Association of Polymorphisms in MACRO Domain Containing 2 With Thyroid-Associated Orbitopathy

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Purpose. Thyroid-associated orbitopathy (TO) is an autoimmune-mediated orbital inflammation that can lead to disfigurement and blindness. Multiple genetic loci have been associated with Graves' disease, but the genetic basis for TO is largely unknown. This study aimed to identify loci associated with TO in individuals with Graves' disease, using a genome-wide association scan (GWAS) for the first time to our knowledge in TO.

Methods. Genome-wide association scan was performed on pooled DNA from an Australian Caucasian discovery cohort of 265 participants with Graves' disease and TO (cases) and 147 patients with Graves' disease without TO (controls). Top-ranked single nucleotide polymorphisms (SNPs) then were genotyped in individual DNA samples from the discovery cohort, and two replication cohorts totaling 584 cases and 367 controls.

Results. In the GWAS of pooled DNA samples, several SNPs showed suggestive association with TO at genome-wide $P \leq 10^{-6}$; rs953128 located on chr10q21.1, rs2867161 on chr7q11.22, rs13360861 on chr5q12.3, rs7636326 on chr3q26.2, rs10266576 on chr 7q11.22, rs60457622 on chr3q23, and rs6110809 on chr20p12.1. However, the only SNP consistently associated with TO on individual genotyping in the discovery and replication cohorts was rs6110809, located within *MACROD2* on chromosome 20p12.1. On combined analysis of discovery and replication cohorts, the minor A allele of rs6110809 was more frequent in TO than in Graves' disease controls without TO ($P = 4.35 \times 10^{-5}$; odds ratio [OR] = 1.77; 95% confidence interval [CI], 1.35–2.32) after adjusting for age, sex, duration of Graves' disease, and smoking.

Conclusions. In patients with Graves' disease, a common genetic variant in *MACROD2* may increase susceptibility for thyroid-associated orbitopathy. This association now requires confirmation in additional independent cohorts.

Keywords: genome-wide association study, genotyping, thyroid-associated orbitopathy

Thyroid-associated orbitopathy (TO) is an autoimmune-mediated orbital inflammation affecting 25% to 50% of patients with Graves' disease (GD).[1] The overall age-adjusted incidence rate of TO is 16 cases per 100,000 per year for females and 2.9 cases per 100,000 population per year for males. The average age at diagnosis is 44.7 years.[2] The onset of TO occurs mostly within 18 months of diagnosis of GD,[3] and the close temporal relationship suggests the two diseases share a common autoantigen.

Genetic susceptibility for GD is complex and involves multiple genetic loci regulating immune system and thyroid-specific genes. The HLA class II alleles, *CTLA4*, *PTPN22*, *CD40*

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polymorphisms were identified early on by candidate gene association studies as genetic risk factors for GD, even though the overall contributions from each of these genetic variants are modest.[4–7] The introduction of high throughput microarray technology, in combination with large cohorts of cases and controls, has seen genome-wide association studies (GWAS) revolutionize the study of the genetics of complex traits, and has allowed detection of association of common genetic variants with many diseases.[8] The earliest GWAS studies of autoimmune thyroid disease reported associations with HLA class I and II regions, and *TSHR* and *FCRL3* genes.[9] Subsequent GWAS studies replicated association of *FCRL3*, HLA loci, *TSHR*, *CTLA4*, *PTPN22*, *IL2RA*, and discovered new susceptibility loci at *SENP1*, *SLAMF6*, *RNASET2-FGFR1OP-CCR6*, an intergenic region at 4p14, *MMEL1*, *LPP*, *BACH2*, *PRICKLE1*, and *ITGAM*.[10–13]

Although genetic risk factors for GD are becoming well defined, limited progress has been made with TO, largely due to small sample sizes limiting power to detect modest genetic effects and failure to replicate genetic findings. Candidate gene studies, predominantly of genes and polymorphisms associated with GD or genes involved in immune response and inflammation, have been investigated for a contribution to TO. While some studies showed *CTLA4*, HLA I, and II alleles, and *IL-23R* rs10889677 and rs2201841 SNPs were associated with TO compared to GD controls, other studies have not supported these associations.[14–21] *TNF-α* alleles were associated significantly with TO in a Japanese but not in a Polish study.[22,23] The adipogenesis-related gene *PPARγ* Pro12Ala polymorphism decreased the risk of TO in one study but not in another, but both studies found the Pro12Ala variant possibly reduces the severity and activity of TO.[24,25] Using tag single nucleotide polymorphisms (SNP), adipocyte-related immediate early genes showed increased risk for TO with genetic variants in *CYR61* (rs1378227), *ZFP36* (rs1057745, rs11083522), and *SCD* (rs1393491) with odds between 1.3 and 1.4 compared to GD, but the findings must be replicated.[26] Similarly, using tag SNPs, SNPs in *IL-1β* did not differ between TO and non-TO in GD in one study but C allele of rs1143634 increased the risk in TO and GD compared to normal controls,[27] whereas another identified rs1800587 polymorphism at *IL-1α* and rs16944 polymorphism at *IL-1β* significantly associated with GD and TO.[28] *PTPN12* (rs1468682, rs4729535, and rs17467232), functionally related to *PTPN22*, was associated with TO compared to non-TO in GD with odds at 1.4, and 2 of the SNPS (rs1468682 and rs4729535) showed interactions with *TSHR* rs2268458 SNP; the findings again require replication.[29] However, *TSHR* rs2268458 SNPs, previously found to be associated with GD, were not associated with TO.[18]

A pooled GWAS study is a cost-effective screening strategy for identifying gene variants in multiple diseases. Pooled DNA strategy has successfully identified known susceptibility loci *CFH*, *ARMS2/HTRA1* in age-related macular degeneration, *LOXL1* in pseudoexfoliation syndrome, and discovered new a susceptibility locus *HGF* in keratoconus.[30,31] We aim to use a GWAS to discover genomic loci associated with TO. We report the first attempts to our knowledge at using a genome-wide association scan for discovering genetic variants in TO, and an association of variants in the *MACROD2* gene suggesting it as a new TO susceptibility locus.

Methods

The genetic study of TO was approved by human research ethics committees at multiple recruitment sites; the Royal Victorian Eye and Ear Hospital, Sunshine and Footscray Hospitals, the Royal Melbourne Hospital, and the Alfred Hospital in Melbourne, Victoria, Australia, and Flinders Medical Centre, and the Royal Adelaide Hospital in Adelaide, South Australia. The genetic research was conducted in accordance with the Declaration of Helsinki.

Australian Thyroid-Associated Orbitopathy Research (ATOR) Cohorts

Definition of Graves' Disease and TO. Graves' disease was defined by the presence of hyperthyroidism based on thyroid function test, including T3, T4, and TSH, and either elevated thyrotropin receptor (TSHR) antibodies or diffuse uptake on technetium-99m pertechnetate thyroid scan. Cases are defined by the presence of TO clinically and controls by the absence of TO.

Thyroid-associated orbitopathy was defined by the presence of symptoms of TO and at least one sign of TO, for example, lid retraction. Early symptoms of TO were detected using the Vancouver Orbitopathy Rule symptom questionnaire, including red eyes, lids swelling, eye protrusion and stare, and blurred vision.[32] Thyroid-associated orbitopathy status was examined and classified using the Vision, Inflammation, Strabismus, and Appearance (VISA) classification.[33] Ophthalmologic measurements included the following: visual acuity, pupil response, color vision, inflammatory index score, extraocular movement, strabismus, lid measurements including palpebral aperture, marginal reflex distance, lid retraction, and Hertel exophthalmometry. The status of TO was determined by an ophthalmologist.

ATOR Discovery Cohort. Thyroid-associated orbitopathy cases ($n = 265$) and GD without TO controls ($n = 147$) of European descent were recruited from endocrine and eye hospital outpatient clinics and private practices of endocrinologists and ophthalmologists from Victoria and South Australia. Whole blood samples in EDTA were stored at 4°C.

Replication Cohorts. A further 319 TO cases and 220 GD controls were recruited from Victoria and Western Australia as replication cohorts. These comprised 231 TO cases and 142 GD controls recruited from Victoria using the same protocol as the ATOR discovery cohort, and 88 TO cases and 78 GD controls of European descent recruited from endocrine and ophthalmology clinics in Western Australia. Thyroid-associated orbitopathy was defined clinically by the presence of eye symptoms and signs with severity classified according to the European group on Graves' orbitopathy (EUGOGO) recommendations (by coauthor JW)[34] or VISA classification (by coauthor AG).[33] DNA samples (50 ng/μL) from venous blood were used for individual genotyping.

A total of 282 normal controls, consisting of healthy hospital volunteers and retirement village residents over 50 years of European descent from South Australia, were used for additional individual genotyping for comparison with the TO and GD cohorts (with and without TO), respectively. The normal control cohort was not actively screened for GD, but no participant reported having this condition.

DNA Pooling for GWAS Study in the Discovery Cohort. Venous blood aliquots from the discovery cohort were stored at 4°C before construction of 2 comparative blood pools (pool number 1, 154 TO cases versus 102 controls; pool number 2, 116 TO cases versus 40 controls), totaling 270 cases and 142 controls. In between the construction of the blood pools and individual genotyping, the phenotypes of 5 individuals were updated (5 from cases to controls); these small numbers do not materially affect the overall blood pool results. Protease and lysis buffer were added to 100 μL of thawed whole blood from each sample, vortexed, and incubated, and then samples were combined in one tube for each pool. DNA was extracted from

the pooled blood using the QIA-amp DNA blood maxi kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. For the remaining discovery samples, equimolar DNA pools were generated in duplicate after stepwise dilutions of genomic DNA extracted using QIA-amp DNA blood maxi kit to 75 ng/μL as previously described.[30,31] The DNA was quantitated using Fluroskan Ascent (ThermoFisher Scientific, Waltham, MA, USA) together with double-stranded DNA quantitation assay using Picogreen reagents. (Invitrogen, Carlsbad, CA, USA). A total of 451 ng of DNA from each sample was added to the DNA pool.

Pooled Genotyping and Analysis

Genome-wide genotyping was conducted by hybridization to HumanOmni5-Quad (Illumina, San Diego, CA, USA) beadchip microarray containing approximately 4.3 million markers, at QIMR Berghofer Medical Research Institute, according to standard protocol for case and control pools on two independent arrays for each pool. Two case pools and two control pools were generated and assigned into two case–control pool comparisons. Each pool was replicated 2 to 4 times to reduce measurement error. For the purpose of this analysis, we retained SNPs with minor allele frequency (MAF) over 1% from the reference panel of EUR samples in the 1000 Genome Project (excluded 1.8 M SNPs). Thus, the number of SNPs was reduced to 2.5 million. The output of the raw red and green bead scores from Illumina Beadstation was used for the pooled data analysis. The data cleaning and quality control procedures in array-based pooling have been described previously.[35–37] Briefly, SNPs with more than 10% negative scores on each array were excluded, as well as the SNPs with the sum of mean red and green scores lower than 1200 across each array to ensure calibration was performed on a precleaned dataset. A normalization/correction factor (corr) was calculated to make the mean value of the pooling allele frequency (PAF) 0.5 across all SNPs on each strands of the array. The PAF then was estimated based on the raw red intensities and the corrected green intensities for all the SNPs [PAF = red/(red + green/corr)].[36] Further quality control included filtering nonautosomal SNPs and any SNPs with a significant variance difference between cases and controls.

After data cleaning, 2.2 M SNPs were left for association testing in the ATOR discovery cohort. A linear model was used to test for allelic association in the two case–control pool comparisons. A test statistic, which corrects for pooling error, was used to rank SNPs, with P values based on a χ^2 distribution with 1 degree of freedom. The application of the test statistic is based upon contrasting case and control pools, the effect of unequal amplification of alleles is minimal as such effects cancel out.[35] Specifically, a final set of autosomal SNPs was determined meeting the following criteria: (1) have sufficient number of probes (i.e., over 1/2 of expected number of probes for MAF 1% to 5%, or over 1/3 of expected number of probes for SNPs >5%; and (2) have no large differences (>0.3) between PAF and reference allele frequency from EUR samples in the 1000 Genome Project. We then performed a meta-analysis weighted by inverse variance, combining results from two case–control pool comparisons. The top-ranked SNPs in the meta-analysis then were counter checked for proxies (linkage disequilibrium r2 > 0.5 with the index SNPs in the EUR populations). To be considered a valid result, if proxy SNPs were available, they also should show evidence of association. A genome-wide significance threshold of 5×10^{-8} was set to account for multiple testing.

Validation Genotyping and Analysis

Top-ranked SNPs with P value threshold $\leq 1 \times 10^{-6}$ indicative of association, or corresponding proxy if failed genotyping, were selected for individual genotyping in the discovery and replication cohorts, to validate pooled genotyping results as well as to replicate the associations in independent samples. In total, 7 SNPs were genotyped on Sequenom iPLEX Gold assay using Sequenom MassARRAY Analyzer in 2 batches, the ATOR discovery cohort and Western Australia replication cohort were genotyped at the Australian Genome Research Facility (Brisbane, Australia), and the ATOR replication cohort at GeneWorks Pty Ltd. (Adelaide, Australia). The allelic association analysis was performed in PLINK,[38] using a χ^2 test and performed separately for the ATOR pooled discovery cohort for validation, the replication cohorts, and for combined discovery and replication cohorts. The odds ratio (OR) and P values were adjusted for age of disease onset, duration of GD, and sex using logistic analysis. Single nucleotide polymorphisms with $P \leq 1 \times 10^{-4}$ were considered validated in validation genotyping. In the replication cohort, $P < 0.05$ was considered associated. In the secondary analysis, we further adjusted the individual genotyping results for smoking status (smoker, ex-smoker, and never smoked) in addition to sex, age, and duration of GD for the discovery, replication, and combined cohorts, bearing in mind there are missing data points for smoking in 10.9% of the replication cohorts. Further individual genotyping for the top ranking SNPS were compared between TO and normal controls; and between GD and normal control to differentiate whether the validated SNPs were associated with TO and GD as a whole or the risk variant is specific for TO in GD. As smoking status data were not available from the healthy controls, the genotyping results for these genotyping analyses were adjusted for sex and age only.

Following individual genotyping in discovery and replication cohorts revealing rs6110809 in *MACROD2* as the locus of interest, further SNPs residing in *MACROD2* with nominal $P \leq 5 \times 10^{-4}$ were extracted from pooled GWAS top ranking data. They were assessed for linkage disequilibrium with rs6110809, to determine if multiple independent risk alleles were potentially present within *MACROD2*. Genotype data limited to a 100 kb region of *MACROD2* capturing all 4 additional SNPs were downloaded from International Hapmap Project (available in the public domain at http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap24_B36/#search). Genotype data were analyzed in HaploView (Version 1.0, Broad Institute) for pairwise comparisons of marker SNPs with minor allele frequency > 0.01.

Results

The age at disease onset, duration of GD, sex, and smoking status distributions of the three cohorts of participants are described in Table 1. Thyroid-associated orbitopathy cases were slightly older compared to GD without TO at diagnosis and the duration of GD in TO cases was longer. The proportions of males to females were similar between the groups and the proportion of smokers was greater in cases compared to controls for ATOR cohorts. The age and sex distribution of the normal controls in comparison with TO as a whole and GD as a whole are shown in Table 2.

Pooled DNA was used to identify genetic variants associated with TO. None of the top ranked SNPs reached the genome-wide significance threshold of 5×10^{-8} (Supplementary Table S1), nevertheless multiple top ranked SNPs approached genome-wide significance with $P \leq 10^{-6}$ and in particular with rs2867161 on chr7q11.22 ($P = 5.94 \times 10^{-8}$) and rs60457622 on chr3q23 ($P = 9.28 \times 10^{-8}$) almost reaching significance (Table 3).

The seven top ranked SNPs from pooled GWAS were individually genotyped in the ATOR discovery cohort.

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Table 1. Clinical Characteristics of GWAS Discovery and Replication Cohorts

	ATOR Discovery Cohort, $N = 412$			ATOR Replication Cohort, $N = 373$			WA Replication Cohort, $N = 166$		
	Case	Control	*P* Value	Case	Control	*P* Value	Case	Control	*P* Value
N	265	147		231	142		88	78	
Mean age, y (SD)	46.7 (13.8)	42.7 (15.2)	0.01	46.4 (13.5)	42.4 (16.2)	0.01	46.5 (14.0)	40.1 (15.5)	0.01
GD Duration, y (SD)	10.91 (9.66)	5.42 (6.83)	<0.001	8.67 (8.83)	5.46 (6.04)	<0.001	8.32 (9.47)	5.56 (7.71)	0.05
Sex									
Male (%)	47 (20.4)	24 (16.3)	0.717	47 (20.4)	36 (25.4)	0.262	13 (15.1)	13 (16.7)	0.79
Female (%)	218 (79.7)	123 (83.7)		184 (79.7)	106 (74.7)		73 (84.9)	65 (83.3)	
Smoking status									
Smoker (%)	66 (25.2)	25 (17.1)	<0.001	69 (29.9)	25 (17.6)	<0.001	15 (21.7)	6 (15.8)	0.02
Ex-smoker (%)	110 (42)	42 (28.8)		79 (34.2)	34 (23.9)		9 (13.1)	14 (36.8)	
Never smoked (%)	86 (32.8)	79 (54.1)		83 (35.9)	83 (58.5)		45 (65.2)	18 (47.4)	
	*3 missing	*1 missing					*19 missing	*40 missing	

rs6110809 was validated with $P < 1 \times 10^{-4}$, the minor allele is associated with TO risk, showing higher frequency in cases compared to controls (Table 3). After further adjustment for smoking, rs6110809 was associated with TO with OR at 2.66 (95% confidence interval [CI], 1.69–4.18; $P = 2.45 \times 10^{-5}$) in the discovery cohort.

All 7 SNPs also were assessed in the replication cohorts. The only SNP consistently associated with TO in replication cohorts was rs6110809, located within an intron of *MACROD2* on chromosome 20p12.1 with an age, duration, sex-adjusted OR 1.42 (95% CI, 1.04–1.95; $P = 0.03$); after further adjustment for smoking, the OR was 1.35 (95% CI, 0.96–1.91; $P = 0.08$). Overall, for the combined discovery and replication cohorts, the *MACROD2* rs6110809 was associated with TO compared to GD controls without TO (OR = 1.81; 95% CI, 1.40–2.34; $P = 6.78 \times 10^{-6}$; Table 4). After adjusting for age, duration of GD, sex, and smoking, the minor A allele was more frequent in TO than in GD without TO with OR 1.77 (95% CI, 1.34–2.32; $P = 4.35 \times 10^{-5}$).

Additional individual genotyping analyses were undertaken to determine if the top ranking SNPs from pooled GWAS in TO versus non-TO in GD were associated with TO or GD as a whole or the risk variant was specific to TO in GD. We found that none of the seven top ranking SNPS was associated with TO compared to normal individuals or GD compared to normal individuals (Supplementary Table S2). Hence, SNP at *MACROD2* locus was an association specific to risk of TO in GD.

Four additional highly ranked SNPs at the *MACROD2* gene locus with $P < 10^{-4}$ extracted from the discovery pooled GWAS data also were assessed for their linkage disequilibrium to rs6110809 in the Caucasian HapMap population. They are rs978767 ($P = 1.8 \times 10^{-4}$), rs175805 ($P = 3.05 \times 10^{-7}$), rs6135575 ($P = 4.32 \times 10^{-4}$), and rs761684 ($P = 3.2 \times 10^{-4}$). Two SNPs were highly correlated with rs6110809 (rs6135575 and rs761684) and two SNPs showed more moderate linkage disequilibrium with rs6110809 (rs978767 and rs175805; Figure). This suggests the top ranking *MACROD2* SNPs may represent two separate loci within *MACROD2* associated with TO, although further genotyping and analysis are required to confirm this.

Discussion

This GWAS discovered a positive association of SNP on chromosome 20 within the *MACROD2* gene with TO with replication in an independent cohort. The genetic variant appeared specific to the risk for TO in patients with GD and not for the risk of GD itself, potentially implicating *MACROD2* as a new genetic susceptibility locus for TO. Multiple genetic loci on chromosome 10, 7, 5, and 3 trended towards positive association with TO but the associations dropped out of significance in subsequent individual genotyping.

To our knowledge, this is the first study to characterize the genetic risk factors for TO using a GWAS approach, where associated loci were discovered a priori and confirmation of these findings in additional independent cohorts was performed. While none of the SNPs reached genome-wide significance, there was evidence of association of *MACROD2* with TO first reported by this study. The evidence of association with MACROD2 stays true overall with adjustment for potential confounders age, duration of GD, sex, and smoking in the combined dataset, bearing in mind that the smoking data are absent in 10.9% of the replication cohorts, and 6.6% in the combined cohorts dataset, which might affect the final estimation of OR and its significance level when smoking was taken into consideration. Hence, we have presented the data in this study for confounder adjustments with and without smoking and found *P* values slightly

Table 2. Demographics of Overall Cohort With GD, TO Compared to Normal Healthy Controls

	TO Compared to Normal Controls			GD Compared to Normal Controls		
	TO Cases	Normal Controls	*P* Value	Graves' Cases	Normal Controls	*P* Value
N	584	282		951	282	
Mean age, y (SD)	46.5 (13.7)	75.9 (8.18)	<0.001	44.8 (14.7)	75.9 (8.18)	<0.001
Sex						
Male (%)	107 (18.38)	116 (42.96)	<0.001	180 (18.97)	116 (42.96)	<0.001
Female (%)	475 (81.62)	154 (57.04)		769 (81.03)	154 (57.04)	
	*2 missing	*12 missing		*2 missing	*12 missing	

Table 3. Top Ranking Pooled Genome-Wide Association Study and Validation Genotyping in ATOR Discovery Cohort

	Location		GWAS Pooled Genotyping Association, Cases = 270, Controls = 142				Validation Individual Genotyping Association (Cases = 265, Controls = 147)			
SNP Name	Chr	bp	Genes	A1	*P*	OR_A1	F_A	F_U	OR_A1 (95% CI) Adjusting for Sex, Age, Duration of GD	*P* Value Adjusting for Sex, Age, Duration of GD
rs953128	Chr10q21.1	56502386	Intronic PCDH15	A	7.13×10^{-7}	2.40	0.133	0.048	3.35 (1.75, 6.51)	2.69×10^{-4}
rs2867161	Chr7q11.2	67637342	Intergenic STAG3L4, AUTS2	A	5.94×10^{-8}	2.50	0.135	0.052	2.59 (1.42, 4.72)	1.97×10^{-3}
rs7636326	Chr3q26.2	170236594	Intronic SLC7A14	T	5.32×10^{-6}	2.78	0.095	0.024	4.34 (1.84, 10.26)	8.15×10^{-4}
rs13360861	Chr5q12.3	65724966	Intergenic SREK1, MAST4	T	1.02×10^{-7}	2.58	0.087	0.024	3.28 (1.44, 7.47)	4.62×10^{-3}
rs60457622	Chr3q23	141549404	Intergenic GRK7, ATP183	A	9.28×10^{-8}	2.85	0.085	0.028	3.18 (1.43, 7.09)	4.68×10^{-3}
rs10266576	Chr7q11.22	67862566	Intergenic STAG3L4, AUTS2	T	4.67×10^{-7}	3.32	0.061	0.014	5.23 (1.75, 15.65)	3.12×10^{-3}
rs6110809	Chr20p12.1	15851077	Intronic MACROD2	A	2.13×10^{-6}	2.14	0.247	0.120	2.77 (1.77, 4.33)	8.17×10^{-6}

P values are uncorrected. F_A, minor allele frequency in TO; F_U, of minor allele A1 in GD without TO control; OR_A1, OR of minor allele in cases versus controls.

Table 4. Replication Individual Genotyping of Top Ranking SNPs From Discovery GWAS in Replication Cohorts and in Combined Discovery and Replication Cohorts

	Location		Replication Cohorts Association Results, Cases = 319, Controls = 220						Combined GWAS and Replication Cohorts Individual Genotyping Results, Cases = 584, Controls = 367			
SNP Name	Chr	bp	Genes	A1	F_A	F_U	OR_A1 (95% CI) Adjusted for Age/Sex/Duration GD	*P* Value Adjusted for Age/Sex/Duration GD	F_A	F_U	OR_A1 (95% CI) Adjusted for Age/Sex/Duration GD	*P* Value Adjusted for Age/Sex/Duration GD
rs953128	Chr10q21.1	56502386	Intronic PCDH15	A	0.114	0.094	1.29 (0.84, 1.98)	0.24	0.122	0.076	1.78 (1.25, 2.51)	1.37×10^{-3}
rs2867161	Chr7q11.2	67637342	Intergenic STAG3L4, AUTS2	A	0.093	0.093	0.90 (0.57, 1.42)	0.65	0.112	0.077	1.40 (0.99, 1.98)	0.06
rs7636326	Chr3q26.2	170236594	Intronic SLC7A14	T	0.069	0.070	0.99 (0.60, 1.63)	0.97	0.081	0.052	1.59 (1.06, 2.39)	0.03
rs13360861	Chr5q12.3	65724966	Intergenic SREK1, MAST4	T	0.104	0.081	1.38 (0.87, 2.19)	0.18	0.096	0.058	1.77 (1.20, 2.60)	3.86×10^{-3}
rs60457622	Chr3q23	141549404	Intergenic GRK7, ATP183	A	0.057	0.075	0.74 (0.44, 1.25)	0.26	0.070	0.056	1.25 (0.83, 1.89)	0.28
rs10266576	Chr7q11.22	67862566	Intergenic STAG3L4, AUTS2	T	0.041	0.048	0.90 (0.48, 1.70)	0.75	0.050	0.034	1.63 (0.97, 2.74)	0.06
rs6110809	Chr20p12.1	15851077	Intronic MACROD2	A	0.239	0.186	1.42 (1.04, 1.95)	**0.03**	0.243	0.160	1.81 (1.40, 2.34)	6.78×10^{-6}

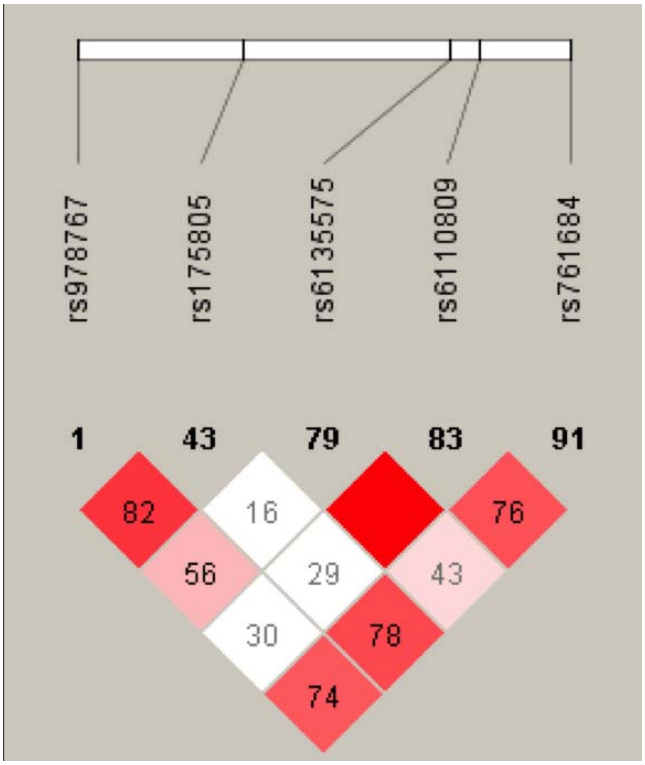

Figure. Haploview linkage disequilibrium plot for 5 highly ranked SNPs within *MACROD2* in HapMap CEU population data.

increased after smoking adjustment. A further limitation of this study is the use of pooled DNA, which, although highly economical, reduces the ability to correct for confounders in the GWAS stage, or to assess population substructure, which may lead to false-positive findings. While we and others find limited evidence for genes with major effects affecting risk for TO,[18,39] the number of cases studied in this study still is relatively small, limiting the power and certainty of the association found. Much larger samples will be required to confirm our findings and to prove genetic associations in TO. Nevertheless, the identification of genetic variants, even of modest effects, will improve our understanding of pathogenesis of TO and identify pathways for therapeutic considerations. In the context of these provisions, we will discuss the results of interest from this study that are potentially relevant to the genetic risk of TO.

The OR for rs6110809 A allele in predicting risk for TO ranges between 2.77 for the pooled discovery cohort and 1.42 in the replication cohorts, with an OR of 1.81 for the extended cohort when all cohorts were combined after adjusting for age at disease onset, duration of GD, and sex. The OR is notably smaller in the replication cohorts, which is consistent with "the winner's curse" where locus-specific size estimate bias upwards in GWAS.[40] Hence, if the association of rs6110809 is true, the estimated conferred risk is more realistic from the replication samples, where the estimated effect size also is more in line with unbiased estimates of ORs discovered by GWAS studies of approximately 1.1 to 1.3.[41]

The *MACROD2* gene encodes a protein with a highly conserved macrodomain binding module in the N-terminal domain that interacts with mono-ADP-ribosylated proteins.[42,43] There are no existing data as to whether *MACROD2* is expressed in the orbital adipocytes or myofibroblasts, and further studies are required to examine this. However, M*ACROD2* is expressed strongly in the brain, and is moderately to strongly expressed in cuboidal epithelium of the lens and the inner nuclear layer of the retina.[42] The association of *MACROD2* with eye diseases has not been previously reported to our knowledge, but genetic variants in *MACROD2* have been reported to be associated with neuropsychiatric disorders. De novo deletion of exon 5 within *MACROD2* was identified in Kabuki syndrome, where the index case suffered from mental retardation and had ophthalmic features including ptosis, hypermetropic astigmatism, alternating strabismus, and blue sclera.[42] *MACROD2* allelic variants and common copy number gain has been linked to several diseases.[41,44–46] Recent GWAS identified SNPs within *MACROD2* as a risk variant in autism spectrum disorder,[41] and possibly a protective factor for MRI-defined brain infarcts.[44] A candidate gene study investigating autistic-like traits also reported an association between rs4141463 SNP in intron 5 of *MACROD2*.[45] Overexpression of *MACROD2* mediates estrogen-independent growth and tamoxifen resistance in breast cancer, suggesting the expressed gene confers advantage to cellular survival.[46]

The MACROD2 protein binds to mono-ribosylated proteins and its catalytic domain functions as O-acetyl-ADP-ribose deacetylases and hydrolases, which reverses mono-ADP ribosylation,[43,47] thus underlying the key role of MACROD2 as an eraser of mono-ADP-ribosylation.[48] The function of macrodomain molecules in general is tightly linked to ADP-ribosylation, which modulate functions of protein by posttranscriptional modification. The addition of ADP-ribose to proteins affects diverse cellular processes including DNA repair, chromatin remodeling, gene transcription, lipid metabolism, and apoptotic processes.[48–50] To understand the role of MACROD2 in disease, we must understand the function of intracellular ADP-ribosyltransferase Cholera toxin-like (ARTD), which inserts ADP-ribose monomers to protein that MACROD2 erases. ARTD10 regulates cell signaling, represses nuclear factor-κβ by mono-ribosylation of NF-κβ essential modulator (NEMO), where NF-κβ signaling pathway promotes inflammation, innate immune response, cellular proliferation, and cell survival.[48,50–53]

Overexpression of ARTD10 promotes apoptosis in HeLa cells whereas ARTD10 knockdown increases cell survival upon DNA damage caused by DNA damaging agents.[54] ARTD10 is inducible by inflammatory and immunogenic stimuli, hence suggesting its role in innate immunity.[48] ARTD10 also inhibits glycogen synthase kinase 3β (GSK3β), a key enzyme that modulates WNT signaling via β-catenin phosphorylation, which regulate apoptosis, immunity, and development of neurodegenerative diseases.[43,48,55,56] Hence, inhibition of GSK3β and NF-κβ signaling by ARTD10 may explain enhanced apoptosis and MACROD2, on the other hand, counteracts the actions of ARTD10.

The role of MACROD2 in modulating DNA repair is yet unknown, but it is recruited to poly-ADP-ribose synthesized by ARTD1 in response to DNA damage.[50] MACROD2 removes the most proximal ADP ribose linked to the target amino acid after partial breakdown of poly-ADP-ribosylation by poly-ADPr-glycohydrolase to fully reverse ARTD1 regulatory modification.[57] This functional link of MACROD2 to ARTD1 becomes more noteworthy in light of the findings that carriers of G allele of *ARTD1* G1672A polymorphism are at risk of GD and a positive association of *ARTD1* C410T polymorphism with TO confers an increased risk of TO by 1.7 times.[58]

MACROD2 and ARTD10 regulation of apoptosis and NF-κβ signaling may be relevant to the mechanism of immune-mediated orbital inflammation in TO. Gene set enrichment analysis of molecular signatures of TO revealed dysregulation of epigenetic signatures, T cell activation, Th1 differentiation,

defensin pathway, apoptosis among a few in active TO.[59] Dysregulation of WNT signaling also was evident in TO from microarray studies.[60,61] Orbital inflammation in TO is mediated by communication of orbital fibroblast and fibrocytes with immune cells via CD40-CD40 ligand. This is inducible by IFN-γ, evidenced by nuclear translocation of NF-κβ and increase secretion of pro-inflammatory cytokine IL-6 and IL-8 upon triggering of CD40 bearing orbital fibroblast by CD40 ligand.[62,63] CD40 ligand significantly increases intercellular adhesion molecule-1 (ICAM-1) protein in a dose- and time-dependent manner, where ICAM-1 has a role in initiating and sustaining inflammatory immune response.[64] The signaling pathways of increased ICAM-1 involve mitogen activated protein kinases and NF-κβ; CD40 ligand at 100 ng/mL induced a moderate activation of NF-κβ, which increases in a time-dependent manner.[64] In addition, polymorphisms of del/ins of *NFκβ1* gene may be related to the development of TO, further supporting the role of NF-κβ in TO pathogenesis.[58] Overexpression of MACROD2 is expected to enhance NF-κβ signaling and increase cell survival, cell proliferation, inflammation and immune response, mechanisms all relevant to the pathogenesis of TO. The functional study of MACROD2 is beyond the scope of this study; this study provided grounds for further candidate gene study and future functional studies looking at expression levels of MACROD2 in orbital fibroblasts culture, and its effects on IL-6, IL-8, ICAM-1, NF-κβ expression levels in vitro.

In conclusion, this exploratory GWAS study of TO suggests that in patients with GD, a common variant at the *MACROD2* locus increases susceptibility to TO. This novel finding should prompt further replication studies to confirm its role in the pathogenesis of TO.

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