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# Association between copy number variation of complement component C4 and Graves' disease

Yu-Huei Liu<sup>1,2</sup>, Lei Wan<sup>1,3,4</sup>, Chwen-Tzuei Chang<sup>5,6</sup>, Wen-Ling Liao<sup>1</sup>, Wen-Chi Chen<sup>2</sup>, Yuhsin Tsai<sup>3</sup>, Chang-Hai Tsai<sup>7,8</sup> and Fuu-Jen Tsai<sup>1,3,7,9,10,11\*</sup>

## 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 effector in peptide-mediated inflammation and phagocytosis, we hypothesized that C4 genetic diversity may partially explain the development of Graves' disease (GD) and the variation in its outcomes.

**Methods:** A case-control study including 624 patients with GD and 160 healthy individuals were enrolled. CNV of C4 isotypes (C4A and C4B) genes were performed by quantitative real-time polymerase chain reaction analysis. Statistical comparison and identification of CNV of total C4, C4 isotypes (C4A and C4B) and C4 polymorphisms were estimated according to the occurrence of GD and its associated clinical features.

**Results:** Individuals with 4, 2, and 2 copies of C4, C4A and C4B genes, especially those with A2B2 polymorphism may associate with the development of GD ( $p = 0.001$ , OR = 10.994, 95% CI: 6.277-19.255;  $p = 0.008$ , OR = 1.732, 95% CI: 1.190-2.520;  $p = 2.420 \times 10^{-5}$ , OR = 2.621, 95% CI: 1.791-3.835; and  $p = 1.395 \times 10^{-4}$ , OR = 2.671, 95% CI: 1.761-4.052, respectively). Although the distribution of copy number for total C4, C4 isotypes as well as C4 polymorphisms did not associate with the occurrence of goiter, nodular hyperplasia, GO and myxedema, <2 copies of C4A may associate with high risk toward vitiligo in patients with GD ( $p = 0.001$ , OR = 5.579, 95% CI: 1.659-18.763).

**Conclusions:** These results may be further estimated for its clinical application on GD and the vitiligo in patients with GD.

## Background

Graves' disease (GD) is an organ-specific autoimmune thyroid disease [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 [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, 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 development of GD as well as the variation in its outcomes. Here we investigated the polymorphic variants of C4 that correlate with predisposition to this disease.

\* Correspondence: d0704@mail.cmuh.org.tw

<sup>1</sup>Department of Medical Genetics and Medical Research, China Medical University Hospital, Taichung, Taiwan

Full list of author information is available at the end of the article

## Methods

### Patients and healthy individuals

A total of 624 patients (227 with GO and 397 without GO) with a confirmed diagnosis of GD and an appropriate control group with 160 healthy volunteers from China Medical University Hospital in Taiwan were enrolled and followed actively. All individuals provided informed consent as approved by the ethics committee of China Medical University Hospital. For the patients, 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. For the healthy individuals, those with matched for gender according to the female predominance of GD including 32 male (20.0%) and 128 female (80.0%). Age was different in healthy ( $27.4 \pm 6.4$  years) as compared to the patients with GD ( $41.1 \pm 12.9$  years) ( $p = 1.96 \times 10^{-34}$ ).

### 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<sup>®</sup> 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<sup>®</sup> Universal PCR Master Mix, No AmpErase<sup>®</sup> uracil-DNA glycosylase (ABI catalog number 4326614), VIC-conjugated TaqMan<sup>®</sup> *RNase P* control reagents (ABI catalog number 4316844), 250 nM of the respective FAM-conjugated TaqMan<sup>®</sup> 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  $nRAW_{C4X} = 2^{-(C_{TRNase P} - (C_{TC4X}) + 1)}$ , 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.). The demographics of patients and healthy individuals were analyzed by the chi-square analysis. For those with  $2 \times 2$  contingency tables, differences in the incidence of individuals with *C4* gene CNs above and below the median or *C4A-C4B* polymorphisms between patients with or without indicated feature were evaluated using Fisher's exact test. For those above  $2 \times 2$  contingency tables, differences in the incidence of individuals with *C4* gene CNs above and below the median or *C4A-C4B* polymorphisms between patients with or without indicated feature were evaluated using Fisher's exact test, and the two-tailed  $p$  value was estimated by 100,000 Monte Carlo simulations with 99% confidence intervals (CI) (99% confidence for the simulation result). Odds ratios (ORs) and 95% CIs were estimated from logistic regression models adjusting for confounding variables as shown in Table 1.

## Results

### CNV of *C4* genes is associated with susceptibility to GD

The distribution of copy number for total *C4*, *C4* isotypes as well as *C4* polymorphisms according to the presence of GD is shown in Table 2. No individuals had a full deficiency of *C4* alleles. After adjusting for age, individuals with 4 copies of *C4* gene were more susceptible to GD ( $p = 0.001$ , OR = 10.994, 95% CI: 6.277-19.255) as compared to those without, whereas those with <4 copies of *C4* gene tended to prevent from GD ( $p = 0.003$ , OR = 0.512, 95% CI: 0.338-0.776) as compared to those without. The distribution of *C4A* and *C4B* among individuals with or without GD was further investigated. For *C4A* gene, individuals with 2 copies of *C4A* increased the risk toward GD ( $p = 0.008$ , OR = 1.732, 95% CI: 1.190-2.520) whereas those with <2 copies of *C4A* reduced the risk toward GD ( $p = 0.01$ , OR = 0.584, 95% CI: 0.360-0.948). For *C4B* gene, individuals with 2 copies of *C4B* increased the risk toward GD ( $p = 2.420 \times 10^{-5}$ , OR = 2.621, 95% CI: 1.791-3.835) whereas those without 2 copies of *C4B* reduced the risk toward GD ( $p = 0.008$ , OR = 0.487, 95% CI: 0.322-0.738 for those with <2 copies *C4B*;  $p = 0.015$ , OR = 0.545, 95% CI: 0.347-0.856 for those with >2 copies *C4B* respectively). Polymorphism analysis indicated that individuals with the most common polymorphism (37.3%), A2B2, with 2.671-fold risk toward GD ( $p = 1.395 \times 10^{-4}$ , OR = 2.671, 95% CI: 1.761-4.052) as compared to

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

Patients' characteristics	Healthy (160)	GD (624)	Myxedema		P-value	GO		P-value	Vitiligo		P-value
			No	Yes		No	Yes		No	Yes	
<b>Age at diagnosis</b>											
≤ 40	145 (90.6)	307 (49.2)	247 (47.0)	59 (60.2)	0.017	182 (45.8)	125 (55.1)	0.027	239 (46.9)	68 (59.6)	0.014
> 40	15 (9.4)	317 (50.8)	278 (53.0)	39 (39.8)		215 (54.2)	102 (44.9)		271 (53.1)	46 (40.4)	
<b>Gender</b>											
Male	32 (20.0)	133 (21.3)	110 (21.0)	22 (22.4)	0.739	74 (18.6)	59 (26.0)	0.031	107 (21.0)	26 (22.8)	0.700
Female	128 (80.0)	491 (78.7)	415 (79.0)	76 (77.6)		323 (81.4)	168 (74.0)		403 (79.0)	88 (77.2)	
<b>Treatment</b>											
Radioiodine											
No		601 (96.3)	504 (96.0)	96 (98.0)	0.345	389 (98.0)	212 (93.4)	0.003	489 (95.9)	112 (98.2)	0.226
Yes		23 (3.7)	21 (4.0)	2 (2.0)		8 (2.0)	15 (6.6)		21 (4.1)	2 (1.8)	
Thyroid gland surgery											
No		564 (90.4)	472 (89.9)	91 (92.9)	0.363	363 (91.4)	201 (88.5)	0.239	457 (89.6)	107 (93.9)	0.164
Yes		60 (9.6)	53 (10.1)	7 (7.1)		34 (8.6)	26 (11.5)		53 (10.4)	7 (6.1)	
<b>Clinical features</b>											
Goiter											
Grade 1-3		146 (23.5)	119 (22.8)	27 (27.6)	0.309	101 (25.5)	46 (20.4)	0.154	117 (23.1)	30 (26.3)	0.462
Grade 4-5		474 (76.5)	403 (77.2)	71 (72.4)		295 (74.5)	179 (79.6)		390 (76.9)	84 (73.7)	
Nodular hyperplasia											
No		483 (77.5)	434 (82.7)	49 (50.5)	$2.880 \times 10^{-12}$	301 (75.8)	182 (80.5)	0.175	430 (84.3)	53 (46.9)	$6.670 \times 10^{-18}$
Yes		140 (22.5)	91 (17.3)	49 (49.5)		96 (24.2)	44 (19.5)		80 (15.7)	60 (53.1)	
Myxedema											
No		525 (74.3)				305 (76.8)	220 (97.3)	$1.35 \times 10^{-11}$	507 (99.4)	18 (15.9)	$8.900 \times 10^{-8}$
Yes		98 (25.7)					92 (23.2)	6 (2.7)		3 (0.6)	95 (84.1)
Graves' ophthalmopathy											
No		397 (63.6)	305 (58.0)	92 (93.9)	$1.350 \times 10^{-11}$				295 (57.8)	102 (89.5)	$2.200 \times 10^{-10}$
Yes		227 (36.4)	220 (41.9)	6 (6.1)					215 (42.2)	12 (10.5)	
Vitiligo											
No		510 (81.7)	507 (96.6)	3 (3.1)	$8.900 \times 10^{-8}$	295 (74.3)	215 (94.7)	$2.204 \times 10^{-10}$			
Yes		114 (18.3)	18 (3.4)	95 (96.9)		102 (25.7)	12 (5.3)				

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

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

Variations	GD		P value, individual <sup>a</sup> [OR (95%CI), individual] <sup>c</sup>	P value <sup>b</sup>	OR (95%CI) <sup>d</sup>
	No, N (%)	Yes, N (%)			
<b>C4 CNV</b>					
4	57 (35.6)	314 (50.3)	0.001 [10.994 (6.277-19.255)]	0.002	(Reference)
< 4	52 (32.5)	134 (21.5)	0.003 [0.512 (0.338-0.776)]		0.389 (0.245-0.615)
> 4	51 (31.9)	176 (28.2)	0.361		0.497 (0.317-0.780)
<b>C4A CNV</b>					
2	83 (51.9)	395 (63.3)	0.008 [1.732 (1.190-2.520)]	0.011	(Reference)
< 2	33 (20.6)	79 (12.7)	0.010 [0.584 (0.360-0.948)]		0.509 (0.307-0.843)
> 2	44 (27.5)	150 (24.0)	0.365		0.628 (0.404-0.977)
<b>C4B CNV</b>					
2	67 (41.9)	377 (60.4)	$2.420 \times 10^{-5}$ [2.621 (1.791-3.835)]	$1.328 \times 10^{-4}$	(Reference)
< 2	53 (33.1)	143 (22.9)	0.008 [0.487 (0.322-0.738)]		0.374 (0.240-0.584)
> 2	40 (25)	104 (16.7)	0.015 [0.545 (0.347-0.856)]		0.391 (0.241-0.636)
<b>C4 polymorphisms</b>					
A2B2	39 (24.4)	254 (40.7)	$1.395 \times 10^{-4}$ [2.671 (1.761-4.052)]	$3.87 \times 10^{-5}$	(Reference)
A2B1	22 (13.8)	78 (12.5)	0.672		0.409 (0.219-0.763)
A3B2	16 (10.0)	64 (10.3)	0.924		0.539 (0.273-1.064)
A2B3	5 (3.1)	44 (7.1)	0.067		0.961 (0.343-2.697)
A3B1	10 (6.3)	32 (5.1)	0.574		0.373 (0.159-0.876)
A1B2	6 (3.8)	34 (5.4)	0.384		0.687 (0.257-1.836)
Other	62 (38.8)	118 (18.9)			0.242 (0.148-0.396)

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

<sup>a</sup>Individual C4 CNVs and polymorphisms between individuals with or without GD were evaluated by Fisher's exact test using 2 × 2 contingency tables.

<sup>b</sup> CNV of C4, C4A and C4B between individuals with or without GD were evaluated by Fisher's exact test using 3 × 2 contingency tables. C4 polymorphisms between individuals with or without GD were evaluated by Fisher's exact test using 7 × 2 contingency tables. The p value was estimated by 100,000 Monte Carlo simulations with 99% confidence intervals (CI).

<sup>c</sup>ORs and 95% CIs were estimated from logistic regression models adjusting for age.

<sup>d</sup>ORs and 95% CIs were estimated from logistic regression models adjusting for age.

those without. These results indicate that individuals with 4, 2 and 2 copies of C4, C4A and C4B genes, especially those with A2B2 polymorphism may have higher risk, whereas those with <4, <2 and ≠2 copies of C4, C4A and C4B genes may have lower risk toward GD, respectively.

#### CNV of C4 genes did not significantly associated with myxedema and GO

We also estimated the association between polymorphism of C4 genes and clinical features of GD. CNV of C4 genes showed association with susceptibility toward GO, vitiligo and myxedema, but not goiter or nodular hyperplasia as estimated by Fisher's exact test (data not shown). After adjusting for age, nodular hyperplasia, GO, and vitiligo, the distribution of copy number for total C4, C4 isotypes as well as C4 polymorphisms did not associate with the occurrence of myxedema (Table 3).

The distribution of copy number for total C4, C4 isotypes as well as C4 polymorphisms according to the presence of GO is shown in Table 4. 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. After adjusting for age, gender, radioiodine treatment, vitiligo and myxedema, neither isotypes nor polymorphisms of C4 was significantly associated with GO, although GD patients with <2 copies (0 or 1) of the C4A gene were less susceptible to GO (p = 0.014, OR = 0.549, 95% CI: 0.303-0.998) as compared to those with 2 copies of C4A, and those with A3B1 polymorphism were less susceptible to GO (p = 0.001, OR = 0.374, 95% CI: 0.146-0.960) as compared to those with A2B2 polymorphism. These results indicate that neither isotypes nor polymorphisms of C4 was significantly associated with GO, however, as compared to GD patients with 2 copies of C4A or those with A2B2 polymorphism, those with <2 copies of C4A or those with A3B1 might be protected against the development of GO, respectively.

#### GD patients with <2 copies of C4A had higher risk toward vitiligo

The distribution of copy number for total C4, C4 isotypes as well as C4 polymorphisms according to the presence of vitiligo is shown in Table 4. After adjusting with age, nodular hyperplasia, GO and myxedema, patients with

**Table 3 Distribution of C4 polymorphisms in Graves' disease patients with or without myxedema**

Variations	Myxedema		P value, individual <sup>a</sup> [OR (95%CI), individual] <sup>c</sup>	P value <sup>b</sup>	OR (95%CI) <sup>d</sup>
	No, N (%)	Yes, N (%)			
<b>C4 CNV</b>					
4	265 (50.5)	48 (49.0)	0.826		(Reference)
< 4	100 (19.0)	34 (34.7)	0.001 [1.884 (0.538-6.597)]	4.900 × 10 <sup>-4</sup>	1.714 (0.447-6.575)
> 4	160 (30.5)	16 (16.3)	0.005 [0.617 (0.166-2.289)]		0.761 (0.186-3.122)
<b>C4A CNV</b>					
2	336 (64.0)	58 (59.2)	0.364		(Reference)
< 2	57 (10.9)	22 (22.5)	0.003 [0.627 (0.164-2.404)]	0.008	0.511 (0.122-2.134)
> 2	132 (25.1)	18 (18.4)	0.159		0.496 (0.117-2.106)
<b>C4B CNV</b>					
2	317 (60.4)	59 (60.2)	1		(Reference)
< 2	115 (21.9)	28 (28.6)	0.152	0.168	1.163 (0.298-4.542)
> 2	93 (17.7)	11 (11.2)	0.072		0.552 (0.125-2.443)
<b>C4 polymorphisms</b>					
A2B2	217 (41.3)	36 (36.7)	0.434	0.050	(Reference)
A2B1	63 (12.0)	15 (15.3)	0.405		1.895 (0.307-11.710)
A3B2	58 (11.0)	6 (6.1)	0.202		1.371 (0.163-11.522)
A2B3	41 (7.8)	3 (3.1)	0.130		0.558 (0.029-10.789)
A3B1	26 (5.0)	6 (6.1)	0.619		0.333 (0.032-3.499)
A1B2	23 (4.4)	11 (11.2)	0.013 [1.094 (0.137-8.709)]		1.009 (0.103-9.841)
Other	97 (18.5)	21 (21.4)			0.735 (0.163-3.310)

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

<sup>a</sup>Individual C4 CNVs and polymorphisms between GD patients with or without myxedema were evaluated by Fisher's exact test using 2 × 2 contingency tables.

<sup>b</sup>CNV of C4, C4A and C4B between GD patients with or without myxedema were evaluated by Fisher's exact test using 3 × 2 contingency tables. C4 polymorphisms between GD patients with or without myxedema were evaluated by Fisher's exact test using 7 × 2 contingency tables. The p value was estimated by 100,000 Monte Carlo simulations with 99% confidence intervals (CI).

<sup>c</sup>ORs and 95% CIs were estimated from logistic regression models adjusting for age, nodular hyperplasia, GO and vitiligo.

<sup>d</sup>ORs and 95% CIs were estimated from logistic regression models adjusting for age, nodular hyperplasia, GO and vitiligo.

<2 copies of C4A had a 5.153-fold increased risk of vitiligo ( $p = 2.650 \times 10^{-4}$ , OR = 5.153, 95% CI: 1.629-16.300). It remained significant even when compared with GD patients with 2 copies of C4A ( $p = 0.001$ , OR = 5.579, 95% CI: 1.659-18.763, Table 5). These results indicate that <2 copies of C4A may increase the risk for vitiligo 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 the CNV of C4, C4A or C4B may associate with the development of GD. In addition, <2 copies of C4A may associate with development of vitiligo in patients with GD. To the best of our knowledge, this is the first study to report that the linkage among CNV of C4 genes, GD and GD-associated vitiligo. Our results provide new information which may be applied clinically.

C4 involves in the classical pathway which is triggered by interaction of the Fc portion of an antibody or C-reactive protein with C1q. It has been shown that the copy number of C4, C4A or C4B positively correlated with the

protein levels of total C4, C4A or C4B, respectively [7]. In our results, individuals with 4, 2, and 2 copies of C4, C4A or C4B have higher risk whereas those with deficiencies of C4, C4A or C4B have lower risk toward GD. One possibility is that a deficiency of complement may lead to ineffective opsonization, lytic activity or impairment of B-cell memory, by which reduce tissue injury [15]. 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 GD needs to be further investigated [16]. This study provides a substantial amount of data that may 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.

However, it may play a different regulatory role in systemic autoimmune diseases. Low level of C4 complements in sera has been found in several autoimmune diseases [17-21]. In addition, the presence of C4A null

**Table 4 Distribution of C4 polymorphisms in Graves' disease patients with or without Graves' ophthalmopathy**

Variations	GO		P value, individual <sup>a</sup> [OR (95%CI), individual] <sup>c</sup>	P value <sup>b</sup>	OR (95%CI) <sup>d</sup>
	No, N (%)	Yes, N (%)			
<b>C4 CNV</b>					
4	196 (49.4)	118 (52.0)	0.561	0.396	(Reference)
< 4	92 (23.2)	42 (18.5)	0.188		0.978(0.614-1.558)
> 4	109 (27.5)	67 (29.5)	0.581		1.029(0.687-1.540)
<b>C4A CNV</b>					
2	238 (39.9)	157 (69.2)	0.025 [1.436 (0.994-2.075)]	0.014	(Reference)
< 2	61 (15.4)	18 (7.9)	0.008 [0.590 (0.328-1.059)]		0.549 (0.303-0.998)
> 2	98 (24.7)	52 (22.9)	0.628		0.772 (0.509-1.169)
<b>C4B CNV</b>					
2	229 (57.7)	148 (65.2)	0.074	0.186	(Reference)
< 2	97 (24.4)	46 (20.3)	0.276		0.806 (0.520-1.249)
> 2	71 (17.9)	33 (14.5)	0.316		0.697 (0.430-1.132)
<b>C4 polymorphisms</b>					
A2B2	149 (37.5)	105 (46.3)	0.035 [1.283 (0.900-1.828)]	0.005	(Reference)
A2B1	53 (13.4)	25 (11.0)	0.451		0.796 (0.449-1.411)
A3B2	37 (9.3)	27 (11.9)	0.338		1.067 (0.596-1.912)
A2B3	29 (7.3)	15 (6.6)	0.871		0.734 (0.366-1.476)
A3B1	25 (6.3)	7 (3.1)	0.091		0.374 (0.146-0.960)
A1B2	28 (7.1)	6 (2.6)	0.026 [0.451(0.176-1.160)]		0.374 (0.153-1.056)
Other	65 (16.4)	40 (17.6)			0.894 (0.549-1.455)

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

<sup>a</sup>Individual C4 CNVs and polymorphisms between GD patients with or without GO were evaluated by Fisher's exact test using 2 × 2 contingency tables.

<sup>b</sup> CNV of C4, C4A and C4B between GD patients with or without GO were evaluated by Fisher's exact test using 3 × 2 contingency tables. C4 polymorphisms between GD patients with or without GO were evaluated by Fisher's exact test using 7 × 2 contingency tables. The p value was estimated by 100,000 Monte Carlo simulations with 99% confidence intervals (CI).

<sup>c</sup>ORs and 95% CIs were estimated from logistic regression models adjusting for age, gender, ever received radioiodine treatment, myxedema and vitiligo.

<sup>d</sup>ORs and 95% CIs were estimated from logistic regression models adjusting for age, gender, ever received radioiodine treatment, myxedema and vitiligo.

allele that results in partial C4 deficiency have shown to be risk factor for susceptibility in systemic lupus erythematosus (SLE) and the SLE-related renal damage [7,19]. A hypothesis is that complement may participate in the presentation of self-antigens to developing B cells by which protects against responses to self-antigens and subsequent promoting the elimination of self-reactive lymphocytes [9]. The pathogenesis of vitiligo, similar to SLE, is characterized by the destruction of cutaneous melanocytes which due to another antibody-induced hypopigmentation. Experiments in knockout mice have demonstrated that complement deficient can cause the destruction of pigment cells leading to vitiligo-like depigmentation [21]. Our results revealed that deficiency of C4A may enhance the development of vitiligo in GD patients, implying exist of an alternative pathway for the deficiency of complement.

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. 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 may be more relevant to the existence of the immune complex than the pathogen. In addition, 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 [22,23]. It may be interesting to investigate the gene-gene interaction between C4 polymorphism and the vitiligo risky locus. Moreover, although confirmation of these results in larger samples is warranted, it would be interesting to further investigate the functional role of C4A in the development of vitiligo.

## Conclusion

This study provides evidence that the CNV of C4, C4A or C4B may associate with the development of GD and <2 copies of C4A may associate with development of vitiligo in patients with GD. These results may be further estimated for its application on predicting the occurrence of GD and the clinical outcome in patients

**Table 5 Distribution of C4 polymorphisms in Graves' disease patients with or without vitiligo**

Variations	Vitiligo		P value, individual <sup>a</sup> [OR (95%CI), individual] <sup>c</sup>	P value <sup>b</sup>	OR (95%CI) <sup>d</sup>
	No, N (%)	Yes, N (%)			
<b>C4 CNV</b>					
4	258 (50.6)	56 (49.1)	0.836	0.002	(Reference)
< 4	97 (19.0)	37 (32.5)	0.002 [1.297 (0.434-3.874)]		1.334 (0.415-4.289)
> 4	155 (30.4)	21 (18.4)	0.011 [0.987 (0.362-2.691)]		1.076 (0.370-3.133)
<b>C4A CNV</b>					
2	330 (64.7)	65 (57.0)	0.133		(Reference)
< 2	52 (10.2)	27 (23.7)	2.650 × 10 <sup>-4</sup> [5.153 (1.629-16.3000)]	0.001	5.579 (1.659-18.763)
> 2	128 (25.1)	22 (19.3)	0.225		1.289 (0.414-4.013)
<b>C4B CNV</b>					
2	310 (60.8)	67 (58.8)	0.751		(Reference)
< 2	112 (22.0)	31 (27.2)	0.267	0.414	1.133 (0.355-3.614)
> 2	88 (17.3)	16 (14.0)	0.487		2.107 (0.687-6.467)
<b>C4 polymorphisms</b>					
A2B2	213 (41.8)	41 (36.0)	0.292	0.03	(Reference)
A2B1	62 (12.2)	16 (14.0)	0.638		0.889 (0.175-4.524)
A3B2	57 (11.2)	7 (6.1)	0.125		0.756 (0.132-4.335)
A2B3	40 (7.8)	4 (3.5)	0.154		1.111 (0.151-8.205)
A3B1	25 (4.9)	7 (6.1)	0.638		1.484 (0.199-11.089)
A1B2	22 (4.3)	12 (10.5)	0.019 [2.035(0.335-12.368)]		2.745 (0.384-19.599)
Other	91 (17.8)	27 (23.7)			3.471 (1.046-11.525)

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

<sup>a</sup>Individual C4 CNVs and polymorphisms between GD patients with or without vitiligo were evaluated by Fisher's exact test using 2 × 2 contingency tables.

<sup>b</sup>CNV of C4, C4A and C4B between GD patients with or without vitiligo were evaluated by Fisher's exact test using 3 × 2 contingency tables. C4 polymorphisms between GD patients with or without vitiligo were evaluated by Fisher's exact test using 7 × 2 contingency tables. The p value was estimated by 100,000 Monte Carlo simulations with 99% confidence intervals (CI).

<sup>c</sup>ORs and 95% CIs were estimated from logistic regression models adjusting for age, nodular hyperplasia, myxedema and GO.

<sup>d</sup>ORs and 95% CIs were estimated from logistic regression models adjusting for age, nodular hyperplasia, myxedema and GO.

with GD which might aid in the diagnosis of the disease and the development of therapeutic strategies.

#### List of abbreviations

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

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#### Author details

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

University, Taichung, Taiwan. <sup>11</sup>Department of Biotechnology and Bioinformatics, Asia University, Taichung, Taiwan.

#### Authors' contributions

YHL designed the study, managed the literature searches, undertook the statistical analysis, and wrote the draft of the manuscript. LW designed and performed the experiments. CTC and WCC recruited and maintained the clinical information of participants. LWLL and TYT undertook the statistical analysis. CHT and FJT directed the study and reviewed the results. All authors contributed to and have approved the final manuscript.

#### Competing interests

The authors declare that they have no competing interests.

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