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Association of *BTG2*, *CYR61*, *ZFP36*, and *SCD* Gene Polymorphisms with Graves' Disease and Ophthalmopathy

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Background: Environmental and genetic factors predispose an individual to the development of Graves' disease (GD). In an expression study of intraorbital tissue, adipocyte-related immediate early genes (IEGs) and immunomodulatory genes were found to be overexpressed in patients with Graves' ophthalmopathy (GO). We hypothesized that genetic variations in these genes could be associated with GD and/or GO.

Methods: A total of 98 single nucleotide polymorphisms (SNPs) in 12 genes were genotyped in 594 GD patients with ($n=267$) or without ($n=327$) GO and 1147 sex- and ethnicity-matched controls from Malmö, Sweden.

Results: Ten SNPs in four genes (*BTG* family, member 2 [*BTG2*], cysteine-rich, angiogenic inducer 61 [*CYR61*], zinc finger protein 36, C3H type, homolog mouse [*ZFP36*], and stearoyl-coenzyme A desaturase [*SCD*]) showed an association with GD and/or GO. SNPs rs12136280 (odds ratio [OR] 1.29, $p=0.002$), rs6663606 (OR 1.26, $p=0.004$), and rs17534202 (OR 1.21, $p=0.02$) in *BTG2* and rs3753793 (OR 1.21, $p=0.03$) in *CYR61* were associated with GD. An association with GO was shown for SNPs rs3753793 (OR 1.45, $p=0.008$), rs6682848 (OR 1.55, $p=0.03$), rs12756618 (OR 1.77, $p=0.049$), and rs1378228 (OR 1.29, $p=0.049$) in *CYR61*, rs1057745 (OR 1.56, $p=0.03$) and rs11083522 (OR 1.32, $p=0.04$) in *ZFP36*, and rs1393491 (OR 1.38, $p=0.048$) in *SCD*. Smoking and *CYR61* rs12756618 interacted to increase the risk of GO.

Conclusions: We found associations of SNPs in *IEGs* and *SCD* with GD and/or GO; however, confirmation in a different population is required.

Introduction

GRAVES' DISEASE (GD) is an autoimmune disorder that develops as a result of the interplay between endogenous, genetic, and environmental factors. The two main groups of autoimmune thyroid disease (AITD)—GD and Hashimoto thyroiditis—share part of the genetic background, and the genetic pattern is complex (1). Although the genetics of GD has been extensively studied, only a few positive results from linkage and candidate gene studies have been replicated. Cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*), a key negative regulator of the T cell-mediated immune response, is linked to and associated with all AITD phenotypes (including GD) in different populations and also with other autoimmune disorders (1).

Virtually all patients with GD display retrobulbar morphological changes, though only approximately one-third of GD patients develop Graves' ophthalmopathy (GO) (2); furthermore, the factors leading to the development of GO in

an individual patient are not entirely known. Therefore, identifying parameters that help to predict GO is of great importance. Nonetheless, genetic studies of GO have thus far given conflicting results. The most promising candidate, *CTLA4*, was associated with GO in some studies (3,4), whereas others found no evidence for a specific risk of GO beyond that conferred for GD (5–8). Known environmental risk factors include smoking (9,10), untreated hyperthyroidism and hypothyroidism (11), and treatment with radioiodine (12–14).

In expression studies, we have shown that a number of genes were upregulated in intraorbital adipose/connective tissue from patients with severe active GO compared to thyroid-healthy controls (15). The major finding involved the overexpression of 14 adipocyte-related immediate early genes (IEGs) in the patient group, and these genes were also overexpressed, though to a lower degree, in chronic ophthalmopathy (16). We hypothesized that genes showing abnormal expression in intraorbital tissue from patients with GO might also play a role in the genetic susceptibility to GD

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and GO. The majority of the genes with a confirmed association with GD are involved in the immune response. Moreover, previous studies have demonstrated abnormalities in monocytes and lymphocytes from patients with AITD (17), and lymphocyte and monocyte infiltration is shared by the thyroid and intraorbital tissue in GD. Therefore, among the genes overexpressed in active GO, IEGs and genes with functions in lymphocytes/monocytes were considered plausible candidates for an association with GO and/or GD.

Materials and Methods

Study population

The study consisted of 594 patients (109 male and 485 female) with GD with ($n=267$) or without ($n=327$) ophthalmopathy and 1147 controls (204 male and 943 female) from one town, Malmö, in southern Sweden (Table 1). A total of 477 patients (80%) were selected from the registry GD2002 in which clinical data on patients with GD have been collected since 2002 by a single endocrinologist. The remaining 117 patients were recruited from the studies MFM (Malmö Preventive Project) (18) and MKC (Malmö Diet and Cancer Study) (19) on the basis of an *International Statistical Classification of Diseases and Related Health Problems*, 10th revision, diagnosis of E05.0 (GD) and H06.2 (GO). These studies were performed in the 1990s as a primary preventive project (MFM) and a study investigating the association of diet and risk of cancer (MKC). As patients with thyrotoxicosis in Malmö are always referred to an endocrinologist, in all cases, diagnosis of GD was made by an endocrinologist based on clinical symptoms and signs and biochemical hyperthyroidism, in combination with the presence of thyrotropin receptor antibodies and/or diffuse uptake by technetium scintigraphy. GO diagnosis was made by an endocrinologist and/or ophthalmologist based on the presence of clinical signs, as previously described (14). Eyelid re-

traction alone was not classified as GO. The controls were recruited from the MFM and MKC databases. The case and control groups were matched for sex and group-matched for ethnicity. Regarding ethnicity, the origin of the individuals was assigned as Swedish, born in Europe other than Sweden, or born outside of Europe. For additional analysis, a total of 116 newly recruited patients with GD from GD2002 ($n=44$, not previously analyzed) and from the TT 96 trial [$n=72$, described in (14)] were used. For these newly recruited patients, data on clinical parameters such as age, ethnicity, smoking and presence of GO, were not available. All patients gave written informed consent, and the study was approved by the local research ethics committee.

Gene selection

Gene selection was based on the results of a previous expression study of active GO (15). Genes with high expression in intraorbital adipose/connective tissue from patients with severe active ophthalmopathy compared to thyroid-healthy controls were chosen, including *BTG* family, member 2 (*BTG2*), cysteine-rich, angiogenic inducer, 61 (*CYR61*), early growth response 1 (*EGRI*), dual specificity phosphatase 1 (*DUSP1*), zinc finger protein 36, C3H type, homolog [mouse] (*ZFP36*), and prostaglandin-endoperoxide synthase 2 (*PTGS2*), representing adipocyte-related IEGs with functions in lymphocytes and/or monocytes. In addition, the following genes with known functions in inflammation and/or adipogenesis were selected: nuclear receptor subfamily 4, group A, member 2 (*NR4A2*), chemokine [C-X-C motif] ligand 2 (*CXCL2*), suppressor of cytokine signaling 3 (*SOC3*), regulator of G-protein signaling 2, 24 kDa (*RGS2*), and stearoyl-coenzyme A desaturase (*SCD*). *CTLA4* was chosen for confirmation in our population on the basis of previously reported associations with GD.

Single nucleotide polymorphism selection

Single nucleotide polymorphisms (SNPs) were selected using data from the Hap Map consortium (20). Applying the tag SNP approach reduces the amount of SNPs that have to be genotyped to cover the genetic variation in a region. Therefore, the region surrounding each of the selected genes, including an extra 10 kb upstream and downstream, was analyzed by Tagger in the Haplowiew program (21), providing tag SNPs for each gene. Additional potentially important SNPs (nonsynonymous SNPs; SNPs that alter an intronic splice site; and SNPs that occur in a predicted promoter, in a region of predicted regulatory potential, or in a region with conservation scores ≥ 99 th percentile genome-wide) were chosen from TAMAL (<http://neoref.ils.unc.edu/tamal/>), as described previously (22), and from the National Center for Biotechnology Information SNP database (www.ncbi.nlm.nih.gov/SNP/). In total, 98 SNPs in twelve genes were selected for the analysis. The minor allele frequency (MAF) for all SNPs was >0.05 .

Power calculations

As the allele frequencies in our population of most SNPs studied in this study were unknown prior to genotyping, we based our power calculations on the *CTLA4* SNPs where the allele frequencies were known from other studies using the

TABLE 1. CHARACTERISTICS OF THE STUDY POPULATION

| | Cases | Controls |
|---------------------------------------|------------|------------|
| <i>N</i> | 594 | 1147 |
| Age at inclusion (years) ^a | 49 ± 14 | 57 ± 6 |
| Sex | | |
| Male (%) | 109 (18.4) | 204 (17.8) |
| Female (%) | 485 (81.6) | 943 (82.2) |
| Ethnicity | | |
| SWE (%) | 434 (73.1) | 835 (72.8) |
| EUR (%) | 100 (16.8) | 177 (15.4) |
| OUT (%) | 59 (9.9) | 84 (7.3) |
| Missing (%) | 1 (0.2) | 51 (4.4) |
| Smoking | | |
| Yes (%) | 229 (38.6) | 345 (30.1) |
| No (%) | 338 (56.9) | 802 (69.9) |
| Missing (%) | 27 (4.5) | 0 (0) |
| Ophthalmopathy | | |
| Yes (%) | 267 (44.9) | 0 (0) |
| No (%) | 327 (55.1) | 1147 (100) |

^aData for age are presented as the mean ± SD.

SWE, Swedish; EUR, born in Europe outside of Sweden; OUT, born outside of Europe.

Genetic Power Calculator (23). Described MAF for the previously associated *CTLA4* SNPs varied between 0.07 and 0.47. Given a control/case ratio of 2, MAF 0.30, and odds ratio (OR) 1.39, as presented in the recent GWAS for GD (24), the number of cases required for 80% power and significance 0.05 is 500. Given MAF 0.40 and OR 1.39, the number of cases required for 80% power and significance 0.05 is 637.

Genotyping

DNA was extracted from whole blood using the MaxiPrep Kit (QIAGEN, Sweden), and SNPs were genotyped using the Sequenom platform (MALDI-TOF) at the DNA/RNA Genotyping Lab, SWEGENE Resource Center for Profiling Polygenic Disease, Lund University, Skåne University Hospital, Malmö, Sweden, and in our laboratory. A total of 15 SNPs failed the genotyping ($n=8$) or Hardy Weinberg equilibrium ($n=7$) analysis. These SNPs were removed from the further analysis, leaving a total of 83 SNPs. The SNPs included in the further analysis had a genotyping success rate >95%.

Statistical analysis

Logistic regression with age and smoking as covariates and standard case/control association for the additional analysis including the 116 newly recruited cases were used for estimating SNP associations. Analyses of the combined effects of SNPs and smoking were performed using additive models of interaction within a logistic regression framework. The data are presented as odds ratios (OR) with 95% confidence intervals (CI). The p -values are based on additive models for the genetic variants. Correction for multiple testing was performed using permutations (maxT permutation method in PLINK). All statistical calculations were performed using PLINK version 1.07 (<http://pngu.mgh.harvard.edu/~purcell/plink/index.shtml>) (25).

Results

SNP association with Graves' disease

Using logistic regression, a total of three tag SNPs in the *BTG2* gene region showed an association with GD: rs12136280 (OR 1.29, $p=0.002$), rs6663606 (OR 1.26, $p=0.004$), and rs17534202 (OR 1.21, $p=0.02$). The promoter SNP rs3753793 in the *CYR61* gene was also associated with GD (OR 1.21, $p=0.03$) (Table 2). A condition analysis revealed that the significant SNPs in *BTG2* influence each other but have an effect that is independent of the *CYR61* SNP rs3753793. None of the investigated SNPs in *CTLA4* were associated with GD. However, including the 116 newly re-

cruited patients with GD, the SNPs rs11571297 (OR 1.15, $p=0.05$) and rs3087243 (OR 1.15, $p=0.05$) in *CTLA4* showed association with GD with borderline significance. The above described significantly associated SNPs (Table 2) remained significant even after inclusion of the new 116 GD cases. Correction for multiple testing was done using permutations, with the maxT method performing 1000 permutations. Without the additional 116 GD individuals, the association of rs12136280 with GD showed borderline significance (corrected empirical p -value of 0.06). Including the additional 116 GD individuals, the association of rs12136280 with GD remained significant (corrected empirical p -value of 0.047). The significance of the association between the remaining SNPs associated with GD (Table 2) disappeared after correction for multiple testing both without and with the additional 116 cases included. However, a new analysis with Haploview using most recent information on the presence of linkage disequilibrium (LD) revealed that many of the analyzed SNPs in *BTG2*, *CYR61*, *ZFP36*, *SCD*, *PTGS2*, *EGR1*, *DUSP1*, *NR4A2*, *RGS2*, *CXCL2*, and *SOCS3* were not independent of each other due to the presence of LD; therefore, adjusting for the number of tested sites rather than the number of tested SNPs might be more appropriate, and with this approach, the reported associations remain significant (data not shown).

SNP association with Graves' ophthalmopathy

Four SNPs in the *CYR61* gene region were associated with GO: the promoter SNP rs3753793 (OR 1.45, $p=0.008$) and the tag SNPs rs6682848 (OR 1.55, $p=0.03$), rs12756618 (OR 1.77, $p=0.049$), and rs1378228 (OR 1.29, $p=0.049$) (Table 3). An association with GO was also shown for the *ZFP36* tag SNPs rs1057745 (OR 1.56, $p=0.03$) and rs11083522 (OR 1.32, $p=0.04$) and for the *SCD* tag SNP rs1393491 (OR 1.38, $p=0.048$) (Table 3). The significance of these associations disappeared after correction for multiple testing. In the condition analysis, the significant SNPs in *CYR61* influenced each other but were independent of the significant SNPs in *ZFP36* and *SCD*. The same result was obtained for the significant SNPs in *ZFP36*, which influenced each other, but were independent of the significant SNPs in *CYR61* and *SCD*. None of the investigated SNPs in *CTLA4* were associated with GO.

Interactions between SNPs and smoking

Because smoking is a risk factor for GD and a strong risk factor for GO, we investigated the possible interactions between smoking and the significant SNPs in GD and GO.

TABLE 2. SINGLE NUCLEOTIDE POLYMORPHISMS ASSOCIATED WITH GRAVES' DISEASE

| Gene | SNP | Selection criteria | Minor allele frequency | Associated allele | OR for associated allele (95% CI) | p-Value |
|--------------|------------|--------------------|------------------------|-------------------|-----------------------------------|---------|
| <i>BTG2</i> | rs12136280 | tag, missense | 0.46 (T) | T | 1.29 (1.10–1.52) | 0.002 |
| | rs6663606 | tag | 0.46 (T) | A | 1.26 (1.08–1.48) | 0.004 |
| | rs17534202 | tag | 0.47 (G) | C | 1.21 (1.03–1.41) | 0.02 |
| <i>CYR61</i> | rs3753793 | tag, promoter | 0.27 (G) | G | 1.21 (1.02–1.45) | 0.03 |

The p -values were calculated using logistic regression with age and smoking as covariates. CI, confidence interval; OD, odds ratio; SNP, single nucleotide polymorphism.

TABLE 3. SINGLE NUCLEOTIDE POLYMORPHISMS ASSOCIATED WITH GRAVES' OPTHALMOPATHY

| Gene | SNP | Selection criteria | Minor allele frequency | Associated allele | OR for associated allele (95% CI) | p-Value |
|--------------|------------|--------------------|------------------------|-------------------|-----------------------------------|---------|
| <i>CYR61</i> | rs3753793 | tag, promoter | 0.27 (G) | T | 1.45 (1.91–2.64) | 0.008 |
| | rs6682848 | tag | 0.10 (A) | A | 1.55 (1.04–2.31) | 0.03 |
| | rs12756618 | tag | 0.06 (T) | C | 1.77 (1.00–3.12) | 0.049 |
| | rs1378228 | tag | 0.36 (T) | G | 1.29 (1.00–1.66) | 0.049 |
| <i>ZFP36</i> | rs11083522 | tag | 0.29 (C) | C | 1.32 (1.02–1.71) | 0.04 |
| | rs1057745 | tag | 0.10 (C) | C | 1.56 (1.05–2.33) | 0.03 |
| <i>SCD</i> | rs1393491 | tag | 0.17 (C) | T | 1.38 (1.00–1.91) | 0.048 |

The *p*-values were calculated using logistic regression with age and smoking as covariates.

Although no interaction with smoking was observed for the SNPs that were associated with GD, an interaction between smoking and the *CYR61* SNP rs12756618 was observed, further increasing the risk of GO (OR 4.75, CI 1.33–17.05, *p* = 0.02).

Discussion

In this study, we used the functional candidate gene approach to examine possible associations with GD and GO. Except for *CTLA4*, the candidate genes were chosen on the basis of our earlier expression study in the same population (15), in which the principal finding was the overexpression of 14 adipocyte-related IEGs in active GO. Variants in three IEGs (*BTG2*, *CYR61*, and *ZFP36*) and in the proadipogenic gene *SCD* showed an association with GD and/or GO.

BTG2 is a member of the *BTG/Tob* gene family with antiproliferative effects and belongs to a group of genes differentially expressed between resting and activated mouse T cells (26). Because *BTG2* is a downstream target of *CD28*, it is tempting to speculate that *BTG2* could be involved in the inhibition of T cell activation, together with other negative regulators of activated T cells associated with GD, such as *CTLA4* and protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*). Associations of three SNPs in the *BTG2* gene region with GD were shown in this study. However, the causative variant is not known, and all SNPs tested were tag SNPs, leaving the possibility of the causative SNP being in LD with any of the associated SNPs. Nonetheless, rs12136280 is a coding SNP leading to an amino acid alteration (Val → Met at position 153; V153M), making it the most promising candidate.

Seven SNPs in three genes, *CYR61*, *ZFP36*, and *SCD*, were associated with GO in this study. *CYR61* is a multifunctional gene with roles in adipogenesis, inflammation, cell proliferation, extracellular matrix production, and fibrosis (27,28). Previous studies have implicated *CYR61* in the pathogenesis of inflammatory diseases, including rheumatoid arthritis (RA), and a microarray analysis of B cells from monozygotic twins revealed a significantly higher expression of *CYR61* in the twin with RA compared to the healthy twin (29). *CYR61* is also overexpressed in RA synovial tissue and plays a critical role in the proliferation of rheumatoid arthritis synovial fibroblasts (30). *CYR61* expression is induced in human skin dermal fibroblasts after exposure to cigarette smoke (31). In our study, four SNPs in *CYR61* were associated with GO, with one, the promoter SNP rs3753793, even

being associated with GD. As smoking is a strong risk factor for GO and even a risk factor for GD, one hypothesis is that the associated risk variants could interact with smoking to increase the risk for GO and GD. In our study, an interaction between rs12756618 in *CYR61* and smoking was observed. However, our study most likely does not have sufficient power to detect all possible gene-environment interactions.

ZFP36 codes for the protein tristetraprolin (TTP), which promotes the destruction of the tumor necrosis factor- α mRNA. (32). TTP-knockout mice exhibit a severe inflammatory phenotype (33), which makes TTP an interesting candidate for associations with autoimmunity. Indeed, the SNP *ZFP36**8 was found to be associated with RA in African Americans in a genetic study of autoimmune diseases (34). In the present study, we demonstrate an association of two SNPs with GO.

IEGs are a group of genes known to be expressed in differentiating preadipocytes and mitogen-stimulated fibroblasts (35,36). The expression of *IEGs* during adipogenesis initiates the mitotic clonal expansion phase of growth-arrested preadipocytes, which is followed by a second growth-arrest phase and then the terminal differentiation phase, leading to the mature adipocyte phenotype (37). In our expression studies of active (15) and chronic (16) ophthalmopathy, *IEGs* showed markedly increased expression in patients with active GO compared to controls and moderately increased expression in patients with chronic GO compared to controls. However, it remains unknown whether the overexpression of an entire group of adipocyte-related *IEGs* contributes to the etiology of GO or is rather a consequence of the disease. The finding of multiple SNPs in the *IEGs* *CYR61*, *ZFP36*, and *BTG2* as being associated with GO supports the hypothesis that genetic variation in this group of genes could contribute to the development of GO.

SCD is an enzyme that catalyzes a rate-limiting step in the synthesis of unsaturated fatty acids. It is a proadipogenic gene, and genetic variations in the *SCD* gene have been associated with body fat distribution and insulin sensitivity in Swedish men (38). In the present study, we showed a borderline association of one *SCD* SNP with GO but not with GD. Thus, it could be speculated that the genetic variant associated with GO promotes adipogenesis, a key pathogenic process in GO.

We were initially not able to confirm the previously reported associations of SNPs in *CTLA4* and GD or GO, including 594 cases and 1147 controls. The allele frequencies in our population were similar to those reported in the other

association studies and the diagnostic criteria for GD and GO the same. For these allele frequencies (MAF around 0.4), the sample size might be slightly small, as demonstrated in the power calculation. Indeed, including additional 116 cases with GD in the analysis resulted in association of two *CTLA4* SNPs with GD (with borderline significance), suggesting that the lack of association between *CTLA4* and GD can probably at least partly be explained by the sample size. However, the Wellcome Trust Case Control Consortium recently performed a large study with a genome-wide set of nonsynonymous coding variants and provided evidence that three previously reported loci (major histocompatibility complex [*MHC*], thyroid stimulating hormone receptor [*TSHR*], and Fc receptor-like 3 [*FCRL3*]) were associated with GD in individuals of European ancestry (39). In this study, associations with the *CTLA4* locus were not shown, suggesting that diversity may exist between different populations.

Lastly, several methodological issues need to be discussed. It is well known that genetic diversity exists between different ethnic groups, and we attempted to overcome this problem by group-matching the cases and controls in three groups, Swedish, European outside of Sweden, and non-European. We then tested the effect of ethnicity in the logistic regression analysis and could not observe any effect. We therefore assumed that the group-matching in our population was sufficient. In contrast, age and smoking, factors known to influence the etiology of GD, were significantly associated with both GD and GO in our population and were therefore used as covariates in the logistic regression analysis to minimize the effect of confounding factors. The number of subjects in the study is in line with most association studies for GD; regardless, recent research has shown that larger numbers of individuals are needed to avoid false-positive results. Our cohort, however, has the advantages of being phenotypically well defined and consisting of a population from a single town. Moreover, the choice of the candidate genes was based on expression data in the same population and the associated genes are biologically relevant to the disease. Furthermore, we have controlled for three known confounding factors in our analyses, which has not been the case in most association studies for GD/GO.

In conclusion, the results of this study suggest associations of SNPs in *BTG2*, *CYR61*, *ZFP36*, and *SCD* with GD and/or GO. Nonetheless, replication in a different population is required.

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Author Disclosure Statement

No competing financial interests exist.

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