C9-R95X Polymorphism in Patients with Neovascular Age-Related Macular Degeneration

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Purpose. A non-sense mutation at codon 95 in the gene encoding complement factor C9 (C9-R95X) is found most frequently among Japanese. The authors investigated the association between C9-R95X and Japanese patients with neovascular age-related macular degeneration (AMD) and polypoidal choroidal vasculopathy (PCV).

Methods. The presence of the C9-R95X polymorphism was assessed by direct sequencing in Japanese patients with either PCV (n = 105) or neovascular AMD (n = 198) and 396 control subjects. Multivariate regression analyses were conducted. Photocoagulation was applied in the eyes of mice with a heterozygous defect in the C3 gene and control wild-type mice. Photocoagulation was also applied to wild-type mice before either anti-C9 antibody or isotype IgG was injected into the eyes. The eyes were collected later for measurement of vascular endothelial growth factor (VEGF) and histological evaluation of choroidal neovascularization (CNV).

Results. The frequency of those with one or two C9-R95X variants was lower in neovascular AMD (2.02%) than in PCV (5.71%) and controls (6.05%). The presence of C9-R95X conferred a 4.7-fold reduction (95% confidence interval, 1.2–18.1; P = 0.021) in the risk for neovascular AMD after adjusting for the major AMD risk factors. A heterozygous defect in the C3 gene was associated with the reduced growth of laser-induced CNV, as was intraocular injection of anti-C9 antibody. This reduced CNV growth was accompanied by a decreased level of secreted VEGF in the intraocular fluid.

Conclusions. These findings support the notion that the haploinsufficiency of C9, a terminal complement complex component, engenders reduced intraocular secretion of VEGF and decreased risk for CNV development. (Invest Ophthalmol Vis Sci. 2012;53:508–512) DOI:10.1167/iovs.11-8425

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Development of choroidal neovascularization (CNV) in age-related macular degeneration (AMD) is a major cause of visual loss in elderly populations of industrialized countries. Results of epidemiologic studies have shown factors such as age, sex, smoking, and genetic background to be important in association with neovascular AMD.¹,² Among the disease-associated genetic variations reported to date, many have been identified in and around complement-related genes, including genes encoding complement factor H (CFH),³,⁴ complement factor B,⁵ complement component 2 (C2),⁶ and complement component 3 (C3).⁷,⁸ In Asian countries, a condition called polypoidal choroidal vasculopathy (PCV) is often distinguished clinically from neovascular AMD without PCV (nAMD). This distinction might be important, at least for patients of East Asian origin, for two reasons. First, though nAMD and PCV share cardinal clinical features and disease-associated genes,¹⁰,¹¹ emerging evidence suggests that they could be distinguished not only by clinical features but also by responses to treatments. This suggests that some difference in basic pathomechanisms may exist between the two conditions. For example, compared with nAMD, photodynamic therapy appears to be more effective for treating PCV,¹²,¹³ whereas neutralizing vascular endothelial growth factor (VEGF), a critical regulator of angiogenesis and CNV formation,¹⁴ might be less effective in reducing vasculopathy in PCV.¹⁵,¹⁶ Second, PCV is diagnosed in 22.3% to 54.7% of neovascular AMD patients in Asian populations, thereby constituting a significant subtype of AMD, whereas it is diagnosed in only 4.0% to 9.8% of Caucasian patients in an equivalent analysis.¹¹

Complement component 9 (C9) is the most downstream component of the terminal complement complex (TCC) cascade that converges as a final effector molecule through activation of all three complement activation pathways: the classical pathway, the alternative pathway, and the lectin pathway. C9 can polymerize and form a pore on the surface cell membrane catalyzed by the TCC constituent, C5b-8.¹⁷ Because the ultrastructure TCC is partially dependent on the amount of C9 molecules relative to those of C5b-8, a significant reduction in C9 can alter the TCC stoichiometry, resulting in reduced cytolytic activity.¹⁷,¹⁸ In mice, TCC has been associated with the development of laser-induced CNV, a model for neovascular development in AMD. For example, abolishment of the gene encoding C3, a component of complement pathway upstream of C9, has been associated with reduced development of laser-induced CNV and deposition of a TCC component, C9, at the laser site.¹⁹ The importance of TCC and C9 in CNV development has been implicated further in mice deficient in CD59, with the C5b-8 catalyzed integration of C9 into the cell surface.²⁰,²¹ Knockout mice showed both accelerated laser-induced CNV development and increased C9 deposition at the lesion.²² In both transgenic mice, suppression of the complement pathway was coupled with the reduced production of
VEGF to account for the regulation of pathologic angiogenesis.19,22 Meanwhile, the association between TCC and AMD in humans is unclear.

In Japan, homozygous defect in the C9 gene is the most common cause of complement deficiency.23 The carrier frequency of a null mutation (R95X) in the C9 gene (C9-R95X) is estimated to be approximately 7%, which is higher than in other populations, among them Koreans (1.6%–2.0%), Chinese (0.4%–1.0%), Thais (3.7%), Germans (0%), and Italians (0%).23–25 The lack of C9 is associated with an increased risk for meningococcal meningitis26 or an inhibition of complement-mediated hemolysis in paroxysmal nocturnal hemoglobinuria,27 indicating its important role in the inflammation mediated by the complement system. Furthermore, the parents of the C9-defective patients, presumed heterozygotes of C9 mutation, were found to have approximately half the TCC-mediated hemolytic activity found in subjects with normal C9,26 suggesting that the haploinsufficiency in C9 significantly compromises the overall function of TCC in humans.

Based on these observations, this study was designed to test the hypothesis that the presence of the C9-R95X variant is associated with a reduced risk for nAMD in Japanese patients. We further assessed the biological effect of haploinsufficiency of C3 and the functional role of C9 in the development of CNV in mice. Our findings suggested that C9-R95X may inhibit the development of CNV and nAMD.

Subjects and Methods

Study Subjects

The research protocol was designed in compliance with the Declaration of Helsinki and was approved by the institutional review board of the Nagoya University School of Medicine (approval number 249–4). Written informed consent for providing medical information and blood samples was obtained from each participant. We studied 303 patients with diagnoses of either PCV (n = 198), and 396 control subjects, 83 of whom were aged 70 years or older. Patients without a clear distinction between nAMD and PCV were excluded from the study before genetic analysis. Most of the samples were collected at the time of treatment induction, which roughly corresponded to the onset of the disease in both the nAMD and the PCV groups. All subjects were Japanese residents of Japanese ancestry from the same area examined at Nagoya University Hospital. Most subjects included in this study for both cases and controls have been reported previously.1,28 Demographic backgrounds of the patients and controls are presented in Supplementary Table S1 (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8425/).

Genotyping

The genotypes of C9-R95X variants (c.543C>T; GenBank accession number, NM_001737) were determined using direct sequencing, as previously described.29 Briefly, genomic DNAs were extracted from peripheral blood using a kit (QIAamp DNA Blood Maxi; Qiagen Inc., Valencia, CA). The DNA fragment including the variant was PCR amplified using a primer pair (forward, TGGCATTGAATCTCAGAAAAGAAA; reverse, GTTGTGGCCAGCTCAGACTC). The sequences were extracted using a cycle sequencing kit (BigDye Terminator v3.1; Applied Biosystems, Foster City, CA) and a genetic analyzer (ABI Prism 3700; Applied Biosystems).

Statistical Analysis

Multivariate regression analyses were conducted to compute the odds ratios and 95% confidence intervals (CIs). Expected risks were calculated after adjustment for major AMD-confounding factors, including age, sex, smoking status, and genotypes of AMD-risk polymorphisms in ARMS2/HTRA1 genes and CFH gene. The allele frequency of the Y402H variant in the CFH gene was low, with no homozygotes identified among nAMD patients. Therefore, application of the additive gene-dosage model was considered inappropriate. Instead, a dominant gene-dosage model was assumed. A t-test was used to compare the difference in the CNV sizes and VEGF levels among groups. Software (SPSS 17.0 for Windows; SPSS Japan Inc., Tokyo, Japan) was used for calculations. P < 0.05 was considered significant.

Animals and Laser-Induced CNV

All experimental procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines for the Use of Animals at Nagoya University School of Medicine. The research protocol for the use of animals was approved by the Animal Facility Committee, Nagoya University School of Medicine (approval number 23043). C3−/− mice on a C57BL background were purchased from The Jackson Laboratory (Bar Harbor, ME). After C3−/− and C57Bl/6j mice (age range, 4–6 weeks) were anesthetized, their pupils were dilated with 1% tropicamide. Then four burns of argon laser photoocoagulation (75-μm spot size; 0.1-second duration; 100 mW) were delivered to each eye in the 3, 9, 6, and 12 o’clock positions of the posterior pole at an equal distance from the optic nerve head, as described previously.23 This was followed by intravitreal injection of an antibody against C6 (0.1 μg/L; HyCult Biotechnology b.v., Uden, The Netherlands) or C9 (0.5 μg/μL; Santa Cruz Biotechnology Inc., Santa Cruz, CA) into one eye and an equal amount of isotype control IgG (Wako Pure Chemical Industries Ltd. [Osaka, Japan] or R&D Systems [Minneapolis, MN]) into the other. C6 is an essential component of TCC, which joins the complex upstream of C9. Age-matched C57BL mice were used as controls for experiments using C3−/− or C3−/− mice. Eyes were collected at 2, 4, or 6 days after laser application for the measurement of VEGF and at 7 days after laser application for histologic evaluation of CNV. The CNV was stained and its size was measured as described previously.30 The images of stained CNV were deblamed and randomized before evaluation by a masked person.

Enzyme-Linked Immunosorbenent Assay of Intraocular Fluids

Intraocular fluids were collected from mice and processed as described previously with some modification of the procedure.31 In brief, the optic nerve was removed and a small incision was made in the enucleated eye through the entry site of the optic nerve. The intraocular fluid was aspirated (~4 μL per eye) using a pipette assisted with a gentle indentation of the globe to facilitate the outflow of the fluid. Samples with contamination by blood, the retina pigment epithelium were excluded from analyses. The collected intraocular fluid samples were centrifuged at 15,000g for 5 minutes, and their supernatants were subjected to analysis. VEGF levels in the intraocular fluid were measured using an ELISA kit purchased from R&D Systems.

Results

Analyses of C9-R25X in nAMD and PCV Patients

In this study, 198 patients with nAMD and 105 with PCV were analyzed together with 396 control subjects, among whom 83 patients were 70 years or older. The characteristics of all participants are presented in Supplementary Table S1 (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8425/).

Sequencing results are summarized and the C9-R95X genotypes for nAMD, PCV, and controls are displayed in Table 1. Four of 198 patients with nAMD (2.02%) and 6 of 105 patients with PCV (5.71%) had heterozygous C9-R95X. Of 396 control subjects, 24 (6.06%) carried one or two C9-R95X. No deviation from the Hardy-Weinberg equilibrium was detected in the
of CNV was observed in C3\(^{+/-}\) mice compared with wild-type mice (P = 0.020; 31.5% reduction; Figs. 1A–D). The suppression of CNV formation was at a similar level in C3 knockout (C3\(^{-/-}\)) mice (P = 0.037; 38.7% reduction; Fig. 1C). This was accompanied by a reduction in the secreted VEGF in the intraocular fluid by 47.6% (P = 0.002) in C3\(^{-/-}\) mice and by 53.1% (P = 0.003) in C3\(^{+/-}\) mice compared with wild-type mice at 4 days after laser (Fig. 1D). These results were consistent with those described in a previous report, showing reduced CNV formation and VEGF expression in the eyes of C3\(^{-/-}\) mice treated with a laser.\(^{19}\)

C6 is an essential component of TCC, which joins the complex upstream of C9. When a neutralizing antibody against C6 or C9 was injected into the eyes, the development of CNV was reduced by 48.4% (P = 0.014) or 38.4% (P = 0.009), respectively, compared with the contralateral eye injected with the isotype IgG, suggesting the involvement of TCC in the development of CNV (Fig. 1E). At the same time, the anti-C9 antibody injection reduced VEGF secretion into the intraocular fluid at 4 days (P = 0.015; 41.8% reduction) and at 6 days (P = 0.016; 26.6% reduction) but not at 2 days after laser treatment (P = 0.037) compared with control eyes (Fig. 1F).

**DISCUSSION**

Results show a lower frequency of C9-R95X in nAMD patients but not in PCV patients compared with controls. This was true when those aged 70 years or older were selected for controls. When the study patients and controls were adjusted further for major disease-confounding factors, including age, sex, CFH Y402H and HTRA1/ARMS2 del-ins genotypes, and smoking, C9-R95X remained associated with reduced risk for nAMD. The frequency of those with C9-R95X was 6.05% for control subjects of all ages (P = 0.002; OR, 0.15; 95% CI, 0.04–0.51). Meanwhile, C9-R95X was significantly lower in nAMD patients than in control subjects (P = 0.016; 26.6% reduction) but not at 2 days after laser injection (P = 0.037; 38.7% reduction; Fig. 1C). This was accompanied by a reduction in the secreted VEGF in the intraocular fluid by 47.6% (P = 0.002) in C3\(^{-/-}\) mice and by 53.1% (P = 0.003) in C3\(^{+/-}\) mice compared with wild-type mice at 4 days after laser (Fig. 1D). These results were consistent with those described in a previous report, showing reduced CNV formation and VEGF expression in the eyes of C3\(^{-/-}\) mice treated with a laser.\(^{19}\)

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In addition to increasing recognition of the complement pathways as the pivotal mediators of AMD pathogenesis, the potential role of C9 in the development of nAMD is supported by the several observations. First, a study showing the results of the systematic screening of 63 complement-related genes in

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**Table 1. Summary of C9-R95X Genotypes in nAMD, PCV, and Controls**

<table>
<thead>
<tr>
<th></th>
<th>Wildtype n (%)</th>
<th>Hetero n (%)</th>
<th>Homo n (%)</th>
<th>Total n</th>
</tr>
</thead>
<tbody>
<tr>
<td>nAMD</td>
<td>194 (97.97)</td>
<td>4 (2.02)</td>
<td>0 (0)</td>
<td>198</td>
</tr>
<tr>
<td>PCV</td>
<td>99 (94.28)</td>
<td>6 (5.71)</td>
<td>0 (0)</td>
<td>105</td>
</tr>
<tr>
<td>PCV + nAMD</td>
<td>293 (96.69)</td>
<td>10 (3.30)</td>
<td>0 (0)</td>
<td>303</td>
</tr>
<tr>
<td>Controls</td>
<td>372 (93.95)</td>
<td>23 (5.80)</td>
<td>1 (0.25)</td>
<td>396</td>
</tr>
<tr>
<td>Controls (age 70 y and older)</td>
<td>77 (92.77)</td>
<td>6 (7.22)</td>
<td>0 (0)</td>
<td>83</td>
</tr>
</tbody>
</table>

Hetero, C9-R95X heterozygote; homo, C9-R95X homozygote.

controls (P = 0.431, \( \chi^2 \) test). Among those aged 70 years or older, 6 of 83 controls (7.22%) had C9-R95X. No significant difference was noted in the frequency of those with C9-R95X between subjects younger than 70 and those 70 or older (P = 0.598, Fisher’s two-sided exact test).

Next, the frequency of the C9-R95X carriers in patients and controls was compared statistically; \( P \) values are presented in Table 2. With Fisher’s exact test, the proportion of those with C9-R95X was significantly lower in nAMD patients than in control subjects of all ages (P = 0.018) and in selected controls aged 70 or older (P = 0.041). These differences were also significant when Pearson’s \( \chi^2 \) test was applied. The frequency of those with C9-R95X showed no difference between PCV patients and control subjects (P = 0.628, Fisher’s exact test).

Similarly, differences were not statistically significant when nAMD and PCV were combined and compared against those of controls (P = 0.064, Fisher’s exact test).

Next, multivariate regression analysis was applied to control for major disease-confounding factors, including age, sex, AMD risk variants in ARMS2/HTRA1 genes and CFH gene, and history of smoking (Table 3). In nAMD patients, the presence of higher, 6 of 83 controls (7.22%) had C9-R95X. No significant difference was noted in the frequency of those with C9-R95X between subjects younger than 70 and those 70 or older (P = 0.598, Fisher’s two-sided exact test).

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**Table 2. Comparison of Frequency of C9-R95X Carriers in Patients and Controls**

<table>
<thead>
<tr>
<th></th>
<th>Fisher Exact Test</th>
<th>( \chi^2 ) Test</th>
<th>Fisher Exact Test</th>
<th>( \chi^2 ) Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>nAMD</td>
<td>0.018</td>
<td>0.028</td>
<td>0.041</td>
<td>0.031</td>
</tr>
<tr>
<td>PCV</td>
<td>0.894</td>
<td>0.554</td>
<td>0.447</td>
<td>0.673</td>
</tr>
<tr>
<td>PCV + nAMD</td>
<td>0.064</td>
<td>0.092</td>
<td>0.104</td>
<td>0.111</td>
</tr>
</tbody>
</table>

Data are presented as \( P \) values.
a cohort of 1162 total case and control subjects identified a significant association between a polymorphism in the C9 gene and AMD.35 This was among the polymorphisms from only five other loci, including four established AMD-risk loci (ARMS2/ HTRA1/PLEKH1, CFH/F13B, C3, and C2), which showed an association with the disease.35 Second, proteome analysis of drusen, a sign of early AMD, from patients with AMD and from age-matched controls, revealed C9 as the most abundant protein component.34 Third, we showed that laser-induced CNV development and VEGF production were reduced even by the intraocular injection of anti–C9 antibody or anti–C6 antibody (n = 5 for each datum).

Although significant advances have been made in characterizing the genetic basis of AMD, the genetic contribution to PCV has received less attention despite its high prevalence among Asians. In this study, the C9-R95X genotypes were found to be associated with nAMD but not with PCV. However, the limited number of PCV subjects analyzed and the relatively low allele frequency of the C9-R95X allele in the general population left the association between PCV and C9-R95X inconclusive. Nevertheless, the contrasting results obtained for nAMD and PCV suggest that these conditions may have some differences in genetic background and pathogenesis. Two pieces of evidence further support this notion. First, the allele frequency of the AMD risk variant in HTRA1/ARMS2 genes is reportedly significantly lower in nAMD than in PCV in both Caucasians and Asians.10,35,36 Moreover, this association was replicated in a cohort of patients that overlaps with the cases analyzed in the present study (data not shown), indicating a quantitative difference in the influence of a shared disease-risk gene between nAMD and PCV. Second, a common variant in the Elastin gene was associated with susceptibility to PCV but not to nAMD. This may be considered a qualitative difference in their genetic backgrounds.37

In conclusion, C9-R95X, a polymorphism that is uniquely frequent among Japanese, might be associated with a reduced risk for nAMD but not for PCV. Results propose C9 as a potential target in the treatment of patients with nAMD.

References


