



The synergic effects of CTLA-4/Foxp3-related genotypes and chromosomal aberrations on the risk of recurrent spontaneous abortion among a Chinese Han population

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Abstract

The current study was aimed to investigate the association of CTLA-4/Foxp3 polymorphisms and chromosomal abnormalities with recurrent spontaneous abortion (RSA) risk in a Chinese Han population. Altogether, 1284 RSA women and 1046 women with normal pregnancy were incorporated in this study. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was implemented to genotype the single-nucleotide polymorphisms (SNPs) located within CTLA4 and Foxp3. Moreover, the cytogenetic diagnosis was performed in line with the standards of G banding karyotype. As a consequence, rs231775 and rs3087243 of CTLA4, as well as rs2232365 and rs2232368 of Foxp3, all appeared to modify the risk of RSA. Besides, significant differences were found between the ratio of structural abnormality and that of numerical abnormality ($P < 0.038$), and chromosome abnormality was associated with higher miscarriage frequency (>3) than normal karyotypes. Of note, the synergic effects of the genotypes and chromosomal abnormality all tallied with the sub-multiplication model ($OR_{\text{chromosome}} \times OR_{\text{SNP}} > OR_{\text{chromosome+SNP}}$), while rs2232365 GG and chromosomal aberration impacted the RSA risk in a super-multiplicative way that $OR_{\text{chromosome}} \times OR_{\text{SNP}} < OR_{\text{chromosome+SNP}}$. In conclusion, susceptibility to RSA was subject to the synthetic regulation of chromosomal aberrations and genetic mutations within CTLA-4 and Foxp3, suggesting that the conduction of karyotype analysis and genetic detection for RSA patients could effectively guide effective RSA counseling and sound child rearing.

Introduction

Recurrent spontaneous abortion (RSA) is referred to that abortions are continuously repeated for ≥ 2 times [1]. At present, ~15% of pregnant women are attacked by abortion, and up to 0.4–2% of the patients are confirmed with RSA [2].

In fact, the pathology of RSA are attributed to several aspects, including heredity, anatomy, immunity, endocrine, infection, thrombophilia, environment and so on, yet the mechanism underlying RSA has not been thoroughly elucidated.

Among the parameters, it has been proved that establishing the maternal–fetal immune tolerance was crucial to maintaining the normal pregnancy, especially that $CD4^+CD25^+Foxp3^+$ regulatory T cell was of vital significance to modulate T cells' tolerance to fetus [3]. The proportion of $CD4^+CD25^+Foxp3^+$ T cells within the peripheral blood was also notably diminished in comparison to the normal pregnant women, further implying the involvement of Foxp3 in facilitating RSA development [4]. Notably, aberration of single-nucleotide polymorphisms (SNPs) that were located in the promoter region of Foxp3, such as rs2232365 and rs3761548, could directly bring about frame shift mutations, and thereby deranging the functions of Foxp3 transcript [5]. Moreover, mutant SNPs situated in the intronic region of Foxp3 (e.g. rs2280883) also affected Foxp3 actions through generating the alternative splice site and imposing effects on RNA processing

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[6–8]. Allowing for the importance of SNPs for *Foxp3*, it has been documented that the alleles of rs2232365 and rs3761548 within a Chinese crowd distributed quite distinct between RSA subjects and normal ones [9]. Besides, the RSA incidence of an Indian population who carried mutations of rs2232365 (A>G), rs3761548 (C>A), and rs2294021 (T>C) climbed to as high as six folds of that within the control Indians [10]. As for CTLA4, its reduced expressions within the placental site were probable to mitigate the inhibition of activated T lymphocytes, suggesting that CTLA-4 might participate in regulating the mother–fetal immunity tolerance [11, 12]. Among a great diversity of SNPs that affected the CTLA4 actions [13–16], it was demonstrated that the mutated genotypes of rs231775 and rs3087243 not merely contributed to depressed level of serum CTLA4, but also created larger risk of RSA [9, 17, 18].

In addition, emerging studies also discovered the relationship between chromosomal polymorphism and fertility-related aberrations, such as spontaneous abortion and embryo damages [19, 20]. Virtually, chromosome polymorphisms were caused by the subtle variances within the heterochromatin region of human chromosomes, including the secondary constriction of chromosomes 1, 9, and 16, the long arm of chromosome Y, the short arm of D/G group (chromosomes 13, 14, 15, 21, and 22), and the heterochromatic region of trisomy [21, 22]. The chromosomal polymorphisms could render genetic silencing or methylation through affecting the activity of restriction enzymes and the nuclear covalent bonds of histones, or by altering siRNA [23]. Hence, the chromosome polymorphisms could be intrinsically connected with abnormal fertilities. For instance, the secondary restrictions of chromosomes 1, 9, and 16 were widely present in the couples with disordered infertilities, which could be explained by the meiotic divisions of chromosomes and the regulatory functions of certain genes [24]. In a word, it was suggested that diverse changes of chromosomes were closely linked with the occurrence of infertilities.

So far, although diverse studies have been concentrated on the role of CTLA-4/*Foxp3* SNPs or chromosomal polymorphisms in regulating the RSA risk, few studies were aimed to study their synthetic effects. Therefore, the current study was purposed to remedy this gap, which might provide evidences for finding novel treatment strategies for RSA.

Materials and Methods

Subjects

Altogether, 1284 RSA women were, retrospectively, recruited from the Reproductive Medical Center during the

period from April 2013 to October 2016. The RSA subjects would be incorporated if: (1) their abortion frequency was ≥ 2 times; (2) they were not subject to the effects of dissection, internal secretion, ABO incompatibility, systemic infection and reproductive tract infection; (3) their husbands were with normal chromosome karyotypes within peripheral blood and normal results after these men examination. Moreover, 1046 subjects with normal pregnancy were also included into the control group, and they were without histories of spontaneous abortion, premature delivery and pregnancy-induced hypertension. The healthy controls should also have at least once of successful pregnancy. All the participants have signed the informed consents, and this study has been approved by the Reproductive Medical Center and the ethics committee of Reproductive Medical Center.

Chromosome examination

About 2 ml venous blood was, respectively, extracted from the puerpera, and then the blood samples were cultured in the 1640 medium at the temperature of 37 °C for 66–68 h. Subsequently, 20 µg/ml colchicine (volume: 8.5 µl) was added to the mixture, which was continuously culture for another 4–6 h. After being centrifugated, the remainder was hypotonically processed with KCl for 35 min, and was fixed for 30 min. Finally, slices were made, and chromosome banding was conducted. Averagely 30 karyotypes were counted for each case, and 5–8 karyotypes were analyzed. As for the abnormal cases, equally 50 karyotypes were counted for each subject, and 30 karyotypes were simultaneously analyzed. The cytogenetic diagnosis was implemented according to the standards of G banding karyotype.

Genotyping

Genomic DNAs were extracted from the peripheral white blood cells utilizing the phenol–chloroform protocol. The reaction primers of CTLA4 [25] and *Foxp3* were designed based on Primer Premier 5.0 software (Supplementary table 1), and were synthesized by Sangon biotechnology corporation (Shanghai, China). Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) was applied to genotype the SNPs of CTLA4 and *Foxp3*. The PCR reaction system (50 µl) mainly consisted of DNA template (4 µl), buffer solution (5 µl), 10 µmol/L sense primer (2 µl), 10 µmol/L anti-sense primer (2 µl), Taq enzyme (0.25 µl) and 32.75 µl deionized water. Besides, the reaction condition was enlisted as: (1) pre-degeneration at 94 °C for 5 min; (2) 30 cycles of degeneration at 94 °C for 40 s, annealing at 55.6 °C for 50 s, and extension at 72 °C for 30 s; (3) re-extension at 72 °C for 7 min.

Table 1 The association of SNPs within CTLA4 and FOXP3 with recurrent spontaneous abortion risk

Gene	Rs number	Allele	Case genotype			Control genotype			Allelic model			Dominant model			Recessive model		
			11	12	22	11	12	22	OR	95% CI	P-value	OR	95% CI	P-value	OR	95% CI	P-value
CTLA4	rs231775	A>G	665	518	101	415	488	143	0.66	0.59–0.75	1.91 × 10 ⁻⁹	0.61	0.52–0.72	1.47 × 10 ⁻⁷	0.54	0.41–0.71	1.44 × 10 ⁻⁴
	rs5742909	C>T	417	629	238	364	506	176	1.09	0.97–1.22	0.17	1.11	0.93–1.32	0.24	1.12	0.91–1.39	0.28
	rs4553808	A>G	761	455	68	653	347	46	1.13	0.98–1.29	0.10	1.14	0.97–1.35	0.12	1.22	0.83–1.78	0.32
	rs3087243	G>A	74	468	742	151	493	402	1.94	1.71–2.20	1.06 × 10 ⁻²³	2.76	2.06–3.69	4.85 × 10 ⁻¹¹	2.19	1.86–2.59	3.94 × 10 ⁻¹⁹
FOXP3	rs2232365	A>G	326	431	527	292	368	386	1.15	1.02–1.29	0.02	1.14	0.95–1.37	0.17	1.19	1.01–1.41	0.04
	rs3761548	C>A	148	576	560	107	455	484	0.91	0.81–1.03	0.15	0.87	0.67–1.14	0.32	0.90	0.76–1.06	0.20
	rs2232368	G>A	697	433	154	551	409	86	1.05	0.93–1.20	0.42	0.94	0.80–1.10	0.44	1.52	1.15–2.01	2.89 × 10 ⁻³
	rs2280883	T>C	594	559	131	512	439	95	1.09	0.96–1.24	0.16	1.11	0.95–1.31	0.20	1.14	0.86–1.50	0.36
	rs2294021	T>C	347	641	296	305	520	221	1.08	0.97–1.22	0.17	1.11	0.93–1.33	0.25	1.12	0.92–1.36	0.27

1 wild allele, 2 mutated allele, *CI* confidence interval, *OR* odds ratio

The bold *P*-value means a significant results

Analysis of the interaction between chromosomal abnormality and genetic mutations

We applied multivariate logistic regression model to detect the interactive action of chromosomal abnormality and SNPs within CTLA4/Foxp3. The regression model included consideration of variants related with genetic mutations, chromosomal abnormality, as well as such concomitant variables as age, sex ratio and so on [26–28]. First, it would be deemed as: (1) multiplication model when $OR_{eg} = OR_e + OR_g$; (2) super-multiplication model when $OR_{eg} > OR_e + OR_g$; (3) sub-multiplication model when $OR_{eg} < OR_e + OR_g$; and (4) additive model when $OR_{eg} = OR_e + OR_g - 1$. Among them, OR_e was representative of the effect size for chromosomal abnormality, and OR_g meant the effect size for genetic mutations. Similarly, OR_{eg} meant the combined effect size of chromosomal abnormality and genetic mutations. Second, γ was regarded as the interaction coefficient of genetic mutations and chromosomal abnormality within case–control studies [26]. If $\gamma > 1$, the genetic factors would enlarge the effects of chromosomal abnormality; if $\gamma < 1$, the genetic factors would weaken the effects of chromosomal abnormality; if $\gamma = 1$, there was hardly any interactions between genetic factors and chromosomal abnormality. Finally, when $\gamma < 0$, chromosomal abnormality was believed as the risky parameter; and genetic mutations would play the protective role.

Statistical analysis

All the data were analyzed via SPSS 17.0 software. The enumeration data were statistically processed through χ^2 -tests, and the measurement data were presented in the

form of (mean \pm SD). The unconditional logistical regression model was applied to evaluate the relationship between SNPs within CTLA4/Foxp3 and development of RSA. It would be deemed as statistically significant when $P < 0.05$ after Bonferroni correction, and 27-time correction were conducted for this study's SNP-based association analyses.

Result

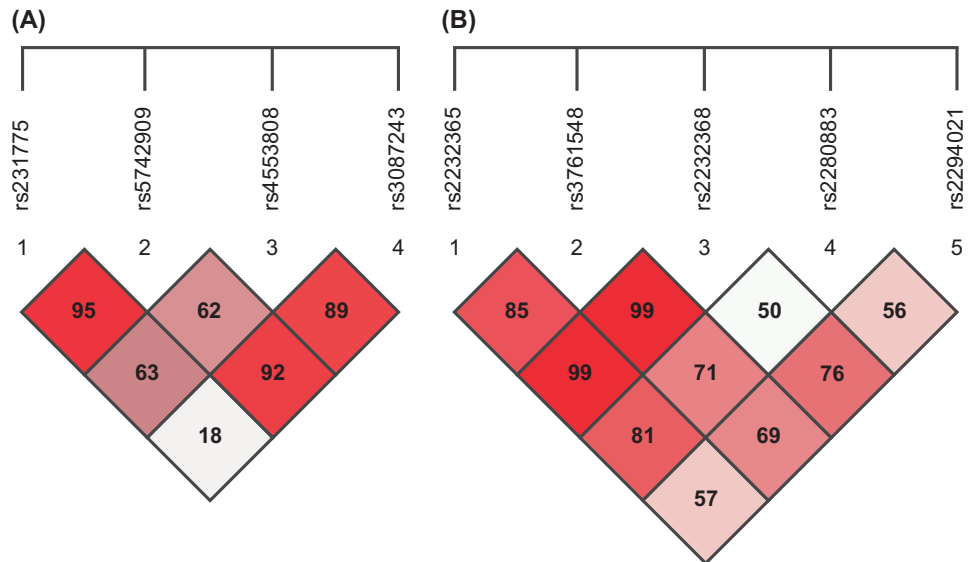
The association of SNPs within CTLA4 and Foxp3 with RSA risk

Regarding CTLA4, the allele G of rs231775 seemed to act as the protective parameter for RSA in comparison to the A allele (odds ratio (OR) = 0.66, 95% confidence interval (CI): 0.59–0.75, $P = 1.91 \times 10^{-9}$) (Table 1). In contrast, the allele A of rs3087243 was tightly linked with higher susceptibility to RSA than allele G (OR = 1.94, 95% CI: 1.71–2.20, $P = 1.06 \times 10^{-23}$). As for Foxp3 rs2232365, carriers of G allele were more ready to suffer from RSA than ones carrying allele A (OR = 1.15, 95% CI: 1.02–1.29, $P = 0.02$). Besides, when compared with genotypes GA and GG of rs2232368, homozygote AA was associated with relatively higher incidence of RSA (OR = 1.52, 95% CI: 1.15–2.01, $P = 2.83 \times 10^{-3}$).

The association of haplotypes within CTLA4 and Foxp3 with RSA risk

After applying SHEsis software for linkage disequilibrium (LD) of SNPs within Foxp3, it was derived that each two of rs231775, rs5742909, rs4553808, and rs3087243 generally

Fig. 1 Linkage disequilibrium (LD) of SNPs within Foxp3 (rs231775, rs5742909, rs4553808 and rs3087243) and CTLA4 (rs2232365, rs3761548, rs2232368 and rs2280883) and rs2294021)



conformed to the rule of LD (all $D' > 0.60$), except the pair of rs231775, and rs3087243 ($D' = 0.18$; Fig. 1a). Regarding CTLA4, each pair of rs2232365, rs3761548, rs2232368, rs2280883, and rs2294021 shared a D' -value of >0.50 (Fig. 1b).

Correspondingly, haplotypes ACAA, ATAG, GTAA and GTAG were associated with reduced susceptibility to RSA ($P = 1.40 \times 10^{-4}$, $P = 7.56 \times 10^{-42}$, $P = 5.23 \times 10^{-29}$, $P = 8.36 \times 10^{-21}$), while haplotypes ACAG ($P = 0.01$), ATAA ($P = 5.94 \times 10^{-19}$), ATGA ($P = 3.95 \times 10^{-48}$) and GCAA ($P = 6.36 \times 10^{-6}$) appeared to remarkably increase the RSA risk (Table 2). For another, haplotypes AAGCT ($P = 3.20 \times 10^{-17}$), GAACT ($P = 5.10 \times 10^{-6}$), GAATT ($P = 1.05 \times 10^{-3}$), GAGCC ($P = 6.90 \times 10^{-14}$), GAGCT ($P = 1.52 \times 10^{-8}$) and GCGTT ($P = 9.63 \times 10^{-19}$) tended to ameliorate the RSA risk, yet other haplotypes, including ACGCT ($P = 7.40 \times 10^{-20}$), ACGTC ($P = 2.94 \times 10^{-17}$), ACGTT ($P = 5.12 \times 10^{-27}$), GAACC ($P = 4.75 \times 10^{-83}$), and GAGTC ($P = 0.01$), were correlated with far greater risk of RSA.

Chromosomal karyotypes of RSA patients

Overall 1284 RSA patients that came for genetic counseling were treated with chromosome G-banding and karyotype analysis, and the detection rate of abnormal karyotypes was found to be 4.67% (60/1284) (Table 3, Supplementary table 2). The mutations of chromosome structure ($n = 23$) were mainly classified as balanced translocation ($n = 18$), Roberston translocation ($n = 4$) and inversion ($n = 1$). Moreover, trisomy chromosome, chromosome mosaicism and marker chromosome successively accounted for 9.10% (1/11), 81.80% (9/11) and 9.10% (1/11) of the abnormal changes in chromosome number. Interestingly, significant

distinctions were found between RSA subjects and healthy controls when the ratio of structural abnormality ($\chi^2 = 4.63$, $P = 0.03$) and numerical abnormality were, respectively, considered ($\chi^2 = 6.52$, $P = 0.01$; Supplementary table 3). Furthermore, as for the chromosomal polymorphism, there were 5 RSA patients with aberrantly increased long arms of chromosomes 1, 9, and 16, as well as 1 case with pericentric inversion of chromosome 9.

The relationship between chromosomal abnormalities with clinical characteristics of RSA patients

In light of the univariate regression analysis, chromosome abnormality was associated with higher miscarriage frequency (>3), when compared with normal karyotypes (OR = 6.07, 95% CI: 3.70–10.61, $P = 3.96 \times 10^{-11}$; Table 4). Nonetheless, hardly any significant correlations could be found between additional parameters, including miscarriage stage, smoking and alcohol, and the unusual karyotypes. Moreover, the results of multivariate regression analysis indicated that chromosome abnormality served as the independent factor leading to relatively high miscarriage frequency (>3 ; OR = 6.18, 95% CI: 3.53–10.83, $P = 5.21 \times 10^{-11}$).

The interactive effects of chromosomal abnormality and genetic mutations on RSA risk

The distributions of chromosomal aberration/non-aberration and CTLA4/Foxp3 genetic mutation/non-mutation among the RSA patients were displayed in Figure S1. Of note, the interactive influences of chromosomal abnormalities and genetic mutations were evaluated imitating the

Table 2 The association of haplotypes within CTLA4 and Foxp3 with RSA risk

Gene	Haplotype*	Case (frequency)	Control (frequency)	Odds ratio	95% CI	P-value
CTLA4	A C A A	345 (0.13)	383 (0.18)	0.72	0.52–0.72	1.40×10^{-4}
	A C A G	433 (0.17)	266 (0.13)	1.47	1.06–1.47	0.01
	A T A A	597 (0.23)	265 (0.13)	2.19	1.59–2.19	5.94×10^{-19}
	A T A G	0 (0.00)	150 (0.07)	0.01	0.00–0.01	7.56×10^{-42}
	A T G A	472 (0.18)	88 (0.04)	5.89	3.67–5.89	3.95×10^{-48}
	G C A A	419 (0.16)	231 (0.11)	1.67	1.18–1.67	6.36×10^{-6}
	G C A G	147 (0.06)	102 (0.05)	1.39	0.83–1.39	0.61
	G C G A	100 (0.04)	61 (0.03)	1.67	0.87–1.67	0.25
	G T A A	20 (0.01)	148 (0.07)	0.15	0.06–0.15	5.23×10^{-29}
	G T A G	15 (0.01)	108 (0.05)	0.17	0.06–0.17	8.36×10^{-21}
FOXP3	A A G C T	1 (0.00)	65 (0.03)	0.07	0.00–0.07	3.20×10^{-17}
	A A G T T	279 (0.11)	181 (0.09)	1.24	0.84–1.24	0.85
	A C G C T	255 (0.10)	61 (0.03)	3.95	2.23–3.95	7.40×10^{-20}
	A C G T C	208 (0.08)	46 (0.02)	4.43	2.31–4.43	2.94×10^{-17}
	A C G T T	329 (0.13)	76 (0.04)	4.09	2.44–4.09	5.12×10^{-27}
	G A A C C	478 (0.19)	19 (0.01)	33.45	13.09–33.45	4.75×10^{-83}
	G A A C T	24 (0.01)	63 (0.03)	0.40	0.15–0.40	5.10×10^{-6}
	G A A T C	142 (0.06)	81 (0.04)	1.55	0.89–1.55	0.27
	G A A T T	52 (0.02)	63 (0.03)	0.78	0.37–0.78	1.05×10^{-3}
	G A G C C	18 (0.01)	87 (0.04)	0.22	0.08–0.22	6.90×10^{-14}
	G A G C T	25 (0.01)	76 (0.04)	0.33	0.13–0.33	1.52×10^{-8}
	G A G T C	375 (0.15)	202 (0.10)	1.53	1.06–1.53	0.01
	G A G T T	301 (0.12)	211 (0.10)	1.13	0.78–1.13	0.51
	G C G T T	17 (0.01)	104 (0.05)	0.17	0.06–0.17	9.63×10^{-19}

*The haplotype of *CTLA4* gene consisted of rs231775, rs5742909, rs4553808, and rs3087243. The haplotype of *FOXP3* gene consisted of rs2232365, rs3761548, rs2232368, rs2280883, and rs2294021

Table 3 Chromosomal karyotypes of recurrent spontaneous abortion patients

Category	Number
Normal chromosome	1224
Abnormal chromosome	60
Structural chromosomal abnormalities	23
Reciprocal translocation	18
Robertsonian translocation	4
Nonhomologous robertsonian translocations	3
Homologous robertsonian translocations	1
Inversion	1
Numerical chromosomal abnormalities	11
Trisomy	1
Chromosomal mosaicism	9
Marker chromosome	1
Polymorphic chromosomal variants	26
1/9/16qh+	7
D/G group chromosomal abnormalities	10
Inv (9)	9

Inv (9) inversion of chromosome 9

appraisal procedures of the interactive effects of environmental exposure and hereditary factors [27, 28]. The interaction co-efficient values in Table 5 suggested that rs231775 AG/GG (AG: $\gamma = -2.85$; GG: $\gamma = -1.43$) and rs2232368 GA ($\gamma = -7.00$) could strongly protect people against RSA risk, and chromosomal aberration highly facilitated RSA onset. Conversely, genotypes GA ($\gamma = 3.38$) and AA ($\gamma = 2.21$) of rs3087243, as well as genotypes GG ($\gamma = 10.25$) and GA ($\gamma = 30.8$) of rs2232365 were positively interacted with chromosomal abnormality. Furthermore, underlying the interaction mechanisms, the synergic effects of chromosomal abnormality and certain genotypes, namely, rs231775 AG ($3.12 < 5.03 \times 0.67$), rs3087243 GA ($9.30 < 6.34 \times 1.93$)/AA ($18.49 < 6.34 \times 3.75$), rs2232365 AG ($4.65 < 1.05 \times 4.65$) and rs2232368 GA ($3.29 < 0.84 \times 5.43$), were conformed to the sub-multiplication model (all $OR_{\text{chromosome}} \times OR_{\text{SNP}} > OR_{\text{chromosome+SNP}}$). Moreover, the homozygote GG of rs2232365 and chromosomal aberration ($7.74 > 1.22 \times 4.65$) acted on the RSA risk in a super-multiplicative manner ($OR_{\text{chromosome}} \times OR_{\text{SNP}} < OR_{\text{chromosome+SNP}}$).

Table 4 The relationship between chromosomal abnormalities with clinical characteristics of recurrent spontaneous abortion patients

Clinical characteristics	Normal chromosome	Abnormal chromosome	χ^2 analysis				Multivariate analysis		
			χ^2	<i>P</i> -value	OR	95% CI	OR	95% CI	<i>P</i> -value
Miscarriage frequency									
≤3	903	19	50.09	3.96 × 10⁻¹¹	6.07	3.47–10.61	6.18	3.53–10.83	5.21 × 10⁻¹¹
>3	321	41							
Miscarriage stage									
Trimester 1	756	42	1.65	0.20	0.69	0.39–1.22	0.68	0.37–1.19	0.17
Trimester 2 or 3	468	18							
Smoking									
No	445	20	0.23	0.63	1.14	0.66–1.98	0.96	0.55–1.68	0.88
Yes	779	40							
Alcohol									
No	433	26	1.58	0.21	0.72	0.40–1.21	1.46	0.90–2.50	0.17
Yes	791	34							

CI confidence interval, *OR* odds ratio

The bold *P*-value means a significant results

Discussion

The predisposing parameters of RSA principally consisted of abnormalities in genital organs (e.g., uterine malformation and hysteromyoma) or endocrine (e.g., inadequate luteal function and hypothyroidism), systematic diseases (e.g., serious infection and high fever), chromosome abnormalities, immunological factors and so on. The previous studies were mostly focused on the correlation between each one of the above factors and RSA development, yet few were intended to figure out their interactive effects on the increased presence of RSA. In consequence, to fill the research gap, this study was firstly aimed to explore the synergic role of genetic mutations relevant to immune tolerance and chromosome abnormalities in modulating the risk of RSA.

The current study deemed CTLA-4 and Foxp3 as the candidate factors for regulating RSA risk, on account of their involvement in the immunity responses (Table 3). Specifically, CTLA-4 combined with B7 not merely inhibited the multiplication and activation of T cells, but also held up the cell cycle within G0/G2 stages, so that T cells were limited by inability of proliferating and secreting cytokines [29]. Moreover, CTLA-4 played a negatively regulatory role in the T-cell response process through facilitating the secretion of TGF- β , reducing the production of IL-2, and mediating the Fas-independent T-cell apoptosis [30–33]. Interestingly, the allele G or homozygote GG of CTLA-4 rs231775 was highly associated with the low CTLA-4 expressions within T lymphocytes, as well as the down-regulated suppression of

activated T lymphocytes [12]. CTLA-4 rs231775 and rs3087243 were also recognized as the risky elements for susceptibility to rheumatoid arthritis, an autoimmune disorder [34–36]. Accordingly, it was comprehensible in this study that the frequency of genotypes GG and AG of rs231775 differed significantly between the RSA and control subjects, and the allele A of CTLA-4 rs3087243 appeared to significantly elevate the incidence of RSA when compared with allele G [37]. Concerning rs5742909, although it was situated in the promoter region and determined the CTLA expression, hardly any correlation was discovered between the SNP and RSA risk [38]. Similarly, CTLA-4 rs4553808 belonged to the binding sites of transcription factor c/EBP β , and thereby affected the cells' proliferation, differentiation and apoptosis [39], yet this investigation also did not exhibit any remarkable correlation between CTLA-4 rs4553808 and RSA development.

Besides, the remarkably declined ratio of Foxp3 (+) Treg cells within the decidua of RSA patients was also believed to partly account for the disequilibrium of maternal immune tolerance, and thus the RSA [40, 41]. The current investigation explored and confirmed the association of Foxp3 rs2232365 and rs2232368 with susceptibility to RSA. In fact, the allele G of rs2232365 was found to be closely tied with the destruction of insulin B cells, and its allele A seemed as a protective factor against type-I diabetes [42]. In addition, the allele A, instead of allele G, could bind to the transcription factor GATA-3, and prompt the transformation of T lymphocytes to Th2 cells [43]. Hence, it was speculated that the G allele of rs2232365 might destroy the Th1/

Table 5 The interactive effects of chromosomal abnormality and genetic mutations on recurrent spontaneous abortion development

Rs	Genotype	Chromosome	Case	Control	OR	95% CI	P-value	Regression of efficient		γ -value
								β_c	β_{eg}	
rs231775	AA	Normal	633	411	Ref.			0.00		
	AG	Normal	495	483	0.67	0.56–0.79	1.57 × 10 ⁻⁴	-0.40		
	GG	Normal	96	142	0.44	0.33–0.59	3.14 × 10 ⁻⁷	-0.82		
	AA	Abnormal	31	4	5.03	1.76–14.36	8.32 × 10 ⁻⁴	0.00	1.62	
	AG	Abnormal	24	5	3.12	1.18–8.24	0.02	-0.40	1.14	-2.85
rs3087243	GG	Abnormal	5	1	3.25	0.38–27.90	0.26	-0.82	1.18	-1.43
	GG	Normal	71	150	Ref.			0.00		
	GA	Normal	446	488	1.93	1.42–2.63	7.18 × 10 ⁻⁴	0.66		
	AA	Normal	707	398	3.75	2.76–5.11	4.45 × 10 ⁻¹⁷	1.32		
	GG	Abnormal	3	1	6.34	0.65–62.04	0.07	0.00	1.85	
rs2232365	GA	Abnormal	22	5	9.30	3.38–25.56	1.54 × 10 ⁻⁵	0.66	2.23	3.38
	AA	Abnormal	35	4	18.49	6.33–54.03	3.97 × 10 ⁻¹⁰	1.32	2.92	2.21
	AA	Normal	311	289	Ref.			0.00		
	AG	Normal	411	364	1.05	0.85–1.30	0.66	0.05		
	GG	Normal	502	382	1.22	0.99–1.50	0.06	0.20		
rs2232368	AA	Abnormal	15	3	4.65	1.33–16.22	8.35 × 10 ⁻³	0.00	1.54	
	AG	Abnormal	20	4	4.65	1.57–13.76	2.43 × 10 ⁻³	0.05	1.54	30.8
	GG	Abnormal	25	3	7.74	2.31–25.93	2.78 × 10 ⁻³	0.20	2.05	10.25
	GG	Normal	664	546	Ref.			0.00		
	GA	Normal	413	405	0.84	0.70–1.01	0.05	-0.17		
rs2232368	AA	Normal	147	85	1.42	1.06–1.90	0.02	0.35		
	GG	Abnormal	33	5	5.43	2.10–14.00	2.52 × 10 ⁻³	0.00	1.69	
	GA	Abnormal	20	5	3.29	1.23–8.82	0.01	-0.17	1.19	-7.00
	AA	Abnormal	7	0	—	—	—			

CI confidence interval, OR odds ratio

The bold P-value means significant results

Th2 balance and raise the RSA risk, by way of decreasing Th2 cells and the immune responses relevant to Th2 cells.

Apart from the genetic mutations, abnormal chromosomal structure, chromosomal number and chromosomal polymorphism (Table 1 and Supplementary tables 2-3) also contributed to high level of RSA risk. Among the aberrations, balanced translocation was confirmed if the relative positions of the translocation fragments were changed within chromosomes [44]. Besides, inversion of chromosomes was called when a chromosome was disrupted for 2 times, and the intermediate fragment that was inverted by

180 degrees was then connected to the other two fragments [45]. Though the hereditary substances were unchanged and most phenotypes were normal, the gametes that were quantitatively and qualitatively aberrant would remarkably affect the next generation. Consequently, patients in the above unusual situations were correlated with higher incidence of habitual abortion, infertility, monster or stillborn fetus due to shaping of monosomes or trisomes [46]. In addition, numerical abnormality (i.e., aneuploidy and euploid) was most commonly caused by Robertsonian translocation [47]. Up to 2/3 of the Robertsonian

translocation carriers would suffer from early miscarriage or their children were born with trisomy 21 syndrome [48]. Meanwhile, there were nine chimeras detected in this study, accounting for 15.00% of all the abnormal karyotypes. The formation of chimera was attributed to by the nondisjunction of homologous chromosomes or sister chromatids, in which way monosome, multimer, or diploid were accordingly formed [49]. Furthermore, chromosomal polymorphisms were also found in this investigation that the long arms of chromosomes 1, 9, and 16 were abnormally increased. In fact, and the chromosomal polymorphisms were commonly involved with the structural heterochromatin region that covered highly repetitive DNA sequences, including the pericentric inversion of chromosome 9, length mutation of the short arms of D/G group chromosomes and the long arm of Y chromosome, as well as enlarged centromeres and secondary constrictions of chromosomes 1, 9, and 16. Since heterochromatins was essential for the integration of sister chromatids and the segregation of chromosomes [50], abnormal heterochromatins were probable to induce chromosome pairing in the meiosis, further affecting the formation of gametes and contributing to infertility.

Nonetheless, this study was also limited in several aspects. First, women were considered playing a greater role in deciding whether RSA would happen than males, for that the incidence of their chromosomal abnormality was hypothesized as similar to that of males, and it was the micro-environment within women's body that might make the living of fetus impossible. Since *CLTA-4/Foxp3* polymorphisms were vital the gestation status of women, we roughly estimated the contribution of females to RSA risk among a Chinese population, through devising the mixed effects of genetic mutations within *CLTA-4/Foxp3* and chromosomal aberrations on the RSA onset. Certainly, the responsibility of males' karyotypes for increased RSA risk also could not be ignored, and if possible, later studies could continue this part. Secondly, merely the genetic mutants of *CTLA-4* and *Foxp3* were explored, additional genes should also been taken into consideration. Lastly, the environmental factors also demanded to be considered, for the unfavorable conditions could facilitate the occurrence of RSA.

All in all, RSA was closely connected to the chromosomal aberrations and immunity-related genetic mutations, so conducting the karyotype analysis and genetic detection for RSA patients could better guide precise RSA diagnosis and sound child-rearing. Therefore, extra studies were needed to remedy the above flaws, and to better verify the conclusions of this study.

Competing interests The authors declare that they have no competing interests.

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