Copy number variation of FCGR3A rather than FCGR3B and FCGR2B is associated with susceptibility to anti-GBM disease

Xu-jie Zhou1, Ji-cheng Lv1, Ding-fang Bu2, Lei Yu1, Yan-rong Yang1,3, Juan Zhao1, Zhao Cui1, Rui Yang1, Ming-hui Zhao1 and Hong Zhang1

1Renal Division, Peking University First Hospital, Peking University Institute of Nephrology and Key Laboratory of Renal Disease, Ministry of Health of China, Beijing 100034, People's Republic of China
2Research Central Institute, Peking University First Hospital, Beijing 100034, People's Republic of China
3Renal Division of the Second Hospital of ShanXi Medical University, Taiyuan 030001, People's Republic of China

Corresponding author: H. Zhang; E-mail: hongzh@bjmu.edu.cn

Transmitting editor: T. Takai

Received 16 August 2009, accepted 31 October 2009

Abstract

Anti-glomerular basement membrane antibody disease (anti-GBM disease) is a rare disorder characteristic of universally poor outcome. Fcγ receptors (FcγRs) play important roles in anti-GBM disease based on evidence from animal models. Copy number variation (CNV) influences disease susceptibility. The FcγRs genes show CNV, and CNV of the FCGR3B gene is associated with glomerulonephritis in systemic lupus erythematosus and anti-neutrophil cytoplasmic antibody-associated small vasculitis. Here, we investigated CNV of three FCGR genes, including two (FCGR3A and FCGR3B) for activating FcγRs and one (FCGR2B) for inhibitory FcγR by duplex quantitative real-time PCR. Copy numbers were analyzed by Applied Biosystems CopyCaller Software v1.0. We first demonstrated the distribution of CNV of FCGR3A, FCGR3B and no CNV of FCGR2B in Chinese population (including 47 anti-GBM patients and 146 healthy controls). The frequency of CNV of FCGR3A was observed to be significantly higher than matched healthy controls (27.7 versus 12.3%, P = 0.013, odds ratio 1.21–6.10). Considering previous report about gene knock-out animal models and CNV effect of FCGR3A, we thus propose that CNV in members of FCGR family should have different roles in the pathogenesis of human anti-GBM disease.

Keywords: anti-glomerular basement membrane antibody disease, copy number variation, FCGR3A

Introduction

Anti-glomerular basement membrane antibody disease (anti-GBM disease) is a rare disorder with an incidence estimated at around one patient per million per year. However, most of patients present with rapidly progressive glomerulonephritis (RPGN) and it accounts for up to 20% of acute renal failure due to RPGN (1–3). Untreated anti-GBM disease has an almost universally poor outcome, with death from renal failure or lung hemorrhage (4). Even with extensive treatment, kidneys are always insufficient to repair themselves; those with severe renal involvement are often left with permanent renal failure and face a life of renal replacement therapy (5–7).

It was hypothesized that disease initiation occurred in response to some sort of environmental trigger in predisposed individuals (8, 9). Environmental factors, including infective agents, hydrocarbon exposure, lithotripsy and urinary obstruction and cigarette smoking, were associated to play a role in triggering the disease (1, 10–12). The role of HLA in disease predisposition with strong positive associations with HLA-DR15 and HLA-DR4 alleles and negative associations with HLA-DR7 and HLA-DR1 was also observed (8, 13–17). However, up-to-date, the clear etiology has not been elucidated.

The family of Fc receptors (FcRs) for IgG (FcγRs), linking the humoral and cellular branches of the immune system, has important functions in the activation and down-modulation of immune responses (18, 19). Recent work in animal models indicated that it mediated the development of autoimmune kidney diseases (20–25). Studies of gene-targeted mice lacking specific FcγR (activating or inhibitory FcγR) have demonstrated the different susceptibility to anti-GBM disease (20, 21, 26–28). And recently, it was also reported...
that the susceptibility of patients with systemic lupus erythematosus and anti-neutrophil cytoplasmic antibody-associated small vasculitis were associated with FCGR3B copy number variation (29–31). However, in humans, seldom study about the association between FcγRs and anti-GBM disease has been reported to date (32).

Newly found copy number variants were considered to be the predominant hereditary variance form regarding total number of base pairs of genetic difference, accounting for at least 5–12% of the human genome (33–35). They may alter gene expression by dosage effect and/or position effect and thus may influence a disease with a genetic etiology (31, 36–38). So we speculated that the copy number variation (loss or gain) in members of FCGR gene family may impact on disease resistance/susceptibility to human anti-GBM disease. We thus selected the members of FCGRs family, activating receptor (FCGR3A and FCGR3B) and inhibitory receptor (FCGR2B) coding genes, as the candidates to investigate the association of their CNVs with anti-GBM disease in a Chinese population.

Methods

Patients and controls

In this study, 47 patients with anti-GBM disease, diagnosed at Peking University First Hospital from 1997 to 2008, and 146 unrelated healthy blood donors as the normal controls were enrolled. Patients with anti-GBM disease were diagnosed by serum with positive anti-GBM antibody, renal biopsy with crescentic glomerulonephritis and linear deposition of IgG along the GBM (1). The study was approved by the medical ethics committee of Peking University and informed consent was obtained from all participants.

DNA extraction

Peripheral blood was collected from patients and healthy controls. Genomic DNA was isolated from white blood cells using a modified salt extraction technique (39), concentrated by ethanol precipitation, re-suspended in Tris–EDTA buffer and stored at −30°C until use. The concentration of DNA was determined by a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Determination of relative copy numbers by quantitative realtime PCR

FCGR2B, FCGR3A and FCGR3B gene copy number (CN) determinations were performed by duplex quantitative real-time PCR (QRT-PCR) on an ABI Prism 7500 Instrument (Applied Biosystems, Foster City, CA, USA) with Sequence Detection Software version1.3.1. Primers and TaqMan probes for QRT-PCR were designed to specifically amplify the target gene and to avoid paralogous or allelic sequence variants. Coagulation factor V gene (F5) was used as a single-CN control (40). Sequencing of the PCR products was undertaken to confirm the specificity of the experiment. The sequences of the primers and probes used in this study are shown in Table 1. In a run of QRT-PCRs, one mixture was prepared and then dispensed into tubes, in which each contained 26.7 μl ddH2O, 5 μl 10× PCR buffer, 4 μl 2.5 mM deoxyribonucleoside triphosphate, 15 pmol/each forward primers, 15 pmol/each reverse primers, 5 pmol/each TaqMan probes and 1.5 U Taq polymerase (Takara, Dalian, China). Genomic DNA sample of 80 ng was added into a tube, and the total reaction volume was 50 μl. For each sample, amplification of the target gene (FCGR2B or FCGR3A or FCGR3B) and the control gene (F5, which has two copies per diploid genome) was performed in the same tube and the sample was determined by triplicate. The thermal cycling condition was as follows: a pre-run at 94°C for 10 min; 40 cycles with a 10-s denaturation step at 94°C, followed by a 62°C (FCGR3A or FCGR3B) or 60°C (FCGR2B) annealing step for 45 s and a 72°C extension step for 10 s.

In a QRT-PCR run, a 96-well plate was used, which contained the measurements for patients, normal controls and the calibrator, as well as negative controls without template. Independent genomic DNA-based standard curves were also run, and the results showed that the amplification efficiencies for target and endogenous control genes are approximately equal (data not shown). CM analyses were undertaken by the CopyCaller Software (Applied Biosystems, version1.0) with a calibrator sample (c0503172) according to the manufacturer’s protocol (Applied Biosystems, Part Number 4400042). The estimate of confidence and absolute z-score provided by the software were also introduced to determine the correct assignment and the intra-assay variation. The inter-assay variable was assessed by the values of three randomly chosen quality controls. The coefficients of variation for their calculated CNs among different assays were <10% and predicted CNs were consistently the same.

Statistical analysis

Results of the measurement data are expressed as mean ± SD. Chi-square tests were used to analyze association of FCGR CNVs with susceptibility to anti-GBM disease. When sample numbers were small, Fisher’s exact tests were used. A P-value of <0.05 was considered statistically significant. Mann–Whitney U-test was applied for abnormal distribution. Statistical analyses were performed by SPSS version 12.0 (SPSS, Chicago, IL, USA).

Results

Clinical features of patients and controls

The average age of patients was 35.0 ± 17.2 years (range 13–82 years), including 30 males and 17 females. All the patients presented with serum-positive anti-GBM antibody and RPGN (1, 3). The mean serum creatinine of patients was 763.0 ± 400.9 μmol l⁻¹ when they were diagnosed. Seven patients died of end-stage renal disease, two patients received transplantation and the rests required sustained hemodialysis.

The mean age of healthy controls was 24.7 ± 7.6 years (range 9–52 years), including 86 males and 60 females. All the healthy controls were geographically and ethnically (Northern Chinese Han population) matched and no gender bias existed between patients and controls (P = 0.55).
The distribution of FCGR2B, FCGR3A and FCGR3B gene copy number variations in controls and patients

Copy number variation in the FCGR gene was studied in 193 Northern Chinese Han people (including 47 anti-GBM disease patients and 146 healthy controls) by means of QRT–PCR assay. As shown in Table 2, FCGR3A and FCGR3B gene copies (Fig. 1B and C) varied both in patients and in controls. CNV for the FCGR3A gene was observed in 31 (16.1%) individuals, with 17 (8.8%) individuals having three gene copies, 4 (2.1%) individuals having four gene copies and 10 (5.2%) carrying only a single FCGR3A gene copy. CNV for the FCGR3B gene was observed in 74 (38.3%) individuals, with 22 (11.4%) individuals having three gene copies, 11 (5.7%) individuals having four gene copies and 41 (21.2%) carrying only a single FCGR3B gene copy. We did not observe any individual with a complete absence of the FCGR3A or FCGR3B gene. The frequency of CNVs was similar to that reported by Hollox et al. (41), which indicating 18.8% for FCGR2B, FCGR3A and FCGR3B high CN in patients and 14.6% for FCGR3B.

As shown in Fig. 1(A), no copy number variances of FCGR2B were detected (n = 113, including 38 anti-GBM disease patients and 75 healthy controls), which was consistent with data about Caucasians (42, 43).

The association of FCGR3A and FCGR3B gene CN with susceptibility to anti-GBM disease

In order to investigate whether patients with variable FCGR3A and FCGR3B gene CNs have different susceptibility to anti-GBM disease, samples were stratified by: (i) individuals with CN (CN = 1, 3, 4) versus individuals without CN (CN = 2), (ii) individuals with high CN (CN = 3, 4) versus individuals with moderate or low CN (CN = 1, 2) and (iii) individuals with low CN (CN = 1) versus individuals with moderate or high CN (CN = 2, 3, 4).

Regarding FCGR3A, (i) the frequency of FCGR3A CNV in anti-GBM disease patients and controls was 27.7% versus 12.3%, P = 0.013, odds ratio (OR) 1.21–6.10; (ii) a significantly higher frequency of high CN was observed in patients (19.1 versus 8.2%, P = 0.036, OR = 1.04–6.75) and (iii) a tendency of higher frequency of low CN was also observed in patients (8.5 versus 3.1%, P = 0.109, OR 0.78–10.72) (Fig. 2A–C).

However, although a similar tendency was observed with regard to FCGR3B, no significance was achieved: (i) the frequency of FCGR3B CN in patients and controls were 46.8 and 35.6% (P = 0.170, OR 0.82–3.10); (ii) the frequency of high FCGR3B CN in patients and controls were 19.1 and 16.4% (P = 0.668, OR = 0.52–2.81) and (iii) the frequency of low FCGR3B CN in patients and controls were 27.7 and 19.2% (P = 0.216, OR 0.75–3.45) (Fig. 2D–F).

When taking into account mean CN of a gene, which may be obfuscated. Since the presence of CNV might lead to skewing of the genotyping errors, therefore, the association between FCGR3A CNV and CNV accounting for genetic heterogeneity

We previously reported that FCGR2B single-nucleotide polymorphism rather than FCGR2A, FCGR3A and FCGR3B was associated with anti-GBM disease in Chinese (32), but the study did not take into account CNV, so the significance of FCGR3A and FCGR3B might be obscured. Since the presence of CNV might lead to skewing of the genotype frequencies by causing genotyping errors, therefore, the association between FCGR3A CNV and FCGR3B NA1/2 and disease susceptibility in subjects with no gene CNV was assessed. However, no association was observed (P = 0.603 for FCGR3A and P = 0.200 for FCGR3B).

### Table 1. Primers and probes used for duplex QRT–PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position</th>
<th>Primer/probe</th>
<th>Sequence of primers and fluorescence labeling probes</th>
</tr>
</thead>
<tbody>
<tr>
<td>F5</td>
<td>Exon 4</td>
<td>Forward</td>
<td>5′-GCTCAGGCGCGAGATACAA-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5′-GTGAGGCTATCGGCTGGT-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probe</td>
<td>5′-FAM-CCACGTTCTCAGTACATTCCATC-BCQ1-3′</td>
</tr>
<tr>
<td>FCGR2B</td>
<td>Exon 6 and intron 6</td>
<td>Forward</td>
<td>5′-GATTGGCTGCGGTTTGGT-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5′-GATTGGCTGCGGTTTGGT-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probe</td>
<td>5′-HEX-CCTCAGGCTACGCAACACGGCGG-BHQ1-3′</td>
</tr>
<tr>
<td>FCGR3A</td>
<td>Intron 1</td>
<td>Forward</td>
<td>5′-AGTTGGATGCTGCTGGT-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5′-AGTTGGATGCTGCTGGT-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probe</td>
<td>5′-HEX-CACACGATTGATGCTGGG-BHQ1-3′</td>
</tr>
<tr>
<td>FCGR3B</td>
<td>Intron 1</td>
<td>Forward</td>
<td>5′-AGTTGGATGCTGCTGGT-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5′-AGTTGGATGCTGCTGGT-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probe</td>
<td>5′-HEX-CACACGATTGATGCTGGG-BHQ1-3′</td>
</tr>
</tbody>
</table>

### Table 2. Distribution of copy number variation of FCGR3A and FCGR3B gene in patients and controls

<table>
<thead>
<tr>
<th>CN</th>
<th>FCGR3A</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1</td>
<td>4 (8.5)</td>
<td>6 (4.1)</td>
</tr>
<tr>
<td>2</td>
<td>34 (72.3)</td>
<td>128 (87.7)</td>
</tr>
<tr>
<td>3</td>
<td>6 (12.8)</td>
<td>11 (7.5)</td>
</tr>
<tr>
<td>4</td>
<td>3 (6.4)</td>
<td>1 (0.7)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CN</th>
<th>FCGR3B</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1</td>
<td>4 (8.5)</td>
<td>6 (4.1)</td>
</tr>
<tr>
<td>2</td>
<td>34 (72.3)</td>
<td>128 (87.7)</td>
</tr>
<tr>
<td>3</td>
<td>6 (12.8)</td>
<td>11 (7.5)</td>
</tr>
<tr>
<td>4</td>
<td>3 (6.4)</td>
<td>1 (0.7)</td>
</tr>
</tbody>
</table>
Although anti-GBM disease is rare, it attracts the nephrologists because of its acute onset, rapid progression and high mortality. It would improve the patients' renal survival or life quality if anti-GBM disease was treated promptly. So investigating the pathogenesis and susceptible factors will give important clues for effective early intervention.

A large amount of evidences supported the role of FcγRs in mediating anti-GBM disease. In murine experimental nephrotoxic serum nephritis (anti-GBM disease model) (44), the glomerular injury (including proteinuria, blood urea nitrogen and histological findings) can be prevented by lack of the activating FcγRs (FcγRI, FcγRII or FcγRIII) and exacerbated by lack of the inhibitory receptor FcγRIIB (20, 21, 45, 46). Mutations

**Fig. 1.** Raw data for the CNV in *FCGR2B* (A), *FCGR3A* (B) and *FCGR3B* (C). The sample CN data were calculated by Applied Biosystems CopyCaller Software v1.0. All the samples were analyzed against calibrator c0503172. The assays were analyzed simultaneously by the software and displayed from multiple experiments. y-axis displayed the CN for each sample. x-axis displayed the sample ID (c indicates control). CN range bars indicated the minimum and maximum CN calculated for the sample replicates. Different colors meant different plates. (A) No copy number variation of *FCGR2B* gene in patients and controls. (B and C) In aim of indicating copy number variation, raw data for *FCGR3A* and *FCGR3B* were shown, which demonstrated *FCGR3A* and *FCGR3B* gene copies varied both in patients and in controls.
within FcγRs are linked to autoimmunity in three ways: (i) failure to clear immune complexes from the circulation and from specific sites such as kidneys, (ii) hyper-responsiveness to circulating immune complexes and (iii) lack of control of antibody production leading to immune complex formation (47, 48).

In this study, we first investigated the role of CN of FCGR genes in human anti-GBM disease. Because CNV frequencies vary among different populations, in the present study we first checked the CNV distribution and have observed similar frequencies to that in the Chinese population examined by Hollox et al. (41). In addition, we found that CNV of FCGR3A was associated with susceptibility to anti-GBM disease in Chinese population. Consistent with the results of animal models, deletion of the mouse activating receptor FCGRIV (mouse FCGRIV are the ortholog of human FCGR3A) protected mice from developing accelerated anti-GBM nephritis (19, 21), more copies of FCGR3A may alter phenotypes by dosage effect (31, 42). In addition, it can also be supported by the recent report that CNV of the FCGR3A gene correlated with FcγRIIIa expression and function on NK cells (42).

In our study, we also found that the CNV of FCGR3B was not statistically associated with human anti-GBM disease and no CNV of FCGR2B. The result that no CN concordance of three genes typed within the FCGR gene cluster was also supported by previous literatures (41–43). It may indicate that copy number variations in different members of FcγR family have different roles in the pathogenesis of human anti-GBM disease. In data presentation, we gave concrete CNs to avoid covering the respective contribution of high/low CNs to disease pathogenesis; we also introduced average means of CNs, which may also have indicating significance from a population perspective. The presented data and speculations may provide preliminary clue for further study.

However, some limitations of the present study should be considered when interpreting our results. First, the number of patients, although larger than previously reported (14, 16, 17), is too small to allow subgroup analysis and makes the P-values seem not quite high or marginal if subjected to correction for multiple comparisons. Second, we cannot rule out the role of low FCGR3A CN may play in the pathogenesis of the disease, for a similar high frequency in patients was also observed despite the weak statistical significance and previous data from transgenic mouse. Third, the implication of similar or opposite tendency of FCGR3B may require more samples, which can hardly be available. We speculated that an optimal receptor level might be protective, whereas both low and high expression might be detrimental (19, 21, 49). We preferred to leave the interpretation to the reader for it was difficult to exclude false-positive association due to limited sample size. Fourth, for limited sample size and all the enrolled patients were in severe clinical manifestation and progressed rapidly to end-stage renal failure, no association between genotype and phenotype (such as renal function) was observed.

To conclude, the present study demonstrated the distribution of CNVs of FCGR2B, FCGR3A and FCGR3B in Chinese population and showed the association of copy number variations of FCGR genes with human anti-GBM disease. It provided a clue for elucidating the role of FCGR genes in anti-GBM disease. The additional study was deserved, by enlarging sample size from multiple populations, and at the same time functional assays comprised of enough individuals with different CNs targeting concrete possible mechanisms about FCGR gene mutation mentioned above.

Disclosures
The authors declare no competing financial interests.

Funding
National Natural Science Foundation of China (30801022, 30825021); Foundation of Ministry of Health of China (200802052).
References


