No association of primary Sjögren's syndrome with Fcγ receptor gene variants

Karstein Haldorsen[1,2], Silke Appel[1], Stephanie Le Hellard[3], Ove Bruland[4], Johan G. Brun[5], Roald Omdal[6], Gudlaug Kristjansdottir[7], Elke Theander[8], Carla P.D. Fernandes[3], Marika Kvarnström[9], Per Eriksson[10], Lars Rönnblom[7], Marie Wahren Herlenius[9], Gunnel Nordmark[7], Roland Jonsson[1,5], Anne Isine Bolstad[2]

[1]Broegelmann Research Laboratory, The Gade Institute, University of Bergen, Bergen, Norway.
[2]Department of Clinical Dentistry, University of Bergen, Bergen, Norway.
[3]Department of Clinical Medicine, University of Bergen, Bergen, Norway.
[4]Center for Medical Genetics and Molecular Medicine, Haukeland University Hospital, Bergen, Norway.
[5]Department of Rheumatology, Haukeland University Hospital, Bergen, Norway
[6]Clinical Immunology Unit, Department of Internal Medicine, Stavanger University Hospital, Stavanger, Norway.
[7] Department of Medical Sciences, Section of Rheumatology, Uppsala University, Uppsala, Sweden.
[8]Department of Rheumatology, Skåne University Hospital, Malmö, Lund University, Sweden.
[9]Rheumatology Unit, Department of Medicine, Karolinska Institute, Stockholm, Sweden.
[10]Rheumatology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Sweden.

Corresponding author:

Karstein Haldorsen, MD, PhD
Broegelmann Research Laboratory
The Gade Institute
University of Bergen
Laboratory Building, 5th floor
N-5021 Bergen
Norway

E-mail: kahn@helse-bergen.no
Telephone: +47 55974648
Fax: +47 55975817

Abstract and keywords

The genetic background of primary Sjögren's syndrome (pSS) is partly shared with systemic lupus erythematosus (SLE). IgG Fc receptors are important for clearance of immune complexes. Fcγ receptor variants and gene deletion have been found to confer SLE risk. In this study, four Fcγ receptor single nucleotide polymorphisms (SNPs) and one copy number variation (CNV) were studied. Swedish and Norwegian pSS patients (N = 527) and controls (N = 528) were genotyped for the Fc receptor gene variant FCGR2A H131R (rs1801274) by the Illumina GoldenGate assay. FCGR3A F158V (rs396991) was analysed in 488 patients and 485 controls, FCGR3B rs447536 was analysed in 471 patients and 467 controls, and FCGR3B rs448740 was analysed in 478 cases and 455 controls, using TaqMan SNP genotyping assays. FCGR3B CNV was analysed in 124 patients and 139 controls using a TaqMan copy number assay. None of the SNPs showed any association with pSS. Also, no FCGR3B CNV association was detected. The lack of association of pSS with Fc receptor gene variants indicates that defective immune complex clearance may not be as important in pSS pathogenesis as in SLE, and may point to important differences between SLE and pSS.

Keywords: Sjögren's syndrome; Fc gamma receptors; single nucleotide polymorphism; DNA copy number variations.

Introduction

Primary Sjögren's syndrome (pSS) is a chronic systemic autoimmune disease mainly affecting the salivary and lacrimal glands. Population-based family studies have not yet been undertaken to ascertain the heritability of this disease. Based on multiplex families, twin case reports, candidate gene studies and clinical considerations, the disease is regarded as a complex genetic trait[1]. pSS shares several genetic susceptibility factors and phenotypic features with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) and twin concordance in pSS is assumed by some investigators to be between that of RA, 15%, and SLE, 25%[1]. Candidate genes showing associations with pSS and other rheumatic diseases include the interferon related genes *IRF5* and *STAT4*, as well as *HLA-DR* and *HLA-DQ* variants[2, 3]. Moreover, a recent study has shown association of pSS with *EBF1*, *BLK* and *TNFSF4*[4].

Immunoglobulin G Fc-receptors (FcγR) are immune cell membrane glycoproteins that bind to the Fc portion of IgG. They have different effects depending on their expression on immune cells. FcγR are important links between the adaptive and innate immune system, and are crucial for phagocytic function (reviewed in reference 5). Low-affinity single nucleotide polymorphisms (SNPs) in *FCGR2A* and *FCGR3A*, and low copy number of *FCGR3B*, have been found to confer risk of SLE or lupus nephritis, probably because of defective clearance of immune complexes, contributing to inflammation[5, 6].

Defective clearance of immune complexes has not been much studied in pSS. The few studies that are available were based on small samples, and showed inconsistent results concerning phagocytosis in pSS patients[7-9]. A preliminary report showed genotyping results for the low-affinity SNPs *FCGR2A* rs1801274 and *FCGR3A* rs396991, in 228 pSS patients and 206 controls[10]. No associations were found in patients versus controls, or related to patient anti-SSA and/or anti-SSB status. Regarding FcγRIIIB, the HNA-1a and HNA-1b receptor forms differ by four amino acids and the corresponding encoding *FCGR3B* gene by five nucleotides, leading to differences in phagocytic activity[11, 12]. Polymerase chain reaction (PCR) analyses of rs447536 and rs448740 have shown good accordance with serotype[13, 14]. Regarding *FCGR3B* copy number, pSS was associated with deletion in a recent study on 174 pSS patients and 162 controls[15], and both deletion and duplication were associated with the disease in a study with a small pSS cohort[16]. The aim of the present study was to explore whether any

of the mentioned FcγR gene SNPs were associated with pSS in a larger case-control association study, and to possibly replicate previous findings concerning *FCGR3B* copy number variation (CNV).

Results

The studied SNPs and gentyping results are shown in Table 1. Genotyping success rates were 90% - 96% for the studied SNPs. None of the SNPs showed any association with pSS at the allelic or at the genotypic level assuming an additive genetic model and correcting for age, gender and nationality. In addition, anti-SSA and/or anti-SSB status among patients showed no association with any SNP. Also, no *FCGR3B* CNV association was detected (Figure 1). In a previous study, we obtained HNA-1a/HNA-1b typing on 282 individuals using an allele specific amplification as described by Bux et al.[13] (data not shown). In the present study, we genotyped rs447536 and rs448740, which discriminate more efficiently between the HNA-1a/HNA-1b and other allelotypes. Indeed, by comparing rs447536 and rs448740 with the previous HNA-1a/HNA-1b obtained by PCR, more than 20% of the individuals showed other allelotypes. Therefore, we chose to keep the rs447536 and rs448740 genotypes rather than the HNA-1a/HNA-1b classical description.

Discussion

In this case-control study, the genotyped FcγR gene polymorphisms showed no association with pSS. To our knowledge, the sample sizes in the present study are larger than those in other reports on rs1801274 and rs396991[10]. We did not replicate the findings of an association with *FCGR3B* CNV previously reported in two studies[15, 16]. The lack of association with pSS versus control status indicates that defective immune complex clearance may not be as important in pSS pathogenesis as in SLE. These two diseases may be looked upon, not as discrete entities, but as partially overlapping phenotypes, with a gradual transition from one to the other, or with both diseases present simultaneously (SLE-SS overlap)[17]. The FcγR variants studied here may be among the genetic factors determining such a transition. Our findings therefore may point to crucial differences between SLE and pSS pathogenesis, and may give directions for future research.

The lack of association regarding *FCGR3B* CNV differs from findings in two other studies in pSS[15, 16]. One possible explanation might be the different methods used to analyse CNV, and

the different stringency for the inclusion of data (we only accepted SD<0.15 between replicates while the study by Nossent et al. used SD<0.2). Moreover, the control group used in the study by Nossent et al. was not gender-matched. However, more analyses are needed in different cohorts to ascertain or disprove any true relationship. Other mechanisms for a possible reduced immune complex clearance in pSS should be studied, especially the role of complement factors and receptors[18], and possible roles of genetic variants in these. Also, replication studies should be performed with a large sample size for all SNPs studied, and could preferentially include other potentially interacting variants. FcγRIIB is an inhibitory Fc receptor important in balancing immune activation, and polymorphisms both in the promoter and in the exon of this receptor's gene have been implicated in SLE[19]. In addition, CNV is found to affect both the *FCGR3A* and *FCGR3B* genes[20], and CNV can falsely skew the SNP genotyping results, blurring any true association of the studied SNPs[21].

The FcγR IIA-H131R polymorphism, rs1801274, confers reduced IgG2 binding by the R131 variant[22]. In a meta-analysis, homozygotes were found to have an increased SLE risk (odds ratio of 1.3), and the population-attributable fraction of SLE cases due to FcγR IIA-R131 variant was estimated to be 13%[5]. In FcγR IIIA, the V158F polymorphism (rs396991) causes reduced binding of most IgG subclasses by the F158 variant[22]. This low-affinity allele is associated with SLE nephritis; the proportion of nephritis cases in SLE that could be attributed to the F158 allele has been estimated to be 10-14%[5]. Low copy number of *FCGR3B* correlates with reduced receptor expression and immune complex uptake, and an association has been found in SLE as well as in other diseases[6,23].

Using TaqMan probes with specificity for rs447536 and rs448740, corresponding to a PCR-based method that reportedly showed good agreement with serological HNA-1a/HNA-1b assessment[13], a relatively high frequency of samples could not be assigned to any of the two *FCGR3B* forms. A possible reason could be inaccuracies of the TaqMan assay, and/or the original PCR method, which showed 100% and 15% accuracy with two different serological phenotyping methods[13]. Another possibility is inherent characteristics in the gene region. According to the literature, the number of SNPs and haplotypes in this area does not seem to have been clarified. Also, sequencing results have shown a confusing picture[24], all of which can affect genotyping efficiency of these regions.

To conclude, FcγR IIA, IIIA and IIIB SNPs and FcγR IIIB CNV were not associated with pSS. The lack of association indicates that defective immune complex clearance may not be as important in pSS pathogenesis as in SLE, indicating differences between SLE and pSS pathogenesis.

Patients and methods

Study populations

For the rs1801274 genotyping, a total of 581 patients with pSS and 595 controls were included. After genotype quality control where individuals with a genotype success rate of less than 90% were removed, 527 cases and 528 controls remained for analysis. The patients were selected from the rheumatology clinics at the Stockholm (n=59), Malmö (n=149), Linköping (n=62) and Uppsala (n=63) University Hospitals in Sweden and the rheumatology clinics at Haukeland University Hospital (n=126) and Stavanger University Hospital (n=68) in Norway. For genotyping of rs396991, rs447536 and rs448740, 519 patient samples and 497 control samples were available. Data on gender, age, and autoantibody profile were collected (Table 2). The patients were unrelated and Caucasians. They fulfilled the American-European consensus criteria[25]. All patients gave their informed consent and the study was approved by the relevant ethical committees.

The controls were healthy blood donors from Uppsala and Linköping (n=114) in Sweden, and Bergen and Stavanger (n=219) in Norway. Controls from Malmö and Stockholm (n=262) were from established population based study cohorts. Controls from Stavanger (n=71) were volunteers recruited as healthy control subjects for participation in a controlled pSS study. *FCGR3B* CNV was analysed in a smaller pSS cohort of 141 patients from Haukeland University Hospital, Bergen, Norway, and 149 healthy blood donor controls.

Genotyping methods

Genotyping of rs1801274 was performed using the GoldenGate assay from Illumina Inc. (San Diego, CA, USA) as described previously[4]. For genotyping of rs396991, a commercially available TaqMan SNP allelic discrimination assay was used. Genotyping of rs447536 and rs448740 was performed using custom TaqMan SNP genotyping assays. TaqMan genotyping was performed using 20 ng of genomic DNA in a total reaction volume of 2 µl. The assays were amplified on an ABI 7900 HT (Applied Biosystems, Foster City, CA, USA), and analysed with the Sequence Detection Systems (SDS) Software v2.3, according to the

supplier's protocol. The clusters were visually examined for each assay and manually corrected if necessary. Gene copy number of *FCGR3B* was estimated by performing a duplex quantitative real time PCR using exon 3 of *PMP22* as a reference gene. PMP22 was verified not to have CNV by comparing PMP22 to exon 25 of *RB1*. Data from 10 patients and 10 controls were removed due to a standard deviation higher than 0.15 between triplicates, leaving 124 patients and 138 controls for further CNV analyses. For further details, see Supplementary method section.

Statistical analysis

An allelic logistic regression was used to evaluate the effect of pSS versus control status (where the affection status is the outcome predicted by the alleles). Unadjusted *p*-values, and odds ratios (OR) with 95% confidence interval (CI) were computed. To estimate any effect of a positive anti-SSA and/or anti-SSB, a corresponding logistic regression was performed with antibody status as the dependent variable. In addition, genotypes were encoded under an additive model: minor alleles "D" and major alleles "d", and DD = 0, Dd = 1 and dd = 2. The numeric genotypes were then used, including age, gender and nationality as covariates in a linear regression. SNP genotyping results were analysed using the Helix Tree SNP Variation Suite software (Golden Helix: http://www.goldenhelix.com). CNV data were analysed using the Mann–Whitney rank sum test with the GraphPad Prism v. 5.0 (GraphPad Software Inc., San Diego, CA, USA) and Fisher's exact test using PASW Statistics 18.0.

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Conflict of interest

The authors declare no conflicts of interests.

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Table 1. Linear regression of genotype effects adjusted for age, gender and nationality

Gene	SNP	Position	Genotype distribution 0/1/2 pSS patients	Controls	p
FCGR2A	rs1801274	161,478,745	104/259/164	120/262/146	0.15
FCGR3A	rs396991	161,514,542	47/216/225	38/207/241	0.62
FCGR3B	rs448740	161,518,336	72/216/190	64/187/205	0.22
	rs447536	161,599,773	62/201/208	49/210/209	0.12

The table shows the distribution of genotypes and *p*-values from a linear regression with pSS disease status as independent variable, and an additive model (using DD = 0, Dd = 1, dd = 2, with D minor allele and d major allele), age, gender and nationality as covariates. Minor allele frequencies in patients/controls were: rs1801274, 0.44/0.47 (A allele); rs396991, 0.32/0.29 (C allele); rs447536, 0.35/0.33 (C allele) and rs448740, 0.38/0.35 (A allele). Positions according to NCBI build GRCh37.

Table 2. Characterisation of the study cohorts in the rs1801274 analysis

Category	Norway	Sweden	Total sample	p value*
Subjects, patients	194	333	527	
Subjects, controls	212	316	528	
Women (%), patients	178 (91.8)	308 (92.5)	486 (92.2)	0.74
Women (%), controls	192 (90.6)	289 (91.5)	481 (91.1)	0.76
Age (+/- SD), patients	57.4 (+/-11.7)	58.1 (+/-14.0)	57.8 (+/-13.2)	0.37
Age (+/- SD), controls	49.3 (+/-13.6)	57.7 (+/-11.4)	54.2 (+/-13.1)	<0.001
ANA, %	144 (74.2)	255 (76.6)	399 (75.7)	0.60
Anti-Ro/SSA, %	132 (71.0)	239 (71.8)	371 (71.5)	0.84
Anti-La/SSB, %	81 (43.5)	152 (45.6)	233 (44.9)	0.71
Anti-Ro/SSA - La/SSB	138 (74.2)	242 (72.7)	380 (73.2)	0.76

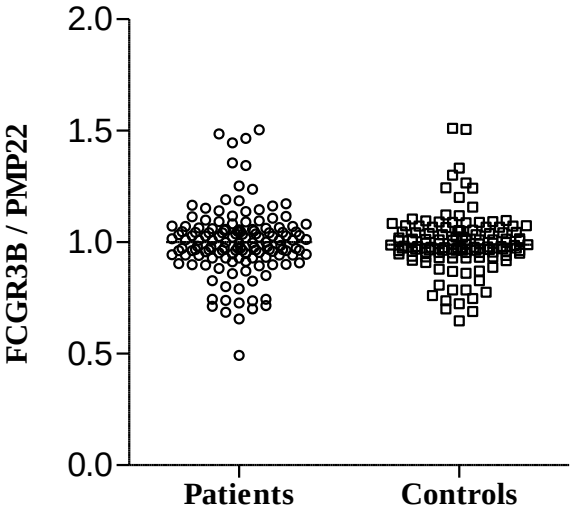
*Frequencies were compared between Norway and Sweden by Chi-square test, age differences were analysed by Mann-Whitney's test.

Figure 1 legend

A total of 141 patients and 149 healthy blood donor controls were included. After exclusion of patients with family relationships and non-Caucasian genetic background, and individuals with a high standard deviation between triplicates, results were obtained in 124 patients and 138 controls. Quantitative real-time PCR was used to determine *FCGR3B/PMP22* ratios. The median ratio (horizontal bar) was 0.99 (patients) and 1.00 (controls), P = 0.8989 (Mann-Whitney test). Using a cut-off ratio of 0.8 for deletion and 1.2 for duplication, copy numbers were similar in patients and controls (p = 1.0 (deletion) and p = 0.79 (duplication), Fisher's exact test).

Figure 1. *FCGR3B* CNV. A total of 141 patients and 149 healthy blood donor controls were included. After exclusion of patients with family relationships and non-Caucasian genetic background, and individuals with a high s.d. between triplicates, results were obtained in 124 patients and 138 controls. Quantitative real-time PCR was used to determine FCGR3B/PMP22 ratios. The median ratio (horisontal bar) was 0.99 (patients) and 1.00 (controls), P=0.90 (Mann–Whitney test). Using a cutoff ratio of 0.8 for deletion and 1.2 for duplication, copy numbers were similar in patients and controls (P=1.0 (deletion) and P=0.79 (duplication), Fisher's exact test).

Figure 1. Relative copy number of *FCGR3B*



Supplementary methods

rs1801274 genotyping:

Considering rs1801274 (Table 1), the SNP genotyping yielded legible results except for two missing control samples and one sample removed due to divergent results (see rs396991 below). Using the GoldenGate assay from Illumina Inc. (San Diego, CA, USA), the sample success rate was 98.8%, and the reproducibility of genotyping was 100% as estimated from a duplicate genotyping of 2.3% of the samples. Individuals with a genotype success rate of <90% were excluded.

rs396991, rs447536 and rs448740 genotyping:

The SNP rs396991 was analysed with TaqMan SNP genotyping assay C__25815666_10. A total of 269 samples were analysed in duplicate; in addition, two samples were sequenced. The sequenced samples were in accordance with the genotyping. In the duplicate run, one sample showed divergent results and was removed from the SNP analyses. The SNPs rs447536 and rs448740 were analysed with TaqMan custom-made assays, sequences of the genotyping probes can be made available upon request to the corresponding author. Two samples each for rs447536 and rs448740 were sequenced and were in accordance with the genotyping.

FCGR3B copy number analysis:

2µl genomic DNA (approximately 30 ng) was used as template for the quantitative duplex PCR in a total volume of 11 µl, with 5 µl 2 x TaqMan universal master mix (Applied Biosystems Inc, ABI), 0.08µl AmpliTaq Gold (ABI), 0.66 µM of each primer and 0.15 µM of each probe. The samples were run in triplicate on 384 well plates on ABI 7900HT real-time PCR system (ABI). Cycling conditions were 50ºC for 2 min, 95ºC for 10 min, and then 40 cycles of 95ºC for 15 sec followed by 60ºC for 1 min. ΔCT (comparative CT method[26]) was calculated between *FCGR3B* and *PMP22*. The results were adjusted so that the median of the control group was equal to one. Standard deviation between the remaining samples was 0.143. Primers and probes were as follows:

FCGR3B (Ex2):

Forward: 5’-AGAAGGACAGTGTGACTCTG-3’

Reverse: 5’-TCTCATTGTGAAACCACTGTG-3’

LNA probe: Hex-5’-TGC(+C)AG(+G)GA(+G)CC(+T)ACT)-3’-BHQ1

PMPM22 (Ex3):

Forward: 5’- GGGCAATGGACACGCAACT-3’

Reverse: 5’- TGATGAGAAACAGTGGTGGACA-3’

TaqMan probe: FAM-5’- CTGGCAGAACTGTAGCACCTCTTCC-3’-BHQ1

RB1 (Ex25)[27]:

Forward: 5’- CCAGAAAATAAATCAGATGGTATGTAACA-3’

Reverse: 5’- TGGTTTAGGAGGGTTGCTTCC-3´

TaqMan probe: FAM-5’- CAGCACTTCTTTTGAGCACACGGTCG-3’-BHQ1

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