model) were significantly associated with RPL. This result consolidates with the findings of Jaber MO et al, Sharif FA et al, who reported a significant association between the above two polymorphisms and unexplained RPL in women from Gaza, Palestine [17,20].

Pooled analysis of studies included from Iran showed that rs2232365 A/G (allele contrast, recessive and homogenous models) and rs2280883 T/C (allele contrast and recessive models) polymorphism were significantly associated with RPL. However, our results contradicted from the individual studies conducted in Iran. While, Naderi-Mahabadi F et al, did found rs2232365 A/G to be significantly associated with RPL, they found no significant association for rs2280883 T/C polymorphism in Iranian population [15]. Further, they found rs3761548 A/C to be significantly associated with unexplained RPL, while our study and Hadinedoushan H et al, found no significant co-relation for the above SNP

with RPL [19]. These contradicted results might be caused by some studies that have small sample size, low statistical power, ethnicity differences, and publication bias.

There were some limitations in this meta-analysis. We have searched literature only in English language; it may cause language bias. Comprehensive analysis could not be conducted because the number of included studies was not sufficient. Data was collected from retrospective research, which may be related to the methodological deficiencies. Finally, environmental and other genetic factors were not taken into account in this meta-analysis. Therefore, the results should be interpreted with caution.

Conclusion

Our meta-analysis is focused on FOXP3 SNPs and examines the relationship between these SNPs in susceptibility to RPL. This study revealed that all the 6 investigated functional polymorphisms proved to be significantly associated with RPL and could represent a potential risk factor for the occurrence of RPL in Asian women. Our meta-analysis supports the notion that immune-related pregnancy complications might be linked to genetic variations in the FOXP3 gene. However, functional and gene expression studies are warranted to prove this hypothesis completely in larger sample size.

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Author Contributions

CB, AK: Literature and data mining, analysis and preparation of manuscript.

MR, AK: quality assessment, editing and critical review of manuscript.

PS: Conceptualisation, quality assessment, and final approval of manuscript

All authors approved the final version of manuscript.

Competing Interest

The authors declare no competing interest.

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Data Availability

The authors have given all the data set in form of tables and supplementary data with the manuscript.

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Supplementary Table 1: PRISMA Checklist

TITLE			
Title	1	Identify the report as a systematic review.	1
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	4
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	5
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	5
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	5
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	5
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Fig.1
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	5
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	6
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	NA
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	5 & Supp Data
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	6
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	Table 1
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	NA
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	NA
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	5,6
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	6
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	9
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Supp Data

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Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	5, 6
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	6,7, Fig 1
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	7
Study characteristics	17	Cite each included study and present its characteristics.	Table 1
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Supp Data
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	Table 2
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	9
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	Table 2, 3
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	Fig 3
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	Fig 4
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	Table 2, 3
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	10,11
	23b	Discuss any limitations of the evidence included in the review.	12
	23c	Discuss any limitations of the review processes used.	12
	23d	Discuss implications of the results for practice, policy, and future research.	12
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	NA
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	Protocol was not prepared
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	NA
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	12
Competing interests	26	Declare any competing interests of review authors.	12
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	5

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Supplementary Table 2: New Castle Ottawa Scale for Quality Assessment

Study	Selection				Comparability	Exposure/Outcome			Overall rating and Total Score
	1	2	3	4		1	2	3	
Jaber MO et al, 2014	*	*		*	**	*	*		7
Abdukassimova M et al, 2021	*	*		*	**	*	*		7
Naderi-Mahabadi F et al, 2015	*	*		*	*	*	*		6
Mishra S et al, 2018	*	*		*	**	*	*		7
Wu Z et al, 2012	*	*		*	*	*	*		6
Saxena D et al, 2015	*	*		*	*	*	*		6
Gu Y et al, 2018	*	*		*	**	*	*		7
Zidan HE et al, 2018	*	*		*	**	*	*		7
Hadinedoushan H et al, 2015	*	*		*	**	*	*		7