

## Association of copy number variation in *Fc gamma receptor IIIb* gene with risk of Graves' ophthalmopathy

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Received 20 Mar 2012

Accepted 9 Aug 2012

**ABSTRACT:** The functional polymorphism that explains the established association of *Fc gamma receptor IIIb* (*FCGR3B*) with Graves' disease (GD) and Graves' ophthalmopathy (GO) remains unidentified, but copy number variation (CNV) might be relevant. The aim of this study was to determine whether CNV for *FCGR3B* is associated with GD and GO. Genotype analysis involved 624 GD patients, including 397 without GO (GD<sub>nonGO</sub>) and 227 with GO (GD<sub>GO</sub>), and 227 healthy controls. The relative copy number (CN) of *FCGR3B* was determined using a relative real-time quantitative polymerase chain reaction. Our findings indicated the distribution of the relative CN of *FCGR3B* significantly differed between the GD<sub>GO</sub> patients and the healthy controls ( $p = 0.02$ ) but not between the total GD patients and the healthy control groups ( $p = 0.06$ ). Individuals with less than 2 CN or more than 2 CN of *FCGR3B* were at significantly decreased risk of developing GD<sub>GO</sub>. In addition, GD patients with less than 2 copies of *FCGR3B* were at reduced risk for developing nodular hyperplasia and vitiligo, but at increased risk for myxedema. Our results suggest that CNV of *FCGR3B* is associated with the development or progression of GD in Taiwan Chinese population.

**KEYWORDS:** thyroid eye disease, autoimmune disease, goitre, exophthalmos

### INTRODUCTION

Graves' disease (GD) is an autoimmune disease characterized by hyperthyroidism, diffuse goitre, thyroid-specific autoantibodies with or without Graves' ophthalmopathy (GO), and dermopathy due to the circulating autoantibodies<sup>1</sup>. GO is the most common extrathyroid manifestation of GD and affects 25%–50% of GD patients<sup>2–5</sup>. Approximately 28% of patients with GO present with severe disease, with restricted mobility, diplopia, keratopathy, and optic neuropathy<sup>6,7</sup>. Numerous functionally relevant single-nucleotide polymorphism (SNP) variants have been characterized in GD and GO, although none have yet proven causative<sup>8</sup>. Other forms of variation that might affect gene expression should also be considered.

Copy number variation (CNV) is recognized as a common form of genomic variation in which DNA segments (> 1 kb) are present in a variable number of copies compared to a reference genome. A CNV caused by genomic rearrangements such as deletions, duplications, inversions, and translocations may contribute to alteration of gene expression and subsequent phenotypic variation, which results in susceptibility or resistance to disease<sup>9–11</sup>. CNV has also been associated with autism<sup>12–15</sup>, schizophrenia<sup>16–19</sup>, cancer<sup>20–25</sup>, and autoimmune disease<sup>11, 26–30</sup>.

*Fc gamma receptor IIIb* (*FCGR3B*), the CD16 cell surface immunoglobulin receptor, located on chromosome 1q23, is a low-affinity IgG receptor that binds with IgG complexes at the Fc region<sup>31</sup>. *FCGR3B* belongs to the Fc gamma receptor fam-

**Table 1** Demographic and clinical characteristics of 624 patients with Graves' disease.

Mean age at diagnosis (S.D.), yrs	41.1 (12.9)
Gender	
Male	132 (21.2%)
Female	490 (78.5%)
Clinical features	
Ophthalmopathy	227 (36.4%)
Goitre	
Grade 2 & 3	474 (76.4%)
Grade 0-1b	147 (23.6%)
Nodular hyperplasia	574 (92.0%)
Myxoedema	114 (18.3%)
Vitiligo	98 (15.7%)

Abbreviations: S.D., standard deviation; yrs, years

ily, which contributes to the regulation of acquired and innate immunity<sup>31</sup>. Studies have shown that a lower copy number of *FCGR3B* can increase the susceptibility to systemic autoimmune diseases such as systemic lupus erythematosus and other autoimmune disorders<sup>27</sup>. Although there have been major advances in understanding the genetic basis of GD<sup>27</sup>, the association between the variation of copy number in *FCGR3B* gene and the risk of GO remains unclear. In the present study, the variant in the *FCGR3B* gene that may explain the association between, and the physiological impact of, CNV in this region and the development of GD and GO was found.

## MATERIALS AND METHODS

### Subjects

A disease group with 624 patients with a confirmed diagnosis of GD and an appropriate control group with 227 healthy volunteers with matched gender according to the female predominance of GD including 32 male (20.0%) and 128 female (80.0%) at China Medical University Hospital in Taiwan were enrolled and actively followed. The methods of diagnosis of GD and classification of GO were as previously described<sup>32</sup>. All individuals classified as affected were interviewed and examined by experienced clinicians. Demographic and clinical characteristics of 624 patients with GD are summarized in Table 1. The study was approved by the institutional review board and the ethics committee of China Medical University Hospital (DMR96-IRB-109; approved 2007 September). Written informed consent was obtained from each participants in accordance with institutional requirements and the Declaration of Helsinki Principles.

### Genomic DNA extraction

The genomic DNA was extracted from peripheral blood leukocytes of participants using the Genomic DNA kit (Qiagen) according to the manufacturer's instructions. Considering that the quantitative polymerase chain reaction (qPCR) technique is sensitive to small variations in DNA quality<sup>33</sup>, the quality of each genomic DNA was verified on an agarose gel and the template DNA were freshly diluted from 200 µg/ml stock and used within 2 weeks.

### Analysis of *FCGR3B* copy number variants

The relative CN of the *FCGR3B* gene in each individual was estimated using a relative real-time qPCR method that is suited to larger-scale genotyping. This method has been tested and proven to provide reliable results. Specific TaqMan probes (Assay ID: Hs04211858\_cn) for *FCGR3B* and for a reference gene *RNase P* (*RNaseP* part number 4403326) were purchased from ABI Biosciences (Applied Biosystems, Foster City, CA, USA). The TaqMan probes for *FCGR3B* and *RNase P* were labelled with FAM and VIC, respectively. Polymerase chain reactions were run on an ABI 9700 machine. Amplification reactions (15 µl) were carried out using genomic DNA (10 ng), TaqMan Copy Number Reference Assay for *FCGR3B* and *RNase P*, and TaqMan Master Mix. Thermal cycling was initiated with an initial denaturation step of 10 min at 95 °C, followed by 40 cycles each of 15 s at 95 °C and of 1 min at 60 °C. A dissociation procedure was performed to generate a melting curve to confirm amplification specificity. The relative *FCGR3B* CN for each individual was estimated using the  $2^{-\Delta\Delta C_t}$  method, where  $C_t$  is cycle threshold,  $-\Delta C_t = (C_{t_{\text{gene}}} - C_{t_{\text{reference}}})$  as described previously<sup>34</sup>. The PCR products were quantified in triplicate, and the standard deviation and coefficient of variation (CV) were calculated based on 3 runs. To control for reaction quality, each reaction plate also included a calibrator, a positive control, and a no-template control (NTC). Data from a plate were included if the calibrator CV was less than 5%, the positive control CV and sample CVs were all less than 10%, and the NTC was negative. To include across-plate data, the CVs of the positive control had to be similar and the NTC had to be negative.

### Statistical analysis

Significance of the differences between the distributions of *FCGR3B* CN in cases and controls was estimated by Chi square test or Fisher's exact test. Odds ratios (OR) and confidence intervals (CI) were

estimated by logistic regression models using SPSS 12.0. The CN equal to two (CN = 2) was chosen as reference category for *FCGR3B*. To estimate OR, bins of CN were grouped as 0–1 CNs for *FCGR3B* (< 2 category), 3–5 CN for *FCGR3B* (> 2 category). We estimated the risk of acquiring disease associated with CNs for *FCGR3B* categories by comparing them with a reference CN category. For each of these categories, an OR > 1 indicates a detrimental effect of CNV on disease susceptibility, and OR < 1 indicates a protective effect of CNV.

## RESULTS

### Correlation between CNV of *FCGR3B* and the development of GD and GO

The distribution of *FCGR3B* copy number (CN) according to the presence of GD or GO is summarized in Table 2. The distribution of *FCGR3B* CN was not statistically significant different between health controls and GD ( $p = 0.06$ ). Further, we separated GD patients into 2 groups according to GO status. The distribution of *FCGR3B* CN was significantly different between healthy control and GD<sub>GO</sub> patients ( $p = 0.0176$ ) but not with GD<sub>nonGO</sub> patients ( $p = 0.14$ ). Our results revealed that individuals with *FCGR3B* copy number not equal to 2 were at significantly decreased risk of developing GD<sub>GO</sub> when compared to individuals with 2 copies of *FCGR3B* (odds ratio (OR) = 0.45, 95% confidence interval (C.I.): 0.24, 0.84 and OR = 0.66, 95% C.I.: 0.42, 1.06 for CN < 2 and CN > 2, respectively) (Table 2). The result (control versus GD<sub>GO</sub> patients) was statistical significant after Bonferroni correction ( $p = 0.035$ ). Regression analysis with or without gender and age as covariates did not affect the significance of the reported association results. The results suggest that *FCGR3B* CNV might not be associated with the development or progression of GD, but could be associated with the development or progression of GD<sub>GO</sub>.

### Correlations between CNVs of *FCGR3B* and clinical phenotypes

The association between clinical features of GD patients and CNV of *FCGR3B* was analysed. The results are summarized in Table 3. No significant difference for *FCGR3B* CNV was found between GD patients and different stages of goitre ( $p = 0.47$ ). Interestingly, the distribution of *FCGR3B* CNV significantly differed between GD patients with different patterns of nodular hyperplasia ( $p = 0.02$ ), myxoedema ( $p = 0.04$ ), and vitiligo ( $p = 0.02$ ). As shown in Table 3, individuals with less than 2 copies of *FCGR3B* were

**Table 2** The distribution of *FCGR3B* copy number in normal, and Graves' disease with or without ophthalmopathy.

CN	Normal <i>N</i> (%)	GD <sub>total</sub> <i>N</i> (%)	GD <sub>nonGO</sub> <i>N</i> (%)	GD <sub>GO</sub> <i>N</i> (%)	OR (95% C.I.) <sup>a</sup>
= 2	144 (63.4)	433 (69.4)	263 (66.2)	170 (74.9)	
< 2	32 (14.5)	54 (8.7)	37 (9.3)	17 (7.5)	0.45 (0.24, 0.84)
> 2	51 (22.5)	137 (22.0)	97 (24.4)	40 (17.6)	0.66 (0.42, 1.06)
<i>P</i> value <sup>b</sup>		0.06	0.14	0.018	

Abbreviations: CN, copy number. GD<sub>total</sub>, total Graves' disease patients. GD<sub>nonGO</sub>, Graves' disease patients without ophthalmopathy. GD<sub>GO</sub>, Graves' disease patients with ophthalmopathy. *N* (%), number (percentage). OR (95% C.I.), odds ratio (95% confidence interval).

<sup>a</sup> Odds ratio and 95% C.I. were estimated by applying unconditional logistic regression when compared GD<sub>GO</sub> with normal control.

<sup>b</sup> *P* value was calculated by chi square test (2 × 3 tables) and was considered significant when less than 0.05.

at reduced risk for the development of nodular hyperplasia (OR = 0.37, 95% C.I.: 0.16, 0.86) and vitiligo (OR = 0.40, 95% C.I.: 0.21, 0.78), but at increased risk for myxoedema (OR = 2.18, 95% C.I.: 1.15, 4.13) compared with individuals with 2 copies of *FCGR3B*.

## DISCUSSION

Several functionally relevant SNP variants are characteristic of GD and GD<sub>GO</sub><sup>8</sup>, but few studies have demonstrated an association between CNV and GD<sup>27,35,36</sup>. Here, we found that CNV in *FCGR3B* significantly differs between GD<sub>GO</sub> patients and healthy controls. Individuals with more or fewer than 2 copies of *FCGR3B* may have reduced susceptibility to GD<sub>GO</sub>. In addition, our data showed the protective effect of *FCGR3B* CNV on GD which was consistent with previous studies in a Caucasian population<sup>27</sup>, although the results in both studies did not reach the statistical significance. The sample size of this study did not reach 80% power for the given effect size and is a limitation. In addition, the genetic backgrounds of ethnically diverse populations should be considered. Niederer et al<sup>37</sup> identified significant differences in *FCGR3B* CNV distribution among Caucasian, East Asian, and Kenyan population. Therefore, confirmation of these results by studies with larger sample sizes and in different population is warranted. More-

**Table 3** Estimated risk of several phenotypes of Graves' disease according to *FCGR3B* gene copy number.

CN	Goitre			Nodular hyperplasia			Myxoedema			Vitiligo		
	Yes <i>N</i> (%)	No <i>N</i> (%)	OR (95% C.I.) <sup>b</sup>									
= 2	325 (68.6)	107 (72.8)	1	406 (70.7)	26 (53.1)	1	70 (61.4)	363 (71.2)	1	58 (59.2)	374 (71.2)	1
< 2	39 (8.2)	13 (8.8)	0.99 (0.51, 1.92)	46 (8.0)	8 (16.3)	0.37 (0.16, 0.86)	16 (14.0)	38 (7.5)	2.18 (1.15, 4.13)	15 (15.3)	39 (7.4)	0.40 (0.21, 0.78)
> 2	110 (23.2)	27 (18.4)	1.34 (0.84, 2.16)	122 (21.3)	15 (30.6)	0.52 (0.27, 1.02)	28 (24.6)	109 (21.4)	1.33 (0.82, 2.17)	25 (25.5)	112 (21.3)	0.70 (0.42, 1.16)
<i>P</i> value <sup>a</sup>	0.47			0.02			0.04			0.02		

Abbreviations: CN, copy number; *N* (%), number (percentage); OR (95% C.I.), odds ratio (95% confidence interval).

<sup>a</sup> *P* values were calculated by chi square test (3 × 2 table) and were considered significant when less than 0.05.

<sup>b</sup> Odds ratio and 95% CIs were estimated by applying unconditional logistic regression.

\* Individuals with missing phenotype data were discarded from the logistic regression analysis.

over, GD patients with 2 copies of *FCGR3B* are at significantly increased risk for the development of nodular hyperplasia and vitiligo, but at reduced risk for myxoedema.

*FCGR3B*, which is expressed by neutrophils and eosinophils, functionally links the humoral and cellular branches of the immune system and plays an important role in the activation and modulation of the immune response<sup>31</sup>. *FCGR3B* CNV correlates with protein expression, soluble serum FcγRIIIb, neutrophil uptake of and adherence to immune complexes, and macrophage overactivity<sup>38</sup>. Dysregulation of FcγRIIIb expression may contribute to the impaired clearance of immune complexes. In GD/GD<sub>GO</sub>, in which immune complexes may not play an important role, the relation between *FCGR3B* CNV, *FCGR3B* expression, and neutrophil activation may require further investigation.

In addition, *FCGR3B* CNV could play a different role involving other co-regulatory genes, in the development of systemic and organ-specific autoimmunity. An association between low *FCGR3B* CNV and systemic lupus erythematosus has been reported in Caucasians<sup>27</sup>. However, anti-neutrophil cytoplasmic antibody-associated systemic vasculitis, an autoimmune disease in which immune complex deposition plays only a minor role, seems to be diversely associated with *FCGR3B* CNV<sup>27</sup>. Also, linkage disequilibrium between multi-allelic *FCGR3B* CNV and SLE-associated SNPs in the *FCGR* locus was defined<sup>37</sup>. However, the associations between NA1/NA2 allelic variant of *FCGR3B* with GO or GD in our study were not significant (*p* value > 0.05). Further study of

the genetic interaction between *FCGR3B* and other genes, such as *FCGR2A*, *FCGR2B*, and complement receptor 3 (CR3) should be considered.

In summary, this study suggests that *FCGR3B* CNV is associated with the development or progression of GD and GO, thereby providing new information on the implications of *FCGR3B* CNV in patients with GD.

**Acknowledgements:** Wen-Ling Liao and Yu-Huei Liu contributed equally to this work. We thank Hsin-Hui Chen for the technical assistance in preparation of DNA and analysing the variations. This study was supported by research grants from China Medical University, Taichung (CMU-98-asia-04 and DMR-101-118), Taiwan and 98-2320-B-039-008-MY3 from National Science Council, Taipei, Taiwan.

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