Copy Number Variation of *FCGR3B* Is Associated with Susceptibility to Idiopathic Pulmonary Fibrosis

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Key Words

Copy number variation · Fc receptors · Gene dosage · Idiopathic pulmonary fibrosis · Neutrophils

Abstract

Background: Several genes exhibit copy number variation (CNV), including *FCGR3B* which encodes the IgG receptor FcγRIIIb. Engagement of Fcγ receptors by IgG complexes may contribute to the pathogenesis of idiopathic pulmonary fibrosis (IPF). **Objectives:** To investigate whether *FCGR3B* CNV is associated with susceptibility to IPF. **Methods:** In a case-control study we compared *FCGR3B* copy number in 142 patients with IPF and in 221 controls by real-time quantitative PCR using *CD36* as gene copy control. **Results:** Significantly increased *FCGR3B:CD36* ratio was evident in the IPF cohort compared to controls ($p = 0.009$). Association of *FCGR3B* copy number with IPF susceptibility was further confirmed by a likelihood ratio statistical approach ($p = 0.003$). *FCGR3B* copy number assignment based on *FCGR3B:CD36* ratios revealed significant skewing in the distribution of *FCGR3B* copy number between IPF patients and controls. In

the IPF cohort, there was increased frequency of >2 *FCGR3B* copies compared to controls (0.30 vs. 0.19; $\chi^2 = 9.27$; d.f. 2; $p = 0.0097$). The presence of >2 *FCGR3B* copies was associated with higher risk of IPF ($p = 0.01$, OR: 1.914, 95% CI: 1.17–3.12). **Conclusions:** These findings support an association of *FCGR3B* copy number with susceptibility to IPF and propose a novel role for Fcγ receptors in IPF disease pathogenesis.

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Introduction

Recent studies on the characterisation of copy number variation (CNV) loci in the human genome revealed that over 12% of the human genome is covered by CNV. CNV thus accounts for a great proportion of genetic diversity between individuals, which might be significantly higher than that attributed to single nucleotide polymorphisms (SNPs) [1]. Indeed, in the database of genomic variants there are currently over 15,000 CNV loci, many of which comprise genes and gene regulatory elements with key roles in several aspects of human physiology [2]. There-

fore, CNV might constitute a significant genetic risk factor for a number of diseases, especially those sensitive to gene dosage due to alterations in their expression.

FCGR3B encodes the low-affinity Fc γ receptor, Fc γ -RIIIb (CD16b), which is exclusively expressed by human neutrophils and recognises IgG-antigen complexes, inducing phagocytosis, cytokine production and generation of reactive oxygen intermediates [3, 4]. *FCGR3B* gene copy number has been shown to be correlated with surface Fc γ RIIIb expression as well as leukocyte functional responses, including adhesion to IgG and uptake of IgG-opsonised particles [5]. Association of *FCGR3B* CNV with systemic lupus erythematosus and systemic vasculitis has been recently reported [5, 6].

Idiopathic pulmonary fibrosis (IPF) is a devastating, non-neoplastic lung disease that carries a poor prognosis and for which no effective treatment is available. IPF involves the gradual loss of lung architecture due to a dys-regulated wound healing response that leads to the excessive deposition of fibrotic tissue within the pulmonary interstitial space, with clear implications for gas transfer [7]. Despite the unknown aetiology of IPF, a significant genetic component that confers disease susceptibility has been described, mainly due to the existence of familial clustering of IPF, even in individuals who were raised in different environments [8]. Association of IPF with a number of SNPs has been reported, mainly in genes involved in pro-inflammatory and pro-fibrotic pathways, including IL-1 β , TNF- α and TGF- β , highlighting the role of inflammatory processes in disease pathogenesis [9].

Although the precise pathogenic mechanisms for IPF are still unclear, several lines of evidence support a link between immune complexes (IgG-antigen) and disease pathogenesis. Indeed, elevated levels of immune complexes have been reported in the blood and the lungs of patients with IPF [10–16]. It is therefore anticipated that pro-inflammatory interactions between immune complexes and leukocyte Fc γ receptors, like Fc γ RIIIb, would constitute an additional determinant for disease pathogenesis. For this reason we have investigated whether *FCGR3B* CNV is associated with IPF susceptibility.

Materials and Methods

Subjects

All subjects were Caucasians and provided written informed consent. Ethical approval was obtained from the Lothian Research Ethics Committee (LREC/2002/4/65). IPF (n = 142) was diagnosed according to the American Thoracic Society/European

Table 1. Characteristics and baseline pulmonary function of IPF patients

Patients, n	142	
Age, years (range)	70 $\pm$ 8.8 (50–87)	
Female/male, n (%)	47/95 (33.1/66.9)	
	Absolute value	% predicted
FEV ₁ , l	2.16 $\pm$ 0.6	87.52 $\pm$ 20.0
FVC, l	2.76 $\pm$ 0.8	87.65 $\pm$ 19.6
FEV ₁ /FVC, % predicted		79.07 $\pm$ 9.9
TLC, l	4.30 $\pm$ 1.0	74.20 $\pm$ 15.3
DL _{CO} , ml/min/mm Hg	4.11 $\pm$ 1.4	52.75 $\pm$ 15.9
K _{CO} , ml/min/mm Hg/l	1.10 $\pm$ 0.3	82.47 $\pm$ 22.8

Values as mean $\pm$ SD. FEV₁ = Forced expiratory volume in 1 s; FVC = forced vital capacity; TLC = total lung capacity; DL_{CO} = diffusing capacity of the lung for carbon monoxide; K_{CO} = DL_{CO} corrected for lung volume.

Respiratory Society international multidisciplinary consensus classification [7], based on the following criteria: (1) exclusion of all known causes or associations with lung fibrosis, including drug toxicities, connective tissue disease or exposure to environmental agents; (2) presence of typical features on high-resolution CT scans, including bibasilar lung honeycombing with minimal ground glass opacities; (3) abnormal pulmonary function with evidence of restriction (reduced FVC) and/or reduced gas transfer measurements [decreased DL_{CO} (diffusing capacity for carbon monoxide)]; (4) age >50 years, and (5) duration of illness >3 months. Baseline characteristics are presented in table 1. Surgical lung biopsy and/or bronchoalveolar lavage (BAL) were performed in cases for which a confident diagnosis on clinical, functional and radiological grounds was not possible. When BAL or trans-bronchial lung biopsy was performed, no features were evident to support alternative diagnosis, revealing a histological profile typical of usual interstitial pneumonia. A consensus diagnosis was made in each case following joint review by 2 respiratory clinicians and a radiologist (and a pathologist for cases in which biopsy was performed). Pulmonary function measurements were recorded at baseline (first radiological evidence for IPF) and at 6 and 12 months ($\pm$ 1 month) following diagnosis to assess disease progression in 121 patients with IPF. The remaining 21 patients were lost to follow-up or were unfit to perform serial testing.

The control group (n = 221) comprised randomly selected age-matched patients (n = 70; mean age 71.4 $\pm$ 10.2) with a number of different lung pathologies without any evidence or history of lung fibrosis that were admitted to the respiratory unit of the Edinburgh Royal Infirmary and healthy blood donors (n = 151).

Quantification of *FCGR3B* Copy Number

Genomic DNA was extracted from peripheral venous blood using a QIAamp® DNA Blood Midi Kit (Qiagen). *FCGR3B* gene copy number was measured by quantitative real-time PCR (qPCR), based on previously described protocols [5, 6]. PCR am-

Fig. 1. Validation of the qPCR-based method for *FCGR3B* copy number quantification. Array comparative genome hybridisation data of the Whole Genome TilePath [1] project (Sanger Institute) were downloaded from www.sanger.ac.uk/humgen/cnv/data/. Plotting of the log intensity ratios of the probe encompassing the *FCGR3B* locus (8H4) from the 270 HapMap individuals revealed distinct clusters corresponding to different copy numbers (0, 1, 2, 3, >3). DNA samples from 10 HapMap individuals (obtained from

the Coriell Institute) were used as templates in our qPCR-based *FCGR3B* quantification method and results compared with the log₂ intensity ratio of the aCGH-based method (**a**). Significant correlation in the signals between the 2 methods was evident ($r^2 = 0.982$, $p < 0.0001$). **b** Comparison of the *FCGR3B*:*CD36* ratios of the HapMap (Coriell/HapMap) individuals with the subjects from this study revealed that both populations are clustered in the same pattern.

plification reactions (25 μ l; 2.5 ng genomic DNA) were performed using QuantiFast[®] SYBR Green PCR Kit (Qiagen), according to manufacturer's recommendations. The following primer pairs were used. For *FCGR3B*: forward 5'-CACCTTGAATCTCATCCCCAGGGTCTTG, reverse 5'-CCATCTCTGTACCTGCAG; for *CD36* (used as a single copy control): forward 5'-TAAGTTCAGGTTCTCTGGAATGC, reverse 5'-CAAATTATGTATGGACTGTGC. Melting curve analysis of the PCR products was performed to verify their specificity and identity. Standard curves were generated by serial 2-fold dilution of a single genomic DNA sample over the range of 25 to 0.78 ng per reaction. Samples and standard curve reactions were performed in quadruplicates on an Applied Biosystems 7500 Fast real-time cyler and data were collected and analysed using the Sequence Detection System software (v1.4; Applied Biosystems). Based on the standard curve analysis, Ct values from each reaction were expressed as amount of DNA (ng). The mean amount of DNA for each sample was calculated from quadruplicate reactions and the ratio of *FCGR3B*- to *CD36*-specific amplification was used to determine *FCGR3B* gene copy number for each sample. For the validation of our qPCR-based method, we obtained array comparative genome hybridisation (aCGH) data (available at www.sanger.ac.uk/humgen/cnv/data/) of the Whole Genome TilePath (WGTP) project (Sanger Institute) [1]. Plotting of the log intensity ratios of the

probe encompassing the *FCGR3B* locus (8H4) from the 270 HapMap individuals revealed distinct clusters corresponding to different copy numbers (0, 1, 2, 3, >3). DNA samples from 10 HapMap individuals (obtained from the Coriell Institute) were used as a template in our qPCR-based *FCGR3B* quantification method and results were compared with the aCGH-based method (fig. 1).

Flow Cytometry

Neutrophil granulocytes were isolated by dextran sedimentation and discontinuous Percoll gradient centrifugation [17] from citrated peripheral venous blood drawn from subjects previously typed as <2, 2, and >2 *FCGR3B* copies. Neutrophils were immunolabelled, as previously described [17], using purified mouse monoclonal antibodies (10 μ g ml⁻¹) against either human Fc γ RIIIb (3G8, mIgG1) or human Fc γ RIIa (IV.3, mIgG2b), followed by Alexa Fluor 488-conjugated goat anti-mouse F(ab')₂ (Invitrogen). Surface expression of Fc γ RIIIb or Fc γ RIIa was assessed by flow cytometry using a BD FACScan flow cytometer (BD Biosciences) and data were analyzed using FlowJo (Treestar) software.

Statistical Analysis

Association of Fc γ RIIIb copy number and IPF susceptibility was assessed by 2 main strategies: (1) direct comparison of *FCGR3B*:*CD36* ratio values between the 2 cohorts by Mann-Whit-

Fig. 2. Association of IPF with increased *FCGR3B* copy number. *FCGR3B* copy number was determined by qPCR in control subjects ($n = 221$) and IPF patients ($n = 142$). *CD36* was used as a single copy control and the *FCGR3B:CD36* ratio was used to determine *FCGR3B* copy number. **a** Comparison of *FCGR3B:CD36* ratio in control and IPF cohorts revealed that IPF was characterised with significantly increased ratios. * $p = 0.009$ (Mann-Whitney t test). Note: due to the diploid nature of the human genome,

an *FCGR3B:CD36* ratio of 1 indicates 2 genomic copies. **b** *FCGR3B* copy number classification using raw *FCGR3B:CD36* ratios was performed using a likelihood ratio statistical test. Significant skewing in the distribution of *FCGR3B* copy number was observed between IPF patients and control subjects and increased frequency of >2 *FCGR3B* copies was noted in the IPF cohort (0.30 vs. 0.19; $\chi^2 = 9.27$; d.f. 2; $p = 0.0097$).

ney non-parametric testing, and (2) assignment of copy number based on *FCGR3B:CD36* ratios. To avoid biases in copy number classification based on a priori binning of ratio values to predefined thresholds, we employed a robust, likelihood ratio statistical test that integrates copy number classification and case-control association testing [18]. The fitting code was obtained from <http://cnv-tools.sourceforge.net/> and copy number assignment of the *FCGR3B:CD36* ratios was performed with 2 fitting models: principal components analysis and linear discriminant function, as previously described [18]. Association between copy numbers and the case-control status was tested based on the likelihood ratio approach, using the Gaussian or T mixture model and the results were distributed as χ^2 with 1 degree of freedom. Alternatively, copy number classification assignment (obtained from the principal components analysis and linear discriminant function fitting models) was used for analysis and differences between control and IPF groups were analysed by a 3×2 contingency table (χ^2 test with 2 degrees of freedom) or by Fisher's exact test (>2 vs. $<2 + 2$). One-way analysis of variance (ANOVA) was used to test for differences in the mean values of quantitative variables, and where statistically significant effects were found, post-hoc analysis using Bonferroni t test was performed. Unless otherwise stated, quantitative data are presented as mean $\pm$ SD and $p < 0.05$ was considered to be statistically significant. Data were analysed with GraphPad Prism software (Graphpad).

Results

Copy Number Variation of FCGR3B Is Associated with Susceptibility to IPF

Using a well-validated qPCR method, we determined *FCGR3B* copy number in patients diagnosed with IPF and control subjects. *FCGR3B* was normalised to the *CD36* gene, which exhibits no copy variation. Direct comparison of the *FCGR3B:CD36* ratio revealed significantly higher ratios in the IPF cohort compared to controls ($p = 0.009$; fig. 2a). In addition, we employed a recently described statistical method for the analysis of disease association with gene copy number, which is based on the likelihood ratio approach and integrates assignment of samples to copy number based on *FCGR3B:CD36* ratios and case-control association testing [18]. Again, we confirmed that *FCGR3B* copy number was associated with IPF susceptibility ($\chi^2 = 8.76$; d.f. 1; $p = 0.003$).

Furthermore, using this likelihood-based method we obtained the assigned *FCGR3B* copy number for each

Fig. 3. Effect of *FCGR3B* gene copy variation on FcγRIIIb surface expression. Neutrophils were isolated from peripheral blood of donors previously typed as <2 (red), 2 (green), and >2 (blue) *FCGR3B* copies and FcγRIIIb expression was assessed by flow cytometry. As control, the expression of CD32 (FcγRIIa) was mea-

sured. Panels show representative flow cytometry histogram overlays of FcγRIIIb (a) and FcγRIIa (b) expression from donors (at least 4 per group) with <2 (red), 2 (green) and >2 (blue) *FCGR3B* copies.

subject and performed additional statistical analyses to confirm the observed association with IPF. Significant skewing in the distribution of the *FCGR3B* copy numbers was observed between patients with IPF and control subjects (fig. 2b). In the IPF cohort, increased frequency of subjects with >2 copies was evident compared to controls (0.30 vs. 0.19; $\chi^2 = 9.27$; d.f. 2; $p = 0.0097$). Similarly, the presence of >2 *FCGR3B* copies was strongly associated with IPF ($p = 0.01$; OR: 1.914, 95% CI: 1.17–3.12). In summary, these findings support an association between *FCGR3B* copy number and susceptibility to IPF, thereby providing evidence on the role of FcγRIIIb in disease pathogenesis.

FCGR3B Copy Number Variation Is Correlated with FcγRIIIb Surface Expression Levels

In order to investigate whether *FCGR3B* copy number variation was also associated with changes in the receptor surface expression, neutrophils were obtained from donors previously typed as <2, 2, and >2 *FCGR3B* copies and FcγRIIIb expression was assessed by flow cytometry. As control, the expression of CD32 (FcγRII), which does not exhibit CNV, was measured. While no differences in the levels of CD32 expression were evident between different donors, we observed substantial variability in the levels of FcγRIIIb expression (fig. 3). In particular, donors with <2 copies displayed lower levels of FcγRIIIb expression, compared with those with >2 *FCGR3B* copies. These findings provide support for the functional significance of *FCGR3B* copy number variation, clearly

indicating that *FCGR3B* copy number is correlated with FcγRIIIb surface expression.

FCGR3B Copy Number Variation Does Not Influence IPF Severity and Progression

We next determined whether variation in the *FCGR3B* copy number could influence IPF severity and progression. We therefore compared *FCGR3B* copy number genotypes with pulmonary function measurements obtained at presentation and following a 12-month follow-up period, in order to assess disease severity and progression, respectively. No significant differences in the baseline pulmonary function were evident for the different *FCGR3B* copy number classes (table 2).

In IPF, a fall from baseline of $\geq 10\%$ in FVC or $\geq 15\%$ in DL_{CO} in the first 6–12 months is strongly associated with poorer prognosis and with a more aggressive form of IPF [7, 19, 20]. For this reason, patients were categorized into either progressive ($n = 49$) or non-progressive ($n = 72$) groups, based on whether they displayed a fall from baseline of $\geq 10\%$ in FVC or $\geq 15\%$ in DL_{CO} in 12 months. No significant skewing in the *FCGR3B* copy number class distribution was noted between progressive and non-progressive groups ($\chi^2 = 0.74$; d.f. 2; $p = 0.69$), and both groups displayed similar copy number frequencies (progressive: <2 copies: 0.04, 2 copies: 0.67, >2 copies: 0.29; non-progressive: <2 copies: 0.05, 2 copies: 0.60, >2 copies: 0.35). In addition, the presence of >2 *FCGR2B* copies was not associated with disease progression ($p = 0.65$; OR: 1.33; 95% CI: 0.6–2.9). Similarly, when we compared the percent

Fig. 4. Role of *FCGR3B* copy number variation in IPF disease progression. In IPF, a drop of $\geq 10\%$ in FVC or $\geq 15\%$ in DL_{CO} from baseline in the first 12 months is generally associated with much higher mortality [7, 19, 20]. Serial measurements of FVC and DL_{CO} were recorded at 12 months following baseline to assess disease

progression. Comparison of the percent change in FVC (a) and DL_{CO} (b) for the different *FCGR3B* copy number variants revealed no significant association (FVC: $p = 0.28$; DL_{CO} : $p = 0.41$). Results are presented as mean $\pm$ 95% CI.

Table 2. Baseline pulmonary function of *FCGR3B* copy number variants of IPF patients

	<i>FCGR3B</i> Copies			p value
	<2	2	>2	
Patients, n	7	91	44	
FEV ₁ , l	2.16 $\pm$ 0.5	2.19 $\pm$ 0.6	2.12 $\pm$ 0.6	0.85
% predicted	87.9 $\pm$ 19.6	86.8 $\pm$ 21.2	88.9 $\pm$ 17.9	0.85
FVC, l	2.86 $\pm$ 0.6	2.76 $\pm$ 0.8	2.73 $\pm$ 0.8	0.90
% predicted	90.2 $\pm$ 15.0	86.0 $\pm$ 20.5	90.4 $\pm$ 18.5	0.45
FEV ₁ /FVC, % predicted	76.1 $\pm$ 8.2	79.5 $\pm$ 9.5	78.7 $\pm$ 10.9	0.64
TLC, l	4.22 $\pm$ 0.7	4.34 $\pm$ 1.1	4.22 $\pm$ 1.0	0.80
% predicted	73.8 $\pm$ 11.8	74.0 $\pm$ 15.8	74.6 $\pm$ 15.0	0.98
DL_{CO} , ml/min/mm Hg	3.84 $\pm$ 1.0	4.19 $\pm$ 1.3	3.99 $\pm$ 1.5	0.65
% predicted	51.2 $\pm$ 17.0	53.1 $\pm$ 15.3	52.2 $\pm$ 17.1	0.93
K_{CO} , ml/min/mm Hg/l	1.00 $\pm$ 0.2	1.12 $\pm$ 0.3	1.08 $\pm$ 0.3	0.53
% predicted	74.1 $\pm$ 17.7	84.3 $\pm$ 22.6	80.2 $\pm$ 23.9	0.42

Values as mean $\pm$ SD. FEV₁ = Forced expiratory volume in 1 s; FVC = forced vital capacity; TLC = total lung capacity; DL_{CO} = diffusing capacity of the lung for carbon monoxide; K_{CO} = DL_{CO} corrected for lung volume.

change in FVC or DL_{CO} observed in 12 months, no significant differences were observed between the *FCGR3B* copy number variants (fig. 4). Collectively, our findings clearly suggest that although the *FCGR3B* copy number variation represents a genetic risk factor for IPF, it is not implicated in disease progression and aggressiveness.

Discussion

Based on the substantial evidence on the involvement of immune complexes in IPF pathogenesis, as well as the association of *FCGR3B* CNV with a number of chronic inflammatory diseases, this study focussed particularly

on this Fc γ receptor and investigated its association with IPF. Although IPF is not classically considered to be a chronic inflammatory disease but rather the result of an abnormal wound healing response, there are several lines of evidence in favour of a significant immunological component that influences IPF pathogenesis and progression. Indeed, a number of pro-inflammatory and pro-fibrotic cytokines have been detected in the BAL fluid of patients with IPF, including TNF- α , TGF- β , MCP-1 and IL-8 [21]. Similarly, an excess of neutrophils, eosinophils and other leukocytes is typically present in the lungs of IPF patients [7, 22].

Since Fc γ RIIIb is expressed exclusively by neutrophils, our findings support the pathogenic potential of this leukocyte subset in IPF. Inappropriate or uncontrolled activation of neutrophils has been shown to be an important pathogenic mechanism in a variety of inflammatory diseases [23]. Engagement of neutrophil Fc γ RIIIb by immune complexes, which have been reported to be present in blood and lung tissue in IPF, can induce a range of effector and immunoregulatory functions, including degranulation, phagocytosis and cell activation [3]. Such processes consequently lead to the production of histotoxic compounds, such as proteolytic enzymes and reactive oxygen and nitrogen intermediates that can trigger damage to the alveolar walls and pulmonary interstitium, leading to aberrant deposition of fibrotic tissue, which is characteristic of IPF. *FCGR3B* gene copy number influences the surface expression of Fc γ RIIIb, as well as neutrophil functional responses, such as superoxide production, adhesion and IgG-mediated phagocytosis [5] (fig. 3). Based on these observations, it is possible that in the context of IPF the observed higher *FCGR3B* copy number could lower the threshold for IgG-mediated neutrophil

activation, thereby increasing their histotoxic capacity and accelerating disease pathogenesis. In addition, since *FCGR3B* copy number was found to be associated only with IPF susceptibility, but not with disease severity or progression, it could be suggested that Fc γ RIIIb-mediated interactions play a role mainly during the early stages of disease pathogenesis. Indeed, neutrophil-mediated cellular damage to the alveolar walls and pulmonary interstitium, as a result of Fc γ receptor engagement, could initiate a strong pro-fibrotic response, leading thereby to fibroblast activation and aberrant deposition of fibrotic tissue, which is characteristic of IPF.

In summary, we have here reported the first copy number variant associated with IPF, which along with previous reports of IPF-associated SNPs [9, 24] highlights the role of genetic variants in disease pathogenesis. However, additional studies should be performed on other IPF cohorts to further validate the observed association between *FCGR3B* copy number variation and IPF disease susceptibility. It is therefore anticipated that multiple genetic factors in combination with a number of environmental and immunological determinants influence disease susceptibility. Identification of such genetic factors could aid the precise characterisation of pathogenic mechanisms and pathways involved in IPF.

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