Original Paper

Polymorphisms of IKZF3 Gene and Autoimmune Thyroid Diseases: Associated with Graves' Disease but Not with Hashimoto's Thyroiditis

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Key Words

Ikaros family of zinc finger3 (*IKZF3*) • Single nucleotide polymorphism (SNP) • Autoimmune thyroid disease (AITD) • Graves' disease (GD) • Hashimoto's thyroiditis (HT)

Abstract

Background/Aims: The *IKZF3* gene encodes a zinc-finger protein that plays an important role in the proliferation and differentiation of B lymphocytes. Autoimmune thyroid diseases (AITDs), mainly include Graves' disease (GD) and Hashimoto's thyroiditis (HT), are probably caused by the aberrant proliferation of B cells. The objective of this study was to explore the association between *IKZF3* polymorphisms and AITDs. **Methods:** We examined 915 AITD patients (604 GD and 311 HT) and 814 healthy controls. *IKZF3* variants (rs2941522, rs907091, rs1453559, rs12150079 and rs2872507) were tested by PCR-ligase detection reaction. **Results:** It was manifested that the minor alleles of the five loci increased susceptibility to GD ($p < 0.05$ for rs2941522, and $p < 0.01$ for rs907091, rs1453559, rs12150079 and rs2872507) but in HT patients, these loci showed no significant difference compared with controls. Similarly, the genotype distributions of GD patients manifested obvious differences in all these loci compared with the control group, whereas no statistical differences were observed between HT patients and controls. Furthermore, bioinformatics tools were used to analyze rs1453559, rs12150079 and rs907091. These variants were believed to be the transcription regulator. **Conclusion:** It is the first time we reported the association between the *IKZF3* polymorphisms and GD, indicating that *IKZF3* gene tends to be an important risk factor for the development of GD.

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Introduction

Autoimmune thyroid diseases (AITDs), including Graves' disease (GD) and Hashimoto's thyroiditis (HT), are the common organ-specific autoimmune disorders affecting the thyroid. Its etiology appears to be the interactions between genetic and environmental factors. GD and HT are characterized by immunocytes' imbalance and autoantibodies' production [1]. The HLA-DR3 gene was identified as a susceptible region for AITD [2]. In addition, several types of non-HLA genes have been reported, including immunoregulatory genes like CD40, CTLA-4, PTPN22 and thyroid-specific genes, such as variants for thyroid stimulating hormone receptor (TSHR) and thyroglobulin (TG). In recent years, with the achievement in Human Genome Project and the genome-wide association (GWA) studies, a number of new loci have been identified as the risk factors of Graves' disease [3]. In this study, we examined the Ikaros family of zinc finger3 (*IKZF3*) gene, novel susceptible variants, in AITDs patients.

The *IKZF3* gene is located on human chromosome 17q12-q21.1, consisting of 9 exons and spanning a region of 104kb. This gene encodes a zinc-finger protein, Aiolos, which is a member of the Ikaros family. The Ikaros family includes 3 members - Ikaros, Aiolos and Helios. Each is hematopoietic-specific transcription factor that regulates the development of lymphocytes. The product of *IKZF3* gene is expressed highly in mature peripheral B cells, and also detected in T and B cell precursors [4]. The *IKZF3* not only participates in chromatin remodeling and histone deacetylation, but also is an important transcription factor inhibiting B cell proliferation and differentiation [5, 6]. The *IKZF3*-deficient mice exhibited an activated cell surface phenotype, leading to the formation of germinal centers and overproduction of serum IgG and IgE [7]. As AITDs are prone to be aroused by the excessive differentiation of plasma cells and overproduction of autoantibodies, the *IKZF3* might be a susceptibility gene for GD or HT.

Many recent studies have suggested several SNPs of *IKZF3* gene have significant associations with systemic lupus erythematosus (SLE), a representative systemic autoimmune disease [8, 9]. It is also demonstrated that the *IKZF3*-mutant mice would developed into the SLE-like phenotypes [10]. Besides SLE, several *IKZF3* SNPs are also associated with rheumatoid arthritis (RA) [11], asthma [12-14] and primary biliary cirrhosis (PBC) [15], indicating its comprehensive involvement with autoimmune or immunological diseases. Up to now, few researches have investigated the relationship between *IKZF3* polymorphisms and AITDs. Therefore, we performed a case-control study to explore the genetic association between *IKZF3* and AITDs.

Materials and Methods

Patients and Controls

In our study, we investigated a total of 1729 subjects, including 915 AITD patients (604 GD patients and 311 HT patients) and 814 healthy controls. Inclusion criteria: Individuals were diagnosed with GD by clinical and laboratory confirmed hyperthyroidism, added with diffuse goiter and positive anti-thyroid stimulating hormone receptor antibody (TRAb). HT was diagnosed by enlarged thyroid, either positive anti-thyroid peroxidase antibody (TPO-Ab) or positive anti-thyroglobulin antibody (Tg-Ab), with/without hypothyroidism. Exclusion criteria: Healthy controls were screened for the absence of goiter and negative TPO-Ab. Controls suffered from or with family history for any immune-related diseases were excluded. Patients with other autoimmune diseases except for AITDs were also excluded, such as rheumatoid arthritis and SLE. All of the subjects were Chinese Han ethnicity and unrelated with each other. Patients with AITDs and controls were recruited separately from the department of Endocrinology and the Health Care Center, Jinshan Hospital of Fudan University in Shanghai. This study received the IRB (Institutional Reviewer Board) approval from Jinshan Hospital of Fudan University. Informed consents were obtained from all participants (included AITD patients and controls). Demographic characteristics of all the participants are presented in Table 1.

DNA extraction and SNP genotyping

We collected peripheral blood from all subjects. Genomic DNA was extracted by RelaxGene Blood DNA System (Tiangen Biotech, Beijing, China). *IKZF3* SNPs were genotyped by PCR-ligase detection reaction (PCR-LDR) platform. *IKZF3* SNPs were selected from the Hapmap CHB database by Haploview software 4.2 using LD-based tagSNP selection by LDSelect. We searched the *IKZF3* chromosomal region and its upper/lower sites to capture the 5' and 3' untranslated regions (UTR). All the SNPs were selected according to the following criteria: minor allele frequency (MAF) > 0.1, Hardy-Weinberg equilibrium (HWE) > 0.05, and logarithm of odds (LOD) > 3.0. Information of selected SNPs was shown in Table 2. The presumed functions of these variants were extracted from the database of Ensembl genome browser (www.ensembl.org). Rs1453559 is located within the 5'- untranslated region (UTR), and rs907091 within the 3'UTR of *IKZF3*. Rs2941522 is a downstream variant located at the 3' of *IKZF3*, and two SNPs of rs12150079 and rs2872507 at the 5' flanking region of the gene. The five SNPs are in one linkage disequilibrium block. A strong linkage disequilibrium (LD) was exhibited in these 5 SNPs ($D' \geq 0.9$ for each SNP combination). All the SNPs were located within the same haplotype, representing a single signal for association. Specific primers for these SNPs are shown in Table 3.

Statistical analysis

Clinical data were expressed as $M \pm SD$ (mean $\pm$ standard deviation). HWE and LD were analyzed by Haploview software 4.2. Statistical analysis was performed using SPSS 21.0. SNP frequencies between the patients and the controls were compared by Pearson chi-square test or Fisher's exact test. Odds ratios (OR), with the 95% confidence intervals (95% CI) were calculated to express the risk of acquiring AITDs. $p < 0.05$ was considered to be statistically significant.

Power Calculation

The allele frequency of the selected SNPs was from 0.186 to 0.443, which is proper for power calculation. The 604 GD patients and 814 controls provided a power of 81.3% with an $OR=1.27$ at $p=0.05$ significance level. The power of the data was evaluated by the Power and Sample Size Calculation Software (<http://biostat.mc.vanderbilt.edu/wiki/bin/view/Main/Power-SampleSize>).

Functional prediction

Potential functions of SNPs were extracted from SNPinfo (<https://snpinfom.nih.gov/snpinfo/snpfunc.html>). We also used bioinformatics databases such as Promo (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) to analyze transcription factor binding sites (TFBS). As

Table 1. Clinical statistics of AITD patients and controls

	GD(%)	HT(%)	Control(%)
Number	604	311	814
Gender			
Male	189(31.29)	39(12.54)	291(35.75)
Female	415(68.71)	272(87.46)	523(64.25)
Age	37.20 $\pm$ 14.69	35.07 $\pm$ 14.04	38.73 $\pm$ 9.05
Onset of age	33.90 $\pm$ 14.81	33.51 $\pm$ 14.40	
Goiter			
None	110(18.21)	42(13.50)	
I degree	103(17.05)	52(16.72)	
II degree	315(52.15)	196(63.02)	
III degree	76(12.58)	21(6.75)	
Ophthalmopathy			
(+)	108(17.88)	3(0.96)	
(-)	496(82.12)	308(99.04)	
Thyroid antibodies			
TPO-Ab	241(39.90)	223(71.70)	
Tg-Ab	322(53.31)	199(63.99)	
Family history			
(+)	126(20.86)	63(20.26)	
(-)	478(79.14)	248(79.74)	
Adolescent (<18)	56(9.27)	19(6.11)	64(7.86)
Smoking			
Male	98(51.85)	12(30.76)	136(46.73)
Female	6(1.45)	1(0.37)	7(1.39)
Hypothyroidism		96(30.87)	

Table 2. Information of chosen SNPs. A strong LD was exhibited in these 5 SNPs ($D' \geq 0.9$ for each SNP combination). Chr, Chromosome; MAF, Minor Allele Frequency; HWE, Hardy-Weinberg Equilibrium

SNP	Base change	Gene name	Chr	MAF		HWE
				Database	Subjects	
rs2941522	A>G	IKZF3 (3' flank)	17	0.443	0.313	0.847
rs907091	T>C	IKZF3	17	0.470	0.314	0.734
rs1453559	A>G	IKZF3	17	0.457	0.279	0.784
rs12150079	G>A	IKZF3 (5' flank) / ZPBP2	17	0.186	0.210	0.543
rs2872507	G>A	IKZF3 (5' flank)	17	0.326	0.277	0.624

Table 3. The primer sequences of chosen SNPs at *IKZF3* gene region

SNP	Primer	Sequences(5'-3')
rs2941522	Forward	GTGGGAACCTCGAGAGACAG
	Reverse	GTCGCGGCTCTTTGAACCTC
rs907091	Forward	TCTTAACCCCTTCAGCCTGTGA
	Reverse	CAGGGACTTGAATTTTATGGAA
rs1453559	Forward	TCCTTGGGTTTGACATTCG
	Reverse	AAAGACCCCGGAGAAAAGAA
rs12150079	Forward	GGCTCAGCTCAAATGTCCTT
	Reverse	CAGAACGATGGTATGCTTGG
rs2872507	Forward	ACATCATGTTGACACTTAGAAAAGTT
	Reverse	TAGCTCTATGCCAGAGTAAATTTG

for potential miRNA target site prediction, data were accessed from mirnada (<http://www.microrna.org/microrna/home.do>).

Results

Association of IKZF3 Polymorphism with GD and HT

Genotype distributions for SNPrs2941522, rs907091, rs1453559, rs12150079 and rs2872507 were in the range of predicted HWE, no matter in the patient group or the control group. Allele and genotype frequencies of the five SNPs of patients and controls were listed in Table 4. Each of the five SNP was associated with AITDs ($p < 0.05$) in allele frequency analysis, and the minor (variant) alleles of all the 5 SNPs were risk alleles, which probably induced individuals to AITDs. Genotyping analysis showed that four of five SNPs exhibited different frequencies between AITD patients and controls. Besides, as shown in Table 5, more individuals in AITD group carried mutant homozygotes than in the control group at the loci of rs907091, rs1453559, rs12150079 and rs2872507.

When GD and HT groups were analyzed separately, we noticed the allele distributions of rs2941522, rs907091, rs1453559, rs12150079 and rs2872507 showed obvious differences between GD individuals and controls ($p = 0.020$, OR = 1.21; $p = 0.006$, OR = 1.25; $p = 0.007$, OR = 1.25; $p = 0.006$, OR = 1.29; $p = 0.004$, OR = 1.27, respectively). Similarly, the mutant

Table 4. Allele and genotype distribution of the 5 SNPs in AITD patients and controls

SNP ID	Allele/genotype frequency n (%)				AITDs vs. C	P value [OR(95%CI)]	
	Control	AITD	GD	HT		GD vs. C	HT vs. C
rs2941522							
G	479(29.42)	605(33.06)	405(33.53)	200(32.15)	0.021[1.19(1.03-1.37)] reference	0.020[1.21(1.03-1.42)] reference	0.207[1.14(0.93-1.39)] reference
A	1149(70.58)	1225(66.94)	803(66.47)	422(67.85)			
GG	69(8.48)	103(11.26)	75(12.42)	28(9.00)			
AG	341(41.89)	399(43.61)	255(42.22)	144(46.30)			
AA	404(49.63)	413(45.13)	274(45.36)	139(44.69)			
rs907091							
C	474(29.12)	609(33.27)	410(33.94)	199(31.99)	0.008[1.21(1.05-1.40)] reference	0.006[1.25(1.07-1.47)] reference	0.182[1.15(0.94-1.39)] reference
T	1154(70.88)	1221(66.73)	798(66.06)	423(68.01)			
CC	65(7.98)	108(11.80)	79(13.07)	29(9.32)			
CT	344(42.26)	393(42.95)	252(41.72)	141(45.33)			
TT	405(49.75)	414(45.24)	273(45.19)	141(45.33)			
rs1453559							
G	423(25.98)	543(29.67)	369(30.55)	174(27.97)	0.016[1.20(1.04-1.39)] reference	0.007[1.25(1.06-1.48)] reference	0.339[1.11(0.90-1.36)] reference
A	1205(74.02)	1287(70.33)	839(69.45)	448(72.03)			
GG	48(5.89)	84(9.18)	62(10.26)	22(7.07)			
AG	327(40.17)	375(40.98)	245(40.46)	130(41.80)			
AA	439(53.93)	456(49.83)	297(49.17)	159(51.12)			
rs12150079							
A	312(19.17)	415(22.68)	283(23.43)	132(21.22)	0.011[1.24(1.05-1.46)] reference	0.006[1.29(1.08-1.55)] reference	0.273[1.14(0.90-1.43)] reference
G	1316(80.83)	1415(77.32)	925(76.57)	490(78.78)			
AA	29(3.56)	52(5.68)	36(5.96)	16(5.14)			
AG	254(31.20)	311(33.99)	211(34.93)	100(32.15)			
GG	531(65.23)	552(60.33)	357(59.11)	195(62.70)			
rs2872507							
A	417(25.61)	541(29.56)	368(30.46)	173(27.81)	0.010[1.22(1.05-1.42)] reference	0.004[1.27(1.08-1.50)] reference	0.289[1.12(0.91-1.38)] reference
G	1211(74.39)	1289(70.44)	840(69.54)	449(72.19)			
AA	46(5.65)	82(8.96)	61(10.09)	21(6.75)			
AG	325(39.92)	377(41.20)	246(40.72)	131(42.12)			
GG	443(54.42)	456(49.83)	297(49.17)	159(51.12)			

Table 5. Comparisons of genotype frequencies in AITD patients and controls

SNP ID	Allele/genotype frequency n (%)				AITDs vs. C	P value [OR(95%CI)]	
	Control	AITD	GD	HT		GD vs. C	HT vs. C
rs2941522							
GG	69(8.48)	103(11.26)	75(12.42)	28(9.00)	0.054[1.37(0.99-1.89)] reference	0.015[1.53(1.08-2.16)] reference	0.778[1.07(0.67-1.69)] reference
AG+AA	745(91.52)	812(88.74)	529(87.58)	283(90.99)			
rs907091							
CC	65(7.98)	108(11.80)	79(13.08)	29(9.33)	0.008[1.54(1.12-2.13)] reference	0.002[1.73(1.23-2.45)] reference	0.468[1.19(0.75-1.87)] reference
CT+TT	749(92.02)	807(88.19)	525(86.92)	282(90.67)			
rs1453559							
GG	48(5.89)	84(9.18)	62(10.26)	22(7.07)	0.010[1.61(1.12-2.33)] reference	0.002[1.86(1.25-2.74)] reference	0.465[1.22(0.72-2.05)] reference
AG+AA	766(94.10)	831(90.82)	542(89.74)	289(92.93)			
rs12150079							
AA	29(3.56)	52(5.68)	36(5.96)	16(5.15)	0.037[1.63(1.03-2.60)] reference	0.033[1.72(1.04-2.83)] reference	0.226[1.47(0.79-2.74)] reference
AG+GG	785(96.44)	863(94.32)	568(94.04)	295(94.85)			
rs2872507							
AA	46(5.65)	82(8.96)	61(10.10)	21(6.75)	0.009[1.64(1.13-2.39)] reference	0.002[1.88(1.26-2.79)] reference	0.485[1.21(0.71-2.06)] reference
AG+GG	768(94.35)	833(91.04)	543(89.90)	290(93.25)			

homozygotes in GD group also showed higher frequencies compared with those in the control group ($p = 0.015$, OR = 1.53 for GG in rs2941522; $p = 0.002$, OR = 1.73 for CC in rs907091; $p = 0.002$, OR = 1.86 for GG in rs1453559; $p = 0.033$, OR = 1.72 for AA in rs12150079; and $p = 0.002$, OR = 1.88 for AA in rs2872507). However, in HT patients group, no locus presented a statistical difference in allele or genotype frequencies compared with controls.

After stratified by gender in GD patients (Table 6), the minor allele frequencies of all the 5 SNPs were much higher in female GDs than in female controls. Similarly, mutant homozygotes (GG in rs2941522; CC in rs907091; GG in rs1453559 and AA in rs2872507) were more frequently detected in the female GD group than those in the female control group. On the contrary, these allelic or genotypic frequencies showed no statistical differences between male GD patients and controls (Table 3, OR was not shown if $p > 0.05$).

Associations between Genotype and Clinical Phenotype

In the correlation analysis, we could not find that alleles or genotypes were correlated with a specific clinical phenotype. In GD patients, no significant difference was observed in GD patients with or without ophthalmopathy (Table 7). There were no correlations between genotypes and other clinical features either ($p > 0.05$, data not shown), such as adolescents (<18 years old) or adults, with or without hypothyroidism in HT group, with or without family history in both GD and HT group.

Functional Predictions

The functions of the 5 variants were extracted from the website of SNPinfo. Rs1453559 and rs12150079 were located

Table 6. Analysis of allelic and genotypic distribution of the 5 SNPs in GD patients and controls after gender stratification (OR was not shown if $P > 0.05$). # Male; * Female

SNP ID	Control numbers (%)		GD numbers (%)		P value	OR(95%CI)
	Male	Female	Male	Female		
rs2941522	G	175(30.07)	304(29.06)	127(33.60)	0.250# 0.039*	1.22(1.01-1.47)*
	A	407(69.93)	742(70.94)	251(66.40)	reference	
	GG	26(8.93)	43(8.22)	23(12.17)	0.253# 0.030*	1.60(1.04-2.45)*
	AG+AA	265(91.07)	480(91.78)	166(87.83)	reference	
rs907091	C	169(29.04)	305(29.16)	127(33.59)	0.135# 0.022*	1.26(1.03-1.53)*
	T	413(70.96)	741(70.84)	251(66.41)	reference	
	CC	24(8.25)	41(7.84)	24(12.69)	0.112# 0.007*	1.80(1.17-2.75)*
	CT+TT	267(91.75)	482(92.16)	165(87.31)	reference	
rs1453559	G	156(26.81)	267(25.53)	114(30.16)	0.259# 0.013*	1.29(1.06-1.59)*
	A	426(73.19)	779(74.47)	264(69.84)	reference	
	GG	21(7.22)	27(5.16)	22(11.64)	0.097# 0.008*	1.96(1.18-3.25)*
	AG+AA	270(92.78)	496(94.84)	167(88.36)	reference	
rs12150079	A	120(20.62)	192(18.36)	85(22.49)	0.490# 0.004*	1.39(1.11-1.74)*
	G	462(79.38)	854(81.64)	293(77.51)	reference	
	AA	10(3.44)	19(3.63)	10(5.29)	0.320# 0.061*	
	AG+GG	281(96.56)	504(96.37)	179(94.71)	reference	
rs2872507	A	156(26.81)	261(24.95)	114(30.16)	0.259# 0.006*	1.33(1.08-1.63)*
	G	426(73.19)	785(75.05)	264(69.84)	reference	
	AA	20(6.87)	26(4.97)	21(11.11)	0.105# 0.006*	2.04(1.22-3.40)*
	AG+GG	271(93.13)	497(95.03)	168(88.89)	reference	

Table 7. The allelic and genotypic frequencies of 5 SNPs in GD patients with or without ophthalmopathy

SNP ID		GD		P value AITDs vs. C
		Ophthalmopathy	Non-Ophthalmopathy	
rs2941522	G	76(35.19)	329(33.17)	0.569
	A	140(64.81)	663(66.83)	
	GG	12(11.11)	63(13.43)	
	AG	52(48.14)	203(40.92)	0.388
	AA	44(40.74)	230(46.37)	
rs907091	C	77(35.65)	333(33.57)	0.559
	T	139(64.35)	659(66.43)	
	CC	13(12.03)	66(13.30)	
	CT	51(47.22)	201(40.52)	0.440
	TT	44(40.74)	229(46.16)	
rs1453559	G	69(31.94)	300(30.24)	0.623
	A	147(68.06)	692(69.76)	
	GG	11(10.18)	51(10.28)	
	AG	47(43.51)	198(39.91)	0.777
	AA	50(46.29)	247(49.79)	
rs12150079	A	55(25.46)	228(22.98)	0.436
	G	161(74.54)	764(77.02)	
	AA	5(4.63)	31(6.25)	
	AG	45(41.66)	166(33.46)	0.253
	GG	58(53.70)	299(60.28)	
rs2872507	A	70(32.41)	298(30.04)	0.493
	G	146(67.59)	694(69.96)	
	AA	10(9.26)	51(10.28)	
	AG	50(46.29)	196(39.52)	0.429
	GG	48(44.44)	249(50.20)

Fig. 1. Changed transcription factor binding sites (TFBS) of rs1453559. G>A mutation was marked by red letter. TFBS were shown below the gene sequence. If mutant A was carried, transcription factor RXR-alpha could not bind to this site.

at transcription factor binding sites (TFBS). For rs1453559, transcription factor of RXR- α could bind on this site when nucleotide A was located. If mutant G was carried, this TFBS was predicted not to exist (Fig. 1). As shown in Fig. 2 for rs12150079, if nucleotide G was changed into A, two new TFBS of GATA-1 and GATA-2 would appear. Rs907091 was a miRNA binding site, which was predicted to be targeted by miRNAs of hsa-miR-326, hsa-miR-330-5p and hsa-miR-450b-3p when nucleotide T was present. However, only hsa-miR-326 could target this locus if nucleotide T was changed into C (Fig. 3). This indicates that polymorphisms of these sites could change the transcription efficient of this gene.

Discussion

AITDs may share some common genetic and environmental etiology, such as Th1/Th2 dysregulations and susceptible genes like *CTLA-4*. It is more likely to manifest familial aggregation among the patient of GD or HT. SNP is the variation of base sequence in genome. Therefore, a changed genotype could exert an influence on receptor's binding or protein structure, thus affect the efficiency of gene transcription. As a result, SNP could arouse the susceptibility of certain diseases due to the changed biological property of genotype. In our research, we hypothesized that the SNPs within *IKZF3* gene could be associated with the occurrence of GD or HT. *IKZF3* has been proven to exert a critical role in the development of immunocytes. This protein is composed of 509 amino acids, therefore forms homodimers, or forms heterodimers with the Ikaros. *IKZF3* deficient mice will present chronically activated B lymphocytes as well as spontaneous production of autoantibodies [16]. Several isoforms of *IKZF3* have been observed in normal or leukaemic B cells [17]. Recent research reported that the protein encoded by *IKZF3* gene could induce the expression of immunosuppressive enzymes [18]. Recently, more and more variants of *IKZF3* were successfully identified in a series of autoimmune diseases.

To our knowledge, this is the first research focused on the relationship between *IKZF3* polymorphisms and the susceptibility with AITDs. Our results showed that the minor alleles of rs2941522, rs907091, rs1453559, rs12150079 and rs2872507 were statistically associated with an increased risk in GD patients, but not in HT patients. As we known, the feature of GD is hyperthyroidism, which is due to the overproduction of thyroid hormone induced by specific autoantibodies against TSHR, and B cell is a key regulator of GD. As the most common cause for hypothyroidism, HT, on the other hand, is characterized by lymphocytic infiltration and TPOAb/TgAb overproduction. Discrepancies exist in the lymphocytic profiles [19] and the Fc receptors expressed on B cells [20] between the two diseases. HT is primarily a T lymphocyte mediated disease, and whether B cells play a pathogenic role is still unclear [21].

Fig. 2. Changed transcription factor binding sites (TFBS) of rs12150079. G>A mutation was marked by red letter. TFBS were shown below the gene sequence. If nucleotide G was changed into A, two new TFBS of GATA-1 and GATA-2 would appear.

Fig. 3. Changed miRNA binding sites of rs907091. T>C mutation was marked by red letter. If mutant C was carried, this miRNA binding region could not be targeted by hsa-miR-330-5p or has-miR-450b-3p.

Given the essential role of *IKZF3* in the regulation of B cells' differentiation and proliferation, it can be presumed that *IKZF3* may be an important element for the pathogenesis of GD rather than HT.

Rs1453559 is located in the 5'UTR, an important area which regulates the transcription efficiency of genes [22]. This polymorphism has been considered as a promising disease-related gene of PBC [15] and asthma [12] in Canadian and Italian [15] or the British [12] populations, suggesting that rs1453559 variant might contribute to autoimmune diseases. Taken our results together, it can be proved that rs1453559 A>G was a susceptible factor for GD. Similarly, the variant of rs12150079 has been reported to be associated with other established Type 1 Diabetes (T1D) SNPs in 419 T1D children [23]. Our results showed that the minor A allele and AA genotype were increased in GD, which were consistent with Cai's report that rs12150079 contributed to the etiology of SLE in Chinese Han population [8]. As autoimmune diseases might share some genetic predispositions, the B-cell affecting gene, *IKZF3* could lead to the occurrence of GD.

Rs907091 is located in the 3'UTR, a region which can be recognized and combined by certain miRNA or siRNA, thereby influencing the mRNA transcription and translation. The aberrant profile of miRNA expressions was proved to be a risk factor for GD [24], though the role of rs907091 has not been clearly illustrated. Recently, rs907091 was also referred to as an expression quantitative trait locus for *IKZF3* in lymphoblastoid cell lines [25]. This SNP has been indicated as a susceptible factor for multiple sclerosis and SLE. It has been observed in Keshari's research that rs907091 was in strong linkage with the multiple sclerosis in Norwegian patients [26]. In consistent with our research, a study confirmed rs907091 was associated with SLE in the Chinese Han population. However, the frequency of C allele and CC genotype appeared to be much lower in the case group [8], which indicated that the mutant genotypes were associated with a lower incidence of disease, in contrast with a higher one in our study. As for rs2872507, a recent GWAS study [27] reported its correlation with Crohn's disease and Type 2 Diabetes (T2D). Another GWAS meta-analysis study of 16,659 RA patients and 49,174 controls reported that rs2872507 surpassed genome-wide significance [11]. However, in the previous SLE cohort [8], this variant did not meet statistical significance. These results suggested the heterogeneity of autoimmune disorders and the *IKZF3* could play different roles in these diseases.

We investigated the association between genotype and phenotype. The female GD patients are more likely to carry more mutant alleles than female controls. In male GD group, the frequency of mutant alleles is also higher than that in male control group, though it did not show a statistical significance. We also noticed that *IKZF3* SNPs showed no difference between patients with Graves' ophthalmopathy (GO) and without GO. A number of studies have proved that the polymorphisms of some genes, such as TSHR and HLA-DR3, could induce GO or thyroid-associated ophthalmopathy (TAO). However, our results suggest *IKZF3* may not be a susceptible gene for GO. It is known that GO has some unique pathogenesis compared with GD, nevertheless, *IKZF3* is probably not the gene functioning as a *divide* for GD and GO. Therefore, more researches about *IKZF3* genotypes with clinical manifestations are required to reveal the underlying mechanisms.

In the previous publications, the sample size of mutant homozygotes was too small, which might lead to a biased result. Therefore, although the homozygotes analysis has many advantages, the heterozygote analysis could be more reliable. Our study was able to recruit a large population including relatively sufficient homozygote carriers, which avoided the bias caused by small sample size. The statistical power was strong enough (larger than 0.8) for assessing the relationship between GD patients and controls. What is more, the sample size of patients and controls was large enough to reduce the type I and II errors.

It is also worthwhile to show the limitations in our study. First of all, the five SNPs of *IKZF3* were selected from a linkage disequilibrium block, which provided limited informations, thus cannot fully represent the feature of *IKZF3* gene. The other locus of *IKZF3* was also reported to be associated with autoimmune diseases. Rs12946510 was reported to be an expressional quantitative trait loci (eQTL) of *IKZF3*, which increased the susceptibility with inflammatory

bowel diseases [28]. Future studies using gene sequencing analysis could provide further evidence of *IKZF3*. Besides, the associations between *IKZF3* polymorphisms and GD, as well as the functional predictions, cannot prove that these SNPs are the true functional variant of the disease. In addition, the roles of the five SNPs have not been completely understood. Therefore, further functional studies are required to reveal the pathomechanism of *IKZF3* polymorphisms.

Conclusion

Our study firstly reported that the polymorphisms of rs2941522, rs907091, rs1453559, rs12150079 and rs2872507 in *IKZF3* are associated with the susceptibility of GD, but not with that of HT. These findings could help to boost the cognition of autoimmune thyroid diseases.

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Author Contributions Statement: Ling Li wrote the main manuscript text. Ling Li and Xiao Lian Ding screened this gene out of susceptibility genes. Xuan Wang, Qiuming Yao and Xiaoqing Shao identified the variant loci. Xiaofei An, Ni Yan, Yanfei Jiang, Wen Wang, Liangfeng Shi and Qiu Qin accomplished DNA extraction and purification. Ronghua Song helped with calculations using Haploview software. Jin-an Zhang* and Peilong Sun* designed this study. All authors have reviewed the manuscript.

The work has not been published previously.

Ethical Approval: This study received the approval of Institutional Reviewer Board of Jinshan Hospital of Fudan University. All experiments were performed in accordance with relevant guidelines and regulations.

Disclosure Statement

The authors declared no conflict of interest.

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