Low Copy Number of the Fc-γ Receptor 3B Gene *FCGR3B* Is a Risk Factor for Primary Sjögren's Syndrome

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ABSTRACT. ***Objective.*** Immune complexes play an important role in the pathogenesis of primary Sjögren's syndrome (pSS). Crosslinking of the neutrophil-specific Fc-γ receptor 3b (FCGR3B) facilitates immune complex clearance, and copy number variation (CNV) of the *FCGR3B* gene is known to reduce the uptake, and potentially clearance, of circulating immune complexes. Our objective was to determine whether *FCGR3B* CNV is a risk factor for pSS.

Methods. This was a cross-sectional study of patients with established pSS (n = 174) and population-matched controls (n = 162). *FCGR3B* CNV was determined by a quantitative real-time polymerase chain reaction assay, using genomic DNA as template and Taqman chemistry. Reactions were performed as a duplex, with RNAse P as the reference gene. Clinical and serological data were analyzed for their association with *FCGR3B* copy number (CN).

Results. Low *FCGR3B* CN (< 2 copies) was a risk factor for pSS in this cohort (p = 0.016), and combined results from this and a previous study yielded an overall OR of 2.3 (95% CI 1.3, 3.9, p = 0.003). Among patients with pSS in our cohort, low *FCGR3B* CN was not associated with anti-Ro ± La autoantibodies, but was associated with lower rheumatoid factor titers (p = 0.001) and serum IgG levels (p = 0.031).

Conclusion. We confirmed that, similarly to other systemic autoimmune diseases, *FCGR3B* CN is a genetic susceptibility factor for pSS. As in rheumatoid arthritis, the mechanism does not appear to be related to seropositivity for characteristic autoantibodies. (J Rheumatol First Release Sept 1 2012; doi:10.3899/jrheum.120294)

Key Indexing Terms:
FCGR3B GENE COPY NUMBER SJÖGRENS' SYNDROME NEUTROPHILS

Primary Sjögren's syndrome (pSS) is a common systemic autoimmune inflammatory condition. The predominant feature is failure of exocrine glands, although extraglandular manifestations, involving skin, lung, heart, kidneys, and nervous system, are common[1,2]. Autoantibodies targeting the ribonuclear proteins, Ro and La, are common in pSS and present in up to 80% of patient sera samples[3]. Although previously thought to be nonpathogenic "epiphenomena," anti-Ro ± La are associated with systemic complications such as vasculitis, skin lesions, and neonatal heart block[4,5]. These autoantibodies are now thought to play a central role in the inflammatory response through immune complexes (IC) that are involved in cytokine release and complement activation[6,7].

There is clearly a genetic component to susceptibility for pSS, or specifically, autoantibody-positive pSS[8,9,10]. Copy number variation (CNV) of the *FCGR3B* gene is a candidate gene of particular interest. CNV is a departure from the normal diploid number of genes (n = 2) that may arise from gene duplication and deletion events, and may contribute substantially to quantitative variation in gene expression. An increasing number of CNV have been characterized in the human genome, with implications for both evolution and disease susceptibility[11]. CNV has been identified in multiple genes within the Fc-γ receptor (FCGR) gene cluster on chromosome 1q23[12]. This cluster carries 5 highly homologous genes that encode for low affinity receptors for IgG-complexed antigens, which are expressed widely throughout the hematopoietic system. These low affinity FCGR are involved in the regulation of a multitude of innate and adaptive immune responses, with implications for both response to infection and susceptibility to autoimmunity[13].

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FCGR3B is an activating glycoprotein found only on the surface of human neutrophils that preferentially binds IgG[14]. Cross-linking between FCGR3B and IC initiates an effector response resulting in the clearance of IC[15,16]. CNV of *FCGR3B* has been well characterized, and there is a clear correlation between gene copy number and FCGR3B cell-surface expression, neutrophil adherence to IgG-coated surfaces, and uptake of IC[17]. Multiple studies have confirmed that low (< 2) *FCGR3B* copy number (CN) is a genetic susceptibility factor for systemic autoimmune diseases such as systemic lupus erythematosus (SLE)[18,19,20] and rheumatoid arthritis (RA)[21,22]. Only 1 study has examined *FCGR3B* CN in pSS, reporting that, similar to their SLE cohort, both low (< 2) and high (> 2) *FCGR3B* CN was associated with pSS[19].

We examined the association between *FCGR3B* CN and pSS in another cohort of patients.

MATERIALS AND METHODS

Study participants. The study population consisted of 174 white patients with pSS (90% females, median age 58 yrs, 84% seropositive for anti-Ro ± La autoantibodies), who met the revised 2002 American–European consensus research classification criteria for pSS[23], and 162 white population-based controls (53% females, median age 56 yrs). Anti-Ro ± La autoantibody specificity and serum B cell activating factor (BAFF) levels were evaluated as described[8,9,24], and patients were also assessed by case note review.

The study was conducted in accord with the Declaration of Helsinki and approved by the Central North Adelaide Health Service Ethics of Human Research Committee. All participants provided informed, written consent.

FCGR3B CNV determination. Genomic DNA was prepared by the salt precipitation method from fresh blood samples, as described[10], and genomic FCGR3B CN was determined using a quantitative real-time polymerase chain reaction (qPCR) method[21]. Briefly, a duplex Taqman® CN assay was performed, using FCGR3B-specific primers (Applied Biosystems, Hs04211858, FAM-MGB dual labeled probe) and RNase P (Applied Biosystems, product 4403326, VIC-TAMRA dual labeled probe) as the reference assay. The assay was performed according to the manufacturer's instructions and PCR were run on an Applied Biosystems 7300 Real Time PCR machine. All samples were tested in triplicate, and fluorescence signals were normalized to ROX dye. CN was determined using Copy Caller software (v.1.0, Applied Biosystems), and results were accepted only when calling confidence for discrete CN assignment was > 80%, and the ΔCq SD between replicates was < 0.20; otherwise, samples were retested.

The Taqman qPCR *FCGR3B* CN assay was initially validated against an endpoint PCR paralog ratio assay, measuring *FCGR2C*/*FCGR2A* CN ratios[20], for which the signal intensity (peak height) for both amplicons (274 and 279 bp) was quantified through a multiplexed fluorescence capillary electrophoresis detection system (Qiaxcel). The rationale for this validation is that several studies have reported complete agreement (i.e., linkage disequilibrium) between FCGR2C and FCGR3B CN[12,20]. Three reference samples (1,2,3 CN) validated by this assay were included on each qPCR assay plate to control for possible batch effects.

Statistical analysis. The association between *FCGR3B* CN and disease susceptibility, and other dichotomous variables, was analyzed by chi-square and logistic regression analysis, with results reported as OR. Testing for ordinal relationships between *FCGR3B* CN and other variables was performed using the nonparametric gamma correlation coefficient, and proportional OR as appropriate. All analyses were performed using Statistica v6 (Statsoft).

A random effects metaanalysis of this study and a previous publication, examining the relationship between low CN of FCGR3B and pSS, was performed using the R metafor library[25,26] and the restricted maximum likelihood method.

P values < 0.05 were considered to indicate statistical significance.

RESULTS

The number of genomic *FCGR3B* copies observed in our study varied from 0 to 4; however, 1–3 copies were the most common. One pSS patient carried no *FCGR3B* (null), and 1 patient and 1 control each carried 4 *FCGR3B* copies. Accordingly, the data were grouped for analysis into < 2, 2, and > 2 copies, respectively.

The distribution of *FCGR3B* CN variants was significantly different between pSS patients and controls (chi-square = 6.10, df = 2, p = 0.047; Table 1). Expressed as OR relative to the normal diploid 2 CN, low (< 2) CN was significantly increased in pSS (OR 2.6, 95% CI 1.2, 5.6, p = 0.016), whereas there was no difference in the frequencies of high (> 2) CN between pSS patients and controls (OR 1.1, 95% CI 0.5, 2.4, p = 0.80). Although the sample sizes in this study were comparatively small, a retrospective power analysis indicated that there was sufficient power (79.6% 1-sided test, 69.5% 2-sided test) to detect a difference in low *FCGR3B* CN frequency between patients and controls.

One previous study evaluated *FCGR3B* CN in pSS[19]. In a metaanalytic approach, the combined results from both studies (Figure 1) yielded OR of 2.3 (95% CI 1.3, 3.9, p = 0.003) for low (< 2) CN, and 1.6 (95% CI 0.8, 3.1, p = 0.20) for high (> 2) CN, both expressed relative to the normal diploid 2 CN. Therefore, the association between low *FCGR3B* CN is confirmed in 2 separate studies, but the additional putative association with high *FCGR3B* CN[19] remains unconfirmed. Our observation that high (> 2) *FCGR3B* CN was more frequent in patients with seronegative pSS (Table 1) may be relevant in this context, although the number of seronegative patients was very small.

Given that FCGR play an important role in the clearance of IC, it is relevant to consider whether low *FCGR3B* CN, as a risk factor for pSS, is specifically associated with seropositivity for Ro ± La autoantibodies within patients with pSS. The distribution of *FCGR3B* CN in Ro ± La-seronegative versus seropositive patients with pSS is also shown in Table 1. Although the number of patients with seronegative pSS in this study was limited, the frequency distribution was different between the 2 groups (p = 0.042), which was primarily attributable to a higher proportion of high (> 2) CN in patients with seronegative pSS. The relevance of this observation is uncertain, given the low numbers of patients with seronegative pSS. There was, however, no suggestion that low *FCGR3B* CN is preferentially associated with Ro ± La-seropositive pSS.

In terms of other clinical/serological features, low *FCGR3B* CN was associated with lower rheumatoid factor (RF) titers and lower serum IgG levels (Table 2, Figure 2). There was also a trend for an inverse ordinal relationship

Table 1. Low *FCGR3B* genomic copy numbers (< 2 CN) is associated with primary Sjögren's syndrome (pSS), but not specifically Ro + La autoantibody (seropositive) pSS. The distribution fo *FCGR3B* CN was significantly different between pSS patients and controls (p = 0.047). When expressed as OR relative to the normal diploid 2 CN, low (< 2) CN was significantly increased in pSS (p = 0.016), wheras there was no difference in the frequencies of high (> 2) CN between pSS patients and controls (p = 0.80). The frequency distribution was also different twhen comparing seropositive versus seronegative pSS patients (p = 0.042), which was primarily attributable to a higher proportion of high (> 2) CN in seronegative patients with pSS. Thee was, however, no suggestion that low (< 2) CN is preferentially associated with Ro + La seropositive pSS.

	pSS (n = 174) vs Controls (n = 162)				Seropositive pSS (n = 146) vs Seronegative pSS (n = 27)			
FCGR3B CN	pSS (%)	Controls (%)	OR (95% CI)	p	Seropositive pSS (%)	Seronegative pSS (%)	OR (95% CI)	p
< 2	25 (14.4)	10 (6.2)	2.6 (1.2, 5.6)	0.016	18 (12.3)	6 (22.2)	0.4 (0.1, 1.2)	0.09
2	135 (77.6)	139 (85.8)	1		119 (81.5)	16 (59.3)	1	
> 2	14 (8.0)	13 (8.0)	1.1 (0.5, 2.4)	0.8	9 (6.2)	5 (18.5)	0.2 (0.1, 0.8)	0.022
Global test	Chi-square = 6.10, df = 2, p = 0.047				Chi-square = 6.34, df = 2, p = 0.042			

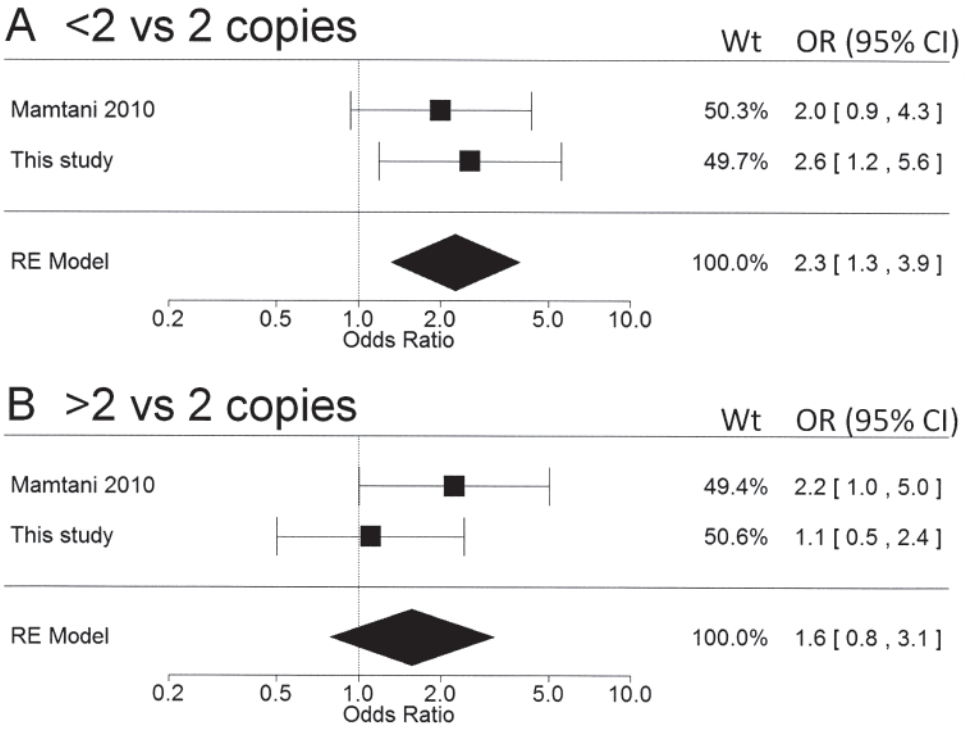

Figure 1. Random effects (RE) metaanalysis of the association between *FCGR3B* copy number variations and primary Sjögren's syndrome. The 2 studies available were Mamtani 2010[19] and the current study. (A) < 2 versus 2 *FCGR3B* copies; p = 0.003. (B) > 2 versus 2 *FCGR3B* copies; p = 0.20. RE model indicates the OR (95% CI), estimated by restricted maximum likelihood, for the 2 studies combined.

between *FCGR3B* CN and the proportion of patients with a history of persistent, presumed autoimmune neutropenia, although the number of such patients was low (26/254 = 17%) and this analysis did not achieve statistical significance (p = 0.12; Table 2, Figure 2). There was no observed relationship with age of onset, sex, Schirmer's test results, sialometry results, or C4 or serum BAFF levels (data not shown).

DISCUSSION

We have confirmed that low *FCGR3B* CN is a genetic susceptibility factor for pSS. Our results for the association with low *FCGR3B* CN were very similar to the single previous study of patients with pSS[19], and collectively, the OR for the association with pSS (relative to the normal diploid 2 CN) was estimated as 2.6 (95% CI 1.2, 5.6, p = 0.003). Therefore low *FCGR3B* CN may be a common genetic risk

Table 2. Within patients with primary Sjögren's syndrome there is a positive ordinal relationship between *FCGR3B* copy number and rheumatoid factor (RF) titer and serum IgG levels, and an inverse ordinal trend with the risk of neutropenia.

Variable	N	Ordinal Test	Coefficient	p
RF titer	140	Gamma correlation	0.33	0.001
Serum IgG	138	Gamma correlation	0.20	0.031
Neutropenia (proportion)	26/154 (17%)	Proportional OR	0.46 (0.18, 1.21)	0.12

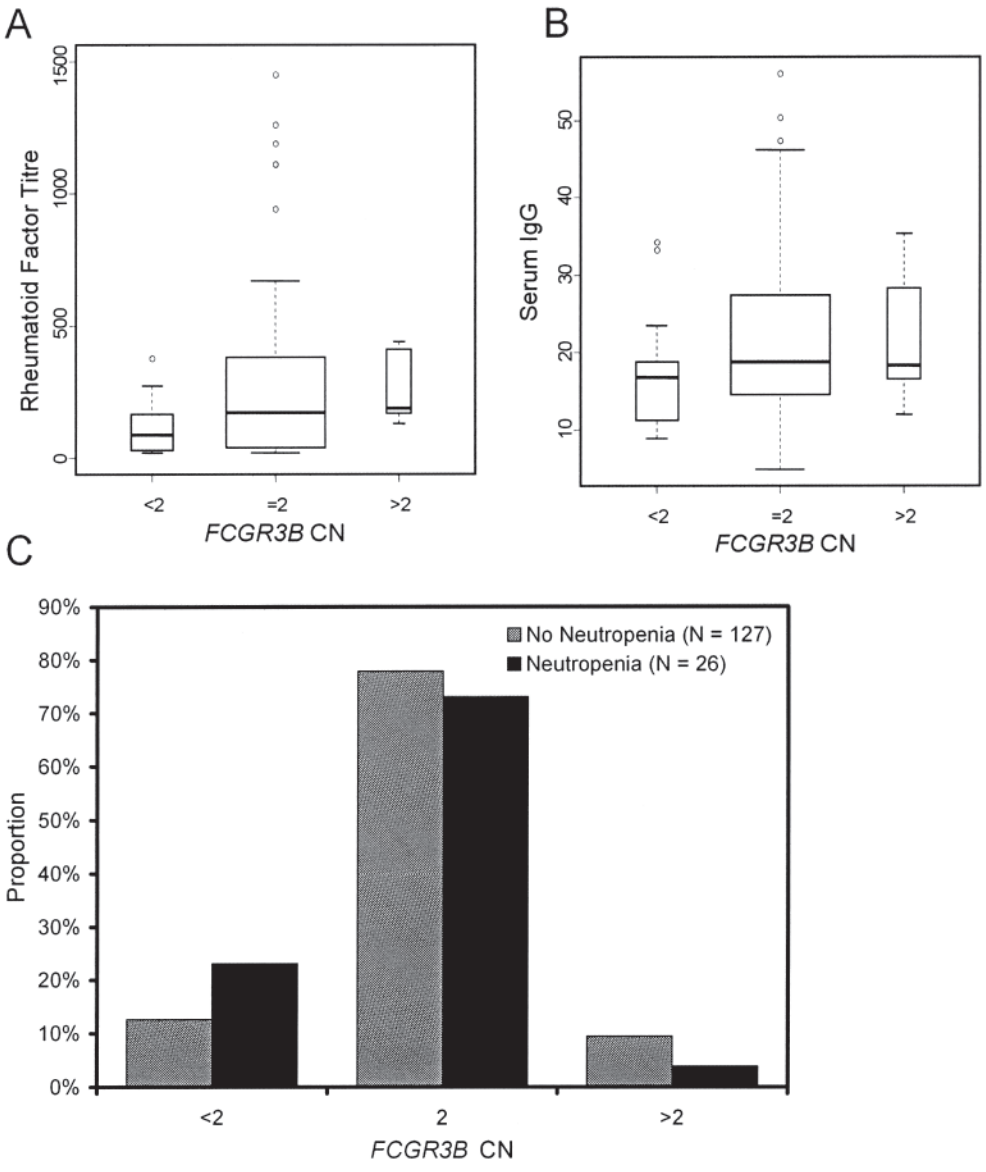

Figure 2. Box plots of *FCGR3B* copy number (CN) by (A) rheumatoid factor titer (n = 140) and (B) serum IgG (n = 138). The width of the box plots reflects the number of observations. It is evident that the ordinal trends reported in Table 3 are primarily due to low (< 2) *FCGR3B* CN, which is associated with lower RF titers and lower serum IgG. (C) The prevalence of low *FCGR3B* CN is slightly higher in patients with a history of neutropenia, and the prevalence of high *FCGR3B* CN slightly lower, indicative of an inverse ordinal trend.

factor for systemic autoimmune diseases such as SLE[18,19,20], RA[21,22], and pSS. However, it is also clear that a relatively low proportion (< 20%) of patients with systemic autoimmune diseases appear to carry this genetic risk factor. This may be indicative of underlying disease heterogeneity, in which low *FCGR3B* CN is a risk factor in an as-yet unidentified subgroup of patients.

The exposure of autoantigens in pSS is thought to occur during apoptosis of salivary gland acinar and ductal epithelial cells, which is accompanied by translocation of autoantigens to apoptotic blebs. This local event may initiate an autoimmune reaction that becomes systemic through upregulation of type I interferon signature and abnormal expression of BAFF[7], and lymphocytic invasion and/or IC-mediated inflammation in other organ systems may result. Circulating IC can be demonstrated in a majority of patients with pSS, and anti-Ro-containing IC are related to continuing systemic immune activation[27,28,29]. As FCGR form an important pathway for IC clearance[15,16,17], it was therefore surprising that we were unable to demonstrate a relation between low *FCGR3B* CN and anti Ro ± La autoantibodies in pSS. In fact we observed that low *FCGR3B* CN was associated with lower RF titers and serum IgG levels in patients with pSS. We previously demonstrated that diversification of the Ro ± La autoantibody response in patients with pSS is under genetic control, and correlates strongly with RF titers and serum IgG levels[5,10]. While our findings in relation to low *FCGR3B* CN are only exploratory, and require replication in future studies, collectively, these results suggest that the autoimmune response may be somewhat attenuated in patients who carried this risk factor. In keeping with our findings, no study to date has been able to demonstrate a relationship between low *FCGR3B* CN and disease-specific autoantibodies in systemic autoimmunity. Therefore, the evidence to date suggests that the pathogenetic mechanisms underpinning the relationship between low *FCGR3B* CN and systemic autoimmunity do not specifically relate to clearance of autoantigen-containing IC. One possibility is that, as FCGR3B is an important defense mechanism for the clearance of microorganisms, low *FCGR3B* CN may delay the resolution of infections, such as Epstein-Barr virus (EBV), which may act as a trigger for systemic autoimmunity in some patients[30]. In this context, it is perhaps relevant that the single *FCGR3B*-null, seronegative patient with pSS in this study had a history of recurrent EBV infections, and our observation that low *FCGR3B* CN is associated with lower IgG levels is also consistent with this hypothesis.

Other pathogenetic mechanisms may also be relevant, but unfortunately the role of neutrophils and FCGR3B in pSS has not been studied extensively. The expression of FCGR3B is upregulated in the saliva of patients with pSS, suggestive of neutrophil activation in salivary glands[31]. Neutropenia may be a relevant hematological finding in pSS[32], and our data were suggestive, but not conclusive, that low *FCGR3B* CN may be a risk factor for neutropenia in patients with pSS. Clinically relevant autoantibodies targeting the human neutrophil FCGR have been described in pSS[33,34], and soluble FCGR3B may act as a ligand for complement receptor activation[35]. However, factors that influence the relative expression of sFCGR3B versus membrane-bound FCGR3B, in both health and disease, are not understood.

It is also not clear whether the association with systemic autoimmunity can be specifically, or solely, attributable to *FCGR3B* CN. The FCGR gene cluster is a complex genomic region, carrying multiple genes, and is characterized by both single-nucleotide polymorphism (SNP) and CNV polymorphism and linkage disequilibrium. For example, *FCGR3B* and *FCGR2C* CNV appear to be in complete linkage disequilibrium[12,20], and *FCGR2C*, although a pseudogene in some individuals, may be expressed in a wider range of cell types, including dendritic and natural killer (NK) cells[12]. Recent evidence also suggests that the *FCGR2C-FCGR3B* insertion/deletion extends into *FCGR2B*, the only inhibitory FCGR, and probably deletes a negative regulatory element in the *FCGR2B* promoter in NK cells[36]. Studies have also implicated SNP in multiple FCGR genes with susceptibility to systemic autoimmunity[16], and 1 study has demonstrated that both *FCGR3B* CNV and SNP polymorphism contribute to SLE susceptibility[37]. The future challenge will be to integrate CNV and SNP data into FCGR gene-cluster haplotypes to systematically evaluate and contrast disease associations.

Our results confirm that, similar to other systemic autoimmune diseases, low *FCGR3B* CN is a genetic risk factor for pSS. However, the underlying pathogenetic mechanism does not appear to relate specifically to clearance of immune complexes formed by anti-Ro ± La autoantibodies. Further studies on immune complex clearance in systemic autoimmunity, with specific respect to the role of neutrophils, are warranted, in addition to careful clinical characterization of patients who carry this genetic risk.

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