Genomic Copy Number Variations of the Complement Component C4B Gene Are Associated With Chronic Central Serous Chorioretinopathy

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PURPOSE. Chronic central serous chorioretinopathy (cCSC) has recently been associated to variants in the complement factor H gene. To further investigate the role of the complement system in cCSC, the genomic copy number variations in the complement component 4 gene (C4) were studied.

METHODS. C4A and C4B copy numbers were analyzed in 197 cCSC patients and 303 healthy controls by using a Taqman copy number determination assay. Copy numbers of C4A, C4B, and the total C4 load were compared between cases and controls, by using a Fisher exact test. For this analysis Bonferroni correction was performed for three tests, and P values <0.017 were considered to be significant. A logistic regression model was constructed to calculate the odds ratios (ORs) of each of the C4B copy numbers, using two copies as a reference. For this model P values <0.05 were considered to be significant.

RESULTS. C4B genomic copy numbers differed significantly between cCSC patients and healthy controls (P = 0.0018). Absence of C4B significantly conferred risk of cCSC (OR = 2.61 [95% confidence interval (CI) = 1.05–6.52]), whereas three copies of C4B significantly decreased the risk of cCSC (P = 0.014, OR = 0.45 [95% CI = 0.23–0.85]). The C4A genomic copy numbers and total C4 load did not significantly differ between cases and controls.

CONCLUSIONS. This study showed that copy numbers of C4B are significantly associated with cCSC. Carrying no copies of C4B significantly increases the risk of cCSC, whereas carrying three C4B copies is protective. These findings reinforce the hypothesis of a possible involvement of the complement system in the pathogenesis of cCSC.

Keywords: chronic central serous chorioretinopathy, cCSC, complement component 4, C4, C4A, C4B

Chronic central serous chorioretinopathy (cCSC) is characterized by fluid accumulation under the neuroretina. It has been postulated that this serous fluid derives from the choroid and that it leaks through a dysfunctional retinal pigment epithelium, causing a detachment of the neuroretina.1–4 Classically, cCSC patients are relatively young (middle-aged) men who are still professionally active.5 Besides male sex, also the use of corticosteroids, type A personality, and stress have been associated with cCSC.4,6 Although the exact pathophysiological mechanism of the disease remains unknown, we and others have previously suggested involvement of the complement system, and in particular the complement factor H gene (CFH) in cCSC.7,8 Interestingly, CFH variants that confer increased risk in cCSC have previously been described to be protective in age-related macular degeneration, and vice versa.8–10

The complement system consists of three major pathways: the classical, the lectin, and the alternative pathway.11 CFH is involved in the alternative pathway and can influence C3b production by blocking one of the two C3-convertases (C3bBb). The classical and lectin pathways also play an important role in C3b production mediated by the other C3-convertase (C4b2a), of which the complement component 4 (C4) protein is a key factor.12

Copy number variations of the C4 gene have been associated with several eye diseases and autoimmune disorders (e.g., Vogt-Koyanagi-Harada and Behçet’s disease).13–15 In addition, Banlaki et al.16 have found that the genomic copy number of C4B is associated with cortisol release after adrenocorticotropic hormone (ACTH) stimulation. This is of particular interest considering that stress, which appears to be associated with cCSC,4 has a strong influence on the hypothalamic–pituitary–adrenal (HPA) axis and increases ACTH release.17

Because of its role in the complement system, and its association with the HPA axis, we hypothesized that copy number variation in the C4 gene may be associated with cCSC. In this study we assessed the copy number variations of the C4 gene in a cCSC cohort.
**Subjects**

In this study, 197 patients diagnosed with cCSC who visited the outpatient clinic of the Department of Ophthalmology at the Radboud University Medical Center, Nijmegen, the Netherlands, were included (Table 1). The diagnosis cCSC was based on an extensive ophthalmologic examination including fundoscopy, spectral-domain optical coherence tomography, fluorescein angiography, and indocyanine green angiography. The definition of typical cCSC used in this study was based on the previously published subgroups by de Jong et al., and patients in this study underwent phenotyping by an experienced retina specialist (CJFB) (Fig. 1A–F). Additionally, a total of 303 control subjects were recruited from the blood bank of the Radboud University Medical Center (n = 154) and the European Genetic Database (EUGENDA, www.eugenda.org; provided in the public domain by the University Hospital of Cologne, Cologne, Germany and the Radboud University Medical Center) (n = 149) (Table 1). For this last group, fundus photographs were graded to rule out any ophthalmologic abnormalities at the moment of inclusion. Informed consent for the use of DNA for genetic studies was obtained from all subjects. This study followed the guidelines of the Declaration of Helsinki and was approved by the local ethics committee.

**Copy Number Determination**

DNA was isolated from peripheral blood by using standard procedures. C4A and C4B copy numbers were determined by real-time PCR using Taqman genotyping assays (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The FAM-labeled C4A (Hs072265349_cn) or C4B (Hs072265350_cn) Taqman copy number assay was combined with the VIC-labeled Ribonuclease P (RNaseP) reference assay (catalog No. 4403326), and Taqman genotyping mastermix (catalog No. 4381656). All samples were tested in duplicate for C4A and C4B on 384-wells plates by using 10 ng DNA in a total reaction volume of 10 µL.

Samples with known copy numbers for either C4A (0–4) or C4B (0–3) were kindly provided by C. Yung Yu. These samples were included as a reference on each plate to facilitate accurate copy number determination, using the method described previously. In each run the amplification efficiencies of the C4A/C4B and RNaseP probes were calculated by using a serial dilution (50–1.56 ng) of a sample with two C4A and C4B copies. The primer efficiencies of the probes were compared and deemed similar if they differed <2%; this was the case in all runs. Therefore, the efficiencies were not incorporated into the calculations of the copy numbers. Polymerase chain reaction was performed with a 7900HT thermocycler (Applied Biosystems, Thermo Fisher Scientific) using the following program: 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Data were analyzed with the Copycaller software (V2.0; Applied Biosystems, Thermo Fisher Scientific). Copy numbers determined by the Copycaller software were corrected by using the trend line based on the reference samples, as described before. If the results were inconsistent between the Copycaller output and the values corrected with the trend line, samples were retested on a new plate in triplicate.

**Statistics**

The comparison of C4A, C4B, and total C4 copy number distribution between cCSC patients and controls was performed by means of a Fisher exact test using SPSS Statistics (V20; IBM Corp., Armonk, NY, USA). Bonferroni correction for multiple testing was performed for three tests and P values < 0.017 were considered to be statistically significant. A logistic regression model was constructed to determine the odds ratios (ORs) for the various copy numbers of C4B. According to previously published studies, two genomic copy numbers of C4B are considered to be most common in the healthy population. We were able to confirm this in our cohort, and therefore this copy number was set as reference. In this model, P values < 0.05 were considered to be significant. Graphs were generated by using Graphpad Prism (V5; Graphpad Software, San Diego, CA, USA).

**RESULTS**

The copy numbers of C4A and C4B were successfully determined in 197 cCSC cases and 303 controls. No significant difference was observed between cases and controls for the C4A genomic copy number (range: 0–6, P = 0.649; Fig. 2A).

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**Table 1. Demographics of the Study Population**

<table>
<thead>
<tr>
<th></th>
<th>cCSC Patients</th>
<th>Controls</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>197</td>
<td>303</td>
<td>NA</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>154/43</td>
<td>226/77</td>
<td>0.392</td>
</tr>
<tr>
<td>Age, mean ± SD, y</td>
<td>53 ± 10</td>
<td>53 ± 11</td>
<td>0.755</td>
</tr>
<tr>
<td>Age range, y</td>
<td>29–74</td>
<td>29–77</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, not annotated; SD, standard deviation.
The $C4B$ distribution was significantly different between cCSC patients and controls (range: 0–4, $P = 0.0018$; Fig. 2B). Overall, cases carried lower copy numbers of $C4B$ than the control population. The total $C4$ genomic copy number was not different in cases compared to controls ($P = 0.148$; Fig. 2C). Age and sex were not associated with $C4A$, $C4B$, or total $C4$ genomic copy number (Table 2; data for $C4A$ and total $C4$ not shown).

To assess the effect size of the different copy numbers of $C4B$ on development of cCSC, a logistic regression was performed (Table 2). The logistic regression model based on the distribution of $C4B$ between cases and controls was significant ($P = 0.0035$; Table 2). Carrying no copies of $C4B$ conferred increased risk of cCSC ($P = 0.059$, OR = 2.61, 95% confidence interval [CI] = 1.05–6.52). A similar trend was observed for carriers of one copy of $C4B$, but the results were not significant ($P = 0.080$, OR = 1.47, 95% CI = 0.96–2.26). Carrying three $C4B$ copies was associated with a significantly decreased risk of cCSC ($P = 0.014$, OR = 0.45, 95% CI = 0.24–0.85), whereas no significant association with cCSC was observed in individuals carrying four copies of $C4B$ ($P = 0.81$).

**DISCUSSION**

Our study results demonstrated that cCSC patients have a significantly different $C4B$ load as compared to healthy controls ($P = 0.0018$). Carrying no copies of $C4B$ was associated with an increased risk of cCSC (OR = 2.61, 95% CI = 1.05–6.52), whereas carrying three $C4B$ copies was associated with a decreased risk of cCSC (OR = 0.45, 95% CI = 0.23–0.85). No association with $C4C$ was observed in individuals carrying four $C4B$ copies, which is likely due to the limited sample size of this group (cases, $n = 4$; controls, $n = 2$). No significant differences were observed between cases and controls for $C4A$ and total $C4$ load.

The $C4$ gene lies within the $RP-C4-CYP21-TNix$ ($RCCX$) locus located in the major histocompatibility complex (MHC) region III on chromosome 6 of the human genome. The MHC region contains an elevated level of genomic copy number variations that are presumably present to increase immunologic diversity. Duplications and deletions in the region have led to the formation of haplotypes containing variable copies of the $RCCX$ locus in the human population (Fig. 3B). Haplotypes containing two or more duplications of the $RCCX$ locus show extensive variability in their gene content, generally with complete duplications of the $C4$ gene (Fig. 3B). The $C4$ gene encodes the $C4$ protein, of which two variants have been described ($C4A$/$C4B$), differing in only four amino acids encoded by exon 26 (Fig. 3A).

Copy number variations of either $C4A$ or $C4B$ have been associated with several systemic diseases with ocular involvement, such as Vogt-Koyanagi-Harada disease, Behçet’s disease, and systemic lupus erythematosus (SLE). Hou et al. have shown that a lower copy number of $C4A$ and $C4B$ increases the risk of Vogt-Koyanagi-Harada disease, an autoimmune disorder characterized by bilateral granulomatous panuveitis. The same group has also demonstrated that higher copy numbers of $C4A$ confer risk of Behçet’s disease, an autoinflammatory disease, which presents with acute anterior uveitis. Several studies have shown an association between low copy numbers of $C4A$ and an increased risk for SLE, an autoimmune disease that is typically mediated by immune complexes. In the past, CSC has been described in SLE patients, but it remains unclear whether this is a primary manifestation of SLE, or whether it is a consequence of corticosteroid treatment for SLE. Several studies have reported a positive linear correlation between serum $C4$ and $C4$ genomic copy number, suggesting that the lower number of $C4B$ copies in cCSC patients leads to lower systemic $C4B$ levels. This may indicate that an overall lower activity of the complement system might be present in cCSC patients.
Recently, low copy numbers of C4B have been associated with hyperreactivity of the HPA axis. Banlaki et al. have shown that in patients with adrenal incidentaloma and low (<2 copies) C4B genomic copy number, baseline ACTH is significantly reduced as compared to high (≥2 copies) genomic copy number of C4B. Moreover, a significantly higher cortisol response is observed after ACTH stimulation in the patients with low C4B genomic copy number.

These results are of interest in the context of cCSC because of the described clinical associations with stress and the use of corticosteroids that both exert physiological effects at the level of the HPA axis. Various relatively small studies have studied cortisol levels in cCSC patients. Although 24-hour urine samples show elevated cortisol levels in cCSC patients in certain studies, these results are not observed in single samples (Center for Molecular and Human Genetics, The Research Institute, City, Ohio, USA). We thank Joannes M. M. Groenewoud, C. Yung Yu, DPhil, for providing the positive control samples (Center for Molecular and Human Genetics, The Research Institute, City, Ohio, USA). We thank Joannes M. M. Groenewoud for providing the positive control samples (Center for Molecular and Human Genetics, The Research Institute, City, Ohio, USA). We thank Joannes M. M. Groenewoud.

![A schematic illustration of the RCCX locus.](image)

**FIGURE 3.** A schematic illustration of the RCCX locus. (A) A display of the two variants of the C4 gene, differing in four amino acids in exon 26 (p.1101-1106). (B) An example of the structure of the RCCX locus with a single copy (upper panel) and two copies (lower panel) of the C4 gene. In case of duplication, a complete C4A or C4B gene and the CYP21 gene are duplicated, while the RP and TNX genes are only partially duplicated. The dotted lines indicate the approximate site where during recombination the duplication has occurred in the past (Figure based on Banlaki et al. 2013).22

**TABLE 2.** Logistic Regression Model for C4B Load

<table>
<thead>
<tr>
<th>C4B Copy Number</th>
<th>Patients, n</th>
<th>Controls, n</th>
<th>P Value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>197</td>
<td>303</td>
<td>0.605</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Sex</td>
<td>197</td>
<td>303</td>
<td>0.346</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>0</td>
<td>13</td>
<td>8</td>
<td>0.039</td>
<td>2.613</td>
<td>1.048–6.518</td>
</tr>
<tr>
<td>1</td>
<td>58</td>
<td>63</td>
<td>0.080</td>
<td>1.469</td>
<td>0.956–2.259</td>
</tr>
<tr>
<td>2</td>
<td>110</td>
<td>179</td>
<td>Ref</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>49</td>
<td>0.014</td>
<td>0.445</td>
<td>0.234–0.849</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>4</td>
<td>0.808</td>
<td>0.809</td>
<td>0.145–4.503</td>
</tr>
</tbody>
</table>

Ref, reference.

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We thank C. Yung Yu, DPhil, for providing the positive control samples (Center for Molecular and Human Genetics, The Research Institute, City, Ohio, USA). We thank Joannes M. M. Groenewoud for providing the positive control samples (Center for Molecular and Human Genetics, The Research Institute, City, Ohio, USA).
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References


