Meta-analysis reveals significant association between *FOXP3* polymorphisms and susceptibility to Graves' disease

**Guiqin Tan[1],*, Xin Wang[2],*,
Guangbing Zheng[1], Juan Du[1], Fangyu Zhou[1],
Zhongzhi Liang[1], Wenwen Wei[1] and
Hongsong Yu[1]** ⓘ

Abstract

Objective: This meta-analysis aimed to determine the associations between the rs3761547, rs3761548, and rs3761549 single-nucleotide polymorphisms (SNPs) of the forkhead box P3 (*FOXP3*) gene and susceptibility to Graves' disease (GD).

Methods: Case–control studies with information on the associations between the rs3761547, rs3761548, and rs3761549 *FOXP3* SNPs and GD published before 01 May 2020 were identified in the PubMed, Embase, Web of Science, and China National Knowledge Infrastructure databases. Data from the studies were analyzed using RevMan version 5.3.

Results: Seven independent case–control studies including 4051 GD patients and 4569 controls were included in the meta-analysis. The overall pooled analysis indicated that *FOXP3*/rs3761548 and *FOXP3*/rs3761549 polymorphisms were significantly associated with GD susceptibility (rs3761548: A vs. C, odds ratio [OR] = 1.32, 95% confidence interval [CI] 1.05–1.67; rs3761549: TT vs. CC, OR = 1.98, 95%CI 1.49–2.65; (TT + TC) vs. CC, OR = 1.44, 95%CI 1.11–1.88). In contrast, the *FOXP3*/rs3761547 polymorphism was not associated with GD susceptibility. Subgroup analysis according to ethnicity showed that rs3761548 was associated with

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[1]Department of Immunology, Special Key Laboratory of Gene Detection and Therapy of Guizhou Province, Zunyi Medical University, Zunyi, China
[2]Department of Basic Medical Education, Zunyi Medical University, Zunyi, China

*These authors contributed equally to this work.

Corresponding author:
Hongsong Yu, Department of Immunology, Special Key Laboratory of Gene Detection and Therapy of Guizhou Province, Zunyi Medical University, No. 6, Xuefu West Road, Xinpu District, Zunyi, Guizhou 563000, China.
Email: yuhongsong@163.com

GD in Asians but not in Caucasians, whereas rs3761549 was associated in both Asians and Caucasians.

Conclusion: This meta-analysis demonstrated that *FOXP3*/rs3761548 and *FOXP3*/rs3761549 SNPs were significantly associated with susceptibility to GD, at least in Asian populations.

Introduction

Graves' disease (GD), also known as toxic diffuse goiter, is a common autoimmune thyroid disease (AITD) and the most common cause of hyperthyroidism, accounting for more than 80% of cases.[1] GD is more common in women (male-to-female ratio of 1:8) and usually occurs between the ages of 20 and 40 years.[2] The prevalence of GD in China is about 1.1% to 1.4%.[3] GD is typically characterized by the unique association of thyrotoxicosis, goiter, ophthalmopathy, and the presence of circulating thyrotropin receptor antibody; however, the exact etiology of GD remains unclear. Numerous recent studies have investigated the roles of epigenetic, environmental, and immunological factors in the pathogenesis of GD, and have suggested that interactions among all these factors play a significant role in the pathogenesis of GD.[4]

Regulatory T cells (Tregs) play a pivotal role in suppression of the immune response and the development of immune tolerance. Tregs are important factors in the pathogenesis of multiple human autoimmune diseases, such as multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, and type 1 diabetes.[5,6] In addition, some studies have described a correlation between Tregs and GD.[5,7] Compared with healthy controls, GD patients show decreased levels of Tregs, thus emphasizing

their significant role in the pathogenesis of GD.[8]

Forkhead box P3 (FOXP3) is an important regulatory factor for the differentiation of T cells into natural Tregs and is a key molecule controlling Treg development and function.[9–15] The locus of *FOXP3* on human chromosome Xp11.23 encodes a protein with 431 amino acids.[16–19] Lack of *FOXP3* impairs the immunosuppressant action of Tregs.[16,20] *FOXP3* regulates T-cell activation and functions as a transcriptional repressor to downregulate cytokine production in T cells.[18,21] Five possible polymorphisms in the *FOXP3* gene may change the expression levels of the protein and thus impair its function, thereby damaging the suppressive ability of Tregs and leading to autoimmune diseases.[22–27] The known functions of *FOXP3* suggest that it might be a candidate susceptibility gene for autoimmune diseases.[9,18,28] *FOXP3* has also been associated with AITDs.[9,29] Some single-nucleotide polymorphisms (SNPs) of the *FOXP3* gene, including -2383 C/T (rs3761549), -3279 C/A (rs3761548), -924 A/G (rs2232365), -1383C/T (rs2232364), and -3499A/G (rs3761547), may influence its expression.[16,24] The SNPs rs3761547, rs3761548, and rs3761549 have been the most commonly tested polymorphisms in previous genetic association studies.

Decreased expression of *FOXP3* can damage the function of Tregs and may result in an increase in autoreactive T cell

activity, leading to destruction of the thyroid gland in GD patients.[9,16] Although some studies have investigated the relationship between *FOXP3* variants (rs3761547, rs3761548, and rs3761549) and the risk of GD in diverse populations,[5,9,16,22,30,31] the results have been inconclusive. To reach a firmer conclusion, we therefore collected data from previous relevant studies and conducted a meta-analysis to examine the possible associations between *FOXP3* polymorphisms and susceptibility to GD.

Methods

Search strategies

We searched the PubMed, Web of Science, Embase, and the China National Knowledge Infrastructure databases for case–control studies examining the relationship between *FOXP3* and GD susceptibility, published before 1 May 2020, with no language restrictions. The keywords were: "forkhead box P3 or *FOXP3*" AND "Graves' disease or Graves disease or Basedow disease."

Inclusion and exclusion criteria

Studies were required to meet the following inclusion criteria: (1) studies examining the association between *FOXP3*/rs3761547/rs3761548/rs3761549 polymorphisms and GD susceptibility; (2) case–control studies; (3) studies concentrating on humans; and (4) detailed genotype data could be obtained to calculate odds ratios (ORs) and 95% confidence intervals (CIs). The exclusion criteria were: (1) not case–control study; (2) duplicate publication; (3) animal study; (4) no original data on allele or genotype frequencies; and (5) article type was a review, letter, case report, meta-analysis, or commentary.

The study did not require ethics committee approval because it was an analysis of previously published studies.

Quality assessment

The qualities of the included studies were evaluated according to the Newcastle–Ottawa assessment scale (NOS). The evaluation criteria included case selection, comparability, exposure, data, and genetic testing method. Each study was awarded a star for each item that was evaluated satisfactorily according to the NOS, up to a maximum of nine stars.

Data extraction

Two reviewers independently extracted data from each eligible article, including the first author, date of publication, original country, race, genotyping method, numbers of cases and controls, frequencies of genotypes and alleles, and *P*-value for the Hardy–Weinberg equilibrium (HWE). Any disagreements were resolved by another reviewer.

Statistical analysis

The meta-analysis examined each SNP according to five genetic comparison models: allele, homozygote, heterozygous, dominant, and recessive. The HWE was assessed for each study using the χ^2 test in healthy subjects. ORs with 95%CIs were calculated using the χ^2 test to evaluate the strength of the association between *FOXP3* polymorphisms (rs3761547, rs3761548, and rs3761549) and GD risk. The statistical significance of the ORs was analyzed by the Z-test, with $Pz < 0.05$ considered a significant association. Heterogeneity was investigated using the Q-test and I^2 statistics. $I^2 < 50\%$ indicated a low degree of heterogeneity, in which case a fixed-effects model was used to calculate the ORs and 95%CIs, otherwise, a random-effect model was used.

Potential publication bias was evaluated by funnel plots. Sensitivity analysis was conducted by removing each individual study to evaluate the robustness of the results. HWE statistical analyses were undertaken using Review Manager (RevMan) Version 5.3 (Cochrane Collaboration, 2014, Copenhagen, Denmark).

Results

Study characteristics

A total of 245 studies were retrieved through an initial search, of which 86 were removed as duplicates and 114 were removed as irrelevant after reading the titles and abstract. We read the full texts of the remaining 18 studies, and finally included seven eligible case–control studies in this meta-analysis. A completed PRISMA flow chart is provided as Supplementary Figure 1. The characteristics of the included studies are summarized in Table 1. The seven included studies involved 4051 GD patients and 4569 controls. Among these, five studies involving 1086 cases and 1157 controls examined rs3761547, six studies involving 1166 cases and 1442 controls examined rs3761548, and seven studies involving 1799 cases and 1970 controls examined rs3761549. For *FOXP3*/rs3761547, four studies were conducted in Asian populations and one in a Caucasian population; for *FOXP3*/rs3761548, five studies were conducted in Asian populations and one in Caucasians; and for *FOXP3*/rs3761549, five studies were conducted in Asian populations and two in Caucasian populations. We subsequently intended to include studies on other *FOXP3* loci and Hashimoto's thyroiditis, as a common AITD, but these studies were not included here because of their small numbers. In terms of NOS evaluation, all articles were awarded more than

six stars, indicating high methodological quality.

Quantitative synthesis

*FOXP3/*rs3761548 polymorphism. Six case–control studies involving a total of 1166 GD cases and 1442 controls assessed the relationship between the *FOXP3*/rs3761548 polymorphism and GD susceptibility. The meta-analysis demonstrated that the rs3761548 polymorphism was significantly associated with GD (A vs. C: $OR = 1.32$, $95\%CI = 1.05–1.67$, $Pz = 0.02$; AC vs. CC: $OR = 1.58$, $95\%CI = 1.01–2.45$, $Pz = 0.04$; (AA+AC) vs. CC: $OR = 1.51$, $95\%CI = 1.02–2.25$, $Pz = 0.04$) (Table 2). Subgroup analysis according to ethnicity indicated that this polymorphism was significantly associated with GD in Asians (A vs. C: $OR = 1.41$, $95\%CI = 1.13–1.77$, $Pz = 0.003$; AC vs. CC: $OR = 1.72$, $95\%CI = 1.06–2.79$, $Pz = 0.03$; (AA + AC) vs. CC: $OR = 1.66$, $95\%CI = 1.10–2.53$, $Pz = 0.02$) but not in Caucasians. A forest plot of the relationship between the rs3761548 polymorphism and the risk of GD is shown in Figure 1.

*FOXP3/*rs3761549 polymorphism. Seven studies including 1799 GD patients and 1970 controls were examined to evaluate the association between the rs3761549 SNP and GD susceptibility. There was a significant relationship between GD and *FOXP3*/rs3761549 genotype (T vs. C: $OR = 1.32$, $95\%CI = 1.03–1.70$, $Pz = 0.03$; TT vs. CC: $OR = 1.98$, $95\%CI = 1.49–2.65$, $Pz < 0.00001$; TC vs. CC: $OR = 1.23$, $95\%CI = 1.03–1.45$, $Pz = 0.02$; (TT+TC) vs. CC: $OR = 1.44$, $95\%CI = 1.11–1.88$, $Pz = 0.006$; TT vs. (TC+CC): $OR = 1.71$, $95\%CI = 1.04–2.81$, $Pz = 0.03$) (Table 3). According to subgroup analysis stratified by ethnicity, we found a significant association between the *FOXP3*/rs3761549 polymorphism and GD risk in both Asians

Table 1. Characteristics of studies included in the meta-analysis.

Study ID	Year	Ethnicity	Genotyping methods	Cases					Controls					P_{HWE}	NOS stars
				CC	CA	AA	C	A	CC	CA	AA	C	A		
rs3761548															
Inoue et al.[31]	2010	Asian	PCR-RFLP	64	27	7	155	41	58	8	5	124	18	<0.05	8
Bossowski et al.[9]	2014	Caucasian	TaqMan	40	43	24	123	91	26	29	19	81	67	0.07	8
Zheng et al.[22]	2015	Asian	PCR-RFLP	196	92	20	484	132	225	70	11	520	92	0.07	8
Yu et al.[5]	2017	Asian	PCR-RFLP	331	166	37	828	240	466	132	32	1064	196	<0.05	8
Fathima et al.[30]	2019	Asian	PCR-RFLP	10	53	17	73	87	17	207	61	241	329	<0.05	7
Shehjar et al.[16]	2018	Asian	PCR-RFLP	29	101	5	159	111	69	69	12	207	93	0.36	7
rs3761549															
Owen et al.[32]	2006	Caucasian	MALDI-TOF-MS	—	—	—	977	142	—	—	—	682	114	—	7
Inoue et al.[31]	2010	Asian	PCR-RFLP	56	24	7	136	38	38	19	5	95	29	0.25	8
Bossowski et al.[9]	2014	Caucasian	TaqMan	78	30	1	186	32	64	11	0	139	11	0.49	8
Zheng et al.[22]	2015	Asian	PCR-RFLP	180	102	26	462	154	188	98	20	474	138	0.15	8
Yu et al.[5]	2017	Asian	PCR-RFLP	304	171	59	779	289	391	202	37	984	276	0.12	8
Fathima et al.[30]	2019	Asian	PCR-RFLP	24	46	10	94	66	132	115	38	379	191	0.11	7
Shehjar et al.[16]	2018	Asian	PCR-RFLP	68	37	30	173	97	102	40	8	244	56	0.14	7
rs3761547															
Inoue et al.[31]	2010	Asian	PCR-RFLP	69	33	7	171	47	46	20	4	112	28	0.37	8
Bossowski et al.[9]	2014	Caucasian	TaqMan	80	25	1	185	27	64	10	0	138	10	0.53	8
Zheng et al.[22]	2015	Asian	PCR-RFLP	190	95	23	475	141	195	92	19	482	130	0.08	8
Yu et al.[5]	2017	Asian	PCR-RFLP	315	171	48	801	267	391	189	50	971	289	<0.05	8
Shehjar et al.[16]	2018	Asian	PCR-RFLP	85	41	9	211	59	98	43	9	239	61	0.16	7

HWE, Hardy–Weinberg equilibrium; NOS, Newcastle–Ottawa scale; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; MALDI-TOF-MS, matrix assisted laser desorption ionization-time of flight mass spectrometry.

Table 2. Meta-analysis of the association between the rs3761548 polymorphism and Graves' disease susceptibility.

Polymorphism	Comparison model	Subgroup	No. of studies	Sample size (cases/controls)	Test of association			Effect model	Test of heterogeneity	
					OR	95% CI	P_Z		I^2	P_H
rs3761548	Allele comparison	Overall	6	2524/3032	1.32	1.05–1.67	0.02	Random	64%	0.02
	(A vs. C)	Asian	5	2310/2884	1.41	1.13–1.77	0.003	Random	57%	0.05
		Caucasian	1	214/148	0.89	0.59–1.36	0.60	–	–	–
	Homozygote comparison	Overall	6	780/1001	1.26	0.92–1.72	0.15	Fixed	39%	0.15
	(AA vs. CC)	Asian	5	716/956	1.37	0.97–1.93	0.07	Fixed	41%	0.15
		Caucasian	1	64/45	0.82	0.38–1.79	0.62	–	–	–
	Heterozygous comparison	Overall	6	1152/1376	1.58	1.01–2.45	0.04	Random	77%	0.0005
	(AC vs. CC)	Asian	5	1069/1321	1.72	1.06–2.79	0.03	Random	79%	0.0007
		Caucasian	1	83/55	0.96	0.49–1.91	0.92	–	–	–
	Dominant model	Overall	6	1262/1516	1.51	1.02–2.25	0.04	Random	75%	0.001
	(AA+AC vs. CC)	Asian	5	1155/1442	1.66	1.10–2.53	0.02	Random	75%	0.003
		Caucasian	1	107/74	0.91	0.49–1.68	0.76	–	–	–
	Recessive model	Overall	6	1262/1516	1.11	0.84–1.47	0.45	Fixed	20%	0.28
	(AA vs. AC+CC)	Asian	5	1155/1442	1.18	0.87–1.60	0.29	Fixed	27%	0.24
		Caucasian	1	107/74	0.84	0.42–1.6	0.61	–	–	–

OR, odds ratio; CI, confidence interval.
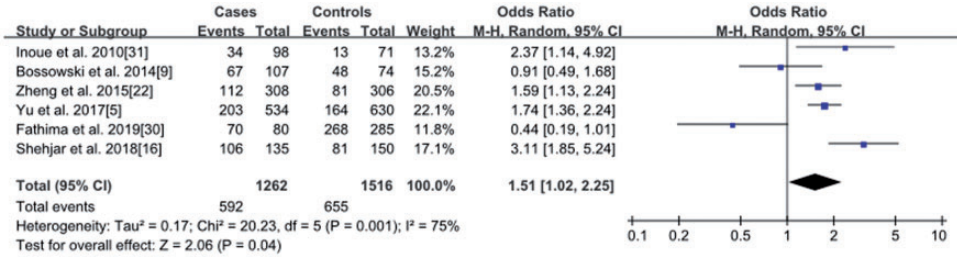
Figure 1. Forest plot of the association between *FOXP3*/rs3761548 polymorphism and risk of Graves' disease in the dominant model (AA + AC vs. CC).
CI, confidence interval; M-H, Mantel–Haenszel.

(T vs. C: OR = 1.38, 95%CI = 1.07–1.78, $Pz = 0.01$; TT vs. CC: OR = 1.92, 95% CI = 1.17–3.15, $Pz = 0.01$; (TT+TC) vs. CC: OR = 1.38, 95%CI = 1.05–1.79, $Pz = 0.02$) and Caucasians (TT+TC vs. CC: OR = 2.31, 95%CI = 1.08–4.96, $Pz = 0.03$).

FOXP3/rs3761547 polymorphism. We finally investigated the relationship between the *FOXP3*/rs3761547 polymorphism and GD susceptibility, but found no significant associations in either the overall or stratified analysis.

Sensitivity analysis and publication bias

We performed sensitivity analysis to assess the influence of individual studies on the pooled results. Sequential omission of individual studies from the pooled analysis had no effect on the overall pooled results, indicating that the results of the analysis were statistically robust. We evaluated possible publication bias by funnel plots, which showed no obvious indication of publication bias.

Discussion

In the present study, we evaluated the associations between *FOXP3*/rs3761547/ rs3761548/rs3761549 polymorphisms and GD susceptibility based on seven eligible case–control studies including 4051 GD patients and 4569 controls. We showed that the rs3761548 and rs3761549 SNPs contributed to GD susceptibility. Furthermore, subgroup analysis according to ethnicity revealed that *FOXP3*/rs3761548 was associated with GD in Asians but not in Caucasians, while *FOXP3*/rs3761549 was associated in both Asians and Caucasians. However, there was no significant association between *FOXP3*/rs3761547 and GD.

FOXP3 is mainly expressed by CD4^{+} and CD25^{+} Tregs, and normal *FOXP3* expression has been shown to be important for maintaining the inhibitory function of Tregs.[13] Genetic variations in the *FOXP3* gene, leading to reduced expression, may thus promote the pathogenesis of GD by weakening the inhibitory function of Tregs and promoting an autoimmune response. The association between *FOXP3* polymorphisms and GD susceptibility has attracted recent attention. *FOXP3* can affect the differentiation of Tregs and is significantly associated with susceptibility to GD.[5,9,16,22,30,31] For instance, Fathima et al.[30] analyzed the correlation between *FOXP3*/rs3761548/rs3761549 polymorphisms and GD in an Indian population and found that presence of the rs3761549 T allele predisposed patients to GD. In addition, a research team from India investigated the association between *FOXP3* promoter SNPs (rs3761547, rs3761548,

Table 3. Meta-analysis of the association between the rs3761549 polymorphism and Graves' disease susceptibility.

Polymorphism	Comparison model	Subgroup	No. of studies	Sample size (cases/controls)	Test of association			Effect model	Test of heterogeneity	
					OR	95% CI	P_Z		I^2	P_H
rs3761549	Allele comparison (T vs. C)	Overall	7	3625/3812	1.32	1.03–1.70	0.03	Random	75%	0.0006
		Asian	5	2288/2866	1.38	1.07–1.78	0.01	Random	69%	0.01
		Caucasian	2	1337/946	1.29	0.53–3.14	0.57	Random	82%	0.02
	Homozygote comparison (TT vs. CC)	Overall	6	843/1023	1.98	1.49–2.65	<0.00001	Fixed	47%	0.09
		Asian	5	764/959	1.92	1.17–3.15	0.01	Random	57%	0.05
		Caucasian	1	79/64	2.46	0.10–61.54	0.58	–	–	–
	Heterozygous comparison (TC vs. CC)	Overall	6	1120/1400	1.23	1.03–1.45	0.02	Fixed	45%	0.11
		Asian	5	1012/1325	1.18	1.00–1.41	0.06	Fixed	39%	0.16
		Caucasian	1	108/75	2.24	1.04–4.81	0.04	–	–	–
	Dominant model (TT+TC vs. CC)	Overall	6	1253/1508	1.44	1.11–1.88	0.006	Random	52%	0.06
		Asian	5	1144/1433	1.38	1.05–1.79	0.02	Random	53%	0.07
		Caucasian	1	109/75	2.31	1.08–4.96	0.03	–	–	–
	Recessive model (TT vs. TC+CC)	Overall	6	1253/1508	1.71	1.04–2.81	0.03	Random	56%	0.05
		Asian	5	1144/1433	1.70	1.00–2.88	0.05	Random	65%	0.02
		Caucasian	1	109/75	2.09	0.08–51.94	0.65	–	–	–

OR, odds ratio; CI, confidence interval.

and rs3761549) and GD in a Kashmiri population, and found significant differences between affected individuals and controls with respect to the genotype and allele frequencies of rs3761548 and rs3761549, but no significant association between rs3761547 and GD.[16] Zheng et al.[22] studied the possible associations of *FOXP3* polymorphisms (rs3761547, rs3761548, rs3761549, and rs2280883) with GD in a Chinese Han population, based on 308 GD patients and 306 healthy controls, and found that the frequencies of the AA/CA genotype of rs3761548 and the CC genotype of rs2280883 were linked to an increased risk of GD. In addition, the AA/CA genotype of rs3761548 was more frequent in female than in male GD patients. With regard to rs3761548, GD patients with higher thyroid-stimulating hormone levels or lower thyrotropin receptor antibody levels were more likely to carry the A allele. Another case–control study including 534 Chinese Han patients with GD and 630 healthy controls showed that heterozygote and minor allele (rs3761548, rs3761549, and rs2280883) frequencies were significantly higher in GD patients than in healthy volunteers. The results suggest that *FOXP3*/rs3761548/rs3761549/rs2280883 polymorphisms are associated with GD susceptibility in the Chinese Han population.[5]

Inoue et al.[31] genotyped *FOXP3* polymorphisms (rs3761547, rs3761548, rs3761549, and rs2280883) in a Japanese population including 65 patients with intractable GD, 44 patients with GD in remission, and 71 healthy subjects. They showed that the CA genotype of rs3761548 was more frequent in patients with GD in remission than in patients with intractable GD, and that the AA genotype of rs3761548, which is related to defective transcription of *FOXP3*, was absent in patients with GD in remission. Bossowski et al.[9] investigated the role of *FOXP3* SNPs (rs3761547, rs3761548, and rs3761549) in

GD susceptibility in a Polish study of 145 GD patients and 161 healthy subjects, and showed that rs3761549 G/A and rs3761547 T/C were more frequent in female GD patients compared with control females. In conclusion, these results suggest that the *FOXP3* rs3761549 G/A variant may contribute to the development of GD in female patients. All of the above findings support a role for *FOXP3* polymorphisms in GD susceptibility.

In contrast to the above results however, Owen et al.[32] investigated the association between *FOXP3* polymorphisms (rs3761549, rs2280883, rs2232365, rs2294021, rs6609857, (GT)n, and (TC)n) and GD in a Caucasian population from the northeast of England (633 GD and 528 controls) and found no link between *FOXP3* polymorphisms and susceptibility to GD in the UK population.

The current meta-analysis differs from two previous meta-analyses examining the association between the *FOXP3*/rs3761548 variant and autoimmune diseases performed by Lee et al.[18] and He et al.[33] Both these previous studies found that the *FOXP3*/rs3761548 SNP was associated with susceptibility to autoimmune diseases in Asian populations. However, their meta-analyses only included a few studies and a small number of GD patients, and they did not carry out an independent analysis of the relationship between *FOXP3*/rs3761548 polymorphism and GD susceptibility. Their results could therefore not fully explain the relationship between the *FOXP3*/rs3761548 polymorphism and susceptibility to GD. The present meta-analysis had several advantages, including being the first meta-analysis to focus on the relationship between the three *FOXP3* SNPs (rs3761547, rs3761548, and rs3761549) and the risk of GD. Additionally, compared with former meta-analyses of autoimmune diseases,[18,33] the current analysis included more studies of

patients with GD and more *FOXP3* loci, as well as carrying out supplementary tests including subgroup and sensitivity analyses. Moreover, the results for rs3761548 and rs3761547 did not change significantly, even after excluding studies with a HWE *P*-value <0.05, indicating that our meta-analysis results were reliable.

This study had several limitations. First, the sample size in our meta-analysis was relatively small due to a shortage of original studies, and only two studies in the subgroup analyses investigated the genetic effects of *FOXP3* polymorphisms in Caucasian populations. Further studies with larger sample sizes and additional ethnic populations are therefore needed to verify the present findings. In addition, raw data such as information on sex, lifestyle, clinical factors, and environmental exposure could not be obtained from all of the included studies. Furthermore, the current analysis did not contain enough data to analyze gene–environment and gene–gene interactions.

Conclusion

The current meta-analysis found significant associations between the *FOXP3*/rs3761548 and *FOXP3*/rs3761549 SNPs and susceptibility to GD in Asian populations.

Author contributions

Yu HS and Wang X conceived the study idea and designed the meta-analysis. Tan GQ and Zheng GB contributed to data acquisition and analysis. Tan GQ drafted the manuscript and interpreted the data. Tan GQ, Zheng GB, Du J, Zhou FY, Liang ZZ, Wei WW, Wang X, and Yu HS revised critically revised the manuscript for important intellectual content. All authors approved the final manuscript.

Declaration of conflicting interests

The authors declare that there is no conflict of interest.

ORCID iD

Hongsong Yu https://orcid.org/0000-0002-1209-2104

Supplemental material

Supplemental material for this article is available online.

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