Title: Germline copy number variation in complement component *C4A* is associated with Graves' ophthalmopathy in the Taiwan Chinese population

Authors: Yu-Huei Liu[1, 2], Lei Wan[1, 3, 4], Chwen-Tzuei Chang[5, 6], Wen-Ling Liao[1], Wen-Chi Chen[2], Yuhsin Tsai[3], Chang-Hai Tsai[7, 8], Fuu-Jen Tsai[1, 3, 7, 9, 10, 11]

[1]Department of Medical Genetics and Medical Research, China Medical University Hospital, Taichung, Taiwan; [2] Graduate Institute of Integrated Medicine, China Medical University, Taichung, Taiwan; [3] School of Chinese Medicine, China Medical University, Taichung, Taiwan; [4]Department of Health and Nutrition Biotechnology, Asia University, Taichung, Taiwan; [5]Division of Endocrinology and Metabolism, Department of Medicine, China Medical University Hospital, Taichung, Taiwan; [6] Department of Endocrinology and Metabolism, College of Chinese Medicine, China Medical University, Taichung, Taiwan; [7]Department of Pediatrics, China Medical University Hospital, Taichung, Taiwan; [8] Department of Biotechnology, Asia University, Taichung, Taiwan; [9]School of Post-Baccalaureate Chinese Medicine, China Medical University, Taichung, Taiwan; [10]Department of Biotechnology, Asia University, Taichung, Taiwan, [11]Department of Biotechnology and Bioinformatics, Asia University, Taichung, Taiwan.

Address for reprints:

Fuu-Jen Tsai, MD, PhD.

Department of Medical Genetics and Medical Research, China Medical University Hospital

No.2 Yuh-Der Road, 404 Taichung, Taiwan

Telephone: 886-4-22062121 Ext 2041; Fax: 886-4-22033295

E-mail: d0704@mail.cmuh.org.tw

E-mail address:

Liu YH: yuhueiliu@mail.cmu.edu.tw

Wan L: leiwan@mail.cmu.edu.tw

Chang CT: chwentic@ms9.hinet.net

Liao WL: wl0129@mail.cmu.edu.tw

Chen WC: wgchen@mail.cmu.edu.tw

Tsai Y: yhtsai@mail.cmu.edu.tw

Tsai CH: chtsai@mail.cmu.edu.tw

Tsai FJ: d0704@mail.cmuh.org.tw

Abstract

Background: Gene copy number of complement component *C4*, which varies among individuals, may determine the intrinsic strength of the classical complement pathway. Presuming a major role of complement as an effecter in peptide-mediated inflammation and phagocytosis, we hypothesized that *C4* genetic diversity may partially explain the variation in Graves' disease (GD) outcomes.

Methods: A case-control study including a total of 624 Taiwan Chinese patients with GD, including 227 with Graves' ophthalmopathy (GO) and 397 without, were enrolled. CNV of total *C4*, *C4* isotypes (*C4A* and *C4B*) genes were performed by quantitative real-time polymerase chain reaction analysis. *Main Outcome Measures:* Statistical comparison and identification of CNV of total *C4* and *C4* isotypes (*C4A* and *C4B*) genes between GD patients with or without GO and other outcomes.

Results: The risk of susceptibility to GO was significantly reduced among GD patients with <2 copies of *C4A* ($p = 0.014$) but not *C4B* ($p = 0.187$). In addition, different C4 polymorphisms may correlated with GO susceptibility ($p = 0.050$). Moreover, although no CNV of *C4A* or *C4B* was significantly associated with goiter or nodular hyperplasia, having <2 copies of *C4A* resulted in a higher risk of vitiligo and myxedema ($p = 0.001$, OR = 2.646, 95% CI: 1.543–4.504 for vitiligo and $p = 0.008$, OR = 2.236, 95% CI: 1.270–3.936 for myxedema, respectively).

Conclusions: CNV of *C4A* may play an important role in the pathogenesis of GO in patients with GD.

Background

Graves' disease (GD) is an organ-specific autoimmune thyroid disease. Graves' ophthalmopathy (GO), characterized by inflammation and fat deposition in the eye muscles and the connective tissue surrounding the eye, is the most common extrathyroid manifestation of GD and affects 25%–50% of GD patients [1]. It has been known that multiple factors, including the host's genetic factors as well as environmental factors, contribute to the etiology and severity of GD [2,3]. However, other forms of variation that might affect gene expression should also be considered.

A new paradigm in human genetics is high frequencies of interindividual variation in the copy number (CN) of specific genomic DNA segments. Copy number variation (CNV) loci often contain genes engaged in host-environment interactions, including those involved in immune functions, which results in susceptibility or resistance to autoimmune diseases [4-7], however, no significant association has been found between CNV and GD or GO [6].

Complement component C4 (*C4*), located on chromosome 6q21.3, is encoded by 2 separate loci in the major histocompatibility complex class III region and derives 2 functionally distinct *C4A* and *C4B* isoforms [8]. The complement system is the main element of innate immunity and is regarded as the first line of defense against intrinsic and extrinsic antigens, leading to peptide-mediated inflammation, opsonization leading phagocytosis, or the direct lysis of antigens [9]. Presuming a major role of complement as an effector in peptide-mediated inflammation and phagocytosis, we hypothesized that *C4* genetic diversity may partially explain the variation in GD outcomes. Here we investigated the polymorphic variants of *C4* that correlate with predisposition to this disease in the Taiwan Chinese population.

Methods

Patients

A total of 624 patients (227 with GO and 397 without GO) with a confirmed

diagnosis of GD from China Medical University Hospital in Taiwan were enrolled and actively followed. All individuals provided informed consent as approved by the ethics committee of China Medical University Hospital. Diagnosis of GD and GO was followed the criteria set previously [10]. Full medical record abstraction was conducted to obtain demographics (age and gender); treatment and clinical features are summarized in Table 1.

Genomic DNA extraction and quantification gene dosage of *C4A* and *C4B*

Genomic DNA was extracted from peripheral blood following the manufactory's suggestions (Qiagen). *C4* gene dosage was assessed by quantitative real-time TaqMan® PCR analysis (Applied Biosystems) as described in the previously published protocols with some modification [11]. Real-time PCR analysis was performed in 96-well optical plates on a 7900HT real-time PCR system (Applied Biosystems). Primers and probes specific for *C4A*, and *C4B* (common *C4A* and *C4B* forward primer "C4F": 5'-GCA GGA GAC ATC TAA CTG GCT TCT-3'; common *C4A* and *C4B* reverse primer "C4R": 5'-CCG CAC CTG CAT GCT CCT-3'; probe "*C4A*": FAM-ACC CCT GTC CAG TGT TAG; probe "*C4B*": FAM-ACC TCT CTC CAG TGA TAC. TaqMan® Universal PCR Master Mix, No AmpErase® uracil-DNA glycosylase (ABI catalog number 4326614), VIC-conjugated TaqMan® *RNase P* control reagents (ABI catalog number 4316844), 250 nM of the respective FAM-conjugated TaqMan® probes (*C4A* or *C4B*), the particular primers (300 nM *C4A* or *C4B*) in distilled water was contained in each of the distinct PCR batches. Appropriately prediluted genomic DNA (threshold cycle [C_T] values for *RNase P* between 24 and 30) was added before start. CN of each target gene in each sample was determined from three separated experiments. Thermal cycler conditions were adjusted as follows: initial denaturation step for 10 minutes at 95°C; 40 cycles including denaturation for 15 seconds at 95 °C; and annealing/extension for 1 minute for 60°C. The data were analyzed using SDS 2.3 software (Applied Biosystems).

The C_T value of *RNase P*, *C4A* or *C4B* was converted into a raw gene dosage by

the formula $nRAW_{C4X} = 2^{(C_T RNase\ P)-(C_T C4X)+1}$, where *C4X* referred to *C4A* or *C4B* . Raw gene dosages of positive controls selected from the reference panel were plotted versus the actual gene dosages, and the resulting calibration curve served for determination of the actual copy number of unknown samples of this particular run.

Statistical analysis

Statistical analysis was performed using the statistical package PASW for Windows (version 18.0; SPSS Inc.). Differences in the incidence of subjects with *C4* gene CNs above and below the median or *C4A*-*C4B* polymorphisms between patients with or without GO were evaluated using Fisher's exact test. Two-tailed *p* values were estimated by 100,000 Monte Carlo simulations with 99% confidence intervals (CI). Odds ratios (ORs) and 95% CIs were estimated from logistic regression models. A *p* value of 0.05 with adjusted with Bonferroni was considered statistically significant for each test.

Results

CNV of *C4A*, but not *C4B*, is associated with susceptibility to GO

The distribution of copy number for total *C4* as well as C4 isotypes according to the presence of GO is shown in Figure 1. The variation in *C4* CN showed a pattern close to a normal distribution, and the majority of patients (50.3%) had 4 copies of the *C4* gene. No GD patient had a full deficiency of C4 alleles. The relationship between C4 CNV status and GO was not significant ($p = 0.396$). The distribution of *C4A* and *C4B* among GD patients with and without GO were further investigated. The median CNs of *C4A* and *C4B* in the study population were 2 and 2, respectively. Although the CNV of *C4B* was not significantly associated with GO ($p = 0.186$), an unexpected finding was that GD patients with <2 copies (0 or 1) of the *C4A* gene were less susceptible to GO ($p = 0.014$, OR = 0.447, 95% CI: 0.255-0.785). The significance remained after applying the Bonferroni correction ($p < 0.05/3$). These results indicate that <2 copies of *C4A*, but not *C4B*, may be a protective factor against the development of GO.

Polymorphism analysis of *C4* genes

The *C4* polymorphism was estimated from the CNs for *C4A* and *C4B*. The polymorphisms with a frequency >5% are summarized in Table 2 according to the presence of GO. The significance of polymorphism remained, although did not meet the Bonferroni correction. The GD patients with the most common polymorphism (40.7%), A2B2, with 1.433-fold risk toward GO ($p = 0.035$, OR = 1.433, 95% CI: 1.029-1.994) as compared to those without. When compared to those with A2B2, those with A3B1 or A1B2 polymorphisms had 60.3% and 69.6% less susceptibility to GO, respectively ($p = 0.05$, OR = 0.397, 95% CI: 0.166–0.953 for A3B1; $p = 0.05$, OR = 0.304, 95% CI: 0.122–0.760, $p = 0.033$ for A1B2). These results suggest the C4 polymorphisms may associate with GO development in the GD patients.

Correlation between *C4* CNV and other clinical features of GD

To extend our finding, we also investigated the linkage among *C4* CNV and clinical features in patients with GD. Although *C4* CNV was not significantly associated with goiter or nodular hyperplasia ($p = 0.103$ and $p = 0.504$, respectively), patients with <4 copies of *C4* had a 1.747-fold increased risk of vitiligo ($p = 0.002$, OR = 1.747, 95% CI: 1.091–2.830) and a 1.877-fold increased risk of myxedema ($p = 4.9 \times 10^{-4}$, OR = 1.877, 95% CI: 1.143–3.082). Only *C4A*, but not *C4B*, contributed to the effect ($p = 0.001$, OR = 2.646, 95% CI: 1.543–4.504 for vitiligo; $p = 0.002$, OR = 2.646, 95% CI: 1.543–4.504 for myxedema). The significance remained after applying the Bonferroni correction ($p < 0.05/3$) (Table 3). No *C4* polymorphism was significantly associated with the clinical features of GD (data not shown). These results indicate that <4 copies of *C4*, especially <2 copies of *C4A*, may increase the risk for vitiligo and myxedema in patients with GD.

Discussion

Several functionally relevant single nucleotide polymorphisms are characteristic of GD and GO [12,13], but no relevant CNV has been reported [14]. In the present study, we found that GD patients with <2 copies of *C4A* had a significantly lower

prevalence of GO but higher prevalence of vitiligo and myxedema. In addition, while polymorphism A2B2 may confer higher risk of GO, A3B1 and A1B2 may confer lower risk of GO. To the best of our knowledge, this is the first study to report that CNV in *C4* genes is associated with the protection of GO in Taiwan's Chinese population. Our results provide new insights into the prediction of and target therapeutics for GO.

Indeed, low level of C4 complements in sera have been found in several autoimmune diseases [15-18]. In addition, the presence of *C4A* and *C4B* null alleles that result in partial C4 deficiency have shown to be associated with systemic lupus erythematosus (SLE).[7] In the patients with SLE, complement deficiency may promote the accumulation of immune complex in the glomerulus of the kidney. Our results also revealed that deficiency of *C4A*, may enhance the development of vitiligo and myxedema in GD patients. This may due to activated complement exacerbated inflammation-driven tissue injury, immunocomplex clearance and autoreactive B cells deletion. However, it may play a different regulatory role in organ-specific autoimmune diseases such as GO. One possibility is that a deficiency of complement may lead to defective processing of immune complexes, impairment of B-cell memory, and help reduce tissue injury [19]. Unfortunately, the mechanisms by which *C4* abnormality contributes to the protection of organ-specific autoimmunity are poorly understood. Nevertheless, whether a potential gene-gene or gene-environment interaction is involved in susceptibility to GO needs to be further investigated [20]. However, this study provides a substantial amount of data that can help to clarify the role of *C4* genes in this disorder. It is only through investigations of diverse populations that researchers can expect to dissect the complex genetics involved. In addition, functional studies of susceptibility genes using appropriate animal models could allow for an assessment of their role in the disease process.

What is interesting is that although we explored the relationship of *C4* CNV to GD as well as other GD clinical features, only the lower copies of *C4A*, but not *C4B*, were associated with higher risk of vitiligo and myxedema. Because it appears that

C4A binds to amino group-containing antigens such as immune complex, whereas C4B binds to hydroxyl group-containing antigens such as bacteria, this result may provide another view to support the hypotheses that the pathogenesis of vitiligo and myxedema may be more relevant to the existence of the immune complex than the pathogen. In addition, although there is no evidence related to gene polymorphism in myxedema, recent studies have identified that the risk locus within the major histocompatibility complex region on chromosome 6q may be associated with vitiligo in both Chinese Han population and American population [21,22]. It may be interesting to investigate the gene-gene interaction between *C4* polymorphism and the vitiligo risky locus in associated with the GD clinical features. Moreover, although confirmation of these results in larger samples is warranted, it would be interesting to further investigate the role of *C4A* in the development of vitiligo and myxedema.

Conclusion

This study provides evidence that the CNV of *C4* genes is associated with risk of the development and progression of GO and the GD-related clinical features. These results might aid in diagnosis during the early stage of the disease and may be valuable in the development of therapeutic agents for the Taiwan Chinese population.

List of abbreviations

(GD): Graves' disease; (GO): Graves' ophthalmopathy; (CNV)" copy number variation; (CN): copy number; (SLE): systemic lupus erythematosus.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Author Liu YH designed the study, managed the literature searches, undertook the statistical analysis, and wrote the draft of the manuscript. Author Wan L designed and

performed the experiments. Author Chang CT and Chen WC recruited and maintained the clinical information of participants. Author Liao WL and Tsai Y undertook the statistical analysis. Author Tsai CH and Tsai FJ directed the study and reviewed the results. All authors contributed to and have approved the final manuscript.

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Figure legends

Figure 1. *C4* copy numbers in Graves' disease (GD) patients with or without Graves' ophthalmopathy (GO). (A) left panel, *C4* gene copy numberhealthy; middle panel, *C4A* gene copy number; right panel, *C4B* copy number. Fisher's exact test was used to assess the distribution of *C4* genes between groups. Two-tailed *p* values for significance were estimated by 100,000 Monte Carlo simulations with 99% confidence intervals. N, number of subjects in each group. (B) Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated by applying unconditional logistic regression between GD patients with or without GO.

Table 1. Background and demographic characteristics of patients with Graves' disease.

Patients' characteristics	GD (624)
Age at diagnosis	
Mean ± SD	41.1 ± 12.9
	N (%)
Gender	
Male	133 (21.3)
Female	491 (78.7)
Treatment	
Radioiodine	
No	601 (96.3)
Yes	23 (3.7)
Thyroid gland surgery	
No	564 (90.4)
Yes	60 (9.6)
Clinical features	
Goiter	
Grade 1	46 (7.4)
Grade 2	38 (6.1)
Grade 3	63 (10.1)
Grade 4	401 (64.6)
Grade 5	73 (11.8)
Nodular hyperplasia	
No	483 (77.5)
Yes	140 (22.5)
Graves' ophthalmopathy	
No	397 (63.6)
Yes	227 (36.4)
Vitiligo	
No	510 (81.7)
Yes	114 (18.3)
Myxedema	
No	525 (74.3)
Yes	98 (25.7)

Abbreviations: GD, Graves, disease; GO, Graves' ophthalmopathy; SD, standard deviation; N, number.

Table 2. Distribution of *C4* polymorphisms in Graves' disease with or without ophthalmopathy.

C4 polymorphisms	GO		*P* value, total [a]	OR (95%CI), total [c]	*P* value, individual [b]	OR (95%CI), individual [c]
	No, N (%)	Yes, N (%)				
A2B2	149 (37.5)	105 (46.3)		(Reference)	0.035	1.433 (1.029-1.994)
A2B1	53 (13.4)	25 (11.0)		0.669 (0.391-1.145)	0.451	0.803 (0.484-1.333)
A3B2	37 (9.3)	27 (11.9)		1.036 (0.594-1.805)	0.338	0.761 (0.450-1.287)
A2B3	29 (7.3)	15 (6.6)	0.050	0.734 (0.375-1.437)	0.871	0.898 (0.471-1.713)
A3B1	25 (6.3)	7 (3.1)		0.397 (0.166-0.953)	0.091	0.473 (0.201-1.113)
A1B2	28 (7.1)	6 (2.6)		0.304 (0.122-0.760)	0.026	0.358 (0.146-0.878)
Other	65 (16.4)	40 (17.6)		0.865 (0.540-1.384)		
Total	397 (100.0)	227 (100.0)				

Abbreviations: GO, Graves' ophthalmology; CNV, copy number variation; OR, odds ratio; CI, confidence interval; N, number.

[a] *C4* polymorphisms between GD patients with or without GO were evaluated by Fisher's exact test using 7 × 2 contingency tables.

[b] Individual C4 polymorphism between those with or without GO were evaluated by Fisher's exact test using 2 × 2 contingency tables.

[c] Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated by applying unconditional logistic regression between GD patients with or without GO.

Table 3. Distribution of *C4* copy numbers in patients with Graves' disease with or without vitiligo/myxedema.

CNV	Vitiligo		*P* value [a]	OR (95%CI) [b]	Myxedema		*P* value [a]	OR (95%CI) [b]
	No, N (%)	Yes, N (%)			No, N (%)	Yes, N (%)		
C4								
=4	258 (50.6)	56 (49.1)	0.002	(Reference)	265 (50.5)	48 (49.0)		(Reference)
<4	97 (19.0)	37 (32.5)		1.747 (1.091-2.830)	100 (19.0)	34 (34.7)	4.900×10^{-4}	1.877 (1.143-3.082)
>4	155 (30.4)	21 (18.4)		0.624 (0.364-1.071)	160 (30.5)	16 (16.3)		0.552 (0.303-1.005)
Total	510 (100.0)	114 (100.0)			525 (100.0)	98 (100.0)		
C4A								
= 2	330 (64.7)	65 (57.0)		(Reference)	336 (64.0)	58 (59.2)		(Reference)
<2	52 (10.2)	27 (23.7)	0.001	2.646 (1.543-4.504)	57 (10.9)	22 (22.5)	0.008	2.236 (1.270-3.936)
>2	128 (25.1)	22 (19.3)		0.873 (0.516-1.475)	132 (25.1)	18 (18.4)		0.790 (0.449-1.391)
Total	510 (100.0)	114 (100.0)			525 (100.0)	98 (100.0)		
C4B								
= 2	310 (60.8)	67 (58.8)		(Reference)	317 (60.4)	59 (60.2)		(Reference)
<2	112 (22.0)	31 (27.2)	0.414	1.281 (0.794-2.064)	115 (21.9)	28 (28.6)	0.168	1.308 (0.795-2.152)
>2	88 (17.3)	16 (14.0)		0.841 (0.464-1.524)	93 (17.7)	11 (11.2)		0.636 (0.321-1.259)
Total	510 (100.0)	114 (100.0)			525 (100.0)	98 (100.0)		

Abbreviations: GO, Graves' ophthalmology; CNV, copy number variation; OR, odds ratio; CI, confidence interval; N, number.

[a] Copy numbers between patients and controls were evaluated by Fisher's exact test using 3×2 contingency tables.

[b] Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated by applying unconditional logistic regression between GD patients with or without vitiligo or myxedema.

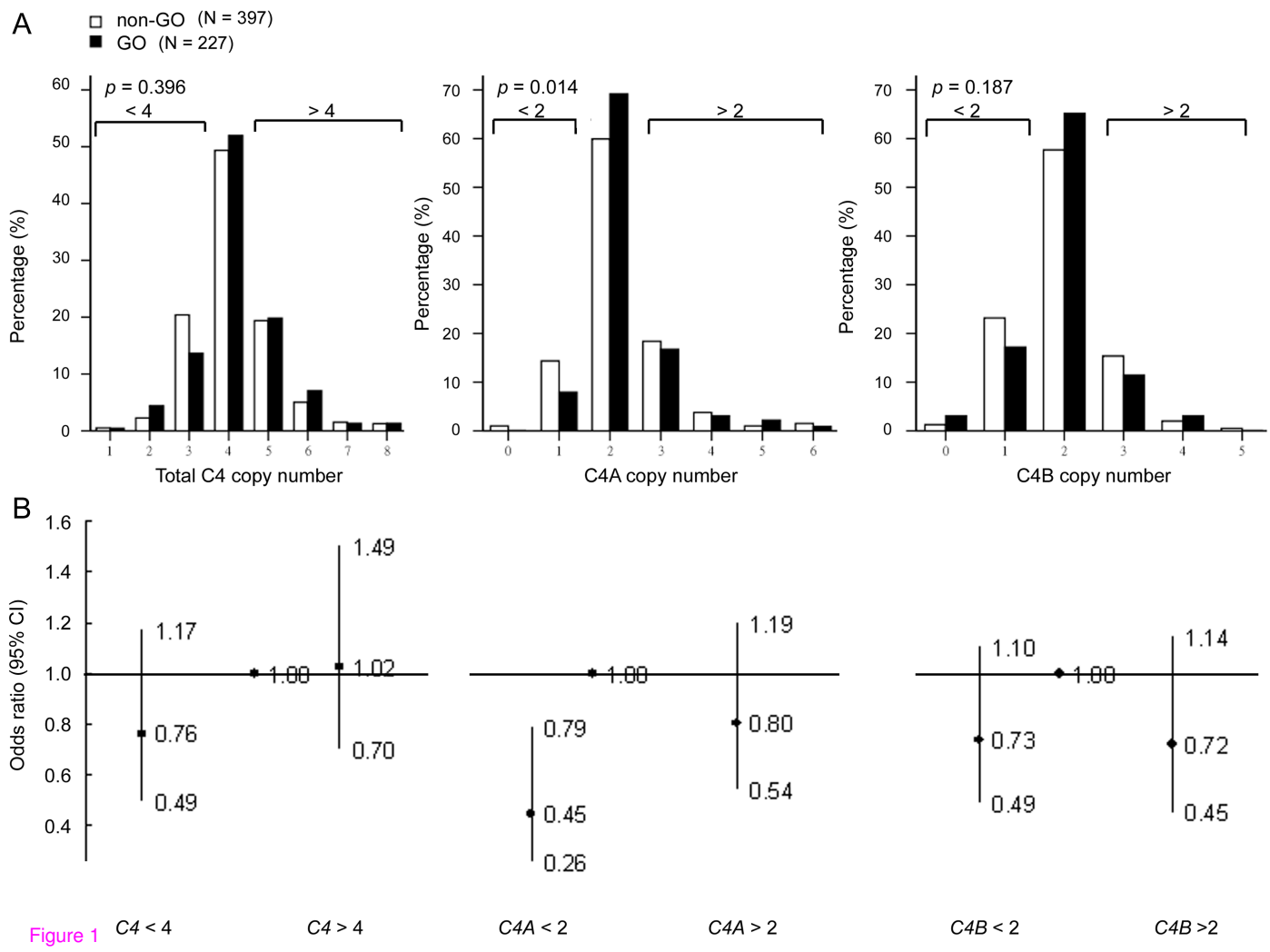
A
non-GO (N = 397)
GO (N = 227)
p = 0.396
< 4
> 4
Percentage (%)
Total C4 copy number
p = 0.014
< 2
> 2
Percentage (%)
C4A copy number
p = 0.187
< 2
> 2
Percentage (%)
C4B copy number
B
Odds ratio (95% CI)
1.17
0.76
0.49
1.00
1.49
1.02
0.70
0.79
0.45
0.26
1.00
1.19
0.80
0.54
1.10
0.73
0.49
1.00
1.14
0.72
0.45
C4 < 4
C4 > 4
C4A < 2
C4A > 2
C4B < 2
C4B >2

Figure 1