Copy Number Variation of \textit{FCGR3B} Is Associated with Susceptibility to Idiopathic Pulmonary Fibrosis

Stylianos Bournazos\textsuperscript{a, b} Irini Bournazou\textsuperscript{a} John T. Murchison\textsuperscript{c}
William A. Wallace\textsuperscript{a, d} Pauline McFarlane\textsuperscript{e} Nikhil Hirani\textsuperscript{a, e} A. John Simpson\textsuperscript{a, e}
Ian Dransfield\textsuperscript{a} Simon P. Hart\textsuperscript{f}

\textsuperscript{a}University of Edinburgh, Medical Research Council Centre for Inflammation Research, Queen’s Medical Research Institute, \textsuperscript{b}Centre for Cardiovascular Science, University of Edinburgh, Queen’s Medical Research Institute, Departments of \textsuperscript{c}Radiology and \textsuperscript{d}Pathology and \textsuperscript{e}Respiratory Medicine Unit, Royal Infirmary of Edinburgh, Edinburgh, \textsuperscript{f}Division of Cardiovascular and Respiratory Studies, Hull York Medical School, University of Hull, Castle Hill Hospital, Cottingham, UK

Abstract

\textbf{Background:} Several genes exhibit copy number variation (CNV), including \textit{FCGR3B} which encodes the \textit{IgG receptor Fc} (FcRIIIb). Engagement of Fc receptors by IgG complexes may contribute to the pathogenesis of idiopathic pulmonary fibrosis (IPF). \textbf{Objectives:} To investigate whether \textit{FCGR3B} CNV is associated with susceptibility to IPF. \textbf{Methods:} In a case-control study we compared \textit{FCGR3B} copy number in 142 patients with IPF and in 221 controls by real-time quantitative PCR using \textit{CD36} as gene copy control. \textbf{Results:} Significantly increased \textit{FCGR3B:CD36} ratio was evident in the IPF cohort compared to controls (p = 0.009). Association of \textit{FCGR3B} copy number with IPF susceptibility was further confirmed by a likelihood ratio statistical approach (p = 0.003). \textit{FCGR3B} copy number assignment based on \textit{FCGR3B:CD36} ratios revealed significant skewing in the distribution of \textit{FCGR3B} copy number between IPF patients and controls. In the IPF cohort, there was increased frequency of >2 \textit{FCGR3B} copies compared to controls (0.30 vs. 0.19; $\chi^2 = 9.27$; d.f. 2; $p = 0.0097$). The presence of >2 \textit{FCGR3B} copies was associated with higher risk of IPF (p = 0.01, OR: 1.914, 95% CI: 1.17–3.12). \textbf{Conclusions:} These findings support an association of \textit{FCGR3B} copy number with susceptibility to IPF and propose a novel role for Fc receptors in IPF disease pathogenesis.

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 Fcγ receptor, FcγRIIIb (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 FcγRIIIb 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 dysregulated 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-1β, 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 Fcγ receptors, like FcγRIIIb, 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 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 DLCO (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 transbronchial 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 (± 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 ± 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].

<table>
<thead>
<tr>
<th>Table 1. Characteristics and baseline pulmonary function of IPF patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, n</td>
</tr>
<tr>
<td>Age, years (range)</td>
</tr>
<tr>
<td>Female/male, n (%)</td>
</tr>
</tbody>
</table>

| FEV1, l | 2.16 ± 0.6 | 87.52 ± 20.0 |
| FVC, l | 2.76 ± 0.8 | 87.65 ± 19.6 |
| FEV1/FVC, % predicted | 79.07 ± 9.9 |
| TLC, l | 4.30 ± 1.0 | 74.20 ± 15.3 |
| DLCO, ml/min/mm Hg | 4.11 ± 1.4 | 52.75 ± 15.9 |
| KCO, ml/min/mm Hg/l | 1.10 ± 0.3 | 82.47 ± 22.8 |

Values as mean ± SD. FEV1 = Forced expiratory volume in 1 s; FVC = forced vital capacity; TLC = total lung capacity; DLCO = diffusing capacity of the lung for carbon monoxide; KCO = DLCO corrected for lung volume.

Respiration 2011;81:142–149
plification reactions (25 µl; 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′-CACCTGATCTCA-TCCCCAGGTCTTG, reverse 5′-CCATCTCTGTCACCTGC-CAG; for CD36 (used as a single copy control): forward 5′-TAAGTTCAGGTTCCTGGAATGC, reverse 5′-CAAATTATG- GTATGGACTGTGC. 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 cycler 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 templates 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 immuno-labelled, as previously described [17], using purified mouse monoclonal antibodies (10 µg ml⁻¹) against either human FcγRIIib (3G8, mlgG1) or human FcγRIIa (IV.3, mlgG2b), followed by Alexa Fluor 488-conjugated goat anti-mouse F(ab')₂ (Invitrogen). Surface expression of FcγRIIib or FcγRIIa 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 FcγRIIib 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-
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 (χ² = 8.76; d.f. 1; p = 0.003).

Furthermore, using this likelihood-based method we obtained the assigned FCGR3B copy number for each
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; χ² = 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 FcγRIIb in disease pathogenesis.

**FCGR3B Copy Number Variation Is Correlated with FcγRIIb 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 (FcγRIIa) was measured. 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.

**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 ≥10% in FVC or ≥15% in DLCO 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 ≥10% in FVC or ≥15% in DLCO in 12 months. No significant skewing in the FCGR3B copy number class distribution was noted between progressive and non-progressive groups (χ² = 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...
change in FVC or DLCO 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.

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

<table>
<thead>
<tr>
<th>FCGR3B Copies</th>
<th>&lt;2</th>
<th>2</th>
<th>&gt;2</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, n</td>
<td>7</td>
<td>91</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>FEV₁, l</td>
<td>2.16 ± 0.5</td>
<td>2.19 ± 0.6</td>
<td>2.12 ± 0.6</td>
<td>0.85</td>
</tr>
<tr>
<td>% predicted</td>
<td>87.9 ± 19.6</td>
<td>86.8 ± 21.2</td>
<td>88.9 ± 17.9</td>
<td>0.85</td>
</tr>
<tr>
<td>FVC, l</td>
<td>2.86 ± 0.6</td>
<td>2.76 ± 0.8</td>
<td>2.73 ± 0.8</td>
<td>0.90</td>
</tr>
<tr>
<td>% predicted</td>
<td>90.2 ± 15.0</td>
<td>86.0 ± 20.5</td>
<td>90.4 ± 18.5</td>
<td>0.45</td>
</tr>
<tr>
<td>FEV₁/FVC, % predicted</td>
<td>76.1 ± 8.2</td>
<td>79.5 ± 9.5</td>
<td>78.7 ± 10.9</td>
<td>0.64</td>
</tr>
<tr>
<td>TLC, l</td>
<td>4.22 ± 0.7</td>
<td>4.34 ± 1.1</td>
<td>4.22 ± 1.0</td>
<td>0.80</td>
</tr>
<tr>
<td>% predicted</td>
<td>73.8 ± 11.8</td>
<td>74.0 ± 15.8</td>
<td>74.6 ± 15.0</td>
<td>0.98</td>
</tr>
<tr>
<td>DLCO, ml/min/mm Hg</td>
<td>3.84 ± 1.0</td>
<td>4.19 ± 1.3</td>
<td>3.99 ± 1.5</td>
<td>0.65</td>
</tr>
<tr>
<td>% predicted</td>
<td>51.2 ± 17.0</td>
<td>53.1 ± 15.3</td>
<td>52.2 ± 17.1</td>
<td>0.93</td>
</tr>
<tr>
<td>KCO, ml/min/mm Hg/l</td>
<td>1.00 ± 0.2</td>
<td>1.12 ± 0.3</td>
<td>1.08 ± 0.3</td>
<td>0.53</td>
</tr>
<tr>
<td>% predicted</td>
<td>74.1 ± 17.7</td>
<td>84.3 ± 22.6</td>
<td>80.2 ± 23.9</td>
<td>0.42</td>
</tr>
</tbody>
</table>

Values as mean ± SD. FEV₁ = Forced expiratory volume in 1 s; FVC = forced vital capacity; TLC = total lung capacity; DLCO = diffusing capacity of the lung for carbon monoxide; KCO = DLCO corrected for lung volume.

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 Fcγ 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 FcγRIIIb 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 FcγRIIIb 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 FcγRIIIb, 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 FcγRIIIb-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 Fcγ 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.

Acknowledgements

The authors wish to thank Dr. Melany Jackson (University of Edinburgh, UK) for advice on quantitative PCR. We are also grateful to all the past members from our group (MRC Centre for Inflammation Research, UK) and all the subjects who participated in this study. This study was supported by the British Heart Foundation (FS/05/119/19568).

References


