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Human leucocyte antigen alleles confer susceptibility and progression to Graves' ophthalmopathy in a Southern Chinese population

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ABSTRACT

Purpose To evaluate the contributions of human leucocyte antigen (*HLA*) class I and II genes in the development of Graves' ophthalmopathy (GO) in a Southern Chinese population.

Methods Eight HLA loci were genotyped and analysed in 272 unrelated patients with Graves' disease (GD) or the proptosis and myogenic phenotypes of GO, and 411 ethnically matched control subjects.

Results The allele frequencies of *HLA-DRB1*16:02* and *-DQB1*05:02* in the GD, proptosis and myogenic groups, *HLA-B*38:02* and *-DQA1*01:02* in the myogenic group were significantly higher than those in the control group, respectively (all corrected p values <0.05, OR >2.5). The haplotype frequencies of *HLA-DRB1*16:02-DQA1*01 :02-DQB1*05:02* and *HLA-DRB1*16:02-DQA1*01:02-DQB1*05:02-DPA1*02:02-DPB1*05:01* in the proptosis and myogenic groups, and *HLA-A*02:03-B*38:02-C*07 :02* and *HLA-A*02:03-B*38:02-C*07:02-DRB1*16:02-DQA1*01:02-DQB1*05:02-DPA1*02:02-DPB1*05:01* in the myogenic group were significantly higher than those in the control group respectively (all corrected p values <0.05, OR >2.5). The potential epitopes ('FLGIFNTGL' of TSHR, 'IRHSHALVS', 'ILYIRTNAS' and 'FVFARTMPA' of IGF-1R) were fitted exactly in the peptide-binding groove between *HLA-DRA1-DRB1*16 :02* heterodimer, and the epitopes ('ILEITDNPY' of THSR, 'NYALVIFEM' and 'NYSFYVLDN' of IGF-1R) were also fitted exactly in the peptide-binding groove between *HLA-DQA1*01:02-DQB1*05:02* heterodimer.

Conclusions The *HLA-DRB1*16:02* and *-DQB1*01:02* alleles might be risk factors for GD including the proptosis and myogenic phenotypes of GO. The alleles *HLA-B*38:02, -DQA1*01:02*, the HLA haplotypes consisting of *HLA-B*38 :02, -DRB1*16:02, -DQA1*01:02* and *-DQB1*05:02* might be susceptibility risk factors for GO. Simultaneously, some epitopes of TSHR and IGF-1R tightly binding to groove of *HLA-DRA1-DRB1*16:02* or *HLA-DQA1*01:02-DQB1*05 :02* heterodimers might provide some hints on presenting the pathological antigen in GO.

INTRODUCTION

Graves' ophthalmopathy (GO) is a common autoimmune inflammatory eye disease of adulthood associated with Graves' disease (GD), whose predisposition is widely attributed to interplay of genetic and environmental factors.[1] The common symptoms of GO are dry, photophobia, double vision and pressure sensation behind the eyes.[1] The clinical phenotypes of GO are proptosis and restrictive extraocular myopathy.[2] Over the years, genome-wide association studies have identified a growing list of credible candidate human leucocyte antigen (*HLA*) loci modestly associated with GD.[3 4] The main function of HLA molecules is to present antigenic peptides to the immune system and thus regulate the immune response.[5] The highly polymorphic HLA system is one of key immunogenetics associated with GO.[6] The genetic variations of classical class I region (eg, *HLA-B*) and the class II region (eg, *HLA-DRB1*, *-DQ1*, and *-DPB1*) are risk or protective factors in some ethnic populations, resulting in differences with GO prevalence.[6] In the Brazilian population, *HLA-DRB1*16* was over-represented in myogenic subtype of GO patients, and *HLA-DRB1*03* was over-represented in non-myogenic subtype of GO patients.[7] In the Polish population, the *HLA-DRB1*03* allele was found to be positively associated with GO.[8] Previous studies have shown that low-resolution HLA genotypes and serological HLA antigens are associated with GO. In the Japanese population, the antigens (HLA-DR14 and -DQ1) might be the genetic markers of predisposition to GO.[9] In the British population, the antigens (HLA-B8, -DQw 3.1 and -DPB 2.1/8) might confer protective effects in patients with GO.[10 11] Associations of *HLA-B8* and *-DR3* genotypes with GO were also reported in the Hungarian population.[12]

Although the initiating trigger of GO is still unclear, the excessive inflammation responses in orbital soft tissue might be the key pathogenesis.[1 13] Thyroid stimulating hormone receptor (TSHR) and type 1 insulin-like growth factor receptor (IGF-1R) might be two major antigens in orbital microenvironment with GO.[14 15] The extracellular domain of TSHR (TSHR-ECD) peptides epitopes bind to the groove of HLA molecules, forming HLA-THSR-complexes, which are presented to $CD4^+$ T cells in Graves' disease (GD).[13 15–18] Krieger *et al* have reported that bidirectional TSHR/IGF-1R cross-talk can mediate GO pathogenesis.[19] Mahdavi *et al* found that some peptides of IGF-1R-ECD (86 and 249 epitopes) were the best HLA-binding peptide epitopes to both HLA class I and II molecules based on molecular docking analyses.[20] As a result, activated orbital fibroblasts and infiltrating lymphocytes might contribute to the pathological phenotype of GO by producing

glycosaminoglycan and differentiate into myofibroblasts or adipocytes.[1 13]

In the present study, we comprehensively conducted high-resolution genotyping for eight HLA loci including *HLA-A, -B, -C, -DRB1, -DQA1, -DQB1, -DPA1* and *-DPB1* in samples collected from 272 unrelated GD patient with and without clinical GO and 411 control subjects with high resolution at both allelic and haplotypic levels using PCR-sequence-based typing (SBT). We aimed to evaluate whether specific alleles, haplotypes and the dock condition of the THSR-ECD or IGF-1R-ECD segment in eight HLA loci contribute to the development of GO in Southern Chinese.

MATERIALS AND METHODS

Participants

A total of 272 unrelated patients with GD were recruited from the patients attending the Clinic of Shenzhen Eye Hospital, China between December 2018 and December 2019. Diagnosis of GD was based on the typical clinical features of hyperthyroidism, diffuse goitre, suppressed TSHR levels, detectable THSR autoantibodies and/or increased radioiodine uptake.[3 8 9] The clinical GO was defined as class 3 or higher in the American Thyroid Association mnemonic NOSPECS scheme. The GO patients divided into proptosis (the proptosis ≥18 mm or the difference between two eyes ≥2 mm) and the myogenic (strabismus caused by extraocular-muscle dysfunction without orbital fat compartment enlargement) groups according to the presence of clinically evident ophthalmopathy, as previously described.[2 8] GD patients without GO were selected from those who did not have any features of ophthalmopathy (including features of class 1–2). The control group consisted of 411 unrelated healthy volunteers randomly chosen from the Shenzhen Blood Centre, China. All patients and controls were Han Chinese from Southern China. The exclusion criteria were setting as follows: the heathy volunteer without autoimmunity systemic disease history including GDor GO, and the patient who was diagnosed as GD or GO without other systemicdiseases were enrolled as the study cohort. Peripheral blood samples (with 5% EDTA as anti-coagulant) were collected from each subject and stored at −20°C. The study protocol was approved by the Ethics Committee of Shenzhen Eye Hospital and was in accordance with the tenets of the Declaration of Helsinki and its later amendments or comparable ethical standards. Written informed consent was obtained from all study participants.

Subgroups of participants

On the basis of the presence of clinically evident ophthalmopathy, four subgroups were considered: Group 1: GD without clinical eye phenotype group (no changes in ophthalmologic findings had been detected according to 3 years follow-up data); Group 2: the proptosis group (the proptosis ≥18 mm or the difference between two eyes ≥2 mm); Group 3: the myogenic group (strabismus caused by extraocular-muscle dysfunction without orbital fat compartment enlargement); Group 4: the control group.

DNA extraction and sequence-based typing for HLA genes

Blood DNAs were obtained from all 683 participants. The IPD-IMGT/HLA Database (http://www.ebi.ac.uk/ipd/imgt/hla/, Release 3.40.0, 2020 April) was applied for the genotyping of each locus. Genotyping for *HLA-A, -B, -C, -DRB1* and *-DQB1* was performed using the AlleleSEQR HLA SBT commercial kit (Atria Genetics, San Francisco) according to the manufacturer's instructions. Exons 2∼4 for *HLA-A, -B, -C* and exons 2, 3 for *-DRB1* and *-DQB1* were sequenced. *HLA-DQA1* SBT covered exons 1∼4 as described by Voorter *et al*.[21] *HLA-DPA1* and *-DPB*1 SBT was performed according to the protocol developed by the 13th International Histocompatibility Workshop. PCR amplification was performed using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). PCR products were purified using ExoSAP-IT (Atria Genetics) and sequenced with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kits (Applied Biosystems, Foster City, CA, USA) on an ABI 3730XL DNA sequencer (Applied Biosystems, Foster City, CA, USA). Four-digit HLA genotypes were assigned with the help of the ASSIGN 3.5 software (Conexio Genomics, Applecross, Australia). Ambiguous allele combinations from the SBT results were further examined using the HLA PCR-SSP kit (Olerup, Stockholm, Sweden).

HLA peptide-binding prediction and in silico docking

The sequences of TSHR and IGF-1R (NP_000360.1 and NP_000866.1) were submitted to the NetMHCIIpan 3.2 server for predicting major HLA-II peptide-binding affinities.[22] *HLA-DRA1-DRB1*16:02* and *HLA-DQA1*01:02-DQB1*05:02* heterodimers were selected for predicting epitopes within the protein. Threshold for weak and strong binding peptides was set as 1% and 10% ranks, respectively. The computing docking of the TSHR-ECD and IGF-1R-ECD segment into *HLA-DRA1-DRB1*16:02* and *HLA-DQA1*01:02-DQB1*05:02* heterodimers in silico was performed using the AutoDock Vina software, as described by Shu *et al*.[23]

Statistical analysis

Statistical analysis was performed using SPSS (version 20.0, SPSS Inc., Chicago, IL). Hardy-Weinberg equilibrium (HWE) was tested using the χ^2 test. Haplotype frequencies were estimated from the genotyping results by the Excoffier-Laval-Balding (ELB) algorithm using the Arlequin software package version 3.5 (Laurent Excoffier, CMPG, Zoological Institute, University of Bern, Switzerland).[24] The difference in allele frequency and haplotype frequency was tested using the χ^2 test or Fisher's exact test. Linkage disequilibrium (LD), defined as D' and r^2, was calculated between each intra-gene haplotype block as described by Lewontin *et al*.[25] Multiple testings were corrected using the Bonferroni method and the corrected p value was calculated by multiplying the p value with the number of tests performed. The corrected p values (Pc) <0.05 were considered statistically significant. OR and 95% CI were calculated whenever applicable.

RESULTS

Demographic and clinical features of participants

In the GD without clinical eye phenotype group, there were 17 (23.0%) males and 57 (77.0%) females (n=74), with an average age of 38.7 (±10.4) years. In the proptosis group (n=82), there were 32 (39.0%) males and 50 (61.0%) females, with an average age of 34.9 (±12.1) years. In the myogenic group (n=116), there were 59 (51.0%) males and 57 (49.1%) females, with an average age of 44.2 (±11.4) years. In the control group (n=411), there were 411 healthy adults in Southern Chinese, with an average age of 40.6 (±12.0) years. No significant difference in age was found between patients with GD and controls (39.9±12.0 vs. 40.6±12.0 years, p value = 0.414).

Allele frequencies of *HLA-A, -B, -C, -DRB1, -DQA1, -DQB1* and *-DPB1*

The genotype distributions of *HLA-A, -B, -C, -DRB1, -DQA1, -DQB1* and *-DPB1* in the control group was in accordance with HWE (all p values >0.05) (data not shown). A total of 31 *HLA-A* alleles, 63 *HLA-B* alleles, 29 *HLA-C* alleles, 40 *HLA-DRB1* alleles, 18 *HLA-DQA1* alleles, 17 *HLA*-DQB1 alleles, 7 *HLA-DPA1*

alleles, and 24 *HLA-DPB1* alleles were identified in all participants including patients and controls (data not shown). The allele frequencies of *HLA-DRB1*16:02* and *-DQB1*05:02* in GD patients without clinical eye phenotype, the proptosis and myogenic groups were significantly higher than those in the control group respectively, which survived the Bonferroni correction (all Pc <0.05, OR >2.5). The allele frequencies of *HLA-B*38:02* and *-DQA1*01:02* in the myogenic group were significantly higher than those in the control group, respectively, which survived the Bonferroni correction (8.6% vs 3.0%, Pc=0.01, OR=3.0; 32.8% vs 19.8%, Pc $=5.9\times10^{-4}$, OR=2.0) (table 1).

Haplotype frequencies

We identified 404 HLA class I haplotypes (A-C-B), 93 *HLA-DRB1-DQA1-DQB1* haplotypes, 44 *HLA-DPA1-DPB1* haplotypes, 338 HLA class II haplotypes (*HLA-DRB1-DQA1-DQB1-DPA1-DPB1*) and 974 HLA eight loci haplotypes (*HLA A–C-B-DRB1-DQA1-DQB1-DPA1-DPB1*) in all participants including patients and controls (data not shown). The haplotype frequencies of *HLA-DRB1*16:02-DQA1*01:02-DQB1*05:02* and *HLA-DRB1*16:02-DQA1*01:02-DQB1*05:02-DPA1*02:02-DPB1*05:01* in the proptosis and myogenic groups were significantly higher than those in the control group, respectively, which survived the Bonferroni correction (all Pc <0.05, OR >4.0). The haplotype frequencies of *HLA-A*02:03-B*38:02-C*07:02* and *HLA-A*02:03-B*38:02-C*07:02-DRB1*16:02-DQA1*01:02-DQB1*05:02-DPA1*02:02-DPB1*05:01* in the myogenic group were significantly higher frequencies than those in the control group, respectively, which survived the Bonferroni correction (6.5 vs 2.7%, Pc=0.02, OR=4.3; 4.7 vs 0.4%, Pc $=1.7\times10^{-3}$, OR=13.6) (table 1).

Since the three disease-associated HLA class II alleles *HLA-DRB1*16:02*, *HLA-DQA1*01:02* and *HLA-DQB1*05:02* formed a haplotype, we further investigated the associations between eight combinations of *HLA-DRB1*16:02*, *HLA-DQA1*01:02* and *HLA-DQB1*05:02* with disease groups. As shown in table 2, *HLA-DRB1*16:02*$^{+}$*-DQA1*01:02*$^{+}$*-DQB1*05:02*$^{+}$ was associated with the proptosis and myogenic groups, respectively, which survived the Bonferroni correction (all Pc <0.05, OR =3.96 and 4.83 respectively). *HLA-DRB1*16:*

Table 1 Allele and haplotype frequencies of HLA in cases and controls

	Controls	GD without clinical eye phenotype group			Proptosis group			Myogenic group		
	(2 n=822, %)	(2 n=148, %)	Pc*	OR	(2 n=164, %)	Pc†	OR	(2 n=232, %)	Pc‡	OR
HLA Allele										
B*38:02	25 (3.0)	4 (2.7)	NS		7 (4.3)	NS		20 (8.6)	0.01	3.0
DRB1*16:02	39 (4.7)	17 (11.5)	0.048	2.6	27 (16.5)	1.7×10^{-6}	4.0	45 (19.4)	1.4×10^{-11}	4.8
DQA1*01:02	163 (19.8)	38 (25.7)	NS		42 (25.6)	NS		76 (32.8)	5.9×10^{-4}	2.0
DQB1*05:02	21 (10.9)	29 (19.6)	1.1×10^{-16}	9.3	36 (22.0)	4.6×10^{-21}	10.7	64 (27.6)	7.2×10^{-34}	14.5
HLA Haplotype										
A*02:03-B*38:02-C*07:02	13 (1.6)	4 (2.7)	NS		0 (0.0)	NS		15 (6.5)	0.02	4.3
DRB1*16:02-DQA1*01:02-DQB1*05:02	39 (4.7)	17 (11.5)	NS		27 (16.5)	3.9×10^{-6}	4.0	45 (19.4)	3.2×10^{-11}	4.8
DRB1*16:02-DQA1*01:02-DQB1*05:02-DPA1*02:02-DPB1*05:01	23 (2.8)	8 (5.4)	NS		17 (10.3)	2×10^{-3}	4.0	37 (15.9)	7.7×10^{-12}	6.6
A*02:03-B*38:02-C*07:02-DRB1*16:02-DQA1*01:02-DQB1*05:02-DPA1*02:02-DPB1*05:01	3 (0.4)	2 (1.6)	NS		1 (0.1)	NS		11 (4.7)	1.7×10^{-3}	13.6

*Pc: GD without clinical eye phenotype group vs controls.
†Pc: proptosis group vs controls.
‡Pc: myogenic group vs controls.
Only HLA alleles with corrected p values <0.05 are shown. The p value was calculated using χ^2 test or Fisher's exact test and corrected for the number of tests performed (Allele B: 63, DRB1: 40, DQA1: 18, DQB1:18; Haplotype A–B-C: 404, DRB1-DQA1-DQB1: 93, DRB1-DQA1-DQB1-DPA1-DPB1: 338, A–B-C-DRB1-DQA1-DQB1-DPA1-DPB1: 1143).
HLA, human leucocyte antigen; NS, not significant; OR, odds ratio; Pc, corrected p value.

Table 2 Association of the DRB1*16:02-DQA1*01:02-DQB1*05:02 haplotype with GD with or without clinical eye disease

			Controls	GD without clinical eye phenotype group			Proptosis Group			Myogenic group		
DRB1*16:02	DQA1*01:02	DQB1*05:02	(2 n=822, %)	(2 n=148, %)	Pc*	OR	(2 n=164, %)	Pc†	OR	(2 n=232, %)	Pc‡	OR
+	+	+	39 (4.7)	17 (11.5)	NS		27 (16.5)	3.9×10^{-6}	4.0	45 (19.0)	3.2×10^{-11}	4.8
–	–	–	627 (76.3)	104 (70.3)	NS		119 (72.6)	NS		146 (63.0)	4.6×10^{-3}	0.5
–	+	–	105 (12.8)	15 (10.1)	NS		9 (5.5)	NS		22 (9.0)	NS	
–	–	+	32 (3.9)	6 (4.1)	NS		3 (1.8)	NS		10 (4.0)	NS	
–	+	+	19 (2.3)	6 (4.1)	NS		6 (3.7)	NS		9 (4.0)	NS	
+	–	–	0 (0)	0 (0)	NS		0 (0)	NS		0 (0.0)	NS	
+	+	–	0 (0)	0 (0)	NS		0 (0)	NS		0 (0.0)	NS	
+	–	+	0 (0)	0 (0)	NS		0 (0)	NS		0 (0.0)	NS	

*Pc: GD without clinical eye phenotype group vs controls.
†Pc: proptosis group vs controls.
‡Pc: myogenic group vs controls.
The p value was calculated using χ^2 test or Fisher's exact test and corrected for 93 tests performed.
HLA, human leucocyte antigen; NS, not significant; OR, odds ratio; Pc, corrected p value.

*02⁻DQA1*01:02⁻DQB1*05:02⁻* was a protective factor for the myogenic group, which survived the Bonferroni correction (Pc <0.05, OR =0.53). Our results suggested that *HLA-DRB1*16:02*, *HLA-DQA1*01:02* and *HLA-DQB1*05:02* were associated with the development of clinical GO.

Comparation of OR and linkage disequilibrium

The OR of significant HLA allele and haplotype between the disease groups and control groups are shown in figure 1, respectively. The ORs of *HLA-DRB1*16:02* and *HLA-DQB1*05:02* alleles of 'the myogenic group vs control group' showed the top odd (OR: 4.8 and 14.5), followed by 'the proptosis group vs control group' (OR: 4.0 and 10.7) and 'GD without clinical eye phenotype group vs control group' (OR: 2.6 and 9.3), respectively. The ORs of *HLA-DRB1*16:02-DQA1*01:02-DQB1*05:02* and *HLA-DRB1*16:02-DQA1*01:02-DQB1*05:02-DPA1*02:02-DPB1* 05:01* haplotypes of 'the myogenic group vs control group' (OR: 4.8 and 6.6) were higher than that in 'the proptosis group vs control group' (OR: 4.0 and 4.0).

Thirty-five alleles had strong LD (r^2 > 0.33, 95% CI 0.70 to 0.98) (data not shown). The allele *HLA-DRB1*16:02* was linked to *HLA-DQA1*01:02* and *HLA-DQB1*05:02* tightly (D'=1, 95% CI 0.95 to 1.00, r^2=0.62; D'=0.99, 95% CI 0.96 to 1.00, r^2=0.34).

Computational epitope prediction and binding of TSHR-ECD and IGF-1R-ECD to HLA

It has been reported that in GO, the pathogenic auto-antigenic peptide epitope of TSHR and IGF-1R could be matched to the antigen-binding pocket of HLA molecule and be recognised by T cell receptors.[13] We predicted the potential epitopes of THSR-ECD (amino acids 1–418, AA 1–418) and IGF-1R-ECD (AA 1–902), which could be recognised by the *HLA-DRA1-DRB1*16:02* and *HLA-DQA1*01:02-DQB1*05:02* heterodimers.

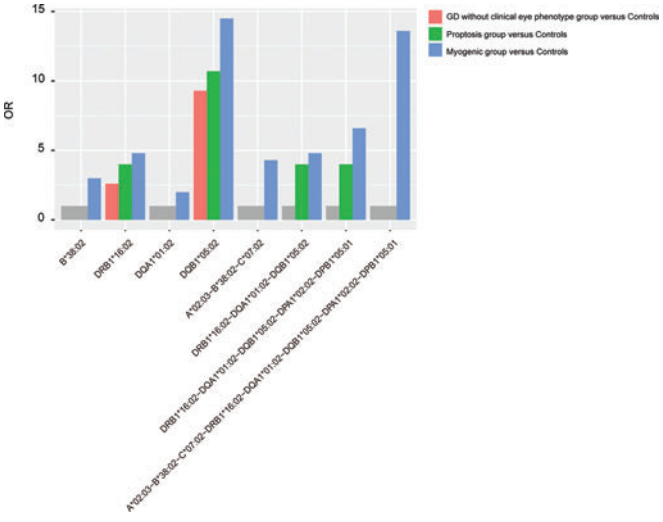

Figure 1 Association analysis OR for significant HLA alleles and haplotypes. The magnitude of OR is indicated by height of column. The OR of 'GD without clinical eye phenotype group vs Controls', 'Proptosis group vs Controls', and 'Myogenic group vs Controls' are shown in pink, blue and green columns, respectively. The grey column represents no significance between two groups.

We identified 1 epitope of THSR-ECD and 3 epitopes of IGF-1R-ECD showing strong binding to *HLA-DRA1-DRB1*16:02* heterodimer respectively, including 'FLGIFNTGL', 'IRHSHALVS', "ILYIRTNAS' and 'FVFARTMPA'. Similarly, 1 epitope of THSR-ECD and 2 epitopes of IGF-1R-ECD also showing strong binding to *HLA-DQA1*01:02-DQB1*05:02* heterodimer respectively, including 'ILEITDNPY', 'NYALVIFEM' and "NYSFYVLDN' (figure 2). The position of the epitopes with the target sequences 'FLGIFNTGL' and 'ILEITDNPY' were located between AA130–138 and AA155–163 of TSHR-ECD, and 'IRHSHALVS', 'ILYIRTNAS', 'FVFARTMPA', 'NYALVIFEM' and 'NYSFYVLDN' were located between AA390–398, AA601–609, AA820–828, AA114–122, and AA417–425 of IGF-1R-ECD. Furthermore, we predicted the binding affinity between the HLA pocket and epitopes of 'FLGIFNTGL', 'IRHSHALVS', 'ILYIRTNAS', 'FVFARTMPA', 'ILEITDNPY', 'NYALVIFEM' and 'NYSFYVLDN'. The potential sequences 'FLGIFNTGL', 'IRHSHALVS', 'ILYIRTNAS' and 'FVFARTMPA' fitted exactly in the peptide-binding groove between *HLA-DRA1-DRB1*16:02* heterodimer, with a docking score (ΔG) of −9.1, −9.6, −5.1 and −5.9 kcal/mol, respectively (figure 3). In addition, the target sequences 'ILEITDNPY', 'NYALVIFEM' and 'NYSFYVLDN' also fitted exactly in the peptide-binding groove between *HLA-DQA1*01:02-DQB1*05:02* heterodimer. The ΔG scores between *HLA-DQB1*05:02* and 'ILEITDNPY', 'NYALVIFEM' and 'NYSFYVLDN' were −6.1, −5.7 and −5.9 kcal/mol, respectively (figure 4).

DISCUSSION

In this study, we investigated the association of eight *HLA* loci with GD and GO in the Southern Chinese population. Our results demonstrated that the alleles *HLA-B*38:02*, *-DRB1*16:02*, *-DQA1*01:02* and *-DQB1*05:02* and the special haplotypes

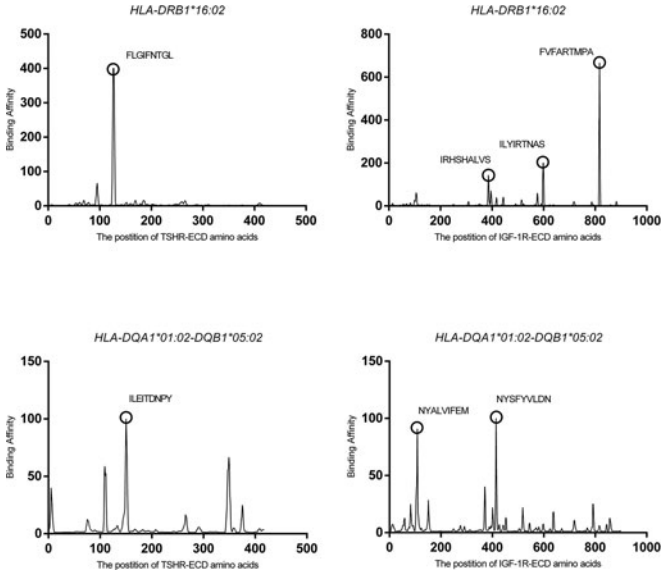

Figure 2 Binding affinity prediction of the TSHR-ECD and IGF-1R-ECD to the *HLA- DRB1*16:02* and *HLA-DQA1*01:02-DQB1*05:02* heterodimers. The NetMHCIIpan 3.2 Server was used to predict epitopes within TSHR and IGF-1R protein (TSHR-NP_000360.1 and IGF-1R-NP _000866.1). The y-axis presents the binding affinity represented as 1 divided by %Rank of predicted affinity compared with random natural peptides, while the x-axis represents the position of amino acids.

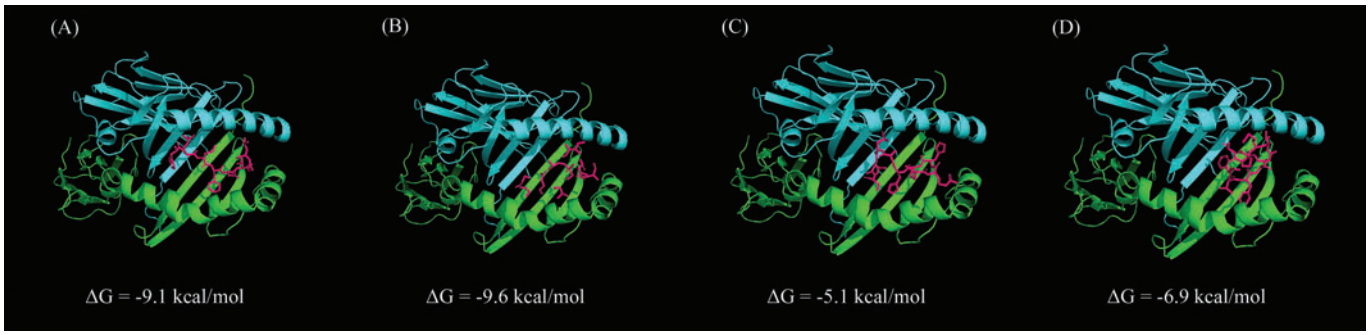

Figure 3 Computational docking of TSHR-ECD and IGF-1R-ECD segments to the HLA-DRA1-DRB1*16:02 heterodimer. (A) 'FLGIFNTGL' of TSHR-ECD binding to the HLA-DRA1-DRB1*16:02 heterodimer; (B) 'IRHSHALVS' of IGF-1R-ECD binding to the HLA-DRA1-DRB1*16:02 heterodimer; (C) 'ILYIRTNAS' of IGF-1R-ECD binding to the HLA-DRA1-DRB1*16:02 heterodimer; (D) 'FVFARTMPA' of IGF-1R-ECD binding to the HLA-DRA1-DRB1*16:02 heterodimer. HLA-DRA1, HLA-DRB1, TSHR-ECD and IGF-1R-ECD segments are shown in blue, green and purple, respectively.

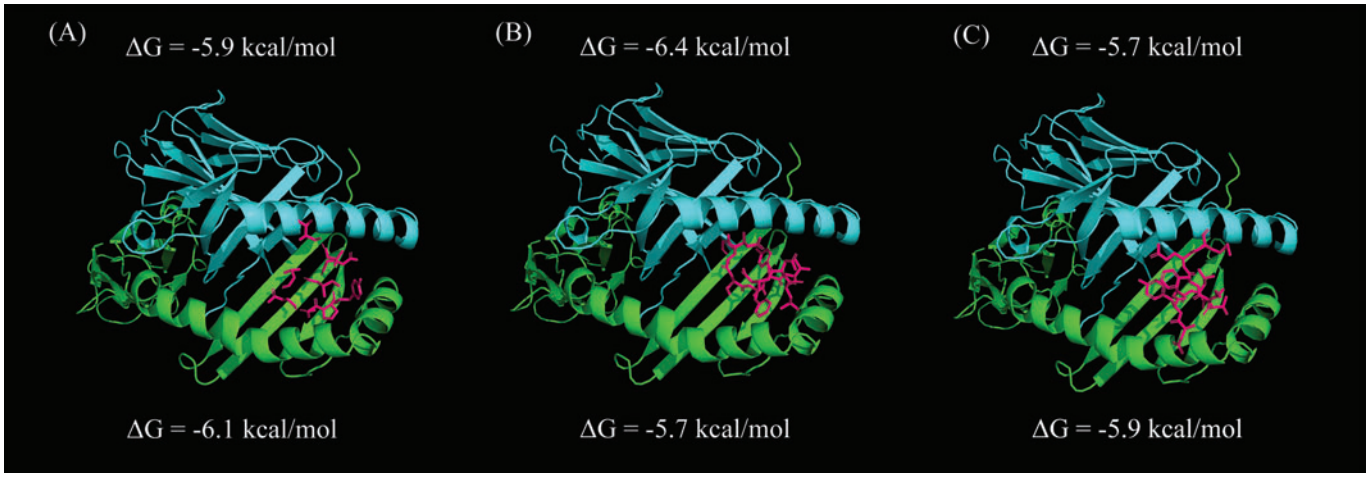

Figure 4 Computational docking of TSHR-ECD and IGF-1R-ECD segments to the HLA-DQA1*01:02-DQB1*05:02 heterodimer. (A) 'ILEITDNPY' of TSHR-ECD binding to the HLA-DQA1*01:02-DQB1*05:02 heterodimer; (B) 'NYALVIFEM' of IGF-1R-ECD binding to the HLA-DQA1*01:02-DQB1*05:02 heterodimer; (C) 'NYSFYVLDN' of IGF-1R-ECD binding to the HLA-DQA1*01:02-DQB1*05:02 heterodimer. HLA-DQA1, HLA-DQB1, TSHR-ECD and IGF-1R-ECD segments are shown in blue, green and purple, respectively.

formed by these HLA alleles might contribute to the development of different types of ocular phenotypes in GD patients.

The allele frequencies of *HLA-DRB1*16:02* and *-DQB1*05:02* in the GD patient in the Southern Chinese population were significantly higher than those in the control group (table 1). Similar findings had been reported in GD patient in Thai, Korea and Han populations in Taiwan.[26–28] We hypothesised that the alleles *HLA-DRB1*16:02* and *-DQB1*05:02* might be risk factors in common for GD in the Southern Chinese population or the other Asian population. However, previous associations did not consider different ocular phenotypes in GD. Chen *et al* revealed that *HLA-DRB1*16:02* carried a unique motif of amino acid residues at position 67–74, which might increase the ability to present immunogenic autoantigens.[29] Alleles *HLA-DRB1*16:02* and *-DQB1*05:02* were tightly linked in our study due to linkage disequilibrium, which has also been reported in the Asian population.[30] The combinations of alleles *HLA-DRB1*16:02* and *-DQB1*05:02* might enhance the susceptibility to some diseases.[23 30 31] *HLA-DRB1*16* has been associated with the pathogenesis of the myopathic process of GO in Brazilian patients.[7] Beside allele *HLA-DRB1*16:02*, the allele frequencies of *HLA-B*38:02* and *-DQA1*01:02* only in the myogenic group in Southern Chinese population were significantly higher than those in the control group (table 1). We hypothesised that alleles *HLA-B*38:02* and *-DQA1*01:02* might be key risk factors in the development of extraocular-muscle dysfunction in Southern Chinese population with GO.

The HLA haplotype combination of alleles *HLA-B*38:02*, *-DRB1*16:02*, *-DQA1*01:02* and *-DQB1*05:02* might contribute to the development of ocular phenotype in GD patients. Firstly, the haplotypes *HLA-DRB1*16:02-DQA1*01:02-DQB1*05:02* and *HLA-DRB1*16:02-DQA1*01:02-DQB1*05:02-DPA1*02:02-DPB1*05:01* might be common risk factors for the proptosis and myogenic phenotypes of GO in Southern Chinese population (table 1). Secondly, the frequencies of class I haplotype *HLA-A*02:03-B*38:02-C*07:02* and eight HLA loci haplotype *HLA-A*02:03-B*38:02-C*07:02-DRB1*16:02-DQA1*01:02-DQB1*05:02-DPA1*02:02-DPB1*05:01* in the myogenic group were significantly higher than those in the control group (table 1). Interestingly, the frequency of the haplotype lacking of alleles *HLA-DRB1*16:02*, *-DQA1*01:02* and *-DQB1*05:02* in the myogenic group was significantly lower than that in the control group (table 2). Lastly, the ORs of the significant HLA alleles and haplotypes in myogenic group were higher than those in the proptosis and GD without clinical eye

phenotype group respectively (table 1, figure 1). Therefore, the combination of alleles *HLA-B*38:02, -DRB1*16:02, -DQA1*01:02* and *-DQB1*05:02* might contribute to different phenotypes of GO with different degrees.

Notably, some disease-causing autoantigenic peptides were only fitted into the peptide- binding pockets of specific kind of *HLA-DRA1-DRB1* and *HLA-DQA1-DQB1* heterodimers, and recognised by the $CD4^+$ T-cell receptor.[13 15–18 23 32] Some previous studies showed that the alleles *HLA-DRB1*16:02* and/or *HLA-DQB1*05:02* might be mediated by autoantibodies in GD, anti-NMDAR encephalitis and anti-IFN-γ disseminated nontuberculous mycobacterial infections myasthenia gravis, and juvenile ocular myasthenia gravis.[23 29 30] Shu *et al* found that the epitope 'FRAITSTLA' of autoantibody to the NR1 subunit of NMDAR binds to *HLA-DRA1-DRB1*16:02* heterodimer on the surface of APCs, which might provoke the pathogenesis of anti-NMDAR encephalitis.[23] Our study also discovered the association between heterodimers (*HLA-DRA1-DRB1*16:02* and *HLA-DQA*01:02-DQB1*05:02*) and the antigens (TSHR and IGF-1R). Interestingly, bioinformatic analyses suggested that some epitopes ('FLGIFNTGL' of TSHR-ECD, 'IRHSHALVS', 'ILYIRTNAS' and 'FVFARTMPA' of IGF-1R-ECD) strongly bind to the HLA-DRα-DRβ1 pocket groove, encoded by the *HLA-DRA1-DRB1*16:02* heterodimer. Simultaneously, the *HLA-DQA*01:02-DQB1*05:02* heterodimer encoded the pocket groove of HLA-DQα1-DQβ1 peptide-binding groove combined with the potential epitopes ('ILEITDNPY' of THSR-ECD, 'NYALVIFEM' and 'NYSFYVLDN' of IGF-1R-ECD), strongly. These peptides were fitted tightly in the peptide-binding groove of HLA-DRα-DRβ1 or HLA-DQα1-DQβ1 with relatively lower docking scores (especial peptides, 'FLGIFNTGL' of TSHR-ECD and 'IRHSHALVS' of IGF-1R-ECD). Previous studies have found that the epitopes ('FLGIFNTGL' and 'ILEITDNPY') of THSR-ECD might be the immunodominant T-cell epitope that binds to the HLA-DR3 molecule in silico and in vitro.[16–18 33 34] These epitopes of TSHR-ECD or IGF-1R-ECD might favour binding to the pocket groove of special heterodimers (HLA-DRα-DRβ1 or HLA-DQα-DQβ1), increasing the capacity in APCs, to present the disease-causing autoantigenic peptides to the T-cell receptors, which might predispose to GD with and without clinical GO in immunogenicity.

We firstly conducted high-resolution genotyping for eight HLA loci in 272 unrelated GDpatient with and without clinical GO in Southern Chinese at both allelic andhaplotypic levels. These results showed that the specific HLA alleles andhaplotypes of GO were prevalent in patients with GD or GO in Southern Chinese,which is a different region and ethnic group from previous studies. Inaddition, we found that the HLA alleles and haplotypes contributed to differentprogression of GO. Furthermore, we performed an in-silico analysis for the bindingaffinities between HLA class II and peptides from the two most extensivelystudied autoantigens primarily. Our results indicated that different HLAalleles and haplotypes could be used as biomarkers and predict the progressionof different phenotypes of GO in Southern Chinese. However, there were some limitations in this study. The number of patients with GD and GO was relatively small, and the participation seems to be population-specific, which might have limited our results generalising to the general population. We found the female frequency of the patients was significantly higher than those in the control group. However, the sex mismatch might not impact the HLAcontribution to GD since the inheritance of HLA loci is autosomal dominant. We should have proven that the binding condition might provoke the progression to GO between the potential peptides of THSR or IGF-1R and *HLA-DRA1-DRB1*16:02* or *HLA-DQA*01:02-DQB1*05:02* heterodimers in vitro and in vivo.

CONCLUSIONS

Taken together, the current study suggested that alleles *HLA-B*38:02, -DRB1*16:02, -DQA1*01:02* and *-DQB1*05:02* and corresponding haplotypes might contribute to the development of GD, proptosis and myogenic phenotypes of GO in the Southern Chinese population. In addition, we predicted that some potential critical epitopes of TSHR-ECD or IGF-1R-ECD favoured binding to *HLA-DRA1-DRB1*16:02* and *HLA-DQA*01:02-DQB1*05:02* heterodimers tightly, by bioinformatic analysis. These findings indicated that the associations of alleles *HLA-B*38:02, -DRB1*16:02, -DQA1*01:02* and *-DQB1*05:02* and corresponding haplotypes with the development of GD and clinical GO might be due to affecting the affinity between HLA peptide-binding groove and antigenic peptides of TSHR-ECD and IGF-1R-ECD.

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REFERENCES

1 Bahn RS. Graves' ophthalmopathy. *N Engl J Med* 2010;362:726–38.
2 Bednarczuk T, Hiromatsu Y, Fukutani T, *et al.* Association of cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) gene polymorphism and non-genetic factors with Graves' ophthalmopathy in European and Japanese populations. *Euro J Endocrinol* 2003;148:13–18.
3 Chu X, Yang M, Song ZJ, *et al.* Fine mapping MHC associations in Graves' disease and its clinical subtypes in Han Chinese. *J Med Genet* 2018;55:685–92.

4 Okada Y, Momozawa Y, Ashikawa K, *et al.* Construction of a population-specific HLA imputation reference panel and its application to Graves' disease risk in Japanese. *Nat Genet* 2015;47:798–802.
5 Dendrou CA, Petersen J, Rossjohn J, *et al.* HLA variation and disease. *Nat Rev Immunol* 2018;18:325–39.
6 Khalilzadeh O, Noshad S, Rashidi A, *et al.* Graves' ophthalmopathy: a review of immunogenetics. *Curr Genomics* 2011;12:564–75.
7 Akaishi PM, Cruz AA, Silva FL, *et al.* The role of major histocompatibility complex alleles in the susceptibility of Brazilian patients to develop the myogenic type of Graves' orbitopathy. *Thyroid* 2008;18:443–7.
8 Bednarczuk T, Hiromatsu Y, Seki N, *et al.* Association of tumor necrosis factor and human leukocyte antigen DRB1 alleles with Graves' ophthalmopathy. *Hum Immunol* 2004;65:632–9.
9 Ohtsuka K, Nakamura Y. Human leukocyte antigens associated with hyperthyroid Graves ophthalmology in Japanese patients. *Am J Ophthalmol* 1998;126:805–10.
10 Kendall-Taylor P, Stephenson A, Stratton A, *et al.* Differentiation of autoimmune ophthalmopathy from Graves' hyperthyroidism by analysis of genetic markers. *Clin Endocrinol (Oxf)* 1988;28:601–10.
11 Weetman A, Zhang L, Webb S, *et al.* Analysis of HLA-DQB and HLA-DPB alleles in Graves' disease by oligonucleotide probing of enzymatically amplified DNA. *Clin Endocrinol (Oxf)* 1990;33:65–71.
12 Frecker M, Stenszky V, Balazs C, *et al.* Genetic factors in Graves' ophthalmopathy. *Clin Endocrinol (Oxf)* 1986;25:479–85.
13 Wang Y, Smith TJ. Current concepts in the molecular pathogenesis of thyroid-associated ophthalmopathy. *Invest Ophthalmol Vis Sci* 2014;55:1735–48.
14 Smith TJ. Recognizing the putative role for TSH receptor expressing fibrocytes in thyroid-associated ophthalmopathy may solve several mysteries. *Nat Rev Endocrinol* 2015;11:171–81.
15 Tsui S, Naik V, Hoa N, *et al.* Evidence for an association between thyroid-stimulating hormone and insulin-like growth factor 1 receptors: a tale of two antigens implicated in Graves' disease. *J Immunol* 2008;181:4397–405.
16 Inaba H, Pan D, Shin YH, *et al.* Immune response of mice transgenic for human histocompatibility leukocyte Antigen-DR to human thyrotropin receptor-extracellular domain. *Thyroid* 2009;19:1271–80.
17 Inaba H, Martin W, Ardito M, *et al.* The role of glutamic or aspartic acid in position four of the epitope binding motif and thyrotropin receptor-extracellular domain epitope selection in Graves' disease. *J Clin Endocrinol Metab* 2010;95:2909–16.
18 Inaba H, De Groot LJ, Akamizu T. Thyrotropin receptor epitope and human leukocyte antigen in Graves' disease. *Front Endocrinol (Lausanne)* 2016;7:120.
19 Krieger CC, Perry JD, Morgan SJ, *et al.* TSH/IGF-1 receptor cross-talk rapidly activates extracellular signal-regulated kinases in multiple cell types. *Endocrinology* 2017;158:3676–83.
20 Mahdavi M, Moreau V, Kheirollahi M. Identification of B and T cell epitope based peptide vaccine from IGF-1 receptor in breast cancer. *J Mol Graph Model* 2017;75:316–21.
21 Voorter CE, van den Berg-loonen EM. Sequence-based typing of the complete coding sequence of DQA1 and phenotype frequencies in the Dutch Caucasian population. *Hum Immunol* 2006;67:756–63.
22 Jensen KK, Andreatta M, Marcatili P, *et al.* Improved methods for predicting peptide binding affinity to MHC class II molecules. *Immunology* 2018;154:394–406.
23 Shu Y, Qiu W, Zheng J, *et al.* HLA class II allele DRB1*16:02 is associated with anti-NMDAR encephalitis. *J Neurol Neurosurg Psychiatry* 2019;90:652–8.
24 Excoffier L, Lischer HE. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under linux and windows. *Mol Ecol Resour* 2010;10:564–7.
25 Lewontin RC. On measures of gametic disequilibrium. *Genetics* 1988;120:849–52.
26 Wongsurawat T, Nakkuntod J, Charoenwongse P, *et al.* The association between HLA class II haplotype with Graves' disease in Thai population. *Tissue Antigens* 2006;67:79–83.
27 Park MH, Park YJ, Song EY, *et al.* Association of HLA-DR and -DQ genes with Graves disease in Koreans. *Hum Immunol* 2005;66:741–7.
28 Chen PL, Fann CS, Chu CC, *et al.* Comprehensive genotyping in two homogeneous Graves' disease samples reveals major and novel HLA association alleles. *PloS One* 2011;6:e16635.
29 Chen Y, Li S, Huang R, *et al.* Comprehensive meta-analysis reveals an association of the HLA-DRB1*1602 allele with autoimmune diseases mediated predominantly by auto-antibodies. *Autoimmun Rev* 2020;19:102532.
30 Chi CY, Chu CC, Liu JP, *et al.* Anti-IFN-γ autoantibodies in adults with disseminated nontuberculous mycobacterial infections are associated with HLA-DRB1*16:02 and HLA-DQB1*05:02 and the reactivation of latent varicella-zoster virus infection. *Blood* 2013;121:1357–66.
31 Terao C, Yoshifuji H, Yamano Y, *et al.* Genotyping of relapsing polychondritis identified novel susceptibility HLA alleles and distinct genetic characteristics from other rheumatic diseases. *Rheumatology (Oxford)* 2016;55:1686–92.
32 Kim TJ, Lee ST, Moon J, *et al.* Anti-LGI1 encephalitis is associated with unique HLA subtypes. *Ann Neurol* 2017;81:183–92.
33 Inaba H, Moise L, Martin W, *et al.* Epitope recognition in HLA-DR3 transgenic mice immunized to TSH-R protein or peptides. *Endocrinology* 2013;154:2234–43.
34 Sawai Y, DeGroot LJ. Binding of human thyrotropin receptor peptides to a Graves' disease-predisposing human leukocyte antigen class II molecule. *J Clin Endocrinol Metab* 2000;85:1176–9.