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Genome-Wide Meta-Analysis Identifies Novel Gender Specific Loci Associated with Thyroid Antibodies Level in Croatians

Short title: Novel Loci Associated with Thyroid Antibodies Level

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Abstract

Autoimmune thyroid diseases (AITD) are multifactorial endocrine diseases most frequently accompanied by Tg and TPO autoantibodies. Both antibodies have a higher prevalence in females and act under a strong genetic influence.

To identify novel variants underlying thyroid antibody levels, we performed GWAS meta-analysis on the plasma levels of TgAb and TPOAb in three Croatian cohorts, as well as gender specific GWAS and a bivariate analysis.

No significant association was detected with the level of TgAb and TPOAb in the meta-analysis of GWAS or bivariate results for all individuals. The bivariate analysis in females only revealed a genome-wide significant association for the locus near *GRIN3A* (rs4457391, $P=7.76 \times 10^{-9}$). The same locus had borderline association with TPOAb levels in females (rs1935377, $P=8.58 \times 10^{-8}$).

In conclusion, we identified a novel gender specific locus associated with TgAb and TPOAb levels. Our findings provide a novel insight into genetic and gender differences associated with thyroid antibodies.

Keywords: genome-wide association study, meta-analysis, thyroid antibody, thyroglobulin, thyroid peroxidase, single nucleotide polymorphism

Introduction

Thyroglobulin (Tg) and thyroid peroxidase (TPO) are major components of the thyroid gland, both engaged in the production of the thyroid hormones (1). Autoimmune thyroid diseases (AITD) are one of the most common autoimmune diseases, affecting 2-5% of the general population (2). The presence of circulating autoantibodies with specificity for Tg and TPO, might represent an early stage in the pathogenesis of AITD (3). Hashimoto thyroiditis (HT) and Graves' disease (GD) are autoimmune diseases in which the immune system turns against the thyroid gland. HT is characterised by destruction of thyroid gland and underproduction of thyroid hormones (hypothyroidism), whereas antibody stimulation of thyroid gland in GD results in overproduction of thyroid hormones (hyperthyroidism) (4, 5). The prevalence of TgAb and TPOAb positivity in the total and disease-free population is greater in females and increases with age, especially among females (6, 7). The prevalence of TgAb positivity is 60–80% in patients with HT and 30–60% in patients with GD. Positivity of TPOAb is detected in 90–95% patients with HT and 80% patients with GD (8).

Many genetic loci seem to be associated with multiple traits in human complex diseases and have the direct biological influence on more than one phenotypic trait (9). HT and GD have some unique loci, as well as some common to both diseases, indicating that there is a shared genetic susceptibility to HT and GD (10). Since various autoimmune diseases often cluster within the same patient, identifying the basis for this shared pathogenesis could be important not only for the fundamental understanding of AITD mechanisms but also in the understanding of other associated diseases (11, 12).

Thyroid antibodies are under the strong genetic influence. Autoimmune prevalence and clinical differences in thyroid function are known to be gender-related (6, 7, 13, 14). According to a twin study, the estimate of genetic influence on serum TgAb concentrations is 39% in males and 75% in females (15). For serum TPOAb concentrations, the estimates are 61% in males and 72% in females (15).

Until now, two genome-wide associations studies were performed on TPO antibody in general population, one in Caucasians (16), and the other in an Asian (Korean) population (17). Also, there is one meta-analysis (18) in which previous GWAS findings (16) were used as a basis for an identification of additional novel genetic variants. Those studies have reported the genome-wide association of several loci with TPOAb level and/or positivity, including variants near *TPO*, *HCP5*, *HLA-DPB1* and in *ATXN2*, *MAGI3*, *KALRN*, *BACH2*, *RERE*, *HLA-DOB* genes (16-18). Although the heritability of TPOAb accounts for around 70% (15), the identified risk loci for AITD accounts for only 4% of the heritability. The genetic association of TgAb has not been analysed on a genome-wide scale so far.

The aim of this study was to identify novel loci associated with thyroid antibodies. We performed genome-wide meta-analysis for TgAb plasma levels in 2629 individuals from three Croatian cohorts for the first time. Genome-wide meta-analysis for TPOAb plasma levels was also performed in 2618 individuals. In addition, we conducted bivariate analysis for these two

correlated traits (i.e., TgAb and TPOAb), gender specific GWAS as well as biological pathway analyses.

Methods

The study was carried out on samples from three Croatian populations: the mainland city of Split and the islands of Vis and Korčula. The samples were obtained from the large-scale project of “10,001 Dalmatians” (19). Cohorts’ description is reported in Table 1. We excluded participants with known thyroid pathologies, the ones that underwent thyroid surgery or were treated for thyroid conditions. After these exclusions, 2629 individuals were included in the analyses for TgAb level, and 2618 for TPOAB level. The study was approved by the Research Ethics Committees in Croatia and Scotland, and all participants provided informed consent.

Genotyping and Imputation

Genotyping platforms and quality control procedures are summarized in Table 2. SHAPEIT2 was used for genotypes pre-phasing, along with duoHMM for refine phasing (20, 21). Samples from Split cohort were collected and genotyped in two rounds (Split1 and Split2) with two different genotyping platforms (Table 2). Cohorts of Vis, Korčula, and Split2 were directly imputed using 1000 Genomes project phase I version 3, whereas for the imputation of Split1 a merged reference panel of 1000 Genomes and Split2 was used. For imputation of Split cohorts, we used SNPTEST, while for Vis and Korčula cohorts IMPUTE2 (22, 23). Variants with minor allele frequency $>5\%$, no significant deviation from HWE ($p > 10^{-7}$), and imputation info score > 0.4 were kept for further analysis. The final number of overlapping SNPs was 5 527 232.

Measurement of Tg and TPO antibodies

Plasma TgAb and TPOAb were determined by a sandwich chemiluminescence immunoassay method in the Laboratory of Biochemistry, Department of Nuclear Medicine, University Hospital Split. The immunoassay was conducted in a fully automated instrument "Liaison" Biomedica Chemiluminescence Analyzer, using LIAISON[®] Anti-Tg and LIAISON[®] Anti-TPO in vitro assays for the quantitative determination of TgAb and TPOAb in the plasma. The reference range of TgAb is 5-100 IU/mL, and for the TPOAb is 1-16 IU/mL.

Statistical analyses

Genome-wide association analyses

TgAb and TPOAb levels were adjusted for age and sex under a linear regression model. Derived residuals were inverse-normal transformed and included in the linear mixed model, which accounts for population structure and relatedness. Association analysis was performed assuming an additive genetic model to test for association between each SNP and adjusted TgAb and TPOAb levels. For the Split sample analysis was carried out using a combination of R-package GenABEL and SNPTEST software, while for the Korčula and Vis samples association analyses were conducted using R-packages GenABEL and VariABEL (24-26). Genomic inflation factors (lambdas) were calculated in each data set prior performing meta-

analysis. There was no need for adjustments ($\lambda_{\text{TgAb_Korčula}}=1.00$, $\lambda_{\text{TgAb_Split}}=0.93$, $\lambda_{\text{TgAb_Vis}}=1.01$; $\lambda_{\text{TPOAb_Korčula}}=0.99$, $\lambda_{\text{TPOAb_Split}}=1.00$, $\lambda_{\text{TPOAb_Vis}}=1.02$).

Meta-analyses

We combined evidence of associations from single GWAS using inverse-variance fixed-effect meta-analysis. Meta-analyses showed no significant evidence for inflated statistics ($\lambda_{\text{TgAb}}=0.97$, $\lambda_{\text{TPOAb}}=1.01$) hence no genomic correction was applied. Manhattan and quantile-quantile (QQ) plots were generated using the qqman R package (27). Regional association plots for loci of interest (400 kb) were created using Locus Zoom based on 1000 genomes EUR population (28). Illumina GenomeStudio software package was used to create cluster plots for confirmation of genotyping quality for associated SNPs. In cases where the SNP of interest was imputed, and not directly genotyped, cluster plots were created for directly genotyped SNPs that were in high LD with the SNP of interest ($r^2 > 0.8$). The genome-wide significance of association was defined as $p\text{-value} \leq 5 \times 10^{-8}$. Meta-analyses were performed with the R-package MetABEL (29).

Gender specific analyses

The prevalence of positive TPOAb and positive TgAb in the general population is higher in females than males (6). To identify gender specific effects we performed GWAS for each gender separately in each cohort. We used the same procedures as in the primary analyses except for the gender covariate. Association results were meta-analysed using the inverse-variance fixed-effects method. The total sample sizes for TgAb were 1596 for women and 1033 for men, while 1593 for women and 1025 for men for the TPOAb.

Bivariate analysis

We applied a multiple-trait analysis method to test the association between each SNP and the two correlated traits TgAb and TPOAb simultaneously, since joint analysis of correlated traits may increase power for identification of novel loci (30). The association was tested using multivariate analysis of variance (MANOVA). Multi-trait association test statistic was calculated on the basis of the summary statistics from single univariate GWAS. We have also performed bivariate analysis of TgAb and TPOAb for females. Correlation amongst antibodies in males was not sufficient for the performance of bivariate analysis.

Pathway analysis

Pathway analysis was performed with ConsensusPathDB (<http://cpdb.molgen.mpg.de>) (31, 32). For each of the loci with $P < 5 \times 10^{-6}$, downstream genes in ± 500 kb window were extracted and used as the input for analysis. The significance of results was defined as a $P < 0.01$. The same analysis was performed separately for males and females.

Results

Meta-analyses

Suggestive associations from genome-wide meta-analyses of TgAb and TPOAb levels are shown in Supplementary Table 1 and 2. We did not find any significant association in the meta-analysis for TgAb or TPOAb level. The Manhattan and Quantile-quantile plots for both traits are shown in Supplementary Figure 1 and 2.

Genome-wide meta-analysis of TgAb levels in females revealed a borderline significant association with rs4710782 genetic variant. SNP is located near protein coding gene *DLL1* (Table 3, Figure 1 and 2).

The rs1935377 locus had borderline significance in females for association with TPOAb (reference allele C, $P=8.58 \times 10^{-8}$) (Table 3, Figure 1 and 2). The SNP is located near protein coding gene *GRIN3A*.

Top findings from bivariate genome-wide meta-analyses are shown in Supplementary Figure 3 and Supplementary Table 3. The bivariate analysis in females revealed a genome-wide significant rs4457391 locus near *GRIN3A* gene (reference allele G, $P=7.76 \times 10^{-9}$) (Table 3, Figure 1 and 2).

Pathway analysis

No enrichment was obtained in general population for neither TgAb nor TPOAb. Results of pathway analyses performed for each gender separately are shown in the Supplementary Table 4 and 5.

For females, there were ten significantly enriched pathways at the $P < 0.01$ for TgAb level, while only one pathway was enriched for the level of TPOAb. For males, the enrichment for TgAb level was obtained for twenty-nine significant pathways at the $P < 0.01$, while two pathways were enriched for the level of TPOAb (Supplementary Table 4 and 5).

Replication of previous GWA findings

We investigated variants that were previously associated with the level or/and positivity of TPOAb (16, 18). Nine loci were reported in previous studies (*TPO*, *ATXN2*, *MAGI3*, *KALRN*, *BACH2*, *RERE*, *HCP5*, *HLA-DOB*, *HLA-DPBI*), however locus *HLA-DOB* could not be tested since the SNPs or any surrogate ($r^2 > 0.5$) were not available in our data. From eight reported variants that were available for the analysis in our data set, two were nominally replicated with $P < 0.05$, SNP rs11675434 for *TPO* gene ($P=0.009$) and SNP rs653178 in *ATXN2* gene ($P=0.035$). All other variants with available data for effect sizes were similar in size and direction as in our study (Supplementary Table 6).

Discussion

Our study confirms the gender specificity of genetic influences on serum thyroid antibody level. In females, a novel significantly associated locus near *GRIN3A* gene was identified in the bivariate analysis of TgAb and TPOAb, and the same locus showed borderline significance in association with TPOAb levels. Furthermore, we detected marginally significant locus associated with variation in TgAb levels in females (*DLL1*). Genetic variants affecting the TgAb level at a genome-wide scale are analysed for the first time in this study.

The levels of TPOAb and TgAb are correlated and participate in the onset and diagnosis of AITD (33). In our study, an intermediate correlation between both antibodies was found in our 3 general populations ($r=0.5-0.7$) as well as in females only ($r=0.6-0.7$), which enabled us to perform the bivariate analysis. The bivariate analysis revealed a significant association for a locus near the *GRIN3A* gene in females, with rs4457391 as a leading SNP ($P=7.76 \times 10^{-9}$). The rs4457391 SNP showed evidence of association for the level of TPOAb and TgAb in females ($P=1.04 \times 10^{-7}$ and $P=1.17 \times 10^{-5}$, respectively). The same locus with rs1935377 as leading SNP was marginally associated with TPOAb levels ($P=8.58 \times 10^{-8}$) and suggestively associated at the bivariate analysis ($P=3.09 \times 10^{-7}$). The rs1935377 is in moderate LD with rs4457391 ($r^2=0.72$, 1000Genomes phase3). Both these polymorphisms showed no association in men with p values < 0.05 , however effect sizes were in the same direction as in females.

GRIN3A gene encodes a subunit of the N-methyl-D-aspartate (NMDA) receptors, which belongs to the superfamily of glutamate-regulated ion channels. *GRIN3A* gene is expressed in several tissues, mostly in brain, bone marrow, immune system, female and male tissue (34). The *GRIN3A* gene is associated with the high-density lipoprotein (HDL) cholesterol levels and suggestively associated with the low-density lipoprotein (LDL) cholesterol and triglyceride levels (35). Decreased levels of thyroid hormones in the liver have an effect on the breakdown of circulating cholesterol, consequently, the higher level of triglycerides, total and LDL cholesterol and lower level of HDL cholesterol are associated with hypothyroidism (36). It is important to emphasize that Eriksson *et al.* found evidence of association of intronic variant (rs9792648, 2.7×10^{-5}) in *GRIN3A* gene with the hypothyroidism (37). A recent study by Joehanes *et al.* showed that SNPs associated with *GRIN3A* have the trans-eQTL effect, thus may act on phenotypes by affecting the expression of distant genes (38). For distant affected genes (*ZNF782*, *LCE2B*, *TEKT5* and *MORF4L2*) from the study (38), polymorphisms with evidence of association with the level of hypothyroidism, LDL, HDL and total cholesterol, as well as with adiponectin level were found (37, 39-42). For polymorphisms in *ZNF782* gene evidence of genome-wide association with hypothyroidism ($P=10^{-5}$), as well as with LDL and total cholesterol level ($P=10^{-2}-10^{-3}$) were detected.

Genes underlying AITD can be divided into thyroid-specific genes and immunoregulatory genes (14). GD and HT, although clinically antithetical, share number of immunological features including thyroid lymphocytic infiltration and autoreactivity against the key thyroid autoantigens (43). Different genes and mechanisms seem to be implicated in autoimmune prevalence in men and woman (13, 14).

Gender specific GWAS meta-analysis identified a novel locus associated with TgAb levels in females. Identified variant rs4710782 ($P=6\times 10^{-8}$) is located on chromosome 6, 9 kb upstream of the protein coding gene *DLL1*. *DLL1* is a human homolog of the Notch Delta ligand and a member of the delta/serrate/jagged family (44). It plays a role in mediating cell fate decisions during lymphopoiesis. Notch ligand Delta-1 inhibits the differentiation of human hematopoietic progenitors into the B cell lineage, while promotes the emergence of cells with a phenotype of T cell/natural killer (NK) precursor (45). Notch1 signalling plays a role in promoting maturation into both the CD4 and CD8 T cell lineages (46). CD4 T cells induce B cells in antibody production both in HD and GD, while CD8 T cells cause the death of thyrocytes in HT (8). Variations near *DLL1* gene are associated with type 1 diabetes (T1D) (47) and suggestively associated with systemic lupus erythematosus (SLE) (48). T1D, as well as SLE, frequently occur with AITD within the same individuals (49, 50).

In general population, bivariate analysis revealed some interesting, suggestive associations with *PDE10A* gene (rs611909, $P=2.37\times 10^{-6}$), which was previously associated with thyroid stimulating hormone (TSH) levels, as well as with the hypothyroidism (43). Likewise, *NFIA* gene (rs17121639, $P=2.97\times 10^{-6}$), previously associated with the level of TSH (43), had the suggestive association in the general population. These findings imply on the possibility of shared genetic susceptibility for thyroid function and autoimmunity.

While there were no functionally enrichment pathways for general populations, interesting findings were obtained for both antibodies in gender specific manner. Most of the enrichments were related with different immune and inflammatory responses. The most interesting pathways enriched for TgAb levels in females were Proteasome Degradation (Wikipathways), Notch, Hedgehog and GPCR signaling-G alpha i (INOH). For TPOAb levels in females, the pathway for Neutrophil degranulation (Reactome) was enriched. The most interesting pathway enriched for TgAb levels in males were Alpha9 beta1 integrin signaling events (PID) and Vitamin D Receptor Pathway (Wikipathways), while for the TPOAb Inflammatory mediator regulation of TRP channels (KEGG) and Vitamin D Receptor Pathway (Wikipathways).

Our study has helped in additional clarification of genetic variants associated with the TgAb and the TPOAb level. Thyroid autoimmunity is a consequence of the complex interaction of multiple genes and pathways, and possibly has different ethology depending on the gender. More GWA studies will be needed in the further enlightenment of this complex trait.

There are several limitations in our study. We had a modest number of participants for genome-wide association analyses, a larger study should be performed in order to replicate our findings and discover novel associated loci. Also, our analysis was restricted to participants of European ancestry, thus further GWAS on populations of different ancestry will be required. We did not perform additional functional studies for identified variants to clarify biological mechanism behind our findings.

Conclusion

We identified gender specific genetic factors associated with thyroid autoimmunity. We detected significantly associated locus (*GRIN3A*) in females with bivariate analysis, and likewise, the same locus was marginally associated in females with variation in TPOAb levels. Furthermore, we found a novel locus (*DLL1*) marginally associated with TgAb levels in females. Overall, our findings add to the knowledge of shared genetic susceptibility affecting thyroid antibodies, as well as of genetic factors that differently affect thyroid autoimmunity in males and females.

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Conflict of Interest

The authors declare no conflict of interest.

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Tables and figures

Table 1. Characteristics of study participants.

Variables for Tg-Ab	Split	Korčula	Vis
Overall sample size	942	819	868
Women, n (%)	587 (62%)	522 (64%)	487 (56%)
Median age, (qL,qU)	52 (40,61)	57 (47,67)	57 (45,69)
Median Tg-Ab, IU/mL (qL,qU)	6.90 (5.00,15.80)	11.90 (8.10, 32.25)	9.90 (5.10,19.20)
Variables for TPO-Ab	Split	Korčula	Vis
Overall sample size	942	819	857
Women, n (%)	587 (62%)	522 (64%)	484 (57%)
Median age, (qL,qU)	52 (40,61)	57 (47,67)	57 (45,69)
Median TPO-Ab, IU/mL (qL,qU)	2.5 (1.3, 7.9)	7.90 (3.85, 18.10)	4.10 (1.80, 11.50)

N: number of individuals; qL: lower quartile, qU: upper quartile.

Table 2. Genotyping methods and quality control procedures.

		Cohorts	Split1 (first 531 individuals from Split sample)	Split2 (other 481 individuals from Split sample)	Korcula	Vis
Genome-wide genotyping	N individuals		531	481	897	960
	Genotyping platform and SNP panel		Illumina HumanHap 370CNV QUAD Phase 1	Illumina HumanOmni ExpressExome8v1- 2_A	Illumina HumanHap 370CNV DUO Phase 1	Illumina Human Hap300v1 BeadChip
	N SNPs		351 514	969 919	346 034	317 509
	Genotype- calling algorithm		Illumina BeadStudio V3	Illumina BeadStudio V3	Illumina BeadStudio V3	Illumina BeadStudio V3
SNP QC (prior to imputation)	Call rate		≥98% per SNP	≥98% per SNP	≥98% per SNP	≥98% per SNP
	MAF		≥1%	≥1%	≥1%	≥1%
	HWE		$p < 10^{-7}$	$p < 10^{-7}$	$p < 10^{-7}$	$p < 10^{-7}$
Sample QC (prior to imputation)	Call rate		≥97%	≥97%	>97%	>95%

MAF: minor allele frequency; HWE: Hardy-Weinberg equilibrium.

Table 3. Associations between genetic variants and TgAb and TPOAb level.

SNP	Chr.	Position GRCh37.p13	Gene	Region of the gene	Min or allele	MA F	β	SE	<i>P</i> value
Bivariate analysis									
<i>Female</i>									
rs44573 91	9	104760468	<i>GRIN3A</i>	260 kb downstream	T	0.4	- 0.02 7	0.00 5	7,76x10 ⁻⁹
Tg-Ab levels									
<i>Female</i>									
rs47107 82	6	170582064	<i>DLL1</i>	9 kb upstream	C	0.32	0.21 0	0.03 9	6,16x10 ⁻⁸
TPO-Ab levels									
<i>Female</i>									
rs19353 77	9	104742291	<i>GRIN3A</i>	241 kb downstream	T	0.37	- 0,20 0	0.03 7	8,58x10 ⁻⁸

SNP - single nucleotide polymorphism

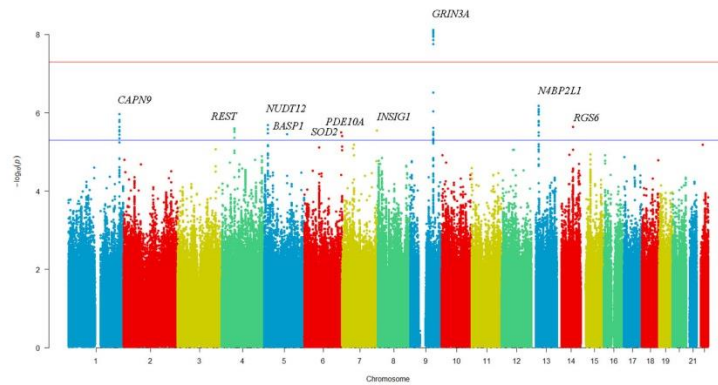
Chr.- chromosome

MAF - minor allele frequency

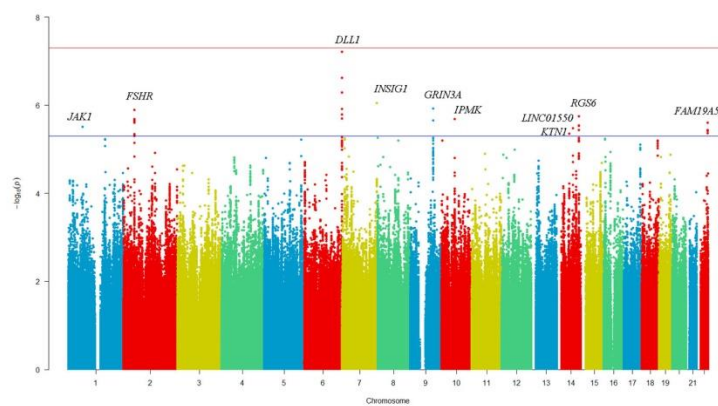
 β - effect size

SE - standard error

A) Bivariate analysis, female



B) TgAT, female



C) TPOAb, female

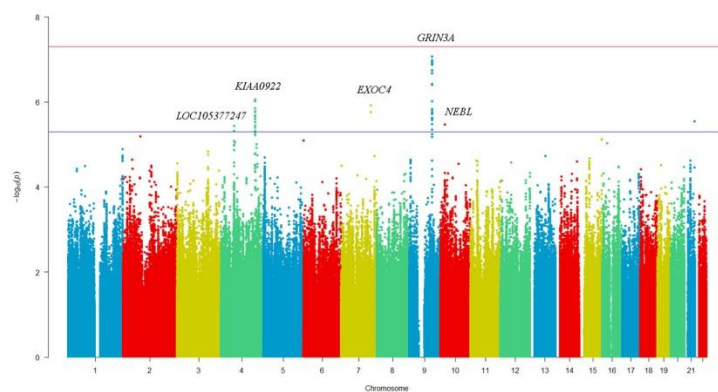


Figure 1. A) Manhattan plot of SNPs for the bivariate and meta-analysis of females in three cohorts. The y-axis shows the $-\log_{10} P$ values of 5 527 232 SNPs, and the x-axis shows their chromosomal positions. The red line indicates the threshold for significant hits ($P=5 \times 10^{-8}$) while the blue line indicates the threshold for suggestive hits ($P=5 \times 10^{-6}$). Gene labels are provided for suggestive hits ($P=5 \times 10^{-6}$) only B) Manhattan plot of SNPs for TgAb levels in the meta-analysis of females in three cohorts C) Manhattan plot of SNPs for TPOAb levels in the meta-analysis of females in three cohorts.

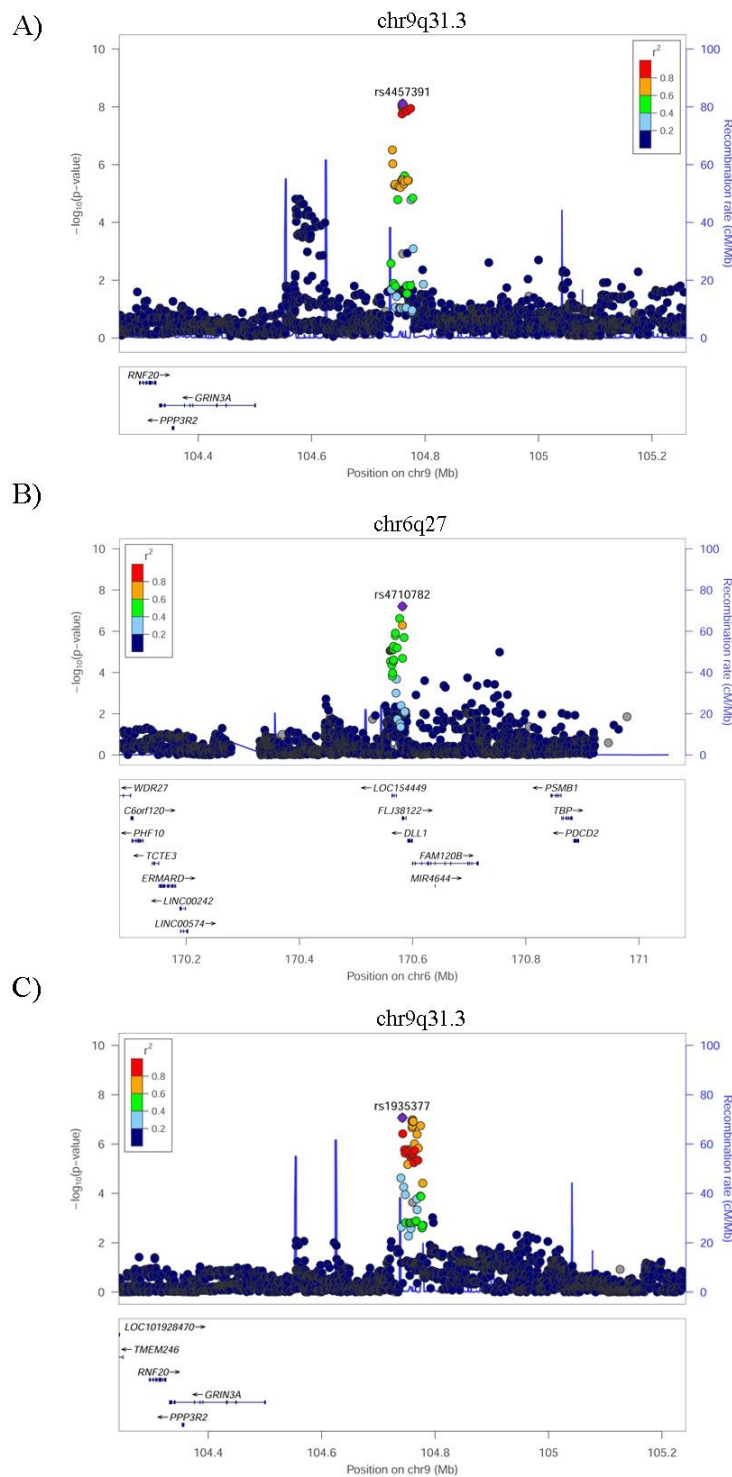


Figure 2. A) Regional association plot for bivariate and meta-analysis of females in three cohorts for the locus rs4457391 on chromosome 9. SNPs are plotted by position against association with two correlated traits TgAb and TPOAb simultaneously ($-\log_{10} P$ values). The purple diamond highlights the most significant SNP in the meta-analysis, whereas the colours of other variant represent LD with most significant SNP. B) Regional association plot for TgAb level in the meta-analysis of females in three cohorts for the locus rs4710782 on chromosome 6. SNPs are plotted by position against association with TgAb ($-\log_{10} P$ values). C) Regional association plot for TPOAb level in the meta-analysis of females in three cohorts for the locus rs1935377 on chromosome 9. SNPs are plotted by position against association with TPOAb ($-\log_{10} P$ values).

Supplementary data

Supplementary Table 1. Associations of suggestively associated single nucleotide polymorphisms ($P=5 \times 10^{-6}$) with TgAb level.

Supplementary Table 2. Associations of suggestively associated single nucleotide polymorphisms ($P=5 \times 10^{-6}$) with TPOAb level.

Supplementary Table 3. Associations of suggestively associated single nucleotide polymorphisms ($P=5 \times 10^{-6}$) with both correlated traits.

Supplementary Table 4. Enrichment results obtained from ConsensusPathDB for TgAb.

Supplementary Table 5. Enrichment results obtained from ConsensusPathDB for TPOAb.

Supplementary Table 6. Association values of the significant results of the previously published study in our data-set for the level of TPOAb.

Supplementary Figure 1. The Manhattan and Quantile-quantile plots for the level of TgAb.

Supplementary Figure 2. The Manhattan and Quantile-quantile plots for the level of TPOAb.

Supplementary Figure 3. The Manhattan and Quantile-quantile plots for both correlated traits.

Highlights

- *GRIN3A* locus was associated with thyroid antibodies levels in females
- *GRIN3A* locus was marginally associated with TPOAb levels in females
- *DLL1* locus was marginally associated with TgAb levels in females
- Gender specificity of genetic influences on thyroid antibody level was confirmed

ACCEPTED MANUSCRIPT