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ORIGINAL ARTICLE

Association and gene–gene interaction analyses for polymorphic variants in *CTLA-4* and *FOXP3* genes: role in susceptibility to autoimmune thyroid disease

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Abstract

Purpose Polymorphic variants of cytotoxic T-lymphocyte antigen-4 (*CTLA-4*) and forkhead box protein P3 (*FOXP3*) genes are implicated in dysregulated immune homeostasis and autoimmune disorders. We analyzed the association between *CTLA-4 rs231775* and *FOXP3 rs3761548, rs3761549* polymorphisms and predisposition to autoimmune thyroid disease (AITD), inclusive of Hashimoto's thyroiditis (HT) and Graves' disease (GD) in South-Indian population.

Methods A total of 355 AITD subjects (comprising 275 HT and 80 GD) and 285 randomly selected age- and sex-matched control subjects were genotyped for the aforementioned polymorphisms by PCR-RFLP method.

Results The rs231775 "G" allele was preponderant in HT and GD subjects when compared with controls and exerted a dominant influence on the susceptibility to HT ($p = 0.009$) and GD ($p = 0.02$), respectively. There was no allelic association of rs3761548 and rs3761549 polymorphisms with AITD susceptibility, albeit a significant difference in genotype distribution with respect to rs3761549. Haplotype analysis revealed an increased frequency of rs3761548 "C"–rs3761549 "T" in HT and GD subjects, thereby associating it with disease predisposition ($p = 0.03$). Epistatic interaction analysis by multifactor dimensionality reduction approach revealed redundancy between *CTLA-4* and *FOXP3* genes in influencing the susceptibility to AITD.

Conclusions The genetic variation in *CTLA-4* gene with reference to rs231775 polymorphism contributes to an increased predisposition to HT and GD. Also, in conjunction with *FOXP3* gene variants it seems to influence the susceptibility to HT and GD respectively. The significance of these findings in combination with antithyroid antibody screening could plausibly contribute towards meticulous case-finding for effective treatment of HT and GD.

Keywords Autoimmune thyroid disease · Hashimoto's thyroiditis · Graves' disease · *CTLA-4* · *FOXP3* · Polymorphism

Introduction

The two commonly prevalent clinical expressions of autoimmune thyroid disease (AITD) or thyroid dysfunction viz., Hashimoto's thyroiditis (HT) and Graves' disease (GD) are characterized by a breach in central and peripheral immune tolerance to thyroid-specific antigenic peptides resulting in an autoimmune response to various thyroid antigens like thyroid peroxidase (TPO), thyroglobulin (Tg), and thyrotropin (TSHR) [1]. This causes an inappropriate immune response through activation of potentially autoreactive T-cell clones in the periphery, which infiltrate thyroid gland and damage thyroid follicular cells through cell-mediated immune mechanisms as well as complement-fixing cytotoxic antibodies [1, 2]. Therein, the most significant feature of AITD is a deficiency in immune regulation, particularly a

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perturbation in the balance between T-effector cells and T-regulatory cells (T-regs) [2–4]. The latter constitute a specified lineage of $CD4^+$ T-cells constitutively expressing the transcription factor forkhead box protein P3 (FOXP3) that suppresses autoimmune response through inhibition of autoreactive T-cell clonal expansion and thereby maintains immune homeostasis [5].

Robust evidence for the contribution of genetic factors to AITD susceptibility emerged from studies on monozygotic twins and transmission disequilibrium analyses [6–8]. Molecular approaches involving analysis of single nucleotide polymorphisms (SNPs) in candidate genes have been adopted to unravel the role of specific genes in predisposition to multifactorial diseases including AITD [7–9]. The importance of genetic studies can be further realized from the significant association of novel susceptibility loci with young age-at-onset GD [10]. In line with this, SNPs in immunoregulatory genes encoding costimulatory molecule cytotoxic T-lymphocyte associated protein (CTLA-4) and FOXP3 have been demonstrated to contribute to altered immune regulation and peripheral immune tolerance [7–14]. CTLA-4 is a negative regulator of T-cell activation, while FOXP3 is essential for the development and functioning of naturally occurring $CD4^+CD25^+$T-regs [5, 15]. Several SNPs in *CTLA-4* gene have been investigated for their association with AITD in various populations [10–14, 16–33]. However, relatively fewer reports are available on association studies between SNPs within *FOXP3* gene and AITD [34–40]. Further, studies on epistatic interactions between these genetic variants that could circumstantially modify the risk of AITD have been sparse. A predominant number of reported genetic variants in these genes associate with AITD susceptibility by altering the functions of the respective proteins (CTLA-4 and FOXP3) resulting in abnormal lympho-proliferation and intractable autoimmune response [3, 5, 35, 41]. SNPs contributing to CTLA-4 dysfunction disrupt organ-specific homeostasis due to an aberrant activation of T-lymphocytes in the periphery which infiltrate thyroid gland causing its dysfunction [15–33]. Similarly, FOXP3 deficiency emanating from the genetic variation impairs the suppressive function of T-regs and promotes autoimmunity [5, 34–40]. In this milieu, the polymorphic variants viz., +49A>G in exon-1 (rs231775) of *CTLA-4* and −3279C>A (rs3761548) and −2383C>T (rs3761549) in the promoter of *FOXP3* genes were selected for the current study in view of their functional significance. It is understood that rs231775 impairs the post-translational modification of CTLA-4 that reduces its density on activated $CD4^+$ Th1 cells and plausibly promotes an autoimmune response [42]. As regards *FOXP3*, the rs3761548 allelic variation supposedly alters the Sp1 transcription factor binding efficiency that in turn influences *FOXP3* gene expression [43]. With respect to rs3761549, owing to its location in the promoter it could significantly alter the expression of FOXP3 that eventually contributes to an autoimmune response [34, 35]. Given the paucity of gene-association studies concerning *CTLA-4* and *FOXP3* in Indian population which has a significant share of AITD patients, we investigated the associative relationship, if any, between the aforementioned gene polymorphisms together with their epistatic interaction and AITD susceptibility in South-Indian AITD population.

Materials and methods

Study participants and phenotypic data

A total of 640 subjects in the age group of 18–75 years, attending Princess Esra Hospital, Hyderabad, inclusive of de novo and established AITD (HT and GD) subjects were recruited for the study. Based on the circulating levels of triiodothyronine (T3) (normal range: 0.6–3.3 nmol/dL), thyroxine (T4) (normal range: 60–120 nmol/dL), and thyroid stimulating hormone (TSH) (normal range: 0.27–5 μIU/mL) levels, subjects were categorized into HT (hypothyroid), GD (hyperthyroid), and control group. Among 640 subjects recruited for the study, 275 subjects were HT, 80 were GD, and 285 were healthy controls. Socio-demographic information inclusive of age, duration of AITD in established subjects, concomitant diseases, and family history of AITD were obtained through a structured questionnaire. All the recruited subjects provided whole blood (2 mL) for biochemical and genotype analysis. The study protocol was approved by the institutional and hospital ethical committees and all the subjects gave written informed consent to participate in this study.

Genotype determination

The isolation of genomic DNA from whole blood was carried out using the rapid salting out method. The genotyping was performed as per the published protocols with minor modifications. The amplification reaction was performed in 25 μL volumes containing the amplimers 5′-CCACGGC TTCCTTTCTCGTA-3′ and 5′-AGTCTCACTCACCTTTG CAG-3′ for rs231775; 5′-CCTCTCCGTGCTCAGTGTAG-3′ and 5′-CTCACCTAGCCCAGCTCTTG-3′ for rs3761548; 5′-CTGAGACTTTGGGACCGTAG-3′ and 5′-TGCGCCGGG CTTCATCGACA-3′ for rs3761549 polymorphisms, respectively, and 0.2 mmol/L of each dNTP (Eppendorf, Hamburg, Germany), 1.5 mM $MgCl_2$, 1× Taq buffer and 1.0 U of Taq DNA polymerase (Bangalore Genei, India). The samples were subjected to an initial denaturation at 94 °C for 5 min, followed by 30 cycles of amplification in a three-step reaction

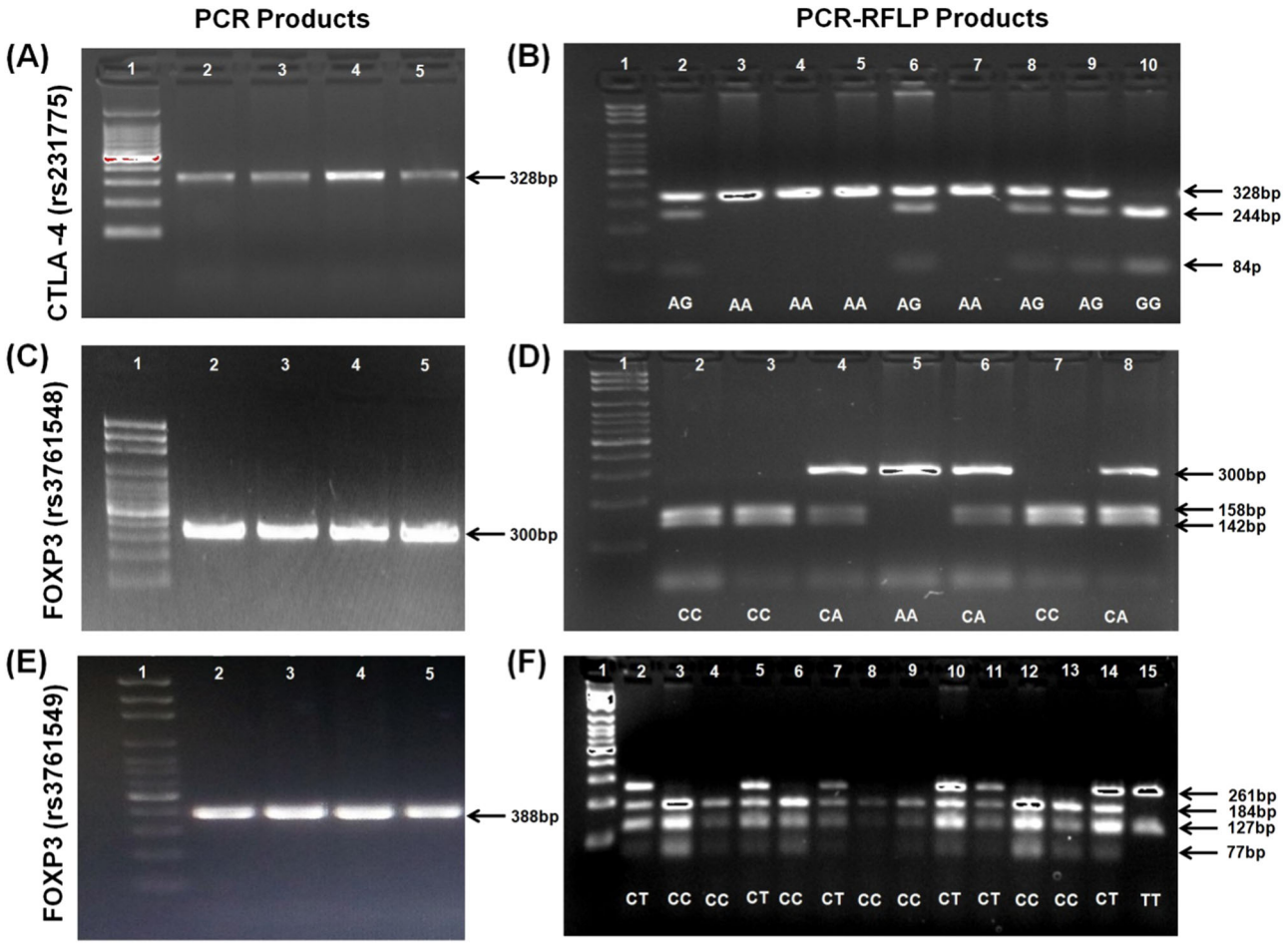

Fig. 1 Representative gel photograph for *CTLA-4* rs231775 **a**–**b** and *FOXP3* rs3761548 **c**–**d**, rs3761549 **e**–**f** polymorphisms. Lane 1 corresponds with 100 base-pair DNA ladder

consisting of denaturation for 30 s at 94 °C, annealing for 30 s at 60°C for rs231775, 68 °C for rs3761548 and 60 °C for rs3761549 and extension for 1 min at 72 °C and a final extension step of 10 min at 72 °C in an iCycler machine (BioRad Laboratories, Hercules, CA, USA). Amplification resulted in a 328-base pair (bp) product for rs231775, a 300-bp fragment for rs3761548, and 388 bp product for rs3761549, respectively, that included BbvI, PstI, and BsrI polymorphic sites, respectively. The resulting PCR products were digested overnight with 1 U of BbvI (rs231775), 10 U of PstI (rs3761548), and 2 U of BsrI (rs3761549) (Thermo Fisher Scientific, Bangalore) at 37 °C overnight. The resulting fragments were separated on 3% agarose gels and visualized under UV light after staining with ethidium bromide (10 mg/mL). The corresponding genotypes were AA (328 bp)/AG (328, 244, 84 bp)/GG (244, 84 bp) for rs231775; CC (158,142 bp)/CA (300, 158, 142 bp)/AA (300 bp) for rs3761548 and CC (184, 127, 77 bp)/CT (261, 184, 127, 77 bp)/TT (261, 127 bp) for rs3761549, respectively (Fig. 1).

Statistical analysis

Percentage distribution of the corresponding genotypes of the polymorphisms entailed in the study was determined and the differences in genotype and allele frequencies between the specified groups were compared using the Pearson's Chi-square (χ^2) (with Yates correction) or Fisher's exact tests as appropriate. The risk estimates for alleles and genotype contrasts were obtained by computing odds ratio (OR) and respective 95% confidence interval (CI). The criterion for statistical significance was a two-tailed p value of <0.05. All these statistical analyses were performed using SPSS version 15.0 software (SPSS, Chicago, IL). Haplotype analysis was performed using Haploview 4.2. The higher order gene-gene interaction analysis in order to assess the epistatic interaction between the SNPs of the genes studied was performed using multifactor dimensionality reduction (MDR) approach 3.0.2 and the best factor model was used to quantify the epistatic relationship

Table 1 Demographic and biochemical characteristics of the study participants

Characteristic	Controls ($N = 285$)	Hashimoto's thyroiditis ($N = 275$)	Graves' disease ($N = 80$)
Gender (female/male) (*n*%)	256 (89.8)/30(10.2)	258 (93.8)/17 (6.2)	74 (92.5)/6(7.5)
Age (mean ± SD) (years)	32.01 ± 12.59	33.89 ± 11.94	33.88 ± 14.73
Tri-iodothyronine (T3) (nmol/dL) (mean ± SD)	1.69 ± 0.54	2.07 ± 1.08	2.32 ± 1.22
Thyroxine (T4) (nmol/dL) (mean ± SD)	87.24 ± 13.61	83.55 ± 24.19	88.46 ± 28.01
Thyroid stimulating hormone (TSH) (μIU/mL) (mean ± SD)	2.54 ± 1.16	8.93 ± 11.91	1.33 ± 5.86
Anti-thyroperoxidase (TPO) positivity (*n*%)		150 (52.9)	43 (55.0)
Anti-thyroglobulin (Tg) positivity (*n*%)		115 (42.9)	40.0 (32)

between *CTLA-4* and *FOXP3* genetic variants that would influence the genetic susceptibility to AITD [44].

Results

The demographic characteristics like mean thyroid hormone levels and thyroid-specific antibody positivity are presented in Table 1. Further, the genotype distribution of *CTLA-4* rs231775 (+49A>G), and *FOXP3* rs3761549 (−2383C>T) were in Hardy–Weinberg Equilibrium (HWE) except for *FOXP3* rs3761548 (−3279C>A). The genotype and allele distribution of the aforementioned SNPs are presented in Table 2. A perusal of the results shows that the genotype distribution of *CTLA-4* rs231775 and *FOXP3* rs3761549 differed significantly between HT and GD patients when compared separately with healthy subjects. However, no significant difference in genotype distribution was observed between control subjects and each of HT and GD groups with respect to *FOXP3* rs3761548. The rs3761548 "CA" heterozygote frequency (79.3%) increased in HT subjects while it was less frequent (66.2%) in GD patients compared to controls (72.6%). As for allele distribution, the frequency of "G" allele of *CTLA-4* rs231775 was significantly higher compared to "A" allele in both HT ($p = 0.009$) and GD ($p = 0.02$) subjects when compared with controls, thereby conferring a 1.41-fold and 1.56-fold increased risk of developing HT and GD, respectively. Nevertheless, there was no significant difference in the allele distribution with respect to the *FOXP3* rs3761548 and rs3761549 polymorphisms, respectively.

The strength of association of the selected SNPs was further examined by comparing different genotype contrasts (Table 3). The presence of *CTLA-4* rs231775 "G" allele (combined genotype of AG and GG) and "T" allele (CT and TT genotypes) of *FOXP3* rs3761549 seemed to exert a dominant effect in conferring an increased risk of 1.58 ($p = 0.009$) and 1.66 ($p = 0.04$); 1.63 ($p = 0.004$) and 2.01 ($p = 0.008$) fold for HT and GD, respectively. The rs231775 "G" allele also seemed to exert a significant effect in the codominant model (GG vs AA) ($p = 0.04$; 0.03) in both HT and GD groups, respectively. The recessive effect of "G" allele on GD susceptibility was observed in the comparison of GG vs AG and AA ($p = 0.04$). With respect to rs3761548, it was observed that the AA genotype was associated with an increased risk for HT in the recessive model ($p = 0.02$). This was nevertheless not observed in GD. Further, haplotype analysis was performed to determine the association of *FOXP3* SNPs with HT/GD susceptibility (Table 4). The C–T haplotype representing the *FOXP3* rs3761548 "C" allele and rs3761549 "T" allele was associated with an increased odds of prevalence for HT [$p = 0.03$; OR = 1.68 (1.05–2.68)] and GD [$p = 0.03$; OR = 2.05 (1.07–3.93)], respectively.

Further, an epistatic interaction between the three polymorphisms was studied by MDR approach which is a non-parametric and model-free alternative to logistic regression that generates a one-dimensional multi-locus genotype variable [44]. The resulting model is evaluated for its ability to classify and predict disease status using cross-validation and permutation testing. Among the set of best multifactor models, the combination of genetic factors that maximize the testing balance accuracy (TBA) and/or that provides the highest cross-validation consistency (CVC) value is chosen. Accordingly, in one-factor model *FOXP3* rs3761549 emerged as the best attribute in risk prediction for HT and GD with a TBA of 0.5514 and 0.5301 for HT and GD, respectively and CVC of 9/10. In our study, the overall best model for the interaction of selected *CTLA-4* and *FOXP3* polymorphisms was the three-factor model depicted in Table 5a with a CVC of 10/10 and TBA of 0.5411 and 0.4996 for HT and GD, respectively. This is indicative of the occurrence of this model once out of 1000 permutations that would otherwise be unlikely under the hypothesis of null association. Further, we also investigated for a possible interaction between the above SNPs and gender, in view of an increased female predisposition for AITD. The overall best model for the interaction between selected *CTLA-4* and *FOXP3* polymorphisms and gender was the four-factor model as indicated in Table 5b with a CVC of 10/10 and TBA of 0.5417 and 0.5304 for HT and GD respectively. In one-factor model, *FOXP3* rs3761549 was the best attribute, recapitulating the above finding in risk prediction for HT and GD with a TBA of 0.5657 and 0.5788 for HT and GD, respectively and a CVC of 10/10.

Table 2 Genotype and allele frequencies of selected *CTLA-4* and *FOXP3* gene polymorphisms in the study groups and healthy subjects

Polymorphism	Controls ($N = 285$) ($n\%$)	Hashimoto's thyroiditis ($N = 275$) ($n\%$)	Grave's disease ($N = 80$) ($n\%$)
rs231775 (*CTLA-4* +49A>G)			
AA	146 (51.2)	110 (40.0)	31 (38.8)
AG	123 (43.2)	140 (50.9)	39 (48.8)
GG	16 (5.6)	25 (9.1)	10 (12.5)
		$\chi^2 = 7.96$; $p = 0.02$	$\chi^2 = 6.6$; $p = 0.037$
A	415 (71.5)	360 (63.2)	101 (63.8)
G	155 (28.5)	190 (36.7)	59 (38.2)
		$\chi^2 = 6.76$; $p = 0.009$*	$\chi^2 = 5.19$; $p = 0.02$*
OR (95%CI)		1.41 (1.1–1.82)	1.56 (1.08–2.27)
rs3761548 (*FOXP3* −3279C>A)			
CC	17 (6.0)	19 (6.9)	10 (12.5)
CA	207 (72.6)	218 (79.3)	53 (66.2)
AA	61 (21.4)	38 (13.8)	17 (21.3)
		$\chi^2 = 5.59$; $p = 0.06$*	$\chi^2 = 3.96$; $p = 0.14$*
C	241 (42.0)	256 (47.0)	73 (46.0)
A	329 (58.0)	294 (53.0)	87 (54.0)
		$\chi^2 = 1.89$; $p = 0.17$*	$\chi^2 = 0.44$; $p = 0.51$*
OR (95%CI)		0.84 (0.66–1.07)	0.87 (0.61–1.24)
rs3761549 (*FOXP3* −2383C>T)			
CC	132 (46.3)	95 (34.5)	24 (30)
CT	115 (40.4)	148 (53.8)	46 (57.5)
TT	38 (13.3)	32 (11.6)	10 (12.5)
		$\chi^2 = 10.51$; $p = 0.004$	$\chi^2 = 8.09$; $p = 0.01$
C	379 (66.0)	338 (61.0)	94 (59.0)
T	191 (34.0)	212 (39.0)	66 (41.0)
		$\chi^2 = 2.87$; $p = 0.09$*	$\chi^2 = 2.95$; $p = 0.08$*
OR (95%CI)		1.24 (0.97–1.59)	1.39 (0.97–1.99)

*p value and OR (95%CI) for comparison between healthy subjects and HT/GD individuals

The graphical image generated from MDR involves the representation of three polymorphisms, each with three genotypes and three-factor combinations. Further, within each multifactor cell (square box), the ratio of number of cases with disease to number of controls was also calculated. Accordingly, approximately three-fold increased risk for HT was observed for the combination of rs231775 "GG", rs3761548 "CA", and rs3761549 "CT" genotypes. (Fig. 2a). As regards GD, the combination of rs231775 "AA" genotype with rs3761548 "CA" and rs3761549 "CC" genotypes displayed eight-fold increased presence in the controls associating with lowered risk for GD (Fig. 3a). With respect to the interaction of selected *CTLA-4* and *FOXP3* SNPs with gender, higher risk was observed for a combination of *CTLA-4* rs231775 "GG", *FOXP3* rs3761548 "CA" and rs3761549 "CT" genotypes in females with HT, while in females with GD the combination of rs231775 "AA" genotype with rs3761548 "CA" and rs3761549 "CC" and genotypes showed a lowered risk (Figs. 4a and 5a, respectively).

Furthermore, two interaction dendrograms that summarize the estimates of interaction information, i.e., entropy for each pair of SNPs were modeled by MDR. This included the genotypic data sets of the three SNPs for HT and GD respectively. Two SNPs connecting by shorter lines suggest a stronger interaction or dependency. A blue or green line implies loss of information, which can be interpreted as redundancy or correlation (for example, linkage disequilibrium). A red or orange line connecting two polymorphisms depicts a positive information gain and hence a synergistic or non-additive relationship, while a yellow line indicates independence or additivity. Accordingly, dendrogram interaction analysis of the data revealed that with respect to HT, *FOXP3* rs3761549 and *CTLA-4* rs231775 are on same branch (green) bifurcating into blue color which indicates a redundant effect or correlation with disease phenotype. Apart from this, rs3761549 of *FOXP3* gene on a separate branch with green color has also shown a lower degree of redundancy or correlation with HT (Fig. 2b). Contrastingly, in GD study set, *FOXP3* rs3761548 and *CTLA-4* rs231775 localized to the same branch (green) and connected by short blue lines imply stronger redundancy (Fig. 3b). *FOXP3* rs3761549 on the adjacent branch (green) exhibited redundancy or correlation with GD. Further, in an interactive analysis of these gene variants with gender rs3761548 showed redundancy with gender in HT subjects, while the redundant interaction between rs231775 and rs3761549 as mentioned above was retained (Fig. 4b). As regards GD, a synergistic or non-additive interaction (red color), albeit not strong (stretched lines), was observed between rs3761549 and gender and a strong redundancy was observed between rs231775 and rs3761548, recapitulating the above finding (Fig. 5b).

Discussion

The possible contribution of the genetic variation (SNPs) in two prominent immunoregulatory genes viz., *CTLA-4* and *FOXP3* to an increased susceptibility for HT and GD was investigated in our study. Accordingly, it was observed that *CTLA-4* rs231775 associated significantly with

Table 3 Analysis of the risk for Hashimoto's thyroiditis and Graves' disease according to the genotype contrasts

Genotype contrast	Hashimoto's thyroiditis		Graves' disease	
	p	OR (95%CI)	*p*	OR (95%CI)
rs231775 *CTLA-4* +49 A> G				
AG and GG vs AA	0.009	1.58 (1.13–2.2)	0.04	1.66 (1.00–2.75)
GG vs AG and AA	0.06	1.68 (0.88–3.22)	0.04	2.40 (1.04–5.52)
GG vs AA	0.04	2.07 (1.06–4.07)	0.03	2.94 (1.22–7.09)
rs3761548 *FOXP3* −3279C>A				
CA and AA vs CC	0.73	1.2 (0.59–2.3)	0.16	0.52 (0.23–1.17)
AA vs CA and CC	0.02	1.7 (1.09–2.65)	0.88	1.0 (0.55–1.85)
CC vs AA	0.17	0.56 (0.26–1.2)	0.19	0.47 (0.18–1.22)
rs3761549 *FOXP3* −2383C>T				
CT and TT vs CC	0.004	1.63 (1.16–2.3)	0.008	2.01 (1.18–3.43)
TT vs CT and CC	0.54	0.86 (0.52–1.41)	0.84	0.93 (0.44–1.96)
TT vs CC	0.66	1.17 (0.68–2.01)	0.49	1.44 (0.63–3.29)

Table 4 Estimated haplotype frequencies for *FOXP3* gene polymorphisms (rs3761548 and rs3761549) in Hashimoto's thyroiditis and Graves' disease subjects

FOXP3 (rs3761548–rs3761549) haplotype	Haplotype frequency			*p**	OR (95%CI)*	*p*-Value#	OR (95%CI)#
	HT	GD	Controls				
A–C	0.42	0.41	0.44	–	1.00	–	1.00
C–T	0.27	0.28	0.20	0.03	1.68 (1.05–2.68)	0.03	2.05 (1.07–3.93)
C–C	0.19	0.17	0.22	0.59	0.86 (0.50–1.48)	0.59	0.80 (0.35–1.81)
A–T	0.11	0.13	0.13	0.67	0.89 (0.51–1.54)	0.48	1.33 (0.61–2.90)

HT Hashimoto's thyroiditis, *GD* Graves' disease

*,#*p*-value and OR (95%CI) (adjusted) for comparison of each haplotype frequency between each of HT and GD groups with controls, respectively

Table 5a Multifactor dimensionality reduction analysis for higher-order gene–gene interaction in subjects with Hashimoto's thyroiditis and Graves' disease

Factor number	Model	Testing balance accuracy (TBA)	Cross-validation consistency (CVC)
Hashimoto's thyroiditis			
Single	rs3761549	0.5514	9/10
Two	rs3761548, rs3761549	0.5163	7/10
Three	rs231775, rs3761548, rs3761549	0.5411	10/10
Graves' disease			
Single	rs3761549	0.5301	9/10
Two	rs3761548, rs3761549	0.5478	7/10
Three	rs231775, rs3761548, rs3761549	0.4996	10/10

susceptibility to HT and GD respectively. The rs231775 (+49A>G) polymorphism in exon 1 of CTLA-4 induces a threonine to alanine amino acid change that affects the post-translational modification of CTLA-4, in terms of reduced glycosylation resulting in its misprocessing in the endoplasmic reticulum [42]. The "G" allele of this *CTLA-4* signal peptide variant is associated with reduced expression of membrane-bound CTLA-4, with a consequential reduction in its density on activated $CD4^+$ Th1 cells as compared to "A" allele [42]. It is well understood that CTLA-4 is a negative regulator of T-cell activation as it acts as a gatekeeper of conjugation timing [15, 45]. Reduced conjugation corresponds with curtailing of prolonged contact periods of cytotoxic T-lymphocytes with auto-antigen defined targets.

Table 5b Multifactor dimensionality reduction analysis for higher-order gene–gender interaction in subjects with Hashimoto's thyroiditis and Graves' disease

Factor number	Model	Testing balance accuracy (TBA)	Cross-validation consistency (CVC)
Hashimoto's thyroiditis			
Single	rs3761549	0.5657	10/10
Two	rs231775, rs3761549,	0.5144	5/10
Three	rs231775, rs3761548, rs3761549	0.5472	9/10
Four	rs231775, rs3761548, rs3761549, Gender	0.5417	10/10
Graves' disease			
Single	rs3761549	0.5788	10/10
Two	rs3761548, rs3761549	0.5569	6/10
Three	rs231775, rs3761548, rs3761549	0.5052	5/10
Four	rs231775, rs3761548, rs3761549, Gender	0.5304	10/10

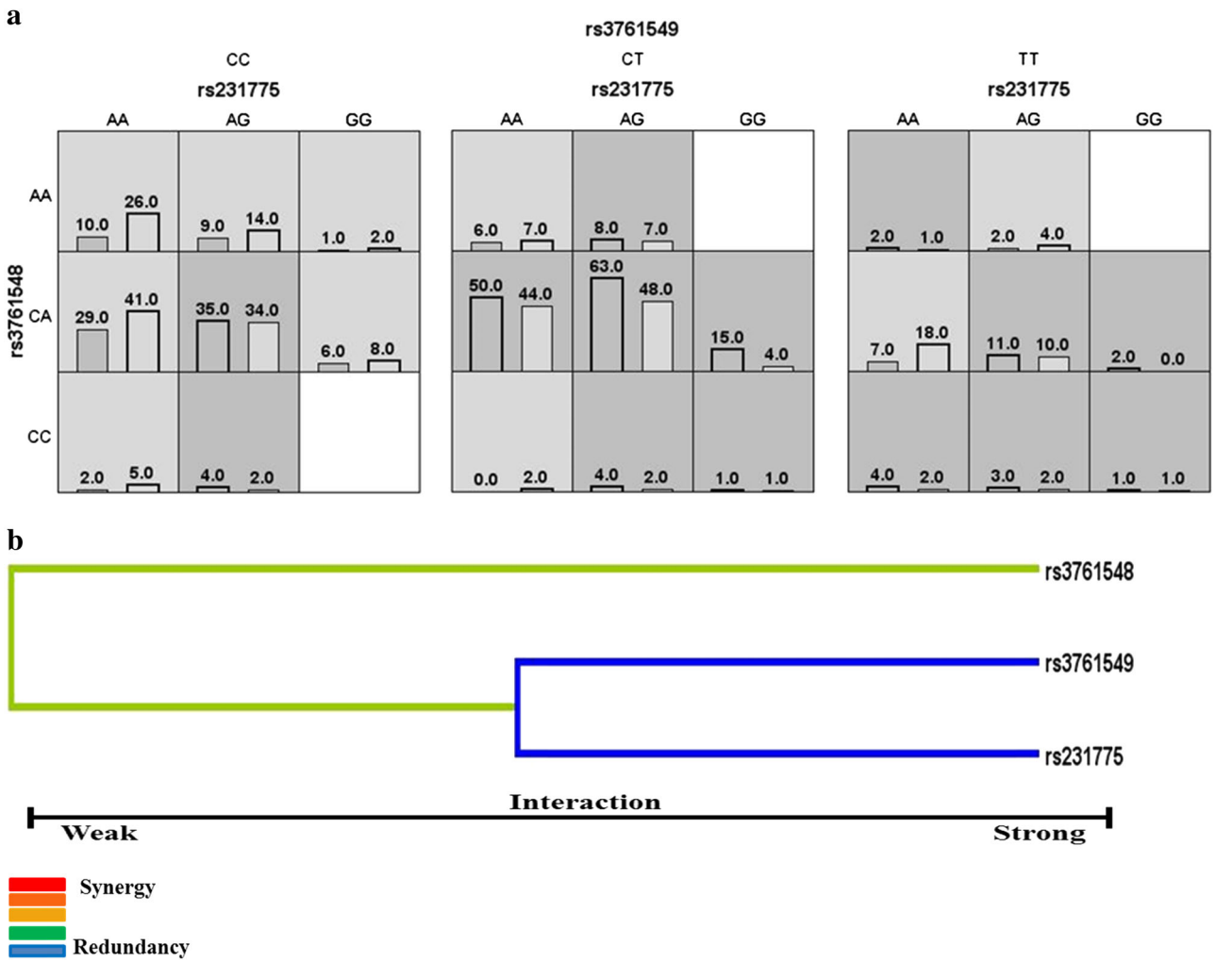

Fig. 2 Epistatic (gene–gene) interactions between rs231775, rs3761548, and rs3761549 polymorphisms in Hashimoto's thyroiditis and healthy subjects. **a** Three-factor model. The light gray bar in each cell represents the frequency of Hashimoto's thyroiditis individuals and white bar represents the frequency of healthy individuals. High-risk genotype combinations are represented by dark gray shade cells, while light gray shade cells represent low-risk genotype combinations. Cells with no shading or white cells represent genotype combination for which no data is observed. **b** Interaction dendrogram analysis between SNPs rs231775, rs3761548, and rs3761549 by MDR analysis in subjects with Hashimoto's thyroiditis

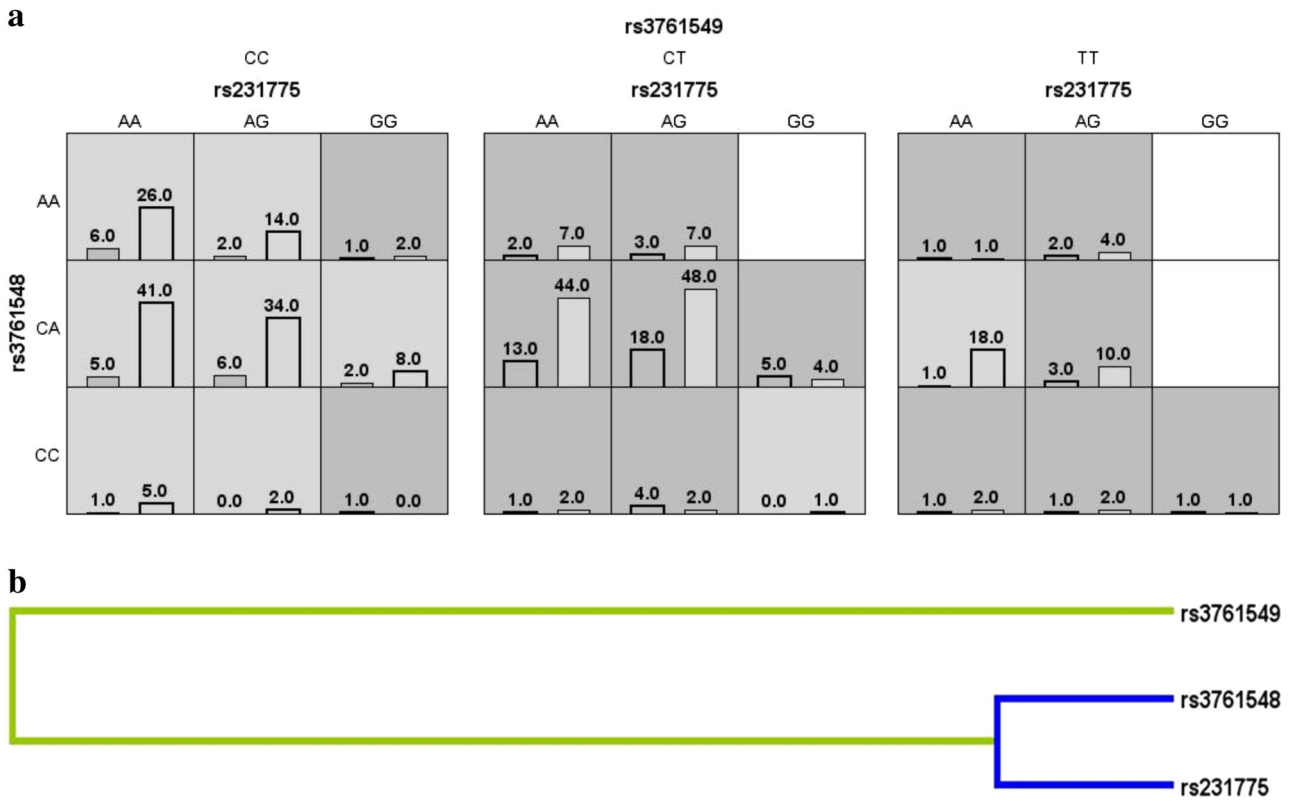

Fig. 3 Epistatic interactions between rs231775, rs3761548, and rs3761549 polymorphisms in Graves' disease and healthy subjects. **a** Three-factor model. The light gray bar in each cell represents the frequency of Graves' disease individuals and white bar represents the frequency of healthy individuals. High-risk genotype combinations are represented by dark gray shade cells, while light gray shade cells represent low-risk genotype combinations. Cells with no shading or white cells represent genotype combination for which no data is observed. **b** Interaction dendrogram analysis between SNPs rs231775, rs3761548, and rs3761549 by MDR analysis in Graves' disease subjects

Therein, higher the density of CTLA-4 on activated Th1 cells, greater is the chance of apoptotic elimination of peripheral Th1-cells upon interaction with B7 costimulatory molecule on antigen-presenting dendritic cells. This could plausibly explain an increased frequency of rs231775 "AA" genotype in controls in our study that associates with optimal CTLA-4 density, which can preclude the protracted expansion of autoreactive T-cell clones [46]. The carriers of "G" allele thereby are characterized by heightened T-cell activation and an exponential expansion of activated autoreactive T-cells, eventually leading to an autoimmune response, clinically manifested as HT or GD [42, 46]. In our study, the presence of variant "G" allele appeared to confer an increased risk of 1.41-fold and 1.56-fold for HT and GD, respectively. Our results are consistent with those reported in Asian (Chinese, Japanese, Koreans, and Taiwanese) and Caucasian (European) populations wherein the "G" allele was associated with an increased risk for GD or HT, respectively [19, 20, 22–25, 28–32, 47–52]. From Indian sub-continent, two recent studies from North (HT) and South Indian (HT and GD) subjects reported an increased risk for HT or/and GD development in "G" allele carriers [53, 54]. The collective association of rs231775 with GD and HT susceptibility in our study is comparable with earlier studies in various other populations [21, 30, 31, 52, 55]. This observation is suggestive of an overlap of the predisposing genetic variants or shared immuno-regulatory defect. This indeed aligns with the observed co-occurrence of these conditions within the family and a transition over time from GD to HT and vice versa within individuals [56].

In our study, a significantly dominant effect of "G" allele in various comparisons of genotype contrasts in HT and GD subjects was observed. This is indeed suggestive of the dominant influence of rs231775 "G" allele in conferring an increasing susceptibility to HT and GD. Our results are in consonance with two meta-analysis studies which reported a significant association between rs231775 and HT risk in Asian population in both allele comparisons and all genetic models [32, 57, 58]. More specifically, our data derives support from the dominant influence of rs231775 "G" allele in conferring an increased HT risk as reflected in similar ORs in these meta-analyses. From this, it could be inferred that the genotype conditioning of exon 1 function by the rs231775 "G" allele (AG/GG) restrains the inhibitory

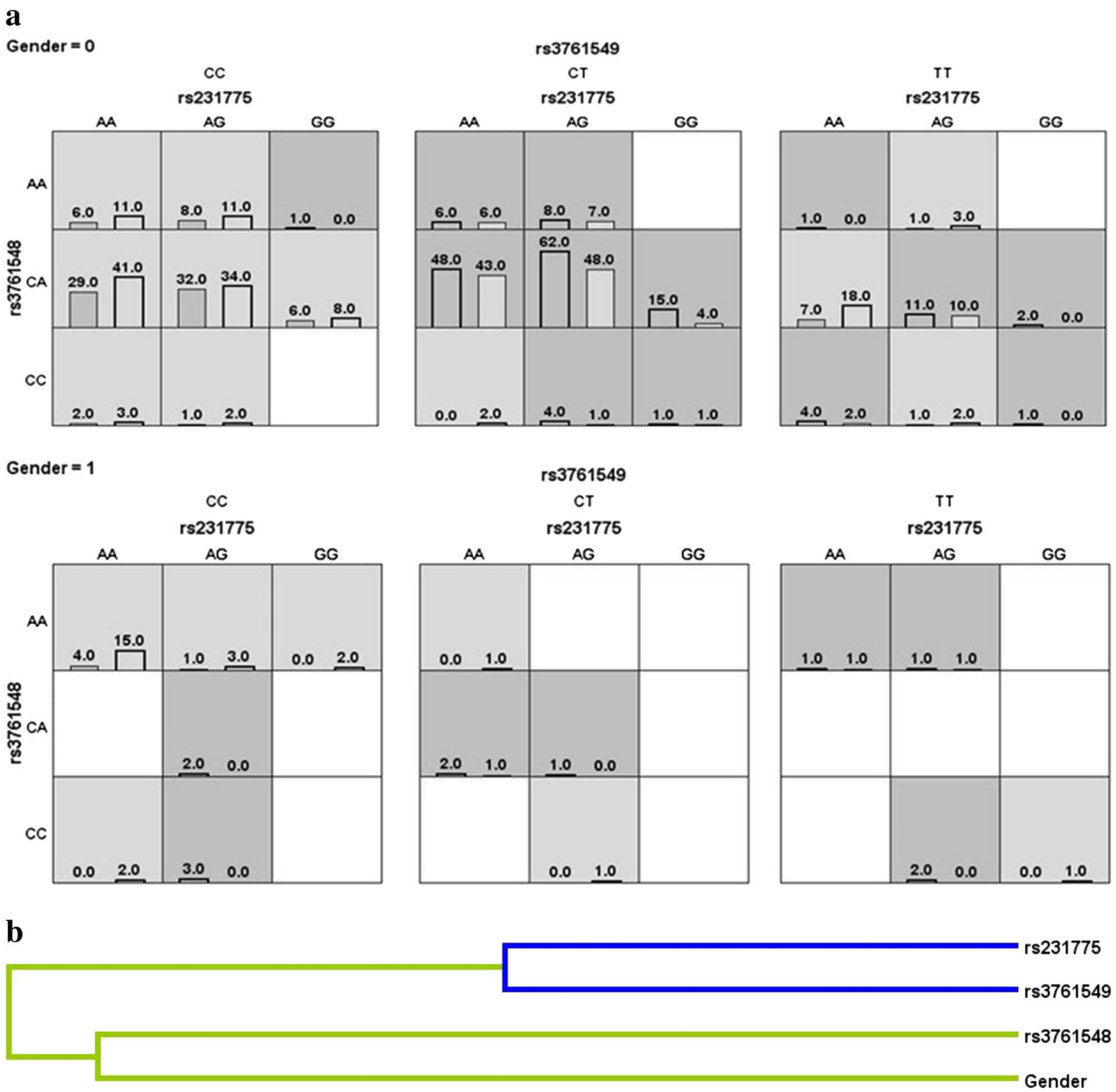

Fig. 4 Multifactor dimensionality reduction analysis for an interaction between rs231775, rs3761548, rs3761549 polymorphisms and gender in Hashimoto's thyroiditis and healthy subjects. **a** Four-factor model. The light gray bar in each cell represents the frequency of Hashimoto's thyroiditis individuals and white bar represents the frequency of healthy individuals. High-risk genotype combinations are represented by dark gray shade cells, while light gray shade cells represent low-risk genotype combinations. Cells with no shading or white cells represent genotype combination for which no data is observed. Gender 0 indicates female cases and 1 indicates male cases. **b** Interaction dendrogram analysis between SNPs rs231775, rs3761548, rs3761549 and gender by MDR analysis in Hashimoto's thyroiditis subjects

effects of CTLA-4 on thyroid-specific T-cell proliferation and expansion. Given the polygenic nature of AITD, it also needs to be mentioned that this effect is not singly imposed by rs231775 and involves the collective complicity of several genetic variants [17, 23, 26, 27, 30, 31, 33, 47, 49–51, 53, 55, 57–59]. Nevertheless, certain meta-analysis and case-control studies reported that rs231775 is not associated with HT and GD in some Caucasian and Asian populations [18, 24, 27, 59]. A study on the association of various SNPs of *CTLA-4* with AITD predisposition by Ueda et al., inferred that a more significant role is played by CT60 A > G variant [12]. However, some authors were critical of these findings and indicated that their data did not exclude the possibility of multiple causative variants, with an intervening influence of ethnic and geographical factors [16]. In line with this, in a study on Italian GD population, the influence of ethnicity and geographic variation on GD susceptibility conferred by genetic variation in *TSHR*, *CTLA-4*, and *TG* genes was observed [16].

With respect to *FOXP3* gene polymorphisms, the generic mechanism operative for many of the SNPs involves precluding of binding of transcription factors to putative binding sites that would eventually affect *FOXP3* transcription [34–40]. With respect to rs3761548, it may be explained that the substitution of C to A possibly interferes with the preferential binding of transcription factor Sp1 at

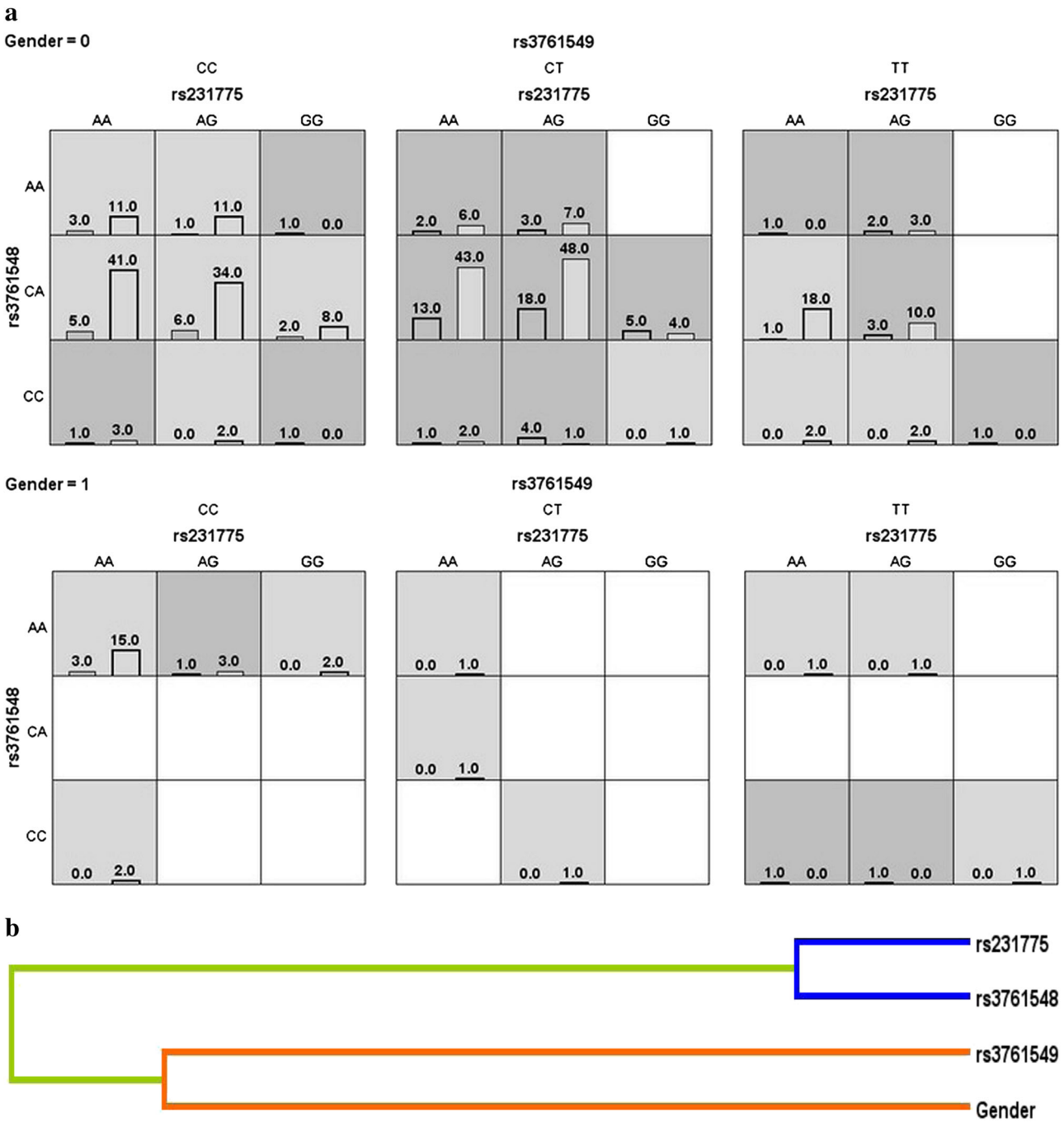

Fig. 5 Multifactor dimensionality reduction analysis for an interaction between rs231775, rs3761548, rs3761549 polymorphisms and gender in Graves' disease and healthy subjects. **a** Four-factor model. The light gray bar in each cell represents the frequency of Hashimoto's thyroiditis individuals and white bar represents the frequency of healthy individuals. High-risk genotype combinations are represented by dark gray shade cells, while light gray shade cells represent low-risk genotype combinations. Cells with no shading or white cells represent genotype combination for which no data is observed. Gender 0 indicates female cases and 1 indicates male cases. **b** Interaction dendrogram analysis between SNPs rs231775, rs3761548, rs3761549 and gender by MDR analysis in individuals with Graves' disease

its cognate site, and plausibly affects *FOXP3* expression [43]. Therein, immune dysregulation is ostensibly secondary to promoter heterogeneity in *FOXP3* gene. A deficiency of FOXP3 expression undermines the T-cell suppressive capacity of T-regs in peripheral sites of immune tolerance [3, 5]. Contrary to the associative relationship of rs3761548 with the development and intractability of GD in the Japanese population and with GD in Chinese or other non-Asians, we did not observe any association of rs3761548 either with HT or GD susceptibility in our study [35, 36,

38–40]. Our results were in concurrence with those in Caucasians [34, 37]. A lack of association of GD with *CD40*, *CD25*, and *FOXP3* genetic variants was also observed in the Italian population [16]. The frequency of CC genotype in GD population in our study was twice that in controls though it did not reach statistical significance. This could be partly ascribed to a relatively lesser sample size owing to very low prevalence of GD in the Indian population (0.67% of GD vs 11% of HT) [60]. Also, this variant could possibly exist in linkage disequilibrium and exert its influence in conjunction with other genetic variants in the proximity. The deviation from HWE observed for this gene variant in control population in our study could be attributed to the phenomenon of "skewed X-chromosome inactivation". A similar observation was observed in studies conducted in the Japanese and Chinese population [35, 40].

Further with respect to rs3761549 (−2383C>T), we could not observe a significant association of "T" allele with HT and GD predisposition, albeit a significant difference in the genotype distribution, denoted by an increased frequency of "CT" heterozygotes in both HT and GD groups compared to controls. However, it is to be noted that in the comparison of genotype contrasts, the presence of "T" allele (CT and TT vs CC) seemed to exert a dominant effect on HT and GD predisposition, which indeed is suggestive of a considerable influence of rs3761549 "T" allele on HT and GD predisposition. A similar finding of an increased frequency of CT heterozygote carriers was reported in female GD patients in Polish population [37]. The observed association of CT heterozygotes with HT and GD predisposition could be predicated on a reduced transcriptional efficiency of *FOXP*3 and corresponding quantitative variation in its transcription in "T" allele carriers [38, 40]. This could plausibly impair the regulatory function of T-regs leading to an uncontrolled clonal expansion of activated T-cells. Conceivably, in an intra-thyroidal milieu, functional genetic variants of *FOXP3* can incapacitate FOXP3-deficient Tregs thereby promoting the expansion of autoreactive T cells specific for thyroid-antigens like Tg, TPO, TSHR, etc. A recent study by Yu et al. demonstrated that the relative expression of FOXP3 was maximum in Chinese GD subjects with rs3761549 "CC" genotype followed by a decline in CT heterozygotes and eventually peters out in TT homozygotes [40]. The CC genotype also has been shown to associate with severe HT in the Japanese population [35]. Further, haplotype analysis between the studied *FOXP3* genetic variants and AITD susceptibility revealed that rs3761548 "C"–rs3761549 "T" was more frequent in HT and GD groups in our study. This could ostensibly support the "liability threshold phenomenon" that proposes the collective contribution of genetic variants and other stochastic factors in precipitating the disease.

As mentioned earlier, the combined association of polymorphic loci of various genes with disease susceptibility is congruent with the polygenic nature of HT and GD. In our study, evidence for an epistatic interaction between *CTLA-4* and *FOXP3* genes was obtained following MDR analysis, employing single-, two-, and three-factor analyses in HT and GD cases. It was shown that the rs231775 "GG" genotype in collaboration with rs3761548 "CA" and rs3761549 "CT" genotypes confer a higher risk for HT. As regards GD, rs231775 "AA", rs3761548 "CA", and rs3761549 "CC" combination was associated with lower risk for GD. This indicates that a combination of three SNPs (factors) is supposedly the best model for predicting susceptibility to HT and GD, respectively. Interaction dendrogram analysis was suggestive of dependence or correlation of these polymorphisms in both HT and GD etiologies. The nature of the relationship seems to be "redundancy", which implies that they could be in linkage disequilibrium in exerting their influence on the disease (HT/GD) susceptibility. Prior to this, there have been no reports of such an epistatic interaction between these genetic variants employing MDR approach. The variation in the MDR results of single and three-factor models appear to be representative of basic differences in the patterns of immune responses directed to thyroid antigens in these two disorders. HT pathology allies with cell-mediated immunity as it is poised for a predominantly $CD4^+$ Th1 response due to the involvement of cytotoxic T-cells and cytotoxic complement-fixing antibodies. Contrastingly, GD represents a predominantly antibody-mediated response with a minimal, if any, damage to the thyrocytes that is suggestive of Th2 response. Perhaps, the observed association of CTLA-4 rs231775 "GG" genotype with GD could be congruent with the protracted clonal expansion of Th2 cells which influence the B-lymphocytes/plasma cells to synthesize and release anti-TSHR-specific antibodies for a long duration. It is in this scenario that we can substantiate as to how the three models (single-, two-, and three-factor) gave contrasting results in combinations of the studied *CTLA-4* (rs231775) and *FOXP3* (rs3761548, 3761549) genotypes in these two diseases. Also, in our study, evidence for an epistatic interaction between the studied *CTLA-4, FOXP3* genetic variants and female gender was obtained following MDR analysis, employing single and four-factor analyses in HT and GD cases. It was shown that the rs231775 "GG" genotype in collaboration with rs3761548 "CA" and rs3761549 "CT" genotypes confer a higher risk in female HT subjects, while rs231775 "AA", rs3761548 "CA", and rs3761549 "CC" was associated with lower risk for GD. The interaction dendrogram further demonstrated a synergistic or non-redundant association between female gender and *FOXP3* rs3761549 in GD subjects. This aligns with the propensity of female gender for AITD development that

subsumes various female gender-related factors like estrogen, skewed X-chromosome inactivation and hormonal imbalance at puberty that are considered to enhance the risk for AITD [61, 62].

Our study has its own strengths and limitations. To date, there are no reports evaluating the risk associated with the SNPs in the *FOXP3* gene from India either singly or in haplotypes. This is seemingly the first international report for an epistatic interaction between the two immunoregulatory genes, i.e., *CTLA-4* and *FOXP3* in AITD subjects. As much as the knowledge of SNP genotyping in selected immunoregulatory genes is imperative, certain limitations persist with respect to the narrow range of SNPs described. Analyzing their association in conjunction with other genetic variants of *CTLA-4* and *FOXP3* and also with other genes that contribute to imbalanced T-cell activation like *CT60*, *TSHR*, *TG*, *CD40*, *CD25*, etc., could reify the influence of genetic variation of *CTLA-4* and *FOXP3* on evolution of AITD and also its tractability [7–9, 12, 13, 16, 63, 64]. It would also enable the generation of bespoke genotyping assays for meticulous case identification as rs231775 has been indicated as a predictive biomarker for determining the tractability and reversal of GD [65, 66]. Also, the inclusion of more SNPs would reinforce the intrinsic value of MDR that is designated for detection of higher order gene–gene interactions, given the perennial possibility of obtaining stronger interactions with several other SNPs. Despite this, evidence for an epistatic interaction was obtained in our study. While the current study did not entail the analysis of gene–environment interaction beyond gender, we advocate acquisition and integration of information on anthropometric parameters and modifiable risk factors in MDR that could disentangle and explain the influence of environment component together with a genetic predisposition on AITD susceptibility. We also acknowledge the sample size constraints as a limitation that could undermine the study power with respect to GD, partly attributable to low prevalence of GD in Indian population. This warrants future investigations in expanded sample sizes to confirm the observed gene associations with statistical significance so as to consolidate the proposition of their clinical utility together with thyroid-specific antibody profiling. In the present scenario of genetic epidemiology of complex diseases like AITD, there is a paucity of information concerning gene–gene interactions and a study of this kind could provide pertinent clues for deciphering the genetic basis of AITD pathologies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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