DOI:
10.1111/exd.2017.26.issue-12

Document Version
Peer reviewed version

Genome-wide association study identifies *GALC* as susceptibility gene for mucous membrane pemphigoid

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Running title: *GALC* is a susceptibility gene for MMP

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Word count: 2776

Figures: 2

Tables: 1

Funding: this work was supported by the DFG (KFO 303 Pemphigoid Diseases - *Molecular Pathways and their therapeutic Potential*)

Abstract

Background: Mucous membrane pemphigoid (MMP) is a rare, chronic, and often aggressive subepidermal autoimmune blistering disease potentially affecting several mucous membranes with blisters and secondary erosions and scars. The pathogenesis of MMP is poorly understood, and the contribution of genetic predispositions, other than HLA class II allele variants to MMP, is unknown.

Objectives: The objective of this study is to identify susceptibility genes for MMP in a British cohort of MMP patients.

Methods: A GWAS was conducted in a British cohort of 106 MMP patients. Publicly available genotypes of 2,900 blood donors of the UK Blood Service and of 6,740 individuals of the *1958 British Birth Cohort* served as control. Subsequently, putative susceptibility genes were independently replicated in a German cohort of 42 MMP patients.

Results: The GWAS found 38 SNPs in 28 haploblocks with an odds ratio >2 reaching genome-wide significance ($p<5.7x10^{-7}$). Replication confirmed an association of MMP with SNPs in rs17203398 (OR: 3.9), located intronically in the β-galactocerebrosidase gene (*GALC*) on chromosome 14, and with recessive polymorphisms in rs9936045 (OR: 3.1) in the intergenic region between *CASC16* and *CHD9* on chromosome 16.

Conclusions: The risk of developing MMP is partially genetically determined. SNPs in *GALC* enhance the risk for MMP, indicating that β-galactocerebrosidase may be involved in the pathogenesis of MMP. Likewise, impacts of polymorphisms in the intergenic region between *CASC16* and *CHD9* on the activity of neighboring genes may facilitate the emergence of MMP. The putative role of both polymorphisms requires functional studies in the future.

Keywords: Susceptibility genes, inflammation, fibrosis

Introduction

Pemphigoid diseases are a group of seven different subepidermal autoimmune blistering diseases driven by autoantibodies directed to proteins of the dermal-epidermal anchoring complex (1). They affect the skin and mucous membranes to a variable extent depending on the specific disease and the individual patient. Mucous membrane pemphigoid (MMP), formerly known as “cicatricial pemphigoid”, is the only pemphigoid disease predominantly affecting mucous membranes, not the skin. Mucous membranes, which can be affected by the disease, are that of the conjunctivae, the oral cavity, the esophagus, the nose, the pharynx, the larynx, the trachea, the anal canal, and the genitalia (2, 3). Disease can simultaneously afflict all these mucous membranes or can be locally restricted to specific surfaces. Frequently, MMP only affects the conjunctiva or the oral cavity and is then designated as ocular and oral pemphigoid, respectively (3). MMP lesions, except for those in the oral cavity, usually scar. The scarring may result in impaired organ function and lead to organ failure. Typical residual states in MMP patients include strictures in the upper digestive, airway and anogenital tracts, as well as conjunctival scarring causing severe dry eye, ingrowing lashes, and corneal stem cell failure, all of which result in corneal opacification and blindness. To prevent the emergence of these debilitating residual states, MMP is usually treated aggressively upon diagnosis with potent, immunosuppressive drugs (2, 4).

The pathogenesis of MMP is still largely unknown, but it is thought to be driven by IgG and/or IgA autoantibodies directed against different proteins of the dermal-epidermal anchoring complex, including BP180, BP230, β_4 integrin, laminin 332 (laminin 5), and type VII collagen (2). Notably, autoantibodies against only one of these antigens are sufficient to induce disease (5). The most frequent autoantigen in MMP is BP180 with 75% of patients producing IgG autoantibodies against it (5). BP180 is also the only major autoantigen in bullous pemphigoid

and pemphigoid gestationis (1, 6), illustrating the close relationship between MMP and these two other pemphigoid diseases.

Binding of MMP autoantibodies to the dermal-epidermal junction initiates effector cell recruitment by still unidentified mechanisms. Thus, in acute disease, the histopathology of MMP lesions feature subepidermal infiltration predominantly by neutrophils, T cells and eosinophils. Products of these effector cells, such as proteases and radical oxygen species, are presumably responsible for the disruption of dermal-epidermal adhesion resulting in mucosal blistering (7-9). In addition, the induction of a profibrotic phenotype in fibroblasts, which consequently leads to severe scarring, is pivotal for the pathogenesis of MMP (10). Inflammation and scarring are mediated by neutrophils, dendritic cells, mast cells, eosinophils, macrophages and T cells. These effects are mediated by their cytokines IL-2, IL-4, IL-5, IL-13, IFNγ (interferon gamma) and the growth factors TNFα (tumour necrosis factor alpha), PDGF (platelet derived growth factor), TGFβ (transforming growth factor beta) and HSP47 (heat shock protein). ALDH/RA (aldehyde dehydrogenase/retinoic acid)-mediated paracrine and autocrine effects initiate and perpetuate fibroblast activation which persists after control of inflammation (11).

Notably, in contrast to most other autoimmune diseases, only very little is known about individual predispositions for pemphigoid diseases in general as well as for MMP specifically. One major reason for this situation is the low prevalence of pemphigoid diseases, with MMP, e.g., occurring with a prevalence as low as 24 per million in Germany (12), and the consequent lack of genome-wide association studies (GWAS). To date susceptibility to MMP has only been associated with three genetic variants of the HLA class II alleles: DQB1*0301, DRB*04, and DRB*11 (5, 13-19). However, whether other genetic polymorphisms, outside the HLA class II locus impact the susceptibility to MMP has been unknown. We therefore performed a GWAS in a cohort of MMP patients and identified 38 SNPs in 28 haploblocks associated with MMP. Replication subsequently corroborated an association of MMP with polymorphisms in *GALC*, the

gene encoding for β-galactocerebrosidase (EC 3.2.1.46). *GALC* has not previously been implicated in the pathogenesis of MMP or other pemphigoid diseases.

Patients and Methods

Study population

For the GWAS, 106 patients (Males: 56; females: 50; age: 18 – 86 years) suffering from MMP were recruited at the Moorfields Eye Hospital (London, UK). The diagnosis of MMP was based on the clinical presentation and a positive finding by direct immunofluorescence (IF) microscopy or typical clinical presentation with MMP autoantibodies detectable in the serum and exclusion of other causes of scarring conjunctivitis, as previously described (20, 21). Clinical data on MMP patients were collected using a case report form asking patients to list all body sites presently or previously affected by MMP lesions. Sites, which exhibited signs of MMP in the past, were classified "affected" irrespective of the current state of disease activity or of the presence or absence of residuals. In addition, patients were clinically examined for signs of MMP at all potential anatomical sites except for the oesophagus, by ophthalmologists, dermatologists, oral medicine specialists, and otolaryngologists. 45 individuals (22 males; 23 females; age: 18 - 83 years), who were having surgery for ocular conditions, without associated systemic disease, including cataract, squint, retinal detachment, Fuchs' corneal dystrophy, keratoconus, and blepharoplasty, as well as the publicly available genotypes of 2,900 blood donors of the UK Blood Service and of 6,740 individuals of the *1958 British Birth Cohort* served as controls. Clinical information on the British cohort are summarized in tables S1 and S2. For replication, a cohort of 42 MMP patients was recruited in Dermatology Departments of University Hospitals in Germany. MMP diagnosis was based on the same criteria, as described above. Clinical information on the German cohort are summarized in table S3. 250 age- and sex-matched controls were provided by the *Popgen* Biobank (Kiel, Germany). The study was approved by the

Moorfields & Whittington research ethics committee (reference number: 09/H0721/54) and the ethic committee of the University of Lübeck (reference number: 08-156).

Genome-wide association study (GWAS)

Genomic DNA of the MMP patient cohort and the internal controls was isolated from whole blood. Genome-wide genotyping was executed using the Illumina Immunochip Array (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. 9,684 samples addressing 196,524 SNPs were genotyped and initially included in this study. Quality control and GWAS analysis of genotyped single nucleotide polymorphism (SNPs) were performed using the software programs GenomeStudio (Illumina), PLINK version 1.07 (22), and R. Comparing internal and public controls, 59,527 SNPs had significantly different genotype distributions ($p < 0.01$), when applying a logistic regression analysis in PLINK, and were subsequently excluded from further analysis. The remaining SNPs were quality-checked. R was used to exclude SNPs from further analysis when exhibiting a call rate < 99% or a 10% GC-Score < $0.8*90^{th}$ percentile, as recommended by Illumina. In addition, using PLINK, SNPs were also excluded if more than 10% of genotypes were missing, if the deviation of Hardy-Weinberg equilibrium was significant ($p_{HWE} < 10^{-5}$), or if the minor allele frequency (MAF) was < 2.5%. After this additional quality check, 88,065 SNPs remained for comparison between MMP patients and controls with 95 MMP patients and 9,646 control samples left for analyses. These SNPs and samples were analyzed for association *via* logistic regression with the first five principal-components as covariates. Principal component analysis was performed using EIGENSOFT version 5.0.2 (23). In this analysis, sex was not considered as covariate due to missing information. A global significance of $p < 5 \times 10^{-8}$ was defined as statistically significant and an odds ratio > 2 was considered biologically relevant.

Replication

DNA for replication was isolated using standard extraction kits (Qiagen, Hilden, Germany). Replication was conducted using the TaqMan® SNP Genotyping Assay (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions applying unlabeled forward and reverse PCR primers in combination with VIC® dye – MGB probes for detection of the Allele 1 sequence and 6-FAM™ dye – MGB probes for the detection of the Allele 2 sequence (Suppl. Tabl. S1). The qPCR was run on the *realplex Mastercycler* (Eppendorf, Hamburg, Germany) with an initiation of 10 min at 95 °C and 40 cycles each of 15 sec at 95°C annealing temperature followed by 1 min at 60 °C for elongation. Samples that repetitively did not yield unambiguous genotypes were excluded from further analysis. Replication results of the MMP patient and the control groups were analyzed for statistical significance by *Chi*-square test, testing for both overall significance, and significance in a dominant or recessive setting for the distinct minor allele. In either case, $p < 0.05$ was regarded as statistically significant.

Results

We conducted a GWAS on 95 MMP samples and 9,646 control samples comparing the frequency of 88,065 SNPs in these two groups. This analysis yielded 38 SNPs in 28 haploblocks with an odds ratio > 2 reaching the genome-wide significance level of $p < 5.7 \times 10^{-7}$ in the analysis with and without 5 principal components as covariates (Fig. 1). These 38 SNPs and their precise location are listed in Table 1. They were scattered over 12 autosomal chromosomes. In addition to these 38 SNPs, our analysis also illustrated the strong association of susceptibility to MMP with certain HLA haplotypes, which was reflected by a distinct peak at the site of this the HLA locus on chromosome 6 in the Manhattan plot (Fig. 1). This finding confirms the validity of our approach. The mean odds ratio of these 38 SNPs for disease was 2.6; the highest odds ratio was 3.9 and was linked to 1kg_14_87519600 on chromosome 14 (Tab. S4).

To select the most promising genes for replication, we conducted an association analysis factoring in SNPs with higher p-values located in the proximity of the 38 identified SNPs. Thus, we exploited the fact that in the vicinity of SNPs of high pathogenic relevance, other SNPs are frequently detected (Fig. 2). By this strategy, those 6 SNPs most likely pathogenetically relevant among the 38 disease-associated SNPs were singled out for replication. In the replication analysis, the frequency distribution between controls and MMP patients for 2 of these 6 SNPs, precisely for the SNPs 1kg_14_87519600 and rs9936045, was statistically significantly different (Tab. 1). Herein, the frequency of the SNP 1kg_14_87519600 (rs17203398) was not only statistically significantly different between the two groups when the general significance ($p = 0.004$) was evaluated, but also when the recessive ($p = 0.003$) and the dominant ($p = 0.011$) model were examined (Tab. 1). rs17203398 is located on chromosome 14 in the intron region of the gene *GALC*. The differences for the SNP rs9936045 was in contrast only significantly different in the recessive model ($p = 0.022$). rs9936045 is located on chromosome 16 between the genes *CASC16* and *CHD9*.

Discussion

Although individual genetic predispositions are in general considered to play a major role in the pathogenesis of autoimmune diseases and a plethora of different polymorphisms of most diverse genes has been identified to modulate susceptibility to autoimmunity, genetic predispositions for pemphigoid diseases, including MMP, are almost completely unknown. Therefore, we set out to conduct the first GWAS for MMP in a British cohort of 106 MMP patients. This study yielded 38 SNPs associated with the occurrence of MMP, suggesting that, as in other autoimmune diseases, polymorphisms in genes other than HLA class II alleles additionally co-determine the individual susceptibility to MMP.

Replication of 6 of these genes identified in the GWAS corroborated the association between SNPs in *GALC* and in a region between the genes *CASC16* and *CHD9* with MMP. Both loci have not previously been implicated in the pathogenesis of MMP or in any other pemphigoid disease, although polymorphisms in GALC were previously associated with susceptibility to inflammatory bowel disease (24, 25).

GALC encodes the lysosomal enzyme β-galactocerebrosidase (EC 3.2.1.46), which is expressed in most tissues and is critically involved in the regulation of cellular sphingolipids (26, 27). Homozygous deletion of *GALC* causes Krabbe disease (globoid cell leukodystrophy), an autosomal-recessive hereditary disorder, featuring multiple neurodegenerative deficits due to diffuse demyelination in the peripheral and central nervous system (28). β-galactocerebrosidase is also involved in cancer cell metabolism, primary open-angle glaucoma, and in the regulation of the hematopoietic stem cell niche (29-31). Its expression in cancer cells blocks apoptosis with β-galactocerebrosidase shifting the balance between the proapoptotic sphingolipid ceramide towards its antiapoptotic derivative galactosylcerebroside (29). The regulation of sphingolipid levels is also pivotal to maintain the hematopoietic stem cell niche. Thus, functional deficiency in β-galactocerebrosidase decreases proliferation of early progenitor cells in mice (31).

Intriguingly, hyperreactive immune responses were identified in the last decade as symptom in several lysosomal storage diseases, including Krabbe disease, and have also been suggested to significantly contribute to the emergence of the neurological hallmark symptoms of these diseases (32). Thus, lymphocytes and monocytes of Krabbe disease patients exhibit a proinflammatory activation state characterized, amongst others, by an increased release of TNF-α upon stimulation (33). Furthermore, in *Twitcher* mice, a mouse model of Krabbe disease, expression of proinflammatory cytokines and chemokines is enhanced and contributes to disease pathogenesis (34, 35).

~~Through these immunoregulatory functions, aberrant β-galactocerebrosidase activity may also directly contribute to the pathogenesis of autoimmune diseases, such as MMP. With TNF-α levels elevated in MMP patients and its inhibition effective in treating the disease, it is tempting to speculate that *GALC* polymorphisms may elevate TNF-α levels predisposing to the development of MMP. In line with this hypothesis, TNF-α induces the release of proinflammatory mediators from conjunctival fibroblasts, which can in turn promote conjunctival fibrosis.~~ Heterozygous deletions of *GALC* are also a significant risk factor for primary open-angle glaucoma. Although the pathogenic mechanism behind this association has not been elucidated, this association suggests that physiological functions of β-galactocerebrosidase are partially gene copy number-sensitive and that heterozygous mutations of *GALC* are already sufficient to disturb organ homeostasis (30).

The second SNP associated with MMP was rs9936045, which is located on chromosome 16 in the intergenic region between *CASC16* and *CHD9*. The significance of this finding and whether the polymorphism impacts the activity of the two neighboring genes is currently unclear. In addition, the function of these two neighboring genes is poorly understood, thus complicating its clarification. The biological function of *Cancer susceptibility candidate 16 (CASC16*; also designated as “LINC00918” or “LOC643714”), the gene located on the reverse strand upstream of rs9936045, has not been characterized, but polymorphisms of the gene have been

associated with the risk for breast and lung cancer (36-39). *CHD9*, the gene downstream of rs9936045, encodes the protein “chromodomain helicase DNA binding protein 9” (CHD9), which has also been referred to as “Chromatin Related Mesenchymal Modulator” (CReMM) and “PPARα-interacting cofactor 320” (PRIC320). CHD9 is a transcription coactivator with DNA helicase activity. Thus, the protein bears both chromatin remodeling and nuclear receptor coactivation functions and serves as coactivator of several transcription factors, including PPAR-α, CAR, ER**α**, RXR, CBP, PRIP, and PBP (40-42), suggesting a wide array of possible regulatory roles for this protein. However, except for a role in osteogenic differentiation (41, 43), the physiological roles of CHD9 are still largely elusive.

The only gene polymorphism outside the HLA class II locus previously associated with MMP is the *IL-4RA*-1902 SNP in a Northern Italian cohort of 41 MMP patients suffering from oral pemphigoid (44). However, the pathogenic relevance of this polymorphism has not been addressed. Carriers of this polymorphism exhibit an attenuated response to IL-4 cell stimulation. As IL-4 exerts profibrotic effects in MMP and oral pemphigoid is the only subtype of MMP typically not developing scarring (45). In our study, focusing on ocular MMP, we did not find an association between the *IL-4RA*-1902 SNP and MMP. The association of this polymorphism to MMP may therefore be limited to oral pemphigoid.

Bullous pemphigoid (BP) is the disease most closely related to MMP. Both diseases share BP180 and BP230 as common autoantigens, and in 20% of BP patients blisters and erosions on mucous membranes emerge (2). Thus, the two diseases are sometimes hard to distinguish and may transform from one to the other. Like in MMP, there is also still a paucity in the identification of gene polymorphisms predisposing to BP. Susceptibility to BP has been associated with SNPs in *IL1B*, the gene for IL-1β, in a Chinese cohort and in *CYP2D6*, the gene for the cytochrome P450 oxidase 2D6, in a Polish cohort (46, 47). In addition, copy number variations (CNVs) in the Fcγ receptor genes *FCGR2B* and *FCGR3B* as well as polymorphisms in the mitochondrial gene *ATP8* have been associated with the risk for BP in a German cohort

(48, 49). While our study design did not allow examining the effect of CNVs or mitochondrial genes to MMP, it could have detected associations to *IL1B* and *CYP2D6* SNPs. The fact that both genes are not among the list of the 49 genes identified in our study suggests that despite the resemblance of the two diseases, genetic predispositions for the two diseases significantly differ.

In sum, this first GWAS in MMP patients identified two gene loci, which had not previously been implicated in autoantibody-driven autoimmune diseases, including pemphigoid diseases. Future studies will address the functional relevance of these polymorphisms for the pathogenesis of MMP. Our findings illustrate that MMP must be considered a complex, multifactorial disease with a previously underappreciated significant contribution of genes outside the HLA locus to the pathogenesis of disease.

Conflict of interest

The authors have no conflict of interest.

Acknowledgements

This research was supported by DFG funding (IB 24/10-1; Sa 1960/5-1) for the Clinical Research Unit 303 *Pemphigoid Diseases – Molecular Pathways and their therapeutic Potential*. J.B., A.D., D.M., and N.v.B. performed the research. C.D.S., J.B., N.v.B., E.S., J.D., D.M., and S.I. analyzed the data. D.Z., E.S., S.B., M.S., M.W., S.M., R.G., B.H., J.S., and J.D. collected and provided patient samples. C.D.S., J.B., N.v.B., D.Z., J.D., E.S., and S.I. wrote the paper.

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Table 1: Replication analysis for 6 selected SNPs

SNP	Chr	BP	Minor allele	OR	Significance (p)	Dominant model (p)	Recessive model (p)	Closest gene(s)
imm_1_181612330	1	181612330	T	2.6	0.280	0.074	0.959	*NMNAT2*
seq-rs523937	2	219145840	G	2.5	0.999	0.707	0.381	*RQCD1*
1kg_14_87519600	14	87519600	C	3.9	*0.004*	***0.011***	***0.003***	*GALC*
rs9936045	16	51472905	G	3.1	0.599	0.175	***0.022***	*CASC16/CHD9*
seq-rs1043680	19	60204159	C	2.9	0.581	0.517	0.816	*NLRP2*
seq-rs4806637	19	60247800	G	2.8	0.669	0.955	n/a	*RDH13*

SNP, single nucleotide polymorphism; Chr, chromosome; BP, base pair number; OR, odd ratio

Figure 1. Manhattan plot of a genome-wide association study in 95 MMP patients and 9,646 control samples. The ordinate displays the $-\log_{10}$ P values of 88,065 SNPs included in this study, and the abscissa shows the chromosomal positions. The horizontal blue line represents the reaching the genome-wide significance level of $p < 5 \times 10^{-8}$. 38 SNPs were above this cut-off and were considered biologically relevant when achieving an odds ratio of > 2.

Figure 2. Detailed view of the four loci most closely associated with MMP. The associated regions are located on (**A**) Chr. 1, (**B**) Chr. 2, (**C**) Chr. 16, (**D**) Chr. 19. The $-\log_{10} p$ values of the genotyped SNPs (left ordinate) and the regional recombination rate (right ordinate) are plotted against their physical position on the chromosomes. In each plot, the solid diamond represents the top-ranked SNP in the region.

Table 1. Replication analysis for 6 selected SNPs. Replication was conducted in a German MMP cohort and controls using qPCR. Both groups were tested for statistically significant differences by X^2-test and were tested for overall significance, as well as for significance in a dominant or recessive setting for the minor allele. $p < 0.05$ was regarded as statistically significant. Significant differences are in bold.