Robust evidence for five new Graves’ disease risk loci from a staged genome-wide association analysis

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Graves’ disease (GD), characterized by autoantibodies targeting antigens specifically expressed in thyroid tissues causing hyperthyroidism, is triggered by a combination of genetic and environmental factors. However, only a few loci for GD risk were confirmed in the various ethnic groups, and additional genetic determinants have to be detected. In this study, we carried out a three-stage study in 9529 patients with GD and 9984 controls to identify new risk loci for GD and found genome-wide significant associations in the overall populations for five novel susceptibility loci: the GPR174-ITM2A at Xq21.1, C1QTNF6-RAC2 at 22q12.3–13.1, SLAMF6 at 1q23.2, ABO at 9q34.2 and an intergenic region harboring two non-coding RNAs at 14q32.2 and one previous indefinite locus, TG at 8q24.22 (Pcombined < 5 × 10−8). The genotypes of corresponding variants at 14q32.2 and 8q24.22 were correlated with the expression levels of C14orf64 and a TG transcript skipping exon 46, respectively. This study

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INTRODUCTION

Graves’ disease (GD) is one of the most common autoimmune disease with a prevalence of hyperthyroidism up to 1.3% in the USA (1) and 0.25–1.09% in China (2). Genetic factors play an important role in the pathogenesis of GD, and recent data from twin studies suggest that up to 79% of the risk for the development of GD could be attributable to genetic factors (3). There is compelling evidence for the association of seven bona fide loci with GD in various ethnic populations. These seven loci included the immune-related genes at 6p21 (MHC), 2q33 (CTLA-4), 5q32 (SCGB3A2), 20q12 (CD40), 1p13 (PTPN22) and 1q23.1 (FCRL3), and the thyroid-specific gene at 14q31 (TSHR) (4–8). Recently, two new risk loci for GD, at 6q27 (RNASET2-FGFR1OP-CCR6) and 4p14 (GDCG4p14), were identified in the Chinese population in our previous genome-wide association study (GWAS) and confirmed in Polish and UK Caucasians (8–10). More recently, Cooper et al. (10) found seven new risk loci for autoimmune thyroid disease (AITD) using ImmunoChip in the UK population. These loci only partially explain the familial clustering of GD, however, suggesting additional genetic determinants have to be detected.

It is well known that GD and the other autoimmune diseases, including type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus and so on share some susceptibility genes, such as MHC, CTLA-4 and PTPN22 (11–13). However, the extent of the susceptibility genes shared among the different autoimmune diseases still remained unclear. Also, GD has a sex difference in prevalence, with a female preponderance. Recent results from a few studies indicate that women with AITD have an enhanced X-monosomy rate in peripheral blood mononuclear cells (PBMCs) and skewed X-chromosome inactivation (XCI), which are associated with an increased risk of developing AITD (14,15).

Built upon our previous GWAS data on 1536 GD cases and 1516 controls (8), the present study was extended to a three-stage design to identify new risk loci for GD in 9529 GD patients and 9984 controls in the Chinese Han population. We identified five new risk loci of GD, including one novel locus on chromosome X and one intergenic locus harboring non-coding RNAs.

RESULTS

Analyses of genome-wide association, replication and combined data

In the first stage, we revisited the GWAS data in our previous GWAS and carried out the imputation analysis. After stringent quality control, we re-analyzed 483 947 genotyped SNPs in 1442 GD cases and 1468 controls using the Cochran–Armitage trend test (Fig. 1A) and analyzed 8 019 905 imputed SNPs using the SNPTEST V2 software. Based on the analysis for the genotyped SNPs in the first stage, we removed the susceptibility regions replicated in the previous GWAS paper (8) and further genotyped 125 SNPs in 2212 GD patients and 2136 sex-matched controls for the second-stage replication since the genotyped SNPs in distinct region had priorities over the imputed SNPs for further study (Table 1, Supplementary Material, Tables S1 and S2 and Fig. 1B). Among the 125 SNPs, 16 SNPs with \( P < 0.0001 \) in the GWAS stage (\( P_{\text{GWAS}} \)) and 67 SNPs with \( 0.0001 \leq P_{\text{GWAS}} < 0.01 \) as well as with the strengthened association with GD in at least one of the following GD sub-phenotypes—thyroid-stimulating hormone receptor autoantibodies (TRAb) levels, the onset age of GD with a cut-off of 40 years old and specific sex (data not shown since we did not confirm these sub-phenotype association in the replication stage)—were selected for the second-stage study. Given the association of TG locus with GD has been controversial for many years, six SNPs with \( P_{\text{GWAS}} < 0.01 \) in the TG-WISPI region were also selected for the second-stage study. Meanwhile, 36 out of 125 SNPs with \( P_{\text{GWAS}} < 0.05 \) as well as in the susceptibility region of autoimmune diseases in previous reports were selected and genotyped in the second stage (Supplementary Material, Table S2) (16).

Next, we selected 11 SNPs (rs1899982 was excluded due to difficulty in probe synthesis) with \( P < 0.05 \) in the second stage (\( P_{\text{2nd}} \)) for our third-stage analysis in 5781 GD cases and 6332 controls based on the sample size calculations using QUANTO (Table 1, Supplementary Material, Table S3 and Fig. 1C). In addition, three SNPs in BACH2 and ABO and showing weak associations with GD in a total of 5530 GD patients and 5026 controls in our previous GWAS (rs9344996: \( P = 7.68 \times 10^{-6} \); rs8176746: \( P = 4.56 \times 10^{-5} \); rs505922: \( P = 8.37 \times 10^{-6} \), respectively) (8), were further genotyped in 4222 GD patients and 4614 controls (Table 1 and Fig. 1C).

Ultimately, seven SNPs located in six chromosome regions showed unequivocal evidence of association with GD, using a genome-wide significance threshold of \( P = 5 \times 10^{-8} \), and one more SNP on 22q13.1 nearly met the genome-wide significance level (\( P = 6.40 \times 10^{-8} \); Fig. 2; Table 2). Of them, five new GD risk loci were identified and one controversial locus, TG region on 8q24.22, was confirmed (Table 2). In addition, there was relatively weak evidence of the association between BACH2 on 6q15 or KLF13 on 15q13.3 and the susceptibility of GD (rs9344996, \( P = 1.90 \times 10^{-5} \); rs4779520, \( P = 3.55 \times 10^{-5} \); Table 2).

Identification of five novel loci and one indefinite locus to GD risk

Among the five new risk loci of GD, the most significant association was detected at rs5912838 on Xq21.1 (Fig. 2). In the original scan, among 25 genotyped and 510 imputed SNPs located in the \( \sim 350 \) kb high-linkage disequilibrium (LD) region at Xq21.1 containing GPR174 and JTM2A, rs5912838 was one of the best association signal and no other SNPs with \( P_{\text{GWAS}} < 0.05 \) was independently associated with GD after accounting for rs5912838 (\( P_{\text{GWAS}} = 6.09 \times 10^{-5} \); Fig. 3A and B; Table 2; Supplementary Material, Table S4). We confirmed...
the association of rs5912838 with GD in the second and third stages \( P_{2nd} = 3.78 \times 10^{-8} \); \( P_{3rd} = 4.55 \times 10^{-12} \); \( P_{combined} = 2.33 \times 10^{-33} \), odds ratio (OR) = 0.76; Table 2 and Fig. 2], rs5912838 is located between GPR174 and ITM2A. Interestingly, restriction fragment-length polymorphism (RFLP) revealed that the mRNA transcripts harboring the heterozygote genotype AC of rs1751094 in ITM2A could be digested into the two different bands and those harboring the genotype TC of rs3827440 in GPR174 solely had one-band digested products, suggesting ITM2A, instead of GPR174, was found to escape from XCI in the PBMCs (Fig. 3C and D).

Table 1. Description of the sample sets in the current study

<table>
<thead>
<tr>
<th>Genotyping stage</th>
<th>Genotyped SNPs no.</th>
<th>Disease status</th>
<th>No.</th>
<th>Sex ratio (M/F)</th>
<th>Age at examination (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GWAS</td>
<td>655,214 (8)</td>
<td>GD</td>
<td>1536</td>
<td>370/1166</td>
<td>39 ± 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>1516</td>
<td>369/1147</td>
<td>45 ± 9</td>
</tr>
<tr>
<td>The second replication</td>
<td>125</td>
<td>GD</td>
<td>2212</td>
<td>451/1761</td>
<td>39 ± 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>2136</td>
<td>467/1669</td>
<td>49 ± 12</td>
</tr>
<tr>
<td></td>
<td>3* (8)</td>
<td>GD</td>
<td>3994</td>
<td>842/3152</td>
<td>39 ± 15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>3510</td>
<td>641/2869</td>
<td>48 ± 12</td>
</tr>
<tr>
<td>The third replication</td>
<td>11 b</td>
<td>GD</td>
<td>5781</td>
<td>1438/4343</td>
<td>40 ± 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>6332</td>
<td>1500/4832</td>
<td>46 ± 14</td>
</tr>
<tr>
<td></td>
<td>3*</td>
<td>GD</td>
<td>4222</td>
<td>1166/3056</td>
<td>40 ± 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>4614</td>
<td>1142/3472</td>
<td>46 ± 14</td>
</tr>
<tr>
<td>Total</td>
<td>11 b</td>
<td>GD</td>
<td>9529</td>
<td>2269/7260</td>
<td>39 ± 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>9984</td>
<td>2338/7646</td>
<td>47 ± 13</td>
</tr>
<tr>
<td></td>
<td>3*</td>
<td>GD</td>
<td>9752</td>
<td>2408/7344</td>
<td>39 ± 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>9640</td>
<td>2147/7493</td>
<td>47 ± 14</td>
</tr>
</tbody>
</table>

M, male; F, female.
*Three SNPs in the BACH2 and ABO genes genotyped in a total of 5530 GD patients and 5026 controls in our previous GWAS (8) were genotyped in the third replication cohorts.
**Eleven SNPs with \( P_{2nd} < 0.05 \) were genotyped in the third replication cohorts.

Figure 1. The flowchart for quality filtering in the three-stage study. (A) Quality filtering in the initial GWAS stage on SNPs and samples before analysis to ensure robust association tests. Of the 655,214 markers assayed, all SNPs on the Y and mitochondrial chromosomes or CNVs and Illumina controls were excluded, leaving 652,029 autosomal and X chromosome SNPs for further analysis. We further excluded SNPs with a low call rate (<98%), an MAF (<0.01), significant deviation from the Hardy–Weinberg equilibrium in the controls \( P \leq 10^{-6} \), or identity-by-descent >0.125. (B and C) No SNPs with call rates of <98% were removed and samples with call rates <98% were also excluded from further analysis in the second and third stages of the study.
GD-risk variant ($P_{\text{GWAS}} = 0.003$; $P_{\text{2nd}} = 0.035$; $P_{\text{3rd}} = 2.07 \times 10^{-6}$; $P_{\text{combined}} = 5.43 \times 10^{-9}$; OR = 0.89; Figs 2 and 4A; Table 2; Supplementary Material, Table S5). There is no annotated gene located in the $\sim 500$ kb LD region surrounding rs1456988; however, two novel full-length cDNAs [1366 and 1097 bp were designated as C14orf64 and GD Candidate Gene at 14q32.2 (GDCG14q32.2), respectively] without predicted open reading frames of more than 100 amino acids were further cloned from Jurkat cells by 5’ and 3’ rapid amplification cDNA ends (Fig. 4B). C14orf64 located 44 kb upstream, whereas GDCG14q32.2 located 114 kb downstream of rs1456988. Interestingly, the expression of these two non-coding RNAs was abundant in the immune-related tissues, such as thymus and PBMC, especially in CD4+ and CD8+ T cells (Fig. 4C and D). The genotypes of rs1456988, however, were correlated with C14orf64 expression levels in CD4+ subsets of PBMCs isolated from healthy volunteers ($P_{\text{ANOVA}} = 0.0003$), but not in PBMCs and CD8+ subsets (Fig. 4E). Additionally, the rs1456988 genotypes were correlated with the expression of GDCG14q32.2 neither in PBMCs nor its CD4+ and CD8+ subsets (Fig. 4F).

A solid evidence for the association of chromosome 22q12.3–13.1 region with GD is provided in the current study. In the first stage of our study, we identified 81 typed and 382 imputed SNPs in the $\sim 200$ kb region harboring C1QTNF6-RAC2 at 22q12.3–13.1 (Fig. 5A and Supplementary Material, Table S6). In the following second-stage cohorts, four SNPs in this region, which were located in the $< 100$ kb upstream/downstream region of the known autoimmune disease risk loci and showed no high LD ($r^2 < 0.70$), were selected for genotyping (Supplementary Material, Table S2). Then we selected two SNPs (rs229527 and rs2284038) with $P_{\text{2nd}} < 0.05$ and no LD ($r^2 = 0.03$; Supplementary Material, Table S7) for further genotyping in the third-stage cohorts (Table 2). The combined results revealed that the non-synonymous SNP rs229527 (Gly21Val) (exon 2 of C1QTNF6) showed the most significant $P$-value in the combined cohorts ($P_{\text{3rd}} = 1.06 \times 10^{-15}$; $P_{\text{combined}} = 4.85 \times 10^{-20}$, OR = 0.81; Fig. 2 and Table 2). Intriguingly, rs2284038 on RAC2 at 22q13.1 nearly met the genome-wide threshold in the combined population ($P_{\text{combined}} = 6.40 \times 10^{-8}$, OR = 0.89; Fig. 2; Table 2). After accounting for rs229527, rs2284038 was still statistically significant in the combined analysis ($P = 0.0002$; Supplementary Material, Table S7). Another new risk locus identified in this study, rs1265883, is located in 1q23.2–23.3 harboring SLAMF and CD244. Among 62 genotyped and 791 imputed SNPs in the $\sim 370$ kb region at 1q23.2–23.3 harboring SLAMF and CD244 in the first stage, 2 SNPs, rs1265883 and rs2779800, were selected to be genotyped in the second stage (Fig. 5B and Supplementary Material, Tables S2, S3 and S8). Only the former SNP showed the statistical significance in the second-stage study ($P_{\text{2nd}} = 0.0095$; Table 2). The association of rs1265883 with GD was confirmed in the
Table 2. GD susceptibility loci confirmed by three-stage association analysis

<table>
<thead>
<tr>
<th>Chr.</th>
<th>SNP</th>
<th>Chr. position</th>
<th>Annotated</th>
<th>Risk GWAS cohort (1442 versus 1468)</th>
<th>Second cohort (2187 versus 2127)</th>
<th>Third cohort (5704 versus 6224)</th>
<th>Combined cohort (9333 versus 9819)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Combined cohort (P-value)</td>
<td>OR (95% CI)</td>
<td>Combined cohort (P-value)</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>10</td>
<td>SLAMF6 rs11744974</td>
<td>96</td>
<td>97</td>
<td>1.25 (1.12–1.39)</td>
<td>0.003</td>
<td>0.40</td>
<td>0.0110</td>
</tr>
<tr>
<td>6q15</td>
<td>rs9344996</td>
<td>90</td>
<td>92</td>
<td>0.24 (0.20–0.30)</td>
<td>0.004</td>
<td>0.28</td>
<td>0.0092</td>
</tr>
<tr>
<td>8q24.22</td>
<td>rs4301434</td>
<td>134</td>
<td>136</td>
<td>0.24 (0.20–0.30)</td>
<td>0.004</td>
<td>0.28</td>
<td>0.0092</td>
</tr>
<tr>
<td>8q24.22</td>
<td>rs2294025</td>
<td>134</td>
<td>145</td>
<td>0.78 (0.74–0.82)</td>
<td>0.02</td>
<td>0.36</td>
<td>2.33</td>
</tr>
<tr>
<td>22q12.3</td>
<td>rs2294025-TG</td>
<td>37</td>
<td>581</td>
<td>4.27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22q13.1</td>
<td>rs2289896-TG</td>
<td>37</td>
<td>635</td>
<td>4.27</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>KLF13</td>
<td>intergenic</td>
<td>22</td>
<td>23</td>
<td>0.78 (0.74–0.82)</td>
<td>0.02</td>
<td>0.36</td>
<td>2.33</td>
</tr>
<tr>
<td>TG 10</td>
<td>BACH2</td>
<td>4.27</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Recently, ABO has been associated with a plethora of diseases, including myocardial infarction, and plasma levels of inflammatory biomarkers (18–22). Here, we provide the first description, to our knowledge, of a significant genome-wide association between ABO and GD, notwithstanding we could not completely exclude the possibility of the effect of population stratification. The data from 37 typed and 522 imputed SNPs in the ~150 kb LD region with ABO revealed that rs505922 was one of the most probable GD-risk variants (Fig. 5C and Supplementary Material, Table S9). We selected two SNPs, rs8176746 and rs505922, that can be used to infer ABO blood types for further genotyping in the third stage (Table 3) (19–22). Among the two SNPs, the A allele of rs8176746 tags the B blood-group allele, the T allele of rs505922, which is a proxy for rs657152, tags the O blood-group allele and the haplotype CC of these two SNPs tags the A blood-group allele (Table 3). The association of rs505922 with GD risk was confirmed in the combined analyses (\( P_{\text{combined}} = 2.45 \times 10^{-10}, OR = 0.88 \); Table 2; Fig. 2). In the overall population, the frequency of the type-O blood group was higher among the GD patients than among the controls (32.6 versus 27.7%; \( P = 4.27 \times 10^{-9} \); Table 3).

Finally, we confirmed an indefinite risk locus, TG, at 8q24.22 as a susceptibility locus of GD in this study. Given the association of TG locus with GD has been controversial for many years, among the 88 typed and 720 imputed SNPs distributed in the ~340 kb region at 8q24.22 harboring TG, SLA and WISP1, 6 SNPs (except rs3739262 due to difficulty in probe synthesis) with \( P_{\text{GWAS}} < 0.01 \) were selected and genotyped in 2187 patients with GD and 2127 healthy controls (Fig. 6A and Supplementary Material, Tables S1, S3 and S10). Four out of six SNPs with significant \( P \)-values in the second cohorts (\( P_{\text{2nd}} < 0.05 \)) were then genotyped in the third-stage cohorts (Table 2 and Supplementary Material, Table S3). Finally, two SNPs with high LD (\( r^2 = 0.78 \) in the combined controls) reached the genome-wide significance in the combined analyses (rs2294025: \( P_{\text{combined}} = 8.09 \times 10^{-9}, OR = 1.16 \); rs4736437: \( P_{\text{combined}} = 1.14 \times 10^{-9}, OR = 1.15 \); Fig. 2; Table 2). After accounting for rs2294025, no other SNPs on 8q24.22 were independently associated with GD in the combined population (Supplementary Material, Table S7). However, rs2294205 added could not significantly improve the model containing rs4736437 (Supplementary Material, Table S7).

Notably, the \textit{in vitro} expression of the full-length TG transcript (with-E46 TG) was 30.5 and 10.5 times higher than that of the TG transcript skipping exon 46 (non-E46 TG) in the cells transfected with pDUP4-rs2294205-CC and pDUP4-rs2294205-TT vectors, respectively (\( P = 0.002 \) and \( P = 0.009 \), respectively; Fig. 6B–D and Supplementary Material, S1A–TG isofrom in HEK-293 cells transfected with pDUP4-rs2294205-TT was higher than that in HEK-293 cells transfected with pDUP4-rs2294205-CC (\( P = 0.003 \); Fig. 6D). The data \textit{in vitro} indicated that the TT allele at rs2294205 could influence the splicing of TG, leading to the increased expression of non-E46 TG isoforms. Interestingly, the real-time PCR results \textit{in vivo} revealed that the expression of the with-E46 TG transcript was about 30 times greater than that of the non-E46 TG transcript in human thyroid tissues (\( P = \) 5.27 \( \times 10^{-33} \)), and the rs2294205 genotypes were correlated with the expression of the non-E46 TG isoforms.
Figure 3. Regional plot of GD association at Xq21.1 and conformation of XCI. (A) GD association of genotyped and imputed SNPs at Xq21.1 in the GWAS samples. The color of each genotyped SNP spot reflects its $r^2$, with the top SNP within each association locus shown as a large red diamond, and smaller values changing from red to white. Genetic recombination rates are shown in cyan. (B) Two-locus conditional logistic regression results at Xq21.1 accounting for rs5912838 from the GWAS scan. The SNPs at Xq21.1, improved by adding rs5912838, are shown in red points, whereas the SNPs improved the model with rs5912838 are shown in blue triangles. (C and D) Results of XCI for the two candidate genes, GPR174 (C) and ITM2A (D), detected by RFLP PCR methods. 1: digested product amplified from genomic DNA; 2: undigested product amplified from cDNA of PBMCs; 3: digested product amplified from cDNA of PBMCs.
isoform, rather than the with-E46 TG isoform ($P_{ANOVA} = 7.62 \times 10^{-12}$; Fig. 6E). However, the genotypes of rs2294025 were not correlated with the expression of SLA in PBMCs (Supplementary Material, Fig. S1D).

Pathway analysis

To further investigate how the GD susceptibility genes were involved in the pathophysiology of GD, we performed the pathway analysis for a total of 16 risk genes of GD using the Ingenuity database (release IPA 6.0) and built the possible functional relationships between these genes on the basis of direct and indirect interactions. A single network of 23 genes, including 14 out of the 16 risk genes, was identified through unsupervised network analysis (Fig. 7). Interestingly, among the 14 risk genes of GD, 4 risk genes (TSHR, RNASET2, PTPN22 and RAC2) were interacted with the cytokines of Th1, such as INFγ and IL2; two genes (C1QTNF6 and SCGB3A2) were interacted with the cytokines of Th2, such as IL4 and IL10; eight genes (MHC, SLAMF6, TG, ITM2A, CD40, CTLA4, ABO and FCRL3) were linked to altered production of both the cytokines of Th1 and Th2 (Fig. 7).
Figure 5. Regional plots of association at three GD risk loci. (A–C) GD association of genotyped and imputed SNPs at 22q12.3–13.1 (A), 1q23.2–23.3 (B) and 9q34.2 (C) in the GWAS samples. The color of each genotyped SNP spot reflects its $r^2$, with the top SNP within each association locus shown as a large red diamond, and smaller values changing from red to white. Genetic recombination rates are shown in cyan.
DISCUSSION

Identification of genes or regulatory pathways associated with complex diseases such as GD is of key importance in the understanding of the genetic architecture for disease predisposition. The knowledge generated should be valuable in designing new preventive and/or therapeutic strategies. Through a three-stage study in ~20,000 subjects, we identified five novel GD risk loci and confirmed one controversial locus, TG, on 8q24.

An interesting finding, for the first time, in this study, is the associations between rs5912838 at Xq21.1 and GD. Although a preponderance of GD risk among the females is beyond doubt, the mechanism remains elusive. It is reasonable to hypothesize that risk locus/loci on X chromosome can underlie the female preponderance of GD. Although the existence of GD risk loci on X chromosome was suggested by earlier studies based on linkage analysis, the exact location has not been confirmed (23,24). Our GWAS data also did not provide a robust evidence for the association of FOXP3 on Xp11.23, which was reported as a GD risk gene in the Caucasian cohort (25). Interestingly, we found that rs5912838 on Xq21.1 was a risk variant for GD in the Chinese Han population. rs5912838 lies between GPR174 and ITM2A. Notably, ITM2A, which has been reported to escape XCI and exhibits different phenotypic effects in females and males (26–28), was also verified to escape from XCI in PBMCs in our study. So far, little is known about GPR174, which encodes a protein belonging to the G protein-coupled receptor superfamily. However, ITM2A is induced during thymocyte selection and T cell activation (29). Overexpression of ITM2A in mice results in partial down-regulation of CD8 in CD4+CD8+ double-positive thymocytes, and a corresponding increase in the number of CD4+CD8+ thymocytes (29). These data suggested that ITM2A may be involved in the etiopathology of GD. Although our data support ITM2A as the risk gene of GD at Xp11.23, we cannot formally exclude possible roles for GPR174 or additional undetected sequence elements in this critical region in contributing to GD susceptibility.

Of note, a region harboring two novel, long non-coding RNAs at 14q32.2 was identified to be associated with GD in this work. Previous reports revealed 14q32.2 was associated with T1D (17). In current study, we found that rs1456988 on 14q32.2 was associated with GD. rs1456988 located in a gene desert region. However, two novel, long non-coding RNAs (C14orf64 and GDCG14q32.2) surrounding rs1456988 were identified from Jurkat cells. Both of them were abundantly expressed in the immune-related tissue such as thymus and PBMC. Interestingly, the genotypes of rs1456988 were correlated with the expression of C14orf64 in CD4+ T cells. Only one previous study with relatively small-sized samples indicated a variant, located in the intron of ZFAT and the promoter region of small antisense transcript of ZFAT, was associated with GD in the Japanese population (30). These findings suggested that the long non-coding RNAs, as novel regulatory targets of SNPs, might be potentially involved in controlling risk or resistance to GD.

In fact, except for rs1456988 at 14q32.2, two other loci (proxy SNPs: rs229527 and rs2284038 at 22q12.3–13.1 and rs165883 at 1q23.2), reportedly associated with autoimmune diseases, were also associated with GD in the present study. C1QTNF6 and RAC2 at 22q12.3–13.1, which were reported to be associated with autoimmune disease, such as type 1 diabetes, vitiligo, multiple sclerosis and Crohn’s disease (31–34), were independently associated with GD in this study. Although the mechanism of how C1QTNF6 gene is involved in the autoimmune has been enigmatic, the human RAC2 gene, a member of the Ras superfamily of GTP-binding proteins, plays a critical role not only in the elicitation of immune responses, but also in the induction of peripheral immune tolerance (35,36), rs1265883 is located within intron 1 of SLAMF6 (CD352, encoding Ly108), which plays a pivotal role in T cell stimulation as a co-stimulatory molecule and may be involved in the pathogenesis of lupus in mice (37,38).

Chromosome 8q24.22 region containing TG, which has been reported to be associated with the susceptibility ofAITD, especially with Hashimoto’s thyroiditis, was not well validated in different ethnic populations yet (39–42). Six SNPs (rs180195, rs180223, rs853326, rs1153583, rs2256366 and rs2687836) in TG were suggested as GD-susceptibility variants (42–44); however, none of these SNPs was associated with GD in our original GWAS (Supplementary Material, Table S10). Previous researches have indicated that several susceptibility genes of autoimmune diseases have been identified as the target antigens in the corresponding patients, such as TSHR in patients with GD, insulin and ZnT8 in patients with type 1 diabetes (45–47). TG, specifically expressed in thyroid tissue, is a key autoantigen in AITD and TG autoantibodies presented in ~40–70% of patients with GD (48). A recent study reported that human TG cDNA immunization may generate a model of Hashimoto thyroiditis, one type ofAITD, in mice (49). Moreover, it was reported that the Tg antibodies associated with AITD facilitated the formation of immune complexes, their binding to B cells and the proliferation of B and hTg-reactive T cells, demonstrating the association between the production of hTg-αAb and AITD progression (50).
More recently, Cooper et al. (10) also identified seven new loci for AITD, using a custom-made ImmunoChip, in the UK population. We compared our data with those in the study by Cooper et al. and found there were six SNPs excluding rs72928083 in BACH2 in our data (Supplementary Material, Table S1). Among the remaining six SNPs in six susceptibility regions, two SNPs showed association with GD in our first-stage GWAS scan (rs13093110: $P_{GWAS} = 0.0190$, and rs4409785: $P_{GWAS} = 0.0080$; Supplementary Material, Table S1). However, we could not completely exclude the association between the remaining five susceptibility loci and GD since a cluster of SNPs except the proxy SNPs showed $P_{GWAS} < 0.01$ in the five regions (data not shown since there was no replication for the data).

In summary, we identified five previously undetected GD risk loci, including a novel locus at Xq21.1 and a locus harboring long non-coding RNA. We also found that long non-coding RNAs might be potentially involved in the pathogenesis of GD. Collectively, these newly identified loci, along with other previously reported loci, demonstrate the growing complexity of the heritable contribution to GD pathogenesis. A complete genetic architecture will be helpful to understand the pathophysiology of GD once we have identified all susceptibility loci and etiological variant/s for each locus by fine-mapping.

**MATERIALS AND METHODS**

**Subjects**

All subjects were recruited from the Chinese Han population through the collaboration with multiple hospitals in China. This study was approved by the local ethics committee from...
Ruijin Hospital, the Central Hospital of Xuzhou, Linyi People’s Hospital, the First Affiliated Hospital of Bengbu Medical College, Medical School Hospital of Qingdao University, the Hospital Affiliated to Jiangsu University and Xin-Hua Hospital. And all subjects in this study provided written informed consent using protocols approved by the local ethics committee. We recruited 1536 GD patients and 1516 sex-matched controls, 2212 GD patients and 2136 sex-matched controls and 5781 GD patients and 6332 sex-matched controls for the first, second and third stages of the study, respectively (Table 1).

For the three SNPs in \textit{BACH2} and \textit{ABO} genotyped in a total of 5530 GD patients and 5026 controls in our previous GWAS (8), 4222 GD patients and 4614 sex-matched controls were further recruited for the third-stage study (Table 1). We collected 5 ml blood samples from each participant for DNA preparation and biochemical measurements.

Diagnosis of GD was based on documented clinical and biochemical evidence of hyperthyroidism, diffuse goiter and the presence of at least one of the following: positive TSH receptor antibody tests, diffusely increased 131I (iodine-131) uptake in the thyroid gland or exophthalmos (5,8,51). All individuals classified as having GD were interviewed and examined by experienced clinicians.

All the controls in the initial GWAS stage were individuals with neither GD nor family history of GD, and without any other autoimmune disorders. Control subjects were matched by sex with cases and were over 35 years old. Given that GD and other AITD have preponderance in the young female

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{A gene-interaction network of genes contributed to the risk of GD. An Ingenuity Pathways Analysis of the 16 risk genes identified a single, closely connected network of interacting genes, which consists of 14 out of the 16 risk genes to GD and 9 signal pathway genes. The lines between genes represent known interactions, with solid lines representing direct interactions and dashed lines representing indirect interactions. Among them, four risk genes, which are correlated to the cytokines of Th1 such as INF\textgamma and IL2, are highlighted in green; two genes, which are correlated to the cytokines of Th2 such as IL4 and IL10, are highlighted in red; eight genes, which are correlated to the cytokines secreted from both of Th1 and Th2, are highlighted in orange.}
\end{figure}
population, this age criteria could reduce the number of controls who might develop GD later on.

Genotyping and quality control in the first-stage GWAS scan

DNA samples from 1536 GD cases and 1516 controls were genotyped using Illumina Human660-Quad BeadChips at the Chinese National Human Genome Center in Shanghai, China (Table 1) (8). Genotype clustering was conducted using the Illumina GenomeStudio V2011.1 software based on the 660W-Quad_v1_H manifest files. This software, which is used to convert the fluorescence intensities into SNP genotypes, was different than the software used in the previously published GWAS paper (8). The mean call rate across all samples was 99.8%. Quality filtering was performed on SNPs and samples before analysis to ensure robust association tests. Cryptic relationships between genotyped individuals were examined using pairwise identity-by-descent estimation using the PLINK software (52). To maintain the maximum number of available samples, all the pairwise relationships were evaluated and the person who formed the node that related to the most other nodes in the family trees was first excluded. This process was iterated several times until the remaining samples were not related to one another.

Of the 655,214 markers assayed, 3185 that were from the Y or mitochondrial chromosomes or were CNV-related were excluded. Next, 168,082 markers with Hardy–Weinberg equilibrium $P \leq 10^{-6}$, with genotype call rates $<98\%$, or with a minor allele frequency (MAF) $<0.01$, were discarded, leaving 483,947 SNPs for subsequent analyses. After removing samples with low call rates ($<98\%$, $n = 23$), gender inconsistencies ($n = 6$) and cryptic relatedness ($n = 113$), 2910 samples were available for further association analysis (Fig. 1A).

SNP selection for the second-stage study

First, we defined the chromosomal regions based on HapMap recombination rates (the recombination rate $>30\text{cM/Mb}$), our GWAS data and LD information. In the previous GWAS paper, we focused on the majority of GD risk loci with strong association ($P_{\text{GWAS}} < 0.0001$) (8). Therefore, we tried to identify the new GD susceptibility loci from those with the middle association with GD in this study. Among 483,947 SNPs in the first stage, 5701 genotyped SNPs with $0.0001 < P_{\text{GWAS}} < 0.01$ were located in 253 non-MHC chromosomal regions. Next, we excluded the susceptibility regions replicated in the previous GWAS paper (8) and first selected the remaining 16 genotyped SNPs with $0.0001 < P_{\text{GWAS}} < 0.0001$ for the second-stage study (Supplementary Material, Table S1). Meanwhile, we further chose 66 SNPs with $0.0001 \leq P_{\text{GWAS}} < 0.01$ as well as with strengthened association with GD in at least one of the following GD sub-phenotypes: TRAb levels, the onset age of GD with the cut-off of 40 years old and specific sex (data not shown due to no confirmation for the sub-phenotype association in the replication stage), for the second-stage study (Supplementary Material, Table S1). Given the association of TG locus with GD has been controversial for many years, 6 SNPs (except rs3739262 due to difficulty in probe synthesis) with $P_{\text{GWAS}} < 0.01$ in the $TG$-$WISP1$ region were also selected and genotyped in the second-stage study (Supplementary Material, Table S1).

Finally, according to a GWAS database (http://jjwanglab.org/gwasdb) (16), 36 genotyped SNPs with $P_{\text{GWAS}} < 0.05$ as well as in the regions harboring the known autoimmune disease susceptibility loci were selected for the second-stage study (Supplementary Material, Table S2).

Sample size and power calculations

Sample size calculations were performed using QUANTO (version 1.2.4). Based on our GWAS data, we found that a minimum of 1644 (rs5912838) and a maximum of 10,635 (rs3765883) sample pairs would be needed, in order to detect the significant difference of the 125 selected SNPs at a genome-wide significance level for each variant with allele frequencies ranging from 0.01 to 0.5 and with at least 80% power. Therefore, we determined that the total sample pairs were $\approx 10,000$ in the current three-stage study. However, when the 125 selected SNPs were genotyped in $\approx 2000$ GD patients and 2000 controls, we further recalculated the sample sizes needed in the following replication stage according to the GWAS and the second replication cohort and power for different case–control pairs. The data showed that the sample pairs needed for the majority of SNPs with $P_{\text{2nd}} > 0.05$ of these 125 selected SNPs were $>10,000$. More specifically, 18 out of 21 SNPs with $0.05 < P_{\text{2nd}} < 0.2$ need $>10,000$ sample pairs for reaching the significant difference at a genome-wide significance level, and the ORs of other three SNPs of the 21 SNPs with $0.05 < P_{\text{2nd}} < 0.2$ were approximately 1 in the second-stage cohort (Supplementary Material, Table S3). However, 10 out of the 15 SNPs with $P_{\text{2nd}} < 0.05$ need $<10,000$ sample pairs for reaching the significant difference at a genome-wide significance level (Supplementary Material, Table S3). According to the above data, we selected SNPs with $P_{\text{2nd}} < 0.05$ for further genotyping in $\approx 6000$ sample pairs.

Genotyping and quality control in the replication study

In the second-stage study, 125 SNPs were genotyped using TaqMan SNP Genotyping Assays on the Fluidigm EP1 platform. All of the 125 SNPs had a genotype call rate $>98\%$ and did not significantly deviate from the Hardy–Weinberg equilibrium (Fig. 1B). After excluding the samples with a call rate $<98\%$, 2187 GD cases and 2127 controls were analyzed in the second cohort (Fig. 1B).

In the third-stage study, 14 SNPs were genotyped using the ABI 7900HT Fast Real-Time PCR System and no SNP was excluded according to the principles of quality control. After removing the samples with a call rate $<98\%$, the remaining samples were analyzed in the trend test (Fig. 1C).

Statistical analysis

After quality control (Fig. 1A), we analyzed the genotypes of 473 139 autosomal SNPs in 1442 GD patients and 1468 controls for association analysis using the Cochran–Armitage trend test in PLINK (52). Also, we analyzed 10,808 SNPs located in X chromosome, using the logistic regression in PLINK according to the following principles: for alleles A and a, males were coded A to 0 and a to 2, and females were coded AA to 0, Aa to 1 and aa
to 2, and additionally sex (male = 0, female = 1) was also automatically included as a covariate (52).

Genotype imputation was performed using the IMPUTE2 software (53) in the first-stage cohort, and the 1000G phase-I interim impute data (March 2012) were used as a reference. Of the imputed SNPs, we analyzed only those that could be imputed with a relatively high confidence (estimated probability > 0.9), had an MAF > 1%, a genotype call rate > 98% and a Hardy–Weinberg equilibrium P-value > 10−6. To take into account the uncertainty of imputed SNPs, the association analysis was carried out utilizing the SNIPTEST v2 software (frequency association tests with score method) (54).

For the autosomal SNPs, we utilized the Cochran–Armitage trend test in the second and third stages and Cochran–Mantel–Hanezel stratification analysis in the combined samples (52). We also examined heterogeneity among studies using the Breslow–Day test (52). For rs5912838 on X chromosome, we used the logistic regression analysis for the replication and combined cohorts (52). Conditional logistic regression analysis was used to test for independent effects of an individual SNP using PLINK (52).

The R package was used to generate the genome-wide P-value plot, and regional plots were generated using the SNAP version 2.2 software (55).

**Real-time PCR**

Blood samples (10 ml) were collected from 258 unrelated healthy Chinese Han volunteers for gene expression analysis in PBMCs. Samples with more blood volume (100 ml) were donated by 85 individuals from the same group for gene expression assay in distinct subpopulations of PBMCs. The CD4+ , CD8+ , CD14+ and CD19+ subsets of PBMCs were isolated using MACS Column kits (Miltenyi Biotec) according to the manufacturer’s instructions. The purity of each cell subpopulation was determined by an LSR II Flow Cytometer (BD Biosciences), and the cell subpopulations with a purity of >90% were used for real-time reverse transcription PCR (RT–PCR) (8). Seventy-five thyroid tissue samples were collected in multiple hospitals in China during surgery from patients with thyroid adenoma or multinodular goiter, but without hyperthyroidism. cDNAs were made from 1 µg RNA templates using reverse transcriptase and oligo(dT) primer (Promega). Real-time quantitative RT–PCRs for a series of genes or transcript units were performed in duplicate using the SYBR Green and ABI 7900HT Fast Real-Time PCR System. Expression levels in all samples were normalized to the relative expression level of GAPDH. Primers used for the amplifications are shown in Supplementary Material, Table S12. We performed statistical analysis of expression data using ANOVA and an unpaired Student’s t-test (the two-tail P-value was indicated on the figures). The genotypes of the SNPs were determined using the ABI 7900HT Fast Real-Time PCR System.

**Confirmation of XCI**

DNAs were extracted from the PBMCs of 23 unrelated healthy female volunteers. The corresponding RNAs were extracted and treated with RNase-Free DNase. The fragments surrounding rs3827440 in GPR174 and rs1751094 in ITM2A were amplified from DNA and cDNA samples and sequenced to detect XCI. The AvrII and EcoRI restriction endonuclease sites were induced at allele C of rs3827440 and allele A of rs1751094, respectively, by mutating one nucleotide on the corresponding primers, to further detect XCI by RFLP (Supplementary Material, Table S12).

**Cloning of new transcript units at 14q32.2**

Further bioinformatic analysis by inspection of the UCSC Genome Browser (http://genome.ucsc.edu) revealed two groups of split expressed sequence tags (ESTs) surrounding rs1456988. C14orf64, a reference gene without known function located 44 kb upstream of rs1456988, has four putative isoforms supported by different split ESTs. These four putative transcripts each contain three exons and share the first and second exons (Supplementary Material, Fig. S2A). Several pairs of primers were designed to amplify the four putative transcripts of C14orf64. Ultimately, only a transcript based solely on EST BC043585 was identified from Jurkat cells (a human T cell lymphoblast-like cell line) using RT–PCR. Although the specific primers for 5′ and 3′ rapid amplification cDNA ends of C14orf64 were designed on exon 2, which was shared by the four putative different transcripts, only one novel 1366 bp full-length cDNA was cloned from Jurkat cells by 5′ and 3′ RACE (Supplementary Material, Table S12). This cDNA was assembled by three exons and contained no open reading frame of >100 amino acids, suggesting that the novel transcript is a non-coding RNA (Fig. 4B).

Homology analysis implying that a region downstream of rs1456988 containing a group of split ESTs is highly conserved in humans and mammals suggests that a novel transcript may exist (Fig. 4B). Of note, a transcript with 1097 bp full-length cDNA was obtained from a Jurkat cell line by using RT–PCR and 5′ and 3′ RACE, and temporarily designated GDCG14q32.2. GDCG14q32.2, located 114 kb downstream of rs1456988, contained no putative open reading frame according to DNA strider analysis (Fig. 4B) (56).

**Cloning of new transcript and functional splicing assay of TG**

Since rs2294025 is located 202 bp upstream of exon 47 of TG, we hypothesized that rs2294025 may affect the splicing of TG, and especially the RNA splicing of exon 46 or 47. We designed a pair of nested RT–PCR primers to amplify a 504 bp fragment from exon 45 to exon 48 of TG (Supplementary Material, Fig. S1A and Table S12). Interestingly, besides a 504 bp fragment (with-E46 TG), an ~370 bp fragment was obtained from human thyroid tissues by nested RT–PCR and identified as a novel transcript skipping exon 46 of TG (non-E46 TG) by direct sequencing (Supplementary Material, Fig. S1A). Next, two pairs of real-time RT–PCR primers were designed to specifically detect the expression of the two isoforms, and the specificity of the primer pairs was confirmed by the sequencing of their corresponding PCR products (Supplementary Material, Table S12, and Fig. S1B and ).
ligated to a pGEMT-Easy vector. The insert with the wild-type CC or mutation TT genotype at rs2294025 was released from the pGEMT-Easy vector with the restriction enzyme ApaI/BamHI, and then ligated to the pDUP4 vector between ApaI and BgII cloning sites to construct the minigene pDUP4-rs2294025-CC or pDUP4-rs2294025-TT vector. The sequence of each insert was verified by direct sequencing. HEK-293T cells were grown in DMEM supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO2 incubator. Cells were seeded onto 12-well plates to 60% confluence and transfected with 1.6 μg of plasmid DNA per well using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocols. Forty hours after transfection, cells were collected and RNA was extracted for cDNA synthesis. Real-time RT–PCR was performed using two pairs of specific primers to amplify the non-E46 TG transcript and the with-E46 TG transcript (Supplementary Material, Table S12).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None declared.

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**REFERENCES**


APPENDIX

THE CHINA CONSORTIUM FOR THE GENETICS OF AUTOIMMUNE THYROID DISEASE MEMBERS

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