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Research Article

Association among cytotoxic T-lymphocyte antigen 4 gene, rs231775 polymorphism, and recurrent pregnancy loss risk

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Cytotoxic T-lymphocyte antigen 4 (*CTLA-4*) is expressed constitutively on regulatory T cells. So far, several studies have focused on association between *CTLA-4* gene polymorphisms and recurrent pregnancy loss (RPL). However, above association between the *CTLA-4* gene polymorphism and RPL susceptibility is uncertain. Therefore, we performed a timely meta-analysis of all current publications to clarify this relationship. We located articles from the PubMed and Chinese language (WanFang) databases that were published up until July 25, 2018. Finally, we obtained six case–control studies, containing 2405 total cases and 2607 total controls, based on search criteria for abortion susceptibility related to the *CTLA-4* +49 G/A polymorphism. The odds ratios (OR) and 95% confidence intervals (CIs) revealed association strengths. There was significantly decreased association between this polymorphism and whole population risk (e.g. AA vs. GG: OR = 0.56, 95% CI = 0.38–0.81, P=0.002). Additionally, in ethnicity subgroups, similar association was found both in China (e.g. AA vs. GG: OR = 0.49, 95% CI = 0.39–0.63, P=0.002) and non-China (e.g. AG vs. GG: OR = 0.46, 95% CI = 0.34–0.63, P<0.001). Current analysis suggested *CTLA-4* +49 G/A polymorphism may weakly decrease RPL risk for women of childbearing age.

Introduction

A pregnancy loss (PL) is defined as the spontaneous demise of a pregnancy before the fetus reaches viability. It includes all PLs [unexplained recurrent spontaneous abortion (RSA), RSA, recurrent miscarriage (RM), idiopathic RM] from the time of conception until 24 weeks of gestation [1,2]. Approximately 15% of pregnant women experience sporadic loss, 2% experience two consecutive PL and 0.4–1% have three consecutive PL [3]. Recurrent PL (RPL), also named as recurrent spontaneous abortion (RSA), is defined as the loss of two or more pregnancies [1,2,4]. In addition, RM is classically defined as the loss of three or more consecutive pregnancies before the 20th weeks of gestation with or without previous live births [5]. In the same time, the definition of RPL and RM exists some discrepancy in opinions, broadly speaking, both are classified as PL or abortion.

A series of pathogenic mechanisms associated with PL has been described, including uterine abnormalities, endocrine and metabolic problems, genetic anomalies, acquired and inherited thrombophilia and immunological factors [6]. More and more studies have focused on the genetic factors, especially the single nucleotide polymorphism (SNP) [7].

The cytotoxic T-lymphocyte antigen 4 (*CTLA-4*, Gene ID: 1493, MIM number: 123890 also known as GSE, ALPS5, CD152) gene maps to band q33 of human chromosome 2, spans about 6.2 kilobases, and contains four exons and three introns [8]. It is well known that *CTLA-4* expressed on human placental regulatory T (T_{reg}) cells in decidual and peripheral dendritic cells may induce the expression of an immune-suppressive enzyme indoleamine 2,3-dioxygenase (ID), particularly during early phases of

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pregnancy [9]. Furthermore, high expression of ID promotes maternal-fetal tolerance [9]. In addition, the expression of T_{reg} cells and *CTLA-4* in peripheral and decidual lymphocytes was down-regulated in human miscarriages in several *in vivo* studies [10]. We predicted *CTLA-4* and its related T_{reg} cells are protective factors for RPL. SNPs are known as the most common type of DNA variation in individuals [11], which may affect DNA promoter activity and influence the translation, and finally may be associated with the susceptibility about human diseases [11]. Therefore, we hypothesized that the reduced number and/or functional deficiency of T_{reg} cells due to the genetic variations in *CTLA-4* gene may increase the risk of RPL.

So far, many studies have investigated the association between *CTLA-4* rs231775 G/A polymorphism (wild-type allele: A; polymorphic allele: G, 49A>G, Thr17Ala) and RPL risk. However, the results were not conclusive or consistent. Thus, we conducted this timely meta-analysis of six case–control studies to derive a more powerful estimation of the association between *CTLA-4* rs231775 G/A polymorphism and RPL susceptibility [12–17].

Materials and methods

Identification and eligibility of relevant studies

Searches were conducted in PubMed and Chinese language (WanFang) databases using the key words 'cytotoxic T-lymphocyte antigen 4 or *CTLA-4*', 'spontaneous abortion or miscarriage or pregnancy loss' and 'polymorphism' or 'variant'. The last search was updated on July 25, 2018. In total, 18 articles were retrieved using the abovementioned terms, and six articles contained the inclusion criteria.

Inclusion criteria and exclusion criteria

Including studies had to meet following criteria: (1) address the correlation between RPL risk and the *CTLA-4* rs231775 G/A SNP; (2) be a case–control study; and (3) have sufficient numbers of genotypes (AA, AG, and GG) for both the cases and controls. The following exclusion criteria were used: (1) lack of a control population; (2) lack of available genotype frequency data; and (3) duplicated studies.

Data extraction

The following items were collected: the last name of first author, the year of publication, the country of origin, the ethnicity of subjects, source of control (SOC), the total and number of each genotype frequency in the case–control groups, the Hardy–Weinberg equilibrium (HWE) of the controls, abortion type, control-type and the genotyping method. Ethnicity was categorized as Asian, China, and non-China.

Quality score assessment

The quality score assessment (Newcastle–Ottawa Scale, NOS) [18] was selected to assess the quality of each study. This measure assesses aspects of the methodologies used in observational studies, which are related to the study quality, including selection of cases, comparability of populations, and ascertainment of exposure to risks. The NOS rating ranges from zero stars (worst) to nine stars (best). Studies with a score of seven stars or greater was considered as a high quality.

Statistical analysis

Odds ratios (OR) with 95% confidence intervals (CI) were used to measure the strength of the association between the *CTLA-4* rs231775 G/A SNP and RPL risk. The statistical significance of the summary OR was determined with the *Z*-test. A heterogeneity assumption was evaluated among studies using a chi-square-based *Q*-test. A *P*-value of more than 0.10 for the *Q*-test indicated a lack of heterogeneity among the studies [19]. If significant heterogeneity was detected, the random-effects model (DerSimonian–Laird method) was used. Otherwise, the fixed-effects model (Mantel–Haenszel method) was chosen [20,21].

We investigated the relationship between genetic variants of the *CTLA-4* rs231775 G/A site and RPL risk by the allelic contrast (A-allele vs. G-allele), homozygote comparison (AA vs. GG), dominant genetic model (AA+AG vs. GG), heterozygote comparison (AG vs. GG), and recessive genetic model (AA vs. AG+GG). A sensitivity analysis was performed by omitting studies, one after another, to assess the stability of results. The departure of the *CTLA-4* rs231775 G/A SNP from expected frequencies under HWE was assessed in controls using the Pearson chi-square test ($P < 0.05$ was considered significant). Funnel plot asymmetry was assessed using Begg's test, and publication bias was assessed using Egger's test [22], both of *P*-value less than is considered as significant. All statistical tests were performed using STATA Software (version 11.0; StataCorp LP, College Station, TX).

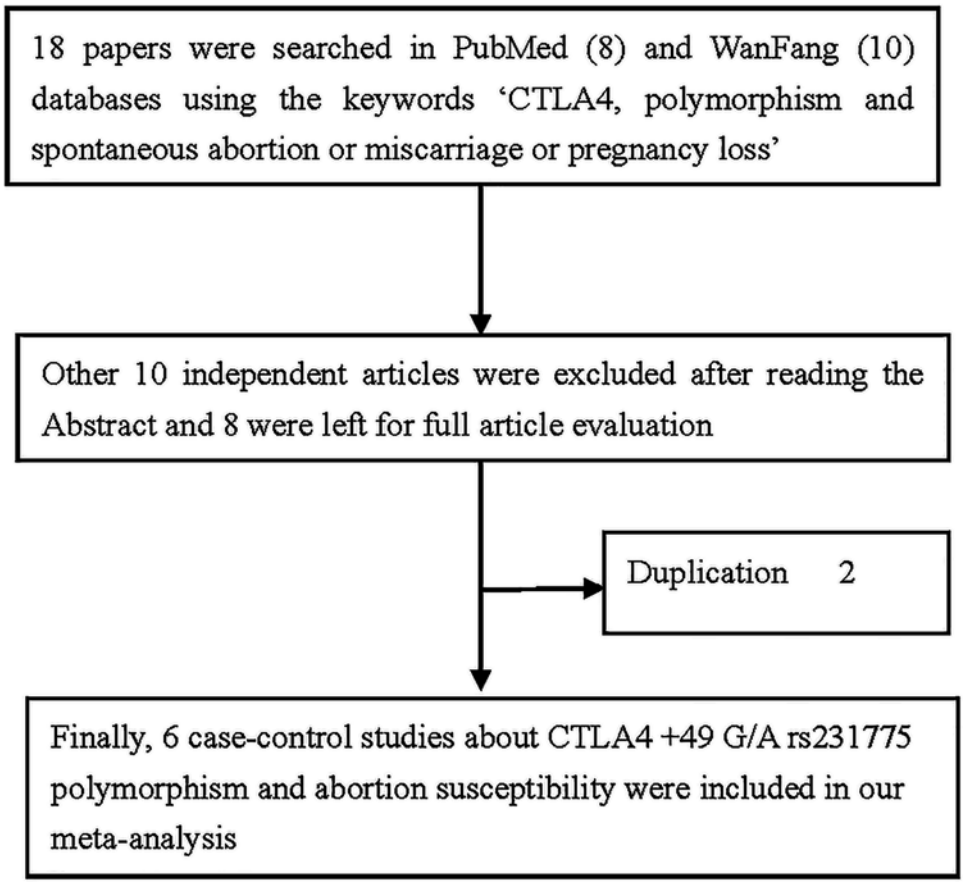

Figure 1. Flowchart illustrating the search strategy used to identify association studies for *CTLA-4* gene rs231775 polymorphism and RPL risk

Network of gene interaction of *CTLA-4* gene

The network of gene–gene interaction for *CTLA-4* gene was utilized through String online server (http://string-db.org/) [23].

Results

Study characteristics

In total, 18 articles were collected from the PubMed and WanFang databases via a literature search using different combinations of key words. As shown in Figure 1, 12 articles were excluded (two were duplications, 10 were irrespective articles). Finally, six different articles were included in current meta-analysis (Figure 1). In total, there were 2405 cases and 2607 controls. Study characteristics from the published studies on the relationship between the *CTLA-4* rs231775 G/A SNP and RPL risk are summarized in Table 1. In all the studies, the controls were women under normal pregnancy. Except one study, all studies were consistent with HWE. Finally, we checked the minor allele frequency (MAF) reported for the five main worldwide populations in the 1000 Genomes Browser [23]: East Asian (EAS), 0.3631; European (EUR), 0.3588; African (AFR), 0.3880; American (AMR), 0.4625; and South Asian (SAS), 0.3098 (Figure 2). The MAF in our analysis was 0.4033 and 0.5213 in the case and control group, respectively, both higher than the results in the EAS from1000 Genomes Browser database.

Quantitative synthesis

There was significantly decreased association between the *CTLA-4* rs231775 G/A SNP and RPL risk susceptibility (AA vs. GG: OR = 0.56, 95% CI = 0.38–0.81, $P_{heterogeneity}$=0.014, P=0.002, Figure 3, AG vs. GG: OR = 0.61, 95% CI = 0.46–0.80, $P_{heterogeneity}$=0.034, P<0.001, and AA+AG vs. GG: OR = 0.61, 95% CI = 0.45–0.82, $P_{heterogeneity}$=0.008, P=0.001) (Table 2).

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Table 1 Study characteristics from previous published studies on the association between *CTLA-4 gene* rs231775 polymorphism and RPL risk

Author	Year	Country	Ethnicity	SOC	Case	Control	Case			Control			HWE	Genotype	Case type	Control type	NOS
							AA	AG	GG	AA	AG	GG					
Wang [17]	2005	China	Asian	HB	168	117	20	66	82	17	61	39	0.38	PCR-RFLP	Unexplained RSA	Normal pregnancy	7
Chai [12]	2010	China	Asian	HB	233	224	28	101	104	35	95	94	0.18	PCR-RFLP	RSA	Normal pregnancy	7
Fan [13]	2018	China	Asian	HB	1284	1046	101	518	665	143	488	415	0.98	PCR-RFLP	RSA	Normal pregnancy	8
Gupta [14]	2012	India	Asian	HB	300	500	140	121	39	227	233	40	0.06	PCR-RFLP	RM	Normal pregnancy	7
Nasiri [16]	2016	Iran	Asian	HB	120	120	94	23	3	68	45	7	0.90	PCR-RFLP	RPL	Normal pregnancy	7
Misra [15]	2016	India	Asian	HB	300	600	105	135	60	264	288	48	0.01	PCR-RFLP	Idiopathic RM	Normal pregnancy	7

Abbreviations: HB, hospital based; HWE: Hardy–Weinberg equilibrium of control group; NOS: Newcastle–Ottawa scale; PCR-RFLP: polymerase chain reaction followed by restriction fragment length polymorphism; SOC; source of control.

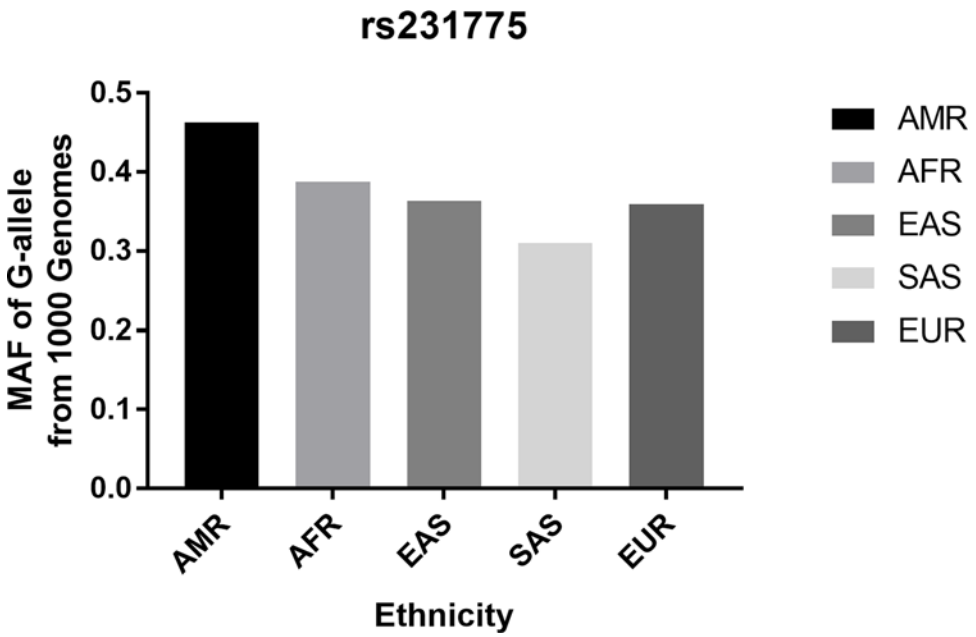

Figure 2. A-allele frequencies for the *CTLA-4* gene rs231775 polymorphism among cases–controls stratified by ethnicity
Vertical line, T-allele frequency; horizontal line, ethnicity type.
Abbreviations: AFR, African; AMR, American; EAS, East Asian; EUR, European; SAS, South Asian.

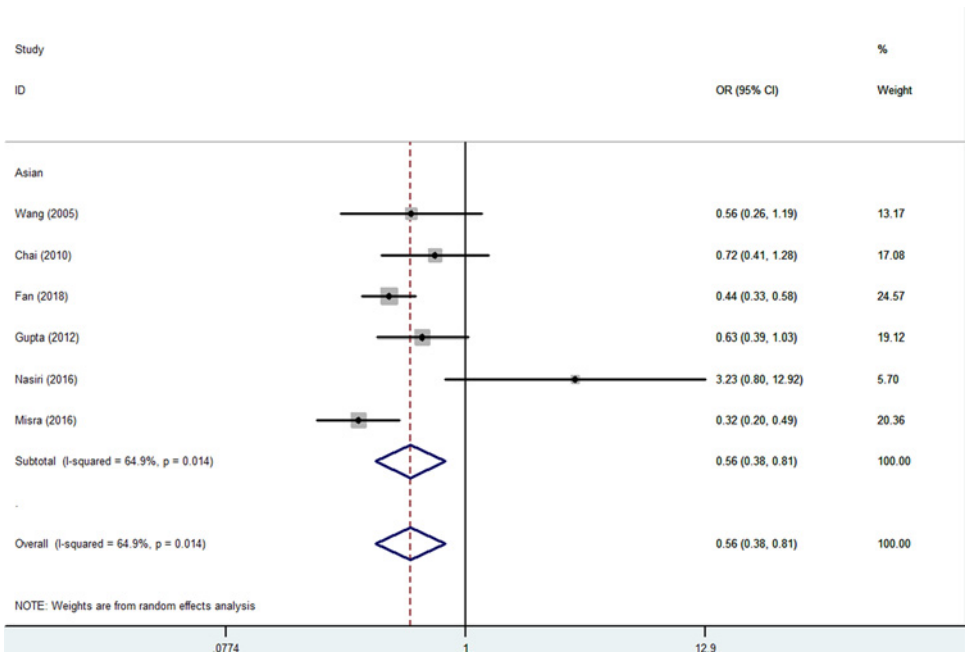

Figure 3. Forest plot of RPL risk associated with *CTLA-4* gene rs231775 polymorphism (AA vs. GG) in the whole
The squares and horizontal lines correspond to the study-specific OR and 95% CI. The area of the squares reflects the weight (inverse of the variance). The diamond represents the summary OR and 95% CI.

When studies were stratified according to ethnicity, there was also similar association found both in China (e.g. A-allele vs. G-allele: OR = 0.69, 95% CI = 0.62–0.77, $P_{heterogeneity}<0.001$, $P<0.001$, AG vs. GG: OR = 0.68, 95% CI = 0.59–0.80, $P_{heterogeneity}=0.126$, $P<0.001$, Figure 4, and AA vs. AG+GG: OR = 0.59, 95% CI = 0.47–0.75, $P_{heterogeneity}=0.406$, $P<0.001$) and non-China risk (AG vs. GG: OR = 0.46, 95% CI = 0.34–0.63, $P_{heterogeneity}=0.237$, $P<0.001$, Figure 5, Table 2).

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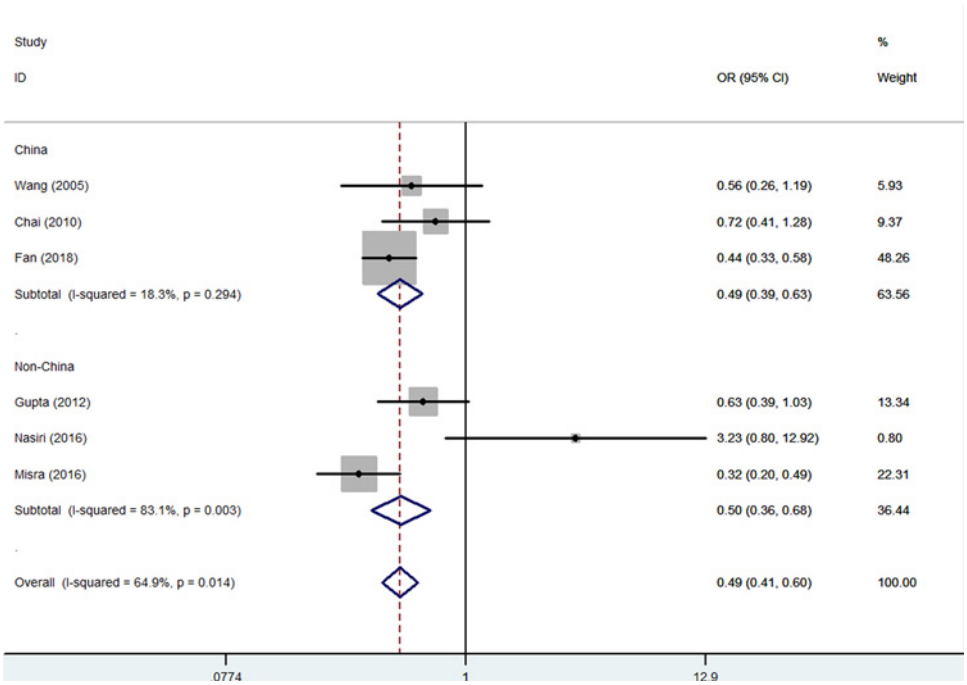

Figure 4. Forest plot of RPL risk associated with *CTLA-4* gene rs231775 polymorphism (AG vs. GG) in China population

The squares and horizontal lines correspond to the study-specific OR and 95% CI. The area of the squares reflects the weight (inverse of the variance). The diamond represents the summary OR and 95% CI.

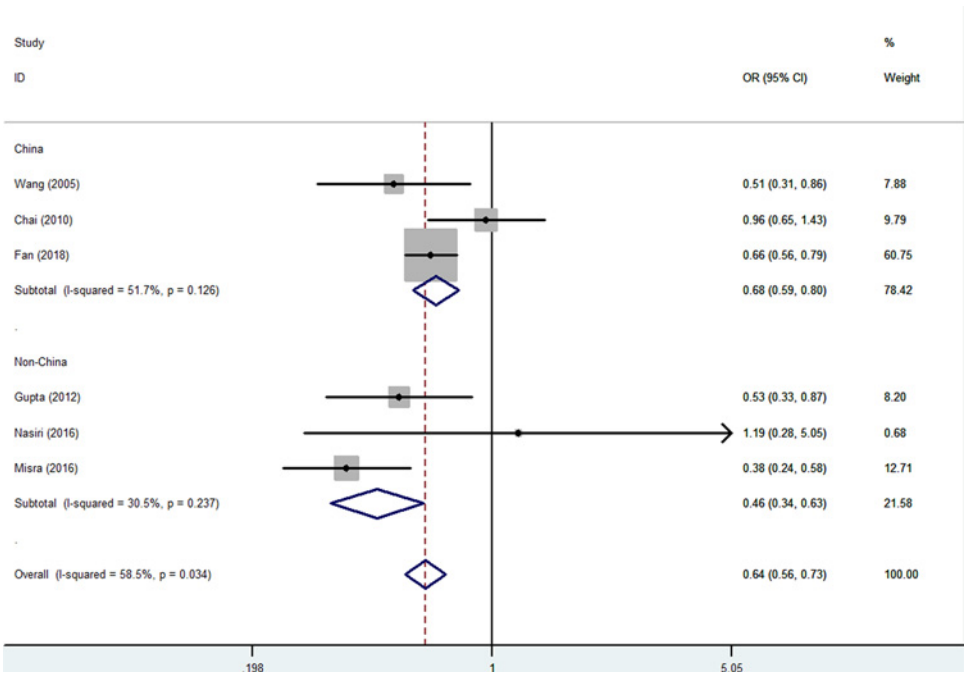

Figure 5. Forest plot of RPL risk associated with *CTLA-4* gene rs231775 polymorphism (AG vs. GG) in non-China population

The squares and horizontal lines correspond to the study-specific OR and 95% CI. The area of the squares reflects the weight (inverse of the variance). The diamond represents the summary OR and 95% CI.

Table 2 Total and stratified analysis of *CTLA-4 gene* rs231775 polymorphism and RPL risk

Total	OR(95% CI) P_h *P* genetic model
A-allele vs. G-allele	0.85(0.66–1.09)0.000 0.193 random model
AA vs. GG	0.56(0.38–0.81)0.014 0.002 random model
AG vs. GG	0.61(0.46–0.80)0.034 0.000 random model
AA+AG vs. GG	0.61(0.45–0.82)0.008 0.001 random model
AA VS. AG+GG	0.90(0.61–1.33)0.000 0.597 random model
Ethnicity subgroup	
China	OR(95% CI) P_h *P* genetic model
A-allele vs. G-allele	0.69(0.62–0.77)0.000 0.000 fixed model
AA vs. GG	0.49(0.39–0.63)0.294 0.002 fixed model
AG vs. GG	0.68(0.59–0.80)0.126 0.000 fixed model
AA+AG vs. GG	0.64(0.55–0.74)0.128 0.000 fixed model
AA VS. AG+GG	0.59(0.47–0.75)0.406 0.000 fixed model
Ethnicity subgroup	
Non-China	OR(95% CI) P_h *P* genetic model
A-allele vs. G-allele	1.06(0.61–1.84)0.000 0.834 random model
AA vs. GG	0.68(0.28–1.68)0.003 0.405 random model
AG vs. GG	0.46(0.34–0.63)0.237 0.000 fixed model
AA+AG vs. GG	0.60(0.29–1.23)0.015 0.166 random model
AA VS. AG+GG	1.20(0.64–2.26)0.000 0.573 random model

P_h: value of *Q*-test for heterogeneity test; *P*: *Z*-test for the statistical significance of the OR

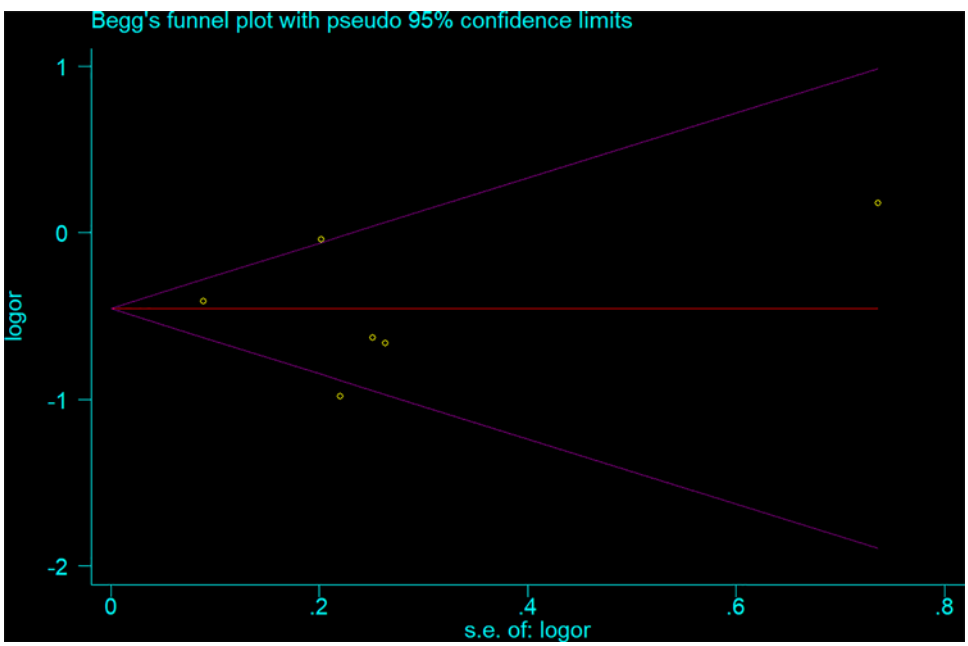

Figure 6. Sensitivity analysis between *CTLA-4* gene rs231775 polymorphism and tuberculosis risk (AG vs. GG)

Sensitivity analysis and bias diagnosis

We used a sensitivity analysis to determine whether modifying the meta-analysis inclusion criteria affected the results. No other single study influenced the summary OR qualitatively (Figure 6). Egger's and Begg's tests were performed to assess publication bias and the funnel plot symmetry was examined. Finally, no proof of publication bias was obtained (e.g. AG vs. GG: $t = -0.23$, P=0.892 for Egger's test; and $z = -0.19$, P=0.851 for Begg's test; Figures 7 and 8, Table 3).

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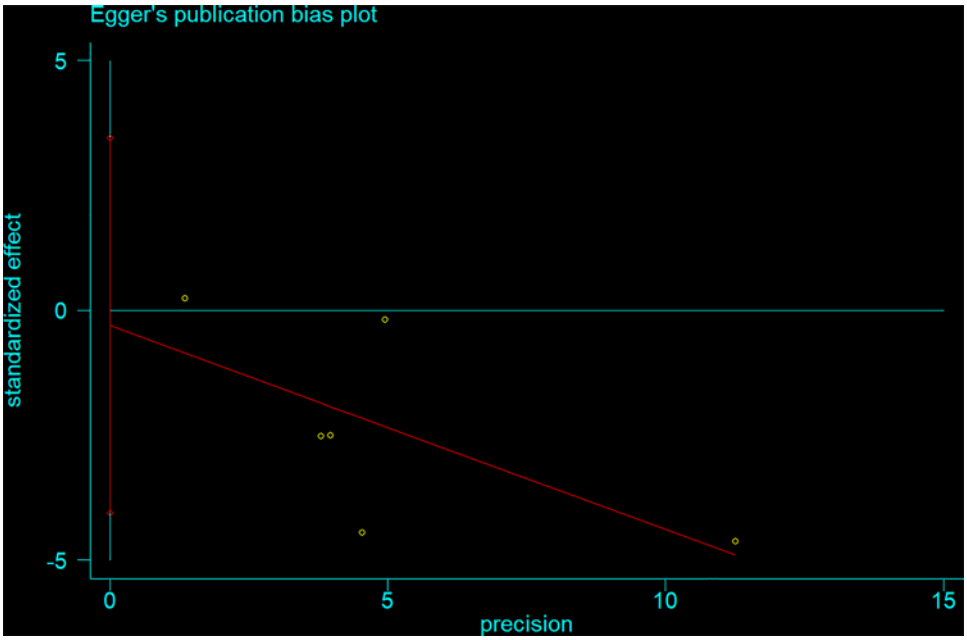

Figure 7. Begg's funnel plot for publication bias test (AG vs. GG)

Each point represents a separate study for the indicated association. Log [OR], natural logarithm of OR. Horizontal line, mean effect size.

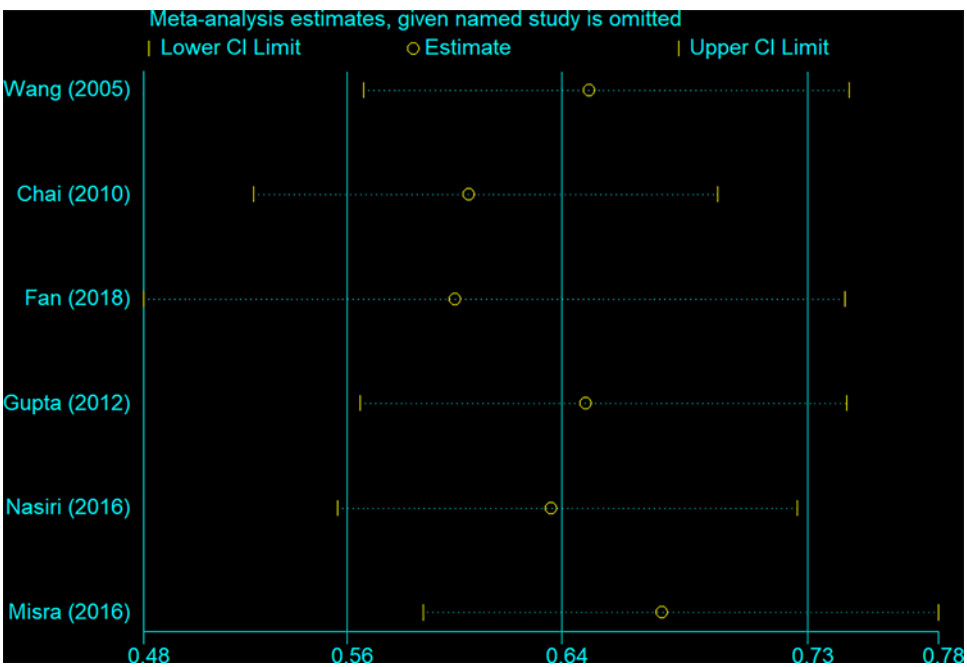

Figure 8. Egger's publication bias plot (AG vs. GG)

Gene–gene interaction of online analysis

String online server indicated that MTR gene interacts with numerous genes. The network of gene–gene interaction has been illustrated in Figure 9.

Table 3 Publication bias tests (Begg's funnel plot and Egger's test for publication bias test) for *CTLA-4 gene* rs231775 polymorphism

Egger's test						Begg's test	
Genetic type	**Coefficient**	**Standard error**	***t***	***P*-value**	**95% CI of intercept**	***z***	***P*-value**
A-allele vs. G-allele	1.093	3.328	0.33	0.759	(−8.147,10.334)	0.75	0.452
AG vs. GG	−0.312	1.354	−0.23	0.892	(−4.072,3.446)	−0.19	0.851
AA vs. GG	0.422	1.461	0.29	0.787	(−3.634,4.479)	0.94	0.348
AA+AG vs. GG	−0.339	1.436	−0.24	0.825	(−4.327,3.647)	0	1
AA vs. AG+GG	2.753	2.308	1.19	0.299	(−3.655,9.162)	0.75	0.452

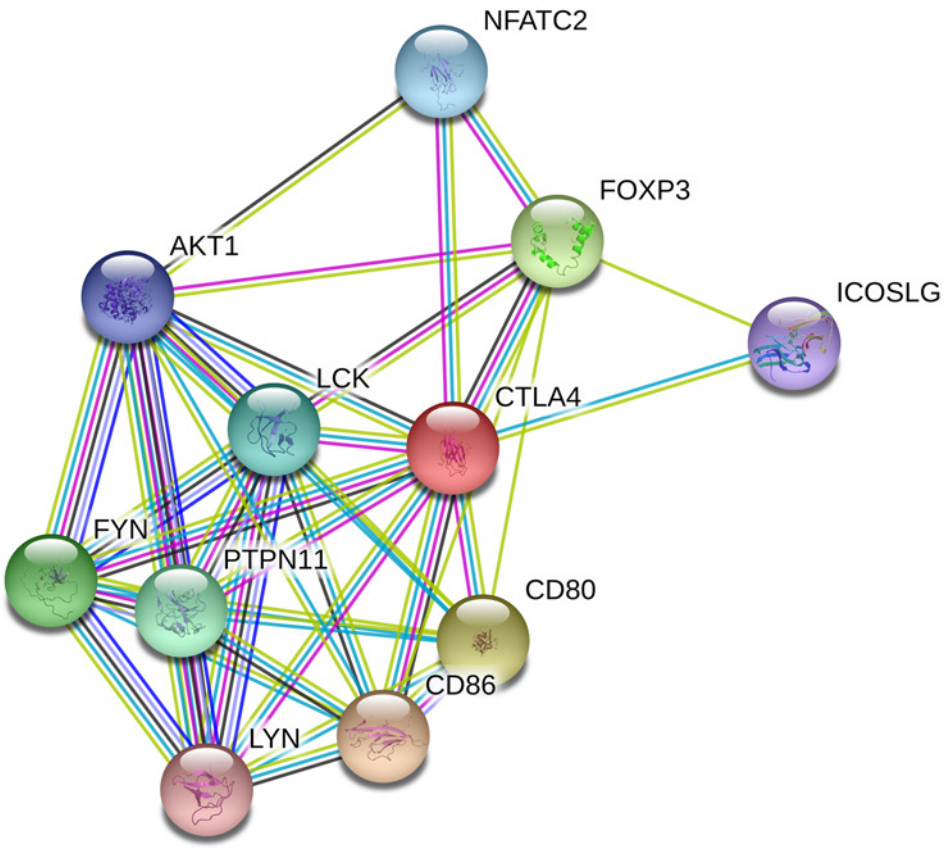

Figure 9. Human *CTLA-4* interactions network with other genes obtained from String server

At least 10 genes have been indicated to correlate with *CTLA-4* gene.

Abbreviations: AKT1, V-akt murine thymoma viral oncogene homolog 1; CD80, CD80 molecule; CD86, CD86 molecule; FOXP3, Forkhead box p3; FYN, FYN oncogene related to SRC, FGR, YES; ICOSLG, inducible T-cell co-stimulator ligand; LCK, lymphocyte-specific protein tyrpsine kinase; LYN, V-yes-1 Yamaguchi sarcoma viral-related oncogene homolog; NFATC2, nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2; PTPN11, protein tyrosine phosphatase, non-reveptor type 11.

Discussion

RPL is a common pregnancy complication affecting 1–3% of couples trying to conceive. Successful pregnancy is a result of maintaining the semi-allograft fetus from maternal immune responses [24]. The decision between tolerance and immunity determines the fate of each pregnancy. A network composed of different immune cells, numerous cytokines, growth factors, and adhesion molecules collaborate to reach the best outcome [25]. T_{reg} cells are cellular components of natural self-tolerance and seem to reduce the chance of pregnancy failure by providing the tolerant environment in the endometrium, where a successful implantation can occur [26]. Confirming evidence, in this regard, came from a significant reduction in circulating and deciduas T_{reg} cells among women with RPL [10,27]. One

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of the proposed mechanisms is the *CTLA-4*-dependent pathway using anti-*CTLA-4*-mAb disrupts the T_{reg} activity *in vivo*, in which T_{reg} provide this tolerance against the fetus [28].

To combine the importance of genetic etiology of RPL, it makes sense to deep study the *CTLA-4* gene polymorphisms. Rs231775 variant is one of common polymorphisms in *CTLA-4* gene. To our best of knowledge, it is the first time to select all published articles to analyze the association between *CTLA-4* gene rs231775 polymorphism and RPL susceptibility. In current study, the major discover is that rs231775 may decrease RPL risk, in other words, individuals carrying A-allele may have a decrease association for RPL, or A-allele is a protective factor for RPL risk, on the other hand, the G-allele is a potential risk factor. We boldly guess that the A-allele may increase the expression of CTLA-4 protein, because CTLA-4 is a protective factor in promoting fetus toleration and RPL [17].

In addition, we used the online analysis system String to predict potential and functional partners (Figure 9). Finally, ten genes were predicted. The highest score of association was CD86 and CD80 (score = 0.999); however, ICOSLG and LYN had the lowest scores (0.963 and 0.949, respectively). CD86 and CD80 are both the natural B7 family ligands of *CTLA-4*, the level of CD86(+) was significantly higher in the RPL group than the normal pregnancy group [29]. Several observations have indicated that CD28/*CTLA-4* and CD86/CD80 are involved in the maternal–fetal immune regulation, which might be potentially useful to immunotherapy for human RPL [30]. Most studies were focused on the association between FOXP3 gene and RPL, including SNPs and immune regulation (such as T_{reg}, CD4+CD25+, Th17) [13]. Karim et al. reported that several novel CNVs/genes (such as 14q32.33/AKT1) in chromosomal abnormalities were associated with RPL risk [31]. Above information predicted that CD86, CD80, FOXP3, and AKT1 may influence *CTLA-4* and regulate the RPL development, which may become intervention and treatment target genes in the future.

Limitations in the present meta-analysis include the suboptimal number of published studies for a comprehensive analysis. Second, interactions between different polymorphic loci of the same *CTLA-4* may modulate RPL risk, which should be included in future research and analysis. In addition, our meta-analysis was based on unadjusted estimates. A more precise analysis should be conducted if individual data are available to adjust for other covariates including age, sex, family history, environmental factors, endocrine abnormalities, each type of PL, and lifestyle.

In summary, in the present meta-analysis, a significant decreased association was found between the *CTLA-4* gene rs231775 SNP and RPL risk. To further confirm the results, larger scale case–control studies with different ethnic groups and multiple PL types are needed.

Author Contribution

Y.S. and Y.C. conceived the study. Y.C. and Q.X. searched the databases and extracted the data. Y.S. and Q.X. analyzed the data. Q.X. wrote the draft of the paper. Y.S. and Y.C. reviewed the manuscript.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Abbreviations

CI, confidence interval; *CTLA4*, cytotoxic T-lymphocyte antigen 4; HWE, Hardy–Weinberg equilibrium; ID, indoleamine 2,3-dioxygenase; MAF, minor allele frequency; NOS, Newcastle–Ottawa Scale; PL, pregnancy loss; RM, recurrent miscarriage; RSA, recurrent spontaneous abortion; SNP, single nucleotide polymorphism.

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