

Predisposition to Graves' Disease and Graves' Ophthalmopathy by Genetic Variants of IL2RA

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Abstract

Previous studies have identified that Th17/Treg cells were involved in Graves' disease (GD). This study aimed at clarifying the association between GD susceptibility and single nucleotide polymorphisms (SNPs) of Th17/Treg cells-related genes, including IL2RA, miR27a, miR182, and FoxO1. A two-stage association study was performed in 650 GD patients and 1300 healthy controls. PCR-RFLP assays, real-time PCR and ELISA were performed. The result demonstrated that significantly increased frequencies of IL2RA/rs3118470 TT genotype ($P_c=2.212\times10^{-3}$) and IL2RA/rs2104286 AA genotype ($P_c=1.754\times10^{-5}$) were observed in GD. Stratification analysis also revealed that rs3118470 TT genotype and rs2104286 AA genotype were associated with Graves' ophthalmopathy (GO) susceptibility ($P_c = 1.848\times10^{-6}$, $P_c = 1.230\times10^{-3}$, respectively). Functional studies showed that carriers of the rs2104286 AA genotype had lower IL2RA mRNA expression than AG genotype carriers ($P=0.021$). Cytokine analyses revealed that carriers with the rs3118470 risk genotype had decreased anti-inflammatory cytokine levels (IL-10 and TGF- β 1) and increased IL-17 secretion than protective genotype individuals ($P < 0.05$). For the rs2104286 locus, AA genotype individuals had lower IL-10 levels ($P=0.015$) and increased IL-17 levels than AG genotype carriers ($P = 1.467\times10^{-4}$). In conclusion, our findings suggested that IL2RA/ (rs3118470, rs2104286) were associated with GD and GO susceptibility in Southwest Chinese Han population.

ABSTRACT

Previous studies have identified that Th17/Treg cells were involved in Graves' disease (GD). This study aimed at clarifying the association between GD susceptibility and single nucleotide polymorphisms (SNPs) of Th17/Treg cells-related genes, including *IL2RA*, *miR27a*, *miR182*, and *FoxO1*. A two-stage association study was performed in 650 GD patients and 1300 healthy controls. PCR-RFLP assays, real-time PCR and ELISA were performed. The result demonstrated that significantly increased frequencies of *IL2RA* /rs3118470 TT genotype ($P_c = 2.212\times10^{-3}$) and *IL2RA* /rs2104286 AA genotype ($P_c = 1.754\times10^{-5}$) were observed in GD. Stratification analysis also revealed that rs3118470 TT genotype and rs2104286 AA genotype were associated with Graves' ophthalmopathy (GO) susceptibility ($P_c = 1.848\times10^{-6}$, $P_c = 1.230\times10^{-3}$, respectively). Functional studies showed that carriers of the rs2104286 AA genotype had lower *IL2RA* mRNA expression than AG genotype carriers ($P = 0.021$). Cytokine analyses revealed that carriers with the rs3118470 risk genotype had decreased anti-inflammatory cytokine levels (IL-10 and TGF- β 1) and increased IL-17 secretion than protective genotype individuals ($P < 0.05$). For the rs2104286 locus, AA genotype individuals had lower IL-10 levels ($P = 0.015$) and increased IL-17 levels than AG genotype carriers ($P = 1.467\times10^{-4}$). In conclusion, our findings suggested that *IL2RA* / (rs3118470, rs2104286) were associated with GD and GO susceptibility in Southwest Chinese Han population.

Keywords: Graves' disease, genetic susceptibility, *IL2RA*, *miR27a*, *miR182*, *FoxO1*

INTRODUCTION

Graves' disease (GD), also known as toxic diffuse goiter, is one of the most common autoimmune thyroid diseases (AITDs), which accounts for more than 85% of all clinical types of hyperthyroidism (Xiaoheng et al., 2017). GD occurs in women aged 30-50 years (Veneti et al., 2019), and it affects approximately 1% of the world's population (J. Li, Teng, Yu, Hou, & Shan, 2019). The ratio of male to female patients is approximately 1:5-1:10 (Liang et al., 2015). Approximately 50% of GD patients can develop Graves' ophthalmopathy (GO), and severe stages can affect vision (Longo & Higgins, 2019). Immunologically, GD is characterized by an increased level of circulating thyroid stimulating antibody (TSAb) against thyroid-stimulating hormone receptor (TSHR), thyroglobulin (Tg) and thyroid peroxidase (TPO), leading to diffuse thyroid enlargement and hyperthyroidism (Fang et al., 2015). The pathogenesis of GD has not yet been fully elucidated, but it is generally believed that the occurrence of GD is the interaction result of genetic susceptibility and environmental factors (Chen et al., 2018).

Studies have shown that the coincidence rate of GD in identical twins was 50%-70%, while the coincidence rate of GD in fraternal twins was 3%-25% (Hwangbo & Park, 2018). Twin studies have indicated that the genes account for 79% of the role in predisposition to GD (Lane et al., 2019). Extensive genetic association studies have identified that many genes are associated with predisposition to GD, for example, human leukocyte antigen (*HLA*), cytotoxic T-lymphocyte-associated antigen-4 (*CTLA4*), protein tyrosine phosphatase 22 (*PTPN22*), Fc receptor like 3 (*FCRL3*), thyroid stimulating hormone receptor (*TSHR*), and vitamin D receptor gene (*VDR*) (Mehraji et al., 2017; Chu et al., 2018; Ting et al., 2016; Li et al., 2017; Khong et al., 2016; Fujii et al., 2017; M. Planck, Shahida, Malm, & Manjer, 2018). At present, antithyroid drugs are regarded as the preferred methods to treat GD in China, but recurrence within two years after retreat of the drug is as high as 50%-60% (J. Li, Sun, Yao, & Xia, 2018). Therefore, it is necessary to elucidate the pathogenesis of GD.

Studies have recognized that T helper 17 (Th17) cells and Regulatory T (Treg) cells are involved in the pathogenesis GD. The proportion of Th17 cells and the expression of related genes (*IL17* mRNA and *POP-γt* mRNA) were increased in patients with GD compared with healthy individuals, and the number of Th17 cells was directly proportional to TSAb, whereas the number of Treg cells and the related *FoxP3* mRNA expression were lower in GD patients than in normal controls (Qin et al., 2017). The human *IL2RA* gene located on the chromosome 10 (10p15-p14), encoded the IL-2RA protein and constitutively expressed on the Treg cell surface, which is essential for mediating both the generation of Treg and the maintenance of immune tolerance (Wang & Chen, 2018). In the context of CD4⁺ T-cell differentiation, FoxO proteins promote the expression of *FoxP3* genes essential for the generation and function of Tregs. In addition, FoxO1 can directly inhibit the differentiation of Th17 by binding to the transcription factor RORγt (Harada et al., 2010; Laine et al., 2015). *MIR27a* and *miR182* can target the 3'UTR of *FoxO1*, suppressing *FoxO1* expression (Guttilla & White, 2009; Wang et al., 2019). A previous study showed that the frequencies of the *IL2RA*/rs3118470 C allele and the CC genotype were higher in the patients with a family history of alopecia areata in a Chinese population (Miao et al., 2013). It has been reported that the *IL2RA*/rs2104286 G allele and the GG genotype increased the risk of multiple sclerosis (Xia, Qin, & Zhao, 2018). An analysis showed that *miR27a*/rs895819 contributed to the occurrence of gastric cancer under a co-dominant model in the Chinese population (Yun et al., 2019). Previous studies have identified that the frequency of the *miR182*/rs76481776 CT/TT genotype was increased in patients with Vogt-Koyanagi-Harada (VKH) syndrome and Behcet's disease, and increased expression of miR-182 in CT/TT cases compared with that in CC cases in anti-CD3 and anti-CD28 antibodies stimulated CD4⁺ T cells (Yu, Liu, Bai, Kijlstra, & Yang, 2014). The *FoxO1*/rs2297626 A allele and the AA genotype showed significantly increased frequencies in acute anterior uveitis patients with ankylosing spondylitis (Yu, Liu, Zhang, et al., 2014). The *FoxO1*/rs17592236 located in 3'-UTR of the *FoxO1* gene and the identified Ars17446614-Crs17592236 haplotype were associated with an increased risk of diabetic nephropathy (Ma et al., 2019). Considering these studies, we selected nine SNPs in Th17/Treg cells-related genes, including *IL2RA*, *miR27a*, *miR182*, and *FoxO1*, to explore the association between gene polymorphisms and GD susceptibility in Southwest Chinese Han population. Our results showed that *IL2RA* gene, but not *miR27a*, *miR182*, and *FoxO1*, contributed to the predisposition

of GD.

MATERIALS AND METHODS

Study subjects

A total of 650 patients with GD and 1300 healthy controls were enrolled in this study.

Diagnosis of GD and GO according to clinical symptom and thyroid hormone level based on American thyroid association guidelines (Ross et al., 2016). Healthy volunteers were recruited from the same region, and they did not have any other autoimmune disease and were matched for sex and age with the patients with GD. The study was registered in the Chinese clinical trial registry (ChiCTR1900022398) and was approved by the Zunyi Medical University Ethics Committee (18-ZY-001). All of the procedures were in accordance with the declaration of Helsinki.

Selection of single nucleotide polymorphisms (SNPs)

Based on the available literature studies, we selected nine SNPs, which were earlier shown to be associated with a variety of autoimmune diseases (Miao et al., 2013; Xia, Qin, & Zhao, 2018; Yun et al., 2019; Yu, Liu, Bai, Kijlstra, & Yang, 2014; Yu, Liu, Zhang, et al., 2014; Ma et al., 2019). Finally, we selected three SNPs (rs3118470, rs2104286, and rs7093069) for *IL2RA*, one SNP (rs895819) for *miR27a*, one SNP (rs76481776) for *miR182*, and four SNPs (rs2297626, rs17592236, rs9549241, and rs12585277) for *FoxO1*.

Extraction of DNA and genotyping of SNPs

Genomic DNA obtained was extracted from the peripheral blood of all of the subjects with the QIAamp DNA Blood Mini kit (Qiagen, Valencia, CA, USA). The targeted sequence was amplified by the PCR using appropriate primers (Yu, Liu, Bai, Kijlstra, & Yang, 2014). Genotyping of the nine SNPs was performed using the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assay. A total of 7 μ L PCR product was digested with 3 U of restriction enzyme according to the manufacturer's instructions, involving Pdi-I, DraIII, RsaI, Eco471, BshNI, HindIII, TscAI, NdeI, and BstNI (ThermoFisher Scientific, Waltham, MA, USA) overnight (Table S1). Digestion products were separated on 4% agarose gels added with goldview I nucleic acid dye (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). To confirm the PCR-RFLP genotyping results, we randomly selected a part of the samples to reperform genotyping using direct sequencing.

Peripheral blood mononuclear cell (PBMC) cultures and stimulation

A total of 5mL whole venous blood was collected in ethylene diaminetetraacetic acid (EDTA) anticoagulant vials from the normal controls. PBMCs were isolated and plated in 24-well plates at 1×10^6 per well density on the day of sample collection, and they were cultured using RPMI1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and 100 U/mL penicillin-streptomycin (Gibco, Carlsbad, CA, USA). Cells were stimulated with the anti-CD3 antibody (0.5 μ g/mL) and anti-CD28 (0.5 μ g/mL) (Miltenyi Biotec, Palo Alto, CA, USA), and then cultured at 37°C in a moist atmosphere containing 5% CO₂ for 72 hours.

RNA isolation and Real-time PCR

Cells were collected for RNA extraction, and supernatants were collected to detect the cytokine levels. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcription was performed using the cDNA synthesis kit according to the manufacturer's instructions (Takara, Dalian, China). Real-time PCR was conducted using BRYT Green. The primers used were as follows: β -*actin* F: 5'-GGATGCAGAAGGAGATCACTG-3', R: 5'-CGATCCACACGGAGTACTT-3'; *IL2RA* F: 5'-ATGGCTGCAACCATGGAGAC-3', R: 5'-TCTGTTCCCGGCT TCTTACC-3'. Real-time PCR parameters were as follows: step one, one cycle at 95°C for 10 min; step two, 40 cycles at 95°C for 15 s, 59°C for 1 min; step three, 95°C for 15 s, 60°C for 15 s and 95°C for 15 s. All of the reactions of real-time PCR were performed in triplicate. The relative expression of candidate genes was calculated using the $2^{-\Delta\Delta C_t}$ method.

ELISA assay

The cytokine levels in supernatants, including IL-6, IL-10, IL-17, and TGF- β 1, were measured by a commercial DuoSet ELISA kit (R&D System, Inc., Minneapolis, MN) according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics version 17.0 (SPSS, Inc., Chicago, IL). The chi-square test was used to determine the difference in sex and distributions of allele as well as the genotype frequency between GD patients and normal controls. The risk effect of each SNP was measured by odds ratios (*OR*) and 95% confidence intervals (95%CI). *P* values were corrected using the Bonferroni correction method considering multiple tests. The corrected *P* (*P_c*) value of the allele was $P \times 18$ and *P_c* of the genotype was $P \times 27$ in the first stage, while the *P_c* value of the allele was $P \times 4$ and *P_c* of the genotype was $P \times 6$ in the second stage and combined study. $P < 0.05$ was defined as indicative of statistical significance. The ttest or Mann-Whitney U test was used to analyze the difference in age, mRNA expression, and cytokine production among various genotypes.

RESULTS

Clinical Characteristics of GD patients and healthy controls

A total of 650 GD patients and 1300 healthy controls were recruited in this study. The average age was 38.8 ± 14.7 years among GD patients and 40.1 ± 12.6 years among controls. The 650 GD patients included 194 (29.8%) males and 456 (70.2%) females. The 1300 healthy controls consisted of 443 (34.0%) males and 857 (66.0%) females. No statistical differences were identified between GD patients and healthy controls in age and gender ($P > 0.05$; Table 1). The distribution of genotype frequencies of nine SNPs did not deviate from the Hardy-Weinberg equilibrium in the control group of this study.

Allele and genotype frequencies of nine SNPs in the first phase study

In the first phase, association between nine SNPs in four genes (*IL2RA*, *miR27a*, *miR182*, and *FoxO1*) and susceptibility to GD were investigated in 300 GD cases and 600 healthy controls. Our analysis revealed a correlation of the *IL2RA* /rs3118470 TT genotype with increased GD risk (TT genotype: $P_c = 0.027$, $OR = 1.688$). For the *IL2RA* /rs2104286 locus, subjects carrying the AA genotype had higher susceptibility to GD ($P_c = 0.027$, $OR = 1.658$), while the AG genotype protected against GD ($P_c = 4.617 \times 10^{-3}$, $OR = 0.530$ (Table 2). No association was identified between the remaining seven SNPs (rs7093069, rs895819, rs76481776, rs2297626, rs17592236, rs9549241, and rs12585277) and susceptibility to GD ($P > 0.05$; Table S2).

Allele and genotype frequencies of two SNPs in the second phase and combined study

To further verify the correlation between two loci (rs3118470 and rs2104286) and susceptibility to GD, we genotyped three loci in another 350 GD patients and 700 healthy controls. The result of rs2104286 was consistent with the first stage results (AA genotype: $P_c = 0.006$, $OR = 1.618$), while no association was observed for rs3118470. The combined data showed that the *IL2RA* /rs3118470 T allele and the TT genotype (T allele: $P_c = 1.490 \times 10^{-3}$, $OR = 1.278$; TT genotype: $P_c = 2.212 \times 10^{-3}$, $OR = 1.453$), and the *IL2RA* /rs2104286 A allele and the AA genotype (A allele: $P_c = 0.008$, $OR = 1.322$; AA genotype: $P_c = 1.754 \times 10^{-5}$, $OR = 1.636$) were significantly associated with an increased risk for GD (Table 2). The sequencing results were consistent with the PCR-RFLP genotyping results.

Allele and genotype frequencies in GO patients and controls

To further verify the relationship between the three candidate genes and susceptibility to GO, we performed a hierarchical analysis. The results showed that the *IL2RA* /rs3118470 T allele and the TT genotype showed increased frequencies in GO patients than in controls (T allele: $P_c = 4.036 \times 10^{-4}$, $OR = 1.612$; TT genotype: $P_c = 1.848 \times 10^{-6}$, $OR = 2.178$), whereas the C allele and the CT genotype showed decreased frequencies in GO patients (C allele: $P_c = 4.036 \times 10^{-4}$, $OR = 0.620$; CT genotype: $P_c = 0.018$, $OR = 0.628$). The *IL2RA*

/rs2104286 AA genotype showed an increased frequency in GO patients than in controls ($P_c = 1.230 \times 10^{-3}$, $OR = 1.851$), while the AG genotype showed a decreased frequency in GO patients ($P_c = 9.420 \times 10^{-6}$, $OR = 0.348$; Table 3).

The effect of SNPs on candidate gene expression

To explore whether the three loci affect the expression of the corresponding gene mRNA, we tested the biological function of various genetic variants. This was performed by measuring the expression of *IL2RA* in anti-CD3 and anti-CD28 antibody stimulated PBMCs obtained from healthy controls since the presence of immunosuppressive drug treatment in GD patients might affect gene expression. Studies revealed that the rs2104286 locus risk AA genotype individuals showed a significantly decreased *IL2RA* mRNA compared with those with the protective AG genotype ($P =$, Figure 1). No significant difference was observed in the *IL2RA* mRNA expression among various genotype individuals at the *IL2RA* /rs3118470 locus in anti-CD3 and anti-CD28 antibody-stimulated PBMC from healthy controls ($P > 0.05$, Figure S1).

Expression of cytokines in different genotypes of the *IL2RA*/rs3118470 locus

For the rs3118470 locus, the homozygous TT genotype individuals had lower IL-10 and TGF- β 1 levels and increased IL-17 level than the CC genotype ($P < 0.05$). No significant difference was identified in the IL-6 level among the rs3118470 TT, CT, and CC genotype individuals in anti-CD3 and anti-CD28 antibody stimulated PBMCs ($P > 0.05$, Figure 2).

Expression of cytokines in different genotypes of the *IL2RA*/rs2104286 locus

A decreased IL-10 level and an increased IL-17 cytokine secretion level were observed in the *IL2RA* /rs2104286 AA genotype individuals than in the AG genotype individuals ($P < 0.05$), while there was no statistical difference in the TGF- β 1 cytokine level ($P > 0.05$). There was no significant difference in the IL-6 secretion levels among the *IL2RA* /rs2104286 AA, AG, and GG genotypes in anti-CD3 and anti-CD28 antibody-stimulated PBMC ($P > 0.05$; Figure 3).

DISCUSSION

In this study, we showed that *IL2RA* /rs3118470 and *IL2RA* /rs2104286 were associated with GD susceptibility, while the remaining seven loci (rs7093069, rs895819, rs76481776, rs2297626, rs17592236, rs9549241, and rs12585277) were not associated with susceptibility to GD in Southwest Chinese Han population. Stratification analysis revealed that two SNPs (rs3118470 and rs2104286) also contributed to the genetic susceptibility of GO in Southwest Chinese Han population.

GD, a common organ-specific autoimmune disease, is caused by genetic susceptibility and environmental factors, resulting in immune dysfunction among individuals and unregulated thyroid hormone production (Sun et al., 2019; Kahaly, Diana, Kanitz, Frommer, & Olivo, 2020). The cytokine IL-2 is essential for the differentiation of Treg and maintenance of the suppressive function. Activated effector T cells produce IL-2 and transiently up-regulate IL-2RA, enabling them to respond to IL-2. IL2RA on the effector T cells can reduce its expression through a negative feedback when the cells come to rest (Sun et al., 2019). A protease can cleave off IL2RA on the T cell surface to produce sIL2RA that can be detected in the serum (Cerosaletti et al., 2013).

Studies have shown that the *IL2RA* mRNA was down-regulated in PBMCs from patients with GD (Ji et al., 2020). A study by Tang et al found that *IL2RA* / rs3118470 was highly significantly associated with type 1 diabetes and was highly acetylated in T cells involved in indirect disruption of *IL2RA* transcription (Tang et al., 2015). Song et al conducted a GWAS study of GD in the Han population in Shanghai and Anhui province of China, and found that the *IL2RA* /rs3118470 polymorphism was significantly associated with susceptibility to GD in the Chinese Han population at the first phase ($P = 0.019$), but the significance was lost in the verification stage (Song et al., 2013). Our study found that the *IL2RA* /rs3118470 TT genotype can increase the GD susceptibility in Southwest Chinese Han population. It should be noted that the P-value of the association between *IL2RA* /rs3118470 TT genotype and GD susceptibility is relatively large in the first stage

($P_c = 0.024$), while lack of the association was found in the second phase. Previous study showed that *IL2RA* /rs2104286 TT genotype was associated with the risk of multiple sclerosis (MS) (Xia et al., 2018). A latest study by Buhelt et al. reported that MS-associated *IL2RA* /rs2104286 was associated with the expression of *IL2RA* in CD4⁺ cells but not CD8⁺T cells, and carriers of the risk genotype had reduced surface expression of *IL2RA* in post-thymic expanded CD4⁺T cells, CD39⁺Treg cells, and memory cell subsets (Buhelt et al., 2019). Chistiakov et al. found that the *IL2RA* /rs2104286 polymorphism was not significantly associated with the GD susceptibility of the Russian population (Chistiakov, Chistiakova, Voronova, Turakulov, & Savost'anov, 2011). Our study identified the *IL2RA* /rs2104286 AA genotype can significantly increase the GD susceptibility in Southwest Chinese Han population, and the *IL2RA* mRNA expression in PBMC is relatively reduced for carrier of rs2104286 AA genotype. The reason for the inconsistent results may be due to the different genetic background of people in different regions. Brand et al found that *IL2RA* /rs7093069 was significantly associated with susceptibility to GD in Caucasian (Brand et al., 2007)., while the results of our study showed that *IL2RA* /rs7093069 was not significantly associated with GD susceptibility in Southwest China. Recent clinical studies have indicated that low-dose recombinant IL-2 can reduce the number of Th17 cells in the peripheral blood of systemic lupus erythematosus patients and the disease activity index, while it can increase the number of Treg cells (He et al., 2016).

The miRNA, short and non-coding RNA, acts mainly via targeting and regulation messenger RNAs (Viuff, Skakkebaek, Nielsen, Chang, & Gravholt, 2019). miR27a and miR182 act on the target molecule, *FoxO1* mRNA, and inhibit the expression of *FoxO1* (Yang et al., 2014). A previous bioinformatic analysis showed that rs895819 is situated in the terminal loop of the pre-miR27a, suggesting that it may affect the expression of matured miR27a and affinity with target mRNAs (Strafella, 2019). However, Takuse et al. reported that there was no difference in the allele and genotype frequencies of *miR27a* /rs895819 between GD patients and healthy controls in a Japanese population, and rs895819 variants did not affect the expression levels of hsa-miR-27a-3p in PBMCs (Takuse et al., 2017). Our results showed that there was a lack of association between *miR27a* /rs895819 variants and susceptibility to GD, which is consistent with the study by Takuse et al. Previous studies have identified that the *miR182* /rs76481776 locus was associated with VKH syndrome and Behçet's disease, and rs76481776 variants influenced the gene expression in CD4⁺ T cells (Yu, Liu, Bai, Kijlstra, & Yang, 2014). In contrast, we did not find any correlation between *miR182* /rs76481776 variants and GD susceptibility. This inconsistent result may be due to the fact that each disease may have specific risk genes.

FoxO1 in the nucleus promotes FoxP3 expression via binding to the FoxP3 conserved non-coding sequence 1 region and decreasing Th17 cells generation as well as transcription of Th17-related genes, including IL-17A, IL-23R and ROR γ t-target genes (Du et al., 2018). The study by Gong et al. and colleagues revealed that *FoxO1* /rs17592236 was not associated with type 2 diabetes in a Chinese Han population (Gong et al., 2017). Previous studies showed that four SNPs (rs2297626, rs17592236, rs9549241, and rs12585277) were not associated with Behçet's disease and VKH Syndrome in a Chinese Han population (Yu, Liu, Bai, et al., 2014). Similarly, our study showed that *FoxO1* / (rs2297626, rs17592236, rs9549241, and rs12585277) were not associated with GD susceptibility in the Chinese Han population. This study is the first to explore *FoxO1* gene polymorphisms and GD susceptibility, and therefore, further research is needed to verify this association in large study samples.

There are some potential limitations in this study. First, this study only investigated a limited number of SNPs, and it is possible that other as yet unknown SNPs of candidate genes might participate in the occurrence of GD. In addition, PBMCs, a large-cell population, were used to compare the difference in the mRNA expression among various genotype carriers. Further study is needed in a more purified cell subgroup, such as CD4⁺ T cells. Moreover, only the Chinese Han population was enrolled in our study, and thus, it is not certain whether this significant association can be generalized to the other ethnic populations.

In summary, our study identified that *IL2RA*/rs3118470 and *IL2RA*/rs2104286 were significantly associated with GD and GO susceptibility in Southwest Chinese Han population, which may be involved in the occurrence of GD and GO by affecting the mRNA expression of *IL2RA* gene and the cytokine production.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

DATA AVAILABILITY

All data relevant to the study are included in the article or uploaded as supplementary information.

AUTHOR CONTRIBUTORS

Yu HS, Wang X and Li H conceived the idea and designed this study. Du J performed the experiment, drafting the manuscript and interpreting the data. Du J, Wan X, Tan GQ, Wei WW, Zhou FY, Liang ZZ, Li H, and Yu HS revised it critically for important intellectual content. Final approval of the version was submitted by all authors.

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Table and Figure legends

Table 1 Characteristics of GD patients and controls in this study.

Table 2 Association between *IL2RA* / rs3118470/ rs2104286 polymorphisms and GD susceptibility.

Table 3 Allele and genotype frequencies of three candidate gene loci and

susceptibility to GO.

Figure 1 The *IL2RA* mRNA expression in anti-CD3/CD28 antibodies-stimulated PBMCs from healthy controls carrying various genotypes of rs2104286 (GG: N=11, AG: N=12, AA: N=12). The data are presented as the mean±SD.

Figure 2 The influence of various rs3118470 genotypes on the IL-6 (A), IL-10 (B), IL-17(C) and TGF-β1(D) production (GG: N=12, AG: N=12, AA: N=12). The production of cytokine was quantified by ELISA in PBMCs, which were obtained from healthy controls with various rs3118470 genotypes and treated with anti-CD3/CD28 antibodies.

Figure 3 The influence of various rs2104286 genotypes on the IL-6 (A), IL-10 (B), IL-17(C) and TGF-β1(D) production (GG: N=11, AG: N=12, AA: N=12). The production of cytokine was quantified by ELISA in PBMCs, which were obtained from healthy controls with various rs3118470 genotypes and treated with anti-CD3/CD28 antibodies.

Table S1 PCR primer sequences and reaction condition in this study.

Table S2 Association between remaining seven SNP polymorphisms and GD susceptibility.

Figure S1 The *IL2RA* mRNA expression difference with rs3118470 locus various genotype individuals.

Table 1 Characteristics of GD patients and controls in this study

Clinical characteristics	Frequency (%)	Frequency (%)	P value
	GD patients	Healthy controls	
Total(N)	650	1300	-
Average age [M±SD]	38.8±14.7	40.1±12.6	0.574
Male	194 (29.8)	443 (34.0)	-
Female	456 (70.2)	857 (66.0)	-
Ratio of male to female	1: 2.35	1: 1.93	0.131

Note: GD: Graves' disease; N: number; M: Mean value; SD: Standard Deviation.

Table 2 Association between *IL2RA*/rs3118470/ rs2104286 polymorphisms and GD susceptibility

SNPs	Stage	Allele /Genotype	GD patients (%)	Healthy controls (%)	<i>P</i> value	<i>P_c</i> value	OR (95% CI)
rs3118470 (<i>IL2RA</i>)	First	C	262(43.667)	611(51.002)	0.004	NS	0.745(0.611-0.907)
		T	338(56.333)	587(48.998)	0.004	NS	1.343(1.103-1.635)
		CC	72(24.000)	165(27.546)	0.262	NS	0.831(0.603-1.14)
		CT	118(39.333)	281(46.912)	0.033	NS	0.734(0.554-0.973)
		TT	110(36.667)	153(25.543)	0.001	0.027*	1.688(1.253-2.274)
	Second	C	299(44.627)	682(49.636)	0.033	NS	0.818(0.679-0.984)
		T	371(55.373)	692(50.364)	0.033	NS	1.223(1.016-1.472)
		CC	69(20.597)	177(25.764)	0.070	NS	0.747(0.545-1.024)
		CT	161(48.060)	328(47.744)	0.924	NS	1.013(0.780-1.315)
		TT	105(31.343)	182(26.492)	0.105	NS	1.267(0.951-1.687)
	Combined	C	561(44.173)	1293(50.272)	3.725×10^{-4}	1.490×10^{-3}	0.783(0.684-0.896)
		T	709(55.827)	1279(49.728)	3.725×10^{-4}	1.490×10^{-3}	1.278(1.116-1.462)
		CC	141(22.205)	342(26.594)	0.037	NS	0.788(0.630-0.986)
		CT	279(43.937)	609(47.356)	0.157	NS	0.871(0.720-1.055)
		TT	215(33.858)	335(26.050)	3.687×10^{-4}	2.212×10^{-3}	1.453(1.182-1.786)
rs2104286 (<i>IL2RA</i>)	First	A	505(84.167)	952(79.333)	0.015	NS	1.385(1.068-1.796)
		G	95(15.833)	248(20.667)	0.015	NS	0.722(0.557-0.936)
		AA	224(74.667)	384(64.000)	0.001	0.027*	1.658(1.217-2.258)
		AG	57(19.000)	184(30.667)	1.710×10^{-4}	4.617×10^{-3} *	0.530(0.379-0.745)
		GG	19(6.333)	32(5.333)	0.543	NS	1.200(0.668-2.155)
	Second	A	569(81.988)	1094(78.143)	0.040	NS	1.273(1.010-1.605)
		G	125(18.012)	306(21.857)	0.040	NS	0.785(0.623-0.990)

SNPs	Stage	Allele /Genotype	GD patients (%)	Healthy controls (%)	<i>P</i> value	<i>P_c</i> value	OR (95% CI)
		AA	250(72.046)	430(61.429)	0.001	0.006	1.618(1.224-2.140)
		AG	69(19.885)	234(33.429)	5.389×10^{-6}	3.233×10^{-5}	0.494(0.364-0.672)
		GG	28(8.069)	36(5.143)	0.063	NS	1.619(0.971-2.700)
	Combined	A	1074(82.998)	2046(78.692)	0.002	0.008	1.322(1.112-1.571)
		G	220(17.002)	554(21.308)	0.002	0.008	0.757(0.636-0.899)
		AA	474(73.261)	814(62.615)	2.924×10^{-6}	1.754×10^{-5}	1.636(1.330-2.012)
		AG	126(19.474)	418(32.154)	4.274×10^{-9}	2.564×10^{-8}	0.510(0.407-0.640)
		GG	47(7.264)	68(5.230)	0.073	NS	1.419(0.966-2.084)

Note: GD: Graves' disease; SNPs: Single nucleotide polymorphisms; *P_c*, Bonferroni corrected *p* value; OR: Odds ratio; 95%CI: 95% Confidence Level; NS, not significant.

Table 3 Allele and genotype frequencies of two candidate gene loci and susceptibility to GO

SNPs (Gene)	Allele /Genotype	GO patients	Healthy controls	<i>P</i> Value	<i>P_c</i> Value	OR(95%CI)
		n=205	n=1300			
rs3118470	C	158(38.537)	1293(50.272)	1.009×10^{-5}	4.036×10^{-4}	0.620(0.501-0.768)
(<i>IL2RA</i>)	T	252(61.463)	1279(49.728)	1.009×10^{-5}	4.036×10^{-4}	1.612(1.303-1.996)
	CC	42(20.488)	342(26.594)	0.063	NS	0.711(0.496-1.020)
	CT	74(36.098)	609(47.356)	0.003	0.018	0.628(0.463-0.852)
	TT	89(43.415)	335(26.050)	3.080×10^{-7}	1.848×10^{-6}	2.178(1.609-2.949)
rs2104286	A	339(82.683)	2046(78.692)	0.064	NS	1.293(0.984-1.698)
(<i>IL2RA</i>)	G	71(17.317)	554(21.308)	0.064	NS	0.773(0.589-1.016)
	AA	155(75.610)	814(62.615)	2.050×10^{-4}	1.230×10^{-3}	1.851(1.320-2.595)
	AG	29(14.146)	418(32.154)	1.570×10^{-7}	9.420×10^{-6}	0.348(0.231-0.524)
	GG	21(10.244)	68(5.230)	0.005	0.030	2.068(1.238-3.455)

Note: GO: Graves' ophthalmopathy; SNPs: Single nucleotide polymorphisms; A: Allele; G: Genotype; Pc, Bonferroni corrected p value; OR: Odds ratio; 95%CI: 95% Confidence Level; NS, not significant.



