Elastin Gene Polymorphisms in Neovascular Age-Related Macular Degeneration and Polypoidal Choroidal Vasculopathy

Naoshi Kondo, Shigeru Honda, Kazuki Ishibashi, Yasutomo Tsukahara, and Akira Negi

PURPOSE. To study and reveal genetic variation in the elastin gene (ELN) that may be associated with neovascular age-related macular degeneration (AMD) and/or polypoidal choroidal vasculopathy (PCV). Eyes with neovascular AMD and PCV exhibit substantially different structural alterations of the elastic layer in the Bruch’s membrane. The hypothesis for the present study was that ELN polymorphisms may play a role in the development of neovascular AMD and PCV and that genetic differences in ELN between these two phenotypes may be a reason for the histopathologic differences. To test these hypotheses, ELN was screened for genetic variation in a Japanese case-control dataset.

METHODS. Two hundred eighty-five subjects were enrolled: 78 with neovascular AMD, 103 with PCV, and 104 control. We genotyped five tagged single nucleotide polymorphisms (SNPs) in ELN, and allele, genotype, and haplotype frequency distributions among neovascular AMD, PCV, and control subjects were compared by χ² tests.

RESULTS. A common ELN variant was significantly associated with susceptibility to PCV. The age- and sex-adjusted odds ratio was 7.56 for individuals homozygous for the risk allele compared with those carrying no more than one copy of the risk allele. Significantly different distributions were found in allele and haplotype frequencies between neovascular AMD and PCV in this region, but no particular ELN SNPs or haplotypes were significantly associated with neovascular AMD.

CONCLUSIONS. The findings implicate ELN as a susceptibility gene for PCV, and suggest that a different pathogenic process may be involved in the phenomenotypic expression of neovascular AMD and PCV. (Invest Ophthalmol Vis Sci. 2008;49:1101–1105) DOI:10.1167/iovs.07-1145

Age-related macular degeneration (AMD) is a leading cause of irreversible loss of vision among older adults in developed countries. The prevalence of this disease increases with the age of the population, and there is no single broadly effective treatment for it. Neovascular or exudative AMD is an advanced form, characterized by progressive breakdown of the macula caused by choroidal neovascularization (CNV).

Polypoidal choroidal vasculopathy (PCV), a peculiar hemorrhagic and exudative disorder of the macula, was first described in 1990 as idiopathic PCV. It has characteristic morphologic features that include vascular networks of choroidal origin with polypoidal lesions at the border, and it can cause irreversible loss of vision.

PCV has been proposed to represent a variant of CNV (i.e., it is a subtype of neovascular AMD), but this is a matter of controversy. Neovascular AMD and PCV share some pathologic similarities: Serum C-reactive protein levels are significantly elevated, vascular endothelial growth factor concentrations in aqueous humor are significantly increased, and surgically excised PCV lesions stain positively for vascular endothelial growth factor and lack pericytes, in a manner similar to CNV lesions in neovascular AMD. However, the histopathologic findings remain confusing, and there are distinct clinical differences between neovascular AMD and PCV, including morphologic features and disease progression, and response to photodynamic therapy with verteporfin.

Although the pathogenic mechanisms of neovascular AMD and PCV remain largely unknown, various lines of evidence suggest that disruption of the elastin matrix may be implicated. The elastic layer in the Bruch’s membrane normally functions as a physical barrier to vessel growth from the choroid to the subretinal pigment epithelial and subretinal space, and therefore its disruption may lead to development of CNV, as shown in a model of laser-induced CNV. Indeed, histopathologically, disruption of the elastic layer in Bruch’s membrane can be seen in neovascular AMD.

In the eye, elastin is also present in the choroidal vessel, providing mechanical support to the vascular wall. Calcification of vascular elastin is a pathologic hallmark of arteriosclerosis, which is known to decrease elasticity and the strength of the vascular wall. The disruption of vascular elastin leads to aneurysm formation. Histopathologic analysis demonstrates marked sclerotic changes in PCV lesions and disruption of the elastic layer within the wall of polypoidal vessels that are of mixed arteriolar and venular origin, suggesting the possible involvement of arteriosclerosis in development of PCV.

Furthermore, we noted a substantial difference in structure between Bruch’s membrane associated with neovascular AMD and PCV. In contrast to neovascular AMD, PCV lesions are located within Bruch’s membrane beneath its elastic layer, suggesting that the elastic layer is relatively intact compared with the neovascular AMD.

We hypothesized that polymorphisms in the elastin (ELN) gene plays a role in the development of neovascular AMD and PCV and that genetic differences in ELN between these two...
phenotypes may be a reason for their histopathologic differences. To test these hypotheses, we used a tag SNP approach to screen ELN sequences for genetic variations in a Japanese case-control population.

METHODS

Study Participants

This study was approved by the Institutional Review Board at Kobe University Graduate School of Medicine and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all subjects. All cases and controls included in the present study were Japanese and were recruited at the Department of Ophthalmology in the Kobe University Hospital.

All patients with neovascular AMD and PCV underwent an ophthalmic examination, including visual acuity measurement, slit lamp biomicroscopy of the fundi, color fundus photography, optical coherence tomography, fluorescein angiography, and indocyanine green (ICG) angiography. To classify patients accurately into neovascular AMD and PCV groups, differential diagnoses were based on ICG angiograms that can clearly image the choroidal circulation through the retinal pigment epithelium and the surrounding exudation.

ICG angiograms showed a choroidal origin of polypoidal lesions in all PCV cases, typically with vascular networks in posterior poles on ICG angiograms and subretinal reddish-orange protrusions corresponding to the polypoidal lesions on the ICG angiograms. In addition, ICG angiograms showed clear images of vascular CNV networks or diffuse staining of the CNV membrane without polypoidal lesions in all neovascular AMD cases. PCV and neovascular AMD were differentially diagnosed by at least three ophthalmologists specializing in macular disease before the selection of subjects for genotyping. Thus, the present study included only clearly defined phenotypes for PCV and neovascular AMD.

Control subjects were 59 years of age or older and were defined as individuals without macular degeneration and without macular changes such as drusen or pigment abnormalities and were categorized as having clinical age-related maculopathy staging system (CARMs) stage 1,22 on the basis of comprehensive ophthalmic examinations.

SNP Selection

Tagged SNPs in ELN were selected using the Tagger tool from the HapMap Project database for the Japanese in Tokyo (JPT) population.23 We selected five tag SNPs with a minor allele frequency above 10% that were correlated at \( r^2 > 0.8 \) with all ELN SNVs of more than 10% minor allele frequency.

Genotyping

Genomic DNA was extracted from the peripheral blood by standard methods. Genotyping was performed with commercial assays (TagMan SNP Genotyping Assays or Custom TagMan SNP Genotyping Assays; Applied Biosystems, Inc. [ABI], Foster City, CA) on real-time PCR systems (model 7500; Applied Biosystems, Inc.) according to the supplier’s recommendations, and the results were analyzed using the SDS software (ABI).

Statistical Analysis

All SNPs were evaluated by \( \chi^2 \) test for Hardy-Weinberg equilibrium (1 degree of freedom; with SNPAllyzer ver. 6.0; Dynacom, Yokohama, Japan). Allele and genotype frequency distributions were compared among neovascular AMD, PCV, and control subjects by using the \( \chi^2 \) test with 1 or 2 degrees of freedom for the allelic and genotype tests, respectively. Odds ratios and 95% confidence intervals (CIs) were calculated (SNPAllyze ver. 6.0; Dynacom).

Linkage disequilibrium (LD) analysis and case-control haplotype analyses were performed (SNPAllyze ver. 6.0; Dynacom). LD is the statistical association in a population along the genome between alleles at two or more sites (SNPs).25 \( D' \) is a standard measure of LD that ranges from 0 (no LD) to 1 (complete LD). \( D' \) is defined to be 1 in the absence of obligate recombination and decreases only due to recombination or recurrent mutation.25,26 In contrast, \( r^2 \), another way of measuring LD, is the square of the correlation coefficient between SNPs and lies between 0 and 1.25 The value of \( r^2 \) is defined to be 1 when two SNPs occur on the same branch of the genealogy without disruption due to recombination, and the value is less than 1 when SNPs occur on different branches, or if an initially strong correlation has been disrupted by crossing over.25 The values of \( D' \) and \( r^2 \) were calculated with the commercial software (SNPAllyze software, Dynacom).

The haplotype frequency was inferred by the expectation-maximization algorithm (the count of iterations was 10,000; SNPAllyze software; Dynacom). To assess the differences in haplotype distribution among neovascular AMD, PCV, and control subjects, we performed a permutation test with 10,000 iterations.27 Permutation probabilities were empirically computed using 10,000 iterations of random sampling with fixed total numbers of both case and control subjects, which is implemented in the software. Haplotype blocks were defined by using the algorithm of Gabriel et al.28 Because only a few neighboring SNPs were tested with high LD between them, the Bonferroni correction, the most conservative correction for multiple testing, might overcorrect the false-positive rate and lead to a statistical power loss.29,30 However, we decided to apply the Bonferroni correction, in which nominal probabilities are multiplied by five (the number of SNPs genotyped) for allelic tests, thereby assuring that the false-positive rate is no greater than the reported probabilities in the present study.

To adjust for age and sex differences between cases and controls, the association analyses were repeated using logistic regression models (implemented by SNPStats software; available at http://bioinfo.iconcologia.net/SNPStats).31 For this adjustment, four genetic models were considered (codominant, dominant, recessive, and log-additive) and the Akaike information criterion (AIC) was used to choose the genetic model that best fits the data. Power

| Table 1. Characteristics of Study Populations

<table>
<thead>
<tr>
<th>Subjects (n)</th>
<th>Neovascular AMD</th>
<th>PCV</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
<td>61/17</td>
<td>83/20</td>
<td>69/35</td>
</tr>
<tr>
<td>Mean age ± SD (y)</td>
<td>76 ± 7.4</td>
<td>74 ± 6.6</td>
<td>71 ± 5.2</td>
</tr>
<tr>
<td>Age range (y)</td>
<td>57–91</td>
<td>57–86</td>
<td>59–83</td>
</tr>
</tbody>
</table>

| Table 2. Minor Allele Frequencies for All SNPs Genotyped

<table>
<thead>
<tr>
<th>SNP</th>
<th>Position*</th>
<th>Context</th>
<th>Neovascular AMD</th>
<th>PCV</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs868005</td>
<td>73075</td>
<td>Intron 1</td>
<td>0.26</td>
<td>0.21</td>
<td>0.24</td>
</tr>
<tr>
<td>rs884843</td>
<td>73748</td>
<td>Intron 1</td>
<td>0.40</td>
<td>0.47</td>
<td>0.40</td>
</tr>
<tr>
<td>rs2301995</td>
<td>740108</td>
<td>Intron 4</td>
<td>0.14</td>
<td>0.26</td>
<td>0.15</td>
</tr>
<tr>
<td>rs13239907</td>
<td>744818</td>
<td>Intron 5</td>
<td>0.33</td>
<td>0.35</td>
<td>0.39</td>
</tr>
<tr>
<td>rs2856728</td>
<td>757107</td>
<td>Intron 20</td>
<td>0.18</td>
<td>0.32</td>
<td>0.24</td>
</tr>
</tbody>
</table>

* The position of each SNP corresponds to the number of the nucleotide position in NW_9234501.
calculations were performed (Quanto, ver. 1.2 (available at http://hydra.usc.edu/gxe)).

RESULTS

A total of 78 neovascular AMD, 103 PCV, and 104 control subjects participated in the study. The demographic details of the study population are given in Table 1. None of the SNPs reported in the present study showed significant deviation from Hardy-Weinberg equilibrium in the entire sample and in control subjects only. No SNPs showed a significant association with neovascular AMD. In comparisons between neovascular AMD and PCV, significant differences in minor allele frequencies were found at rs2301995 (P = 0.0069) and rs2856728 (P = 0.0025). These differences remained significant, even after Bonferroni correction (Bonferroni-corrected P = 0.0125).

The pair-wise LD structure was constructed with all SNPs genotyped (Table 5). Three SNPs, rs868005, rs884843, and rs2301995, were placed within one haplotype block, and we examined haplotypes based on the three SNPs in the haplotype block. Table 6 presents details of the haplotypes and their frequencies in neovascular AMD, PCV, and control subjects. Haplotype analyses identified a common haplotype associated with increased risk of PCV that was present on 25% of chromosomes of individuals with PCV and 15% of chromosomes of control subjects (permutation P = 0.006), with an odds ratio of 2.06 (95% CI: 1.19–3.56; age- and sex-adjusted OR = 2.06, allelic test), with an odds ratio of 1.75.

DISCUSSION

We identified ELN polymorphisms associated with an increased risk of PCV through the candidate gene approach.

Table 3. Results of Single-SNP Association Study

<table>
<thead>
<tr>
<th>SNP</th>
<th>Neovascular AMD vs. Control</th>
<th>PCV vs. Control</th>
<th>Neovascular AMD vs. PCV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Allelic Nominal P</td>
<td>Genotypic Nominal P</td>
<td>Allelic Nominal P</td>
</tr>
<tr>
<td>rs868005</td>
<td>0.65</td>
<td>0.75</td>
<td>0.51</td>
</tr>
<tr>
<td>rs884843</td>
<td>1</td>
<td>1</td>
<td>0.20</td>
</tr>
<tr>
<td>rs2301995</td>
<td>0.73</td>
<td>0.82</td>
<td>0.0092</td>
</tr>
<tr>
<td>rs13259907</td>
<td>0.23</td>
<td>0.46</td>
<td>0.40</td>
</tr>
<tr>
<td>rs2856728</td>
<td>0.19</td>
<td>0.42</td>
<td>0.054</td>
</tr>
</tbody>
</table>

Nominal probabilities were generated by the χ² test with 1 and 2 degrees of freedom for the allelic and genotypic tests, respectively.

Table 4. Age and Sex Adjusted Association of rs2301995 with PCV

<table>
<thead>
<tr>
<th>Model</th>
<th>Genotype</th>
<th>Age and Sex Adjusted Odds Ratio (95% CI)</th>
<th>P</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codominant</td>
<td>C/C</td>
<td>1.00</td>
<td>0.015</td>
<td>270.4</td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>1.23 (0.65–2.34)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>8.02 (1.57–40.95)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dominant</td>
<td>C/C</td>
<td>1.00</td>
<td>0.13</td>
<td>274.4</td>
</tr>
<tr>
<td></td>
<td>C/T, T/T</td>
<td>1.60 (0.88–2.93)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recessive</td>
<td>C/C, C/T</td>
<td>1.00</td>
<td>0.0048</td>
<td>268.8</td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>7.56 (1.49–38.26)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log-additive</td>
<td>—</td>
<td>1.77 (1.08–2.89)</td>
<td>0.022</td>
<td>271.5</td>
</tr>
</tbody>
</table>

Logistic regression analyses were performed to calculate odds ratios with 95% CI and corresponding P, with age and sex controlled for as covariates. The codominant model compared heterozygous C/T and homozygous T/T genotypes to the homozygous for the most frequent allele C/C. The dominant model compared a combination of C/T + T/T genotypes to the homozygous C/C. The recessive model compared a combination of C/C + C/T genotypes to the homozygous T/T. The log-additive model is equivalent to calculating the odds ratio for the risk T allele. The Akaike information criterion (AIC) is useful to choose the genetic model that best fits the data. The lowest value of AIC indicates the best fit.
with a tag SNP approach. Furthermore, we found significantly different distributions in allele and haplotype frequencies between neovascular AMD and PCV in this region. No particular ELN SNPs or haplotypes showed a significant association with neovascular AMD.

ELN maps to chromosome 7, region q11, and contains 34 exons. Since elastin is a major component of the vascular wall, ELN dysfunction may be expected to cause vascular problems. Allelic variants in this gene have been linked to increased risk of intracranial aneurysm,\textsuperscript{32,33} and mutations in this gene causes supravalvar aortic stenosis\textsuperscript{34,35} and Williams-Beuren syndrome\textsuperscript{36} that are characterized by fibrocellular stenoses in large arteries such as the aorta, coronary arteries, and carotid arteries and other peripheral arteries. ELN is not merely a structural molecule, but is a potent and specific regulator of the migration and proliferation of vascular smooth muscle cells.\textsuperscript{37} A dysfunction of this signaling leads to the development of vascular proliferative diseases such as atherosclerosis.\textsuperscript{38} Thus, ELN is critical for stabilizing vascular structure.

Asians have a higher incidence of PCV than whites: 54.7% of patients with findings suggestive of neovascular AMD in Japanese\textsuperscript{40} and 24.5% in Chinese,\textsuperscript{41} compared with only 8% to 13% in whites.\textsuperscript{7} Although significant advances have been made recently in identifying and characterizing the genetic basis of AMD, the genetic contribution to PCV has received less attention, despite its considerable prevalence. Recently, we demonstrated for the first time that the AMD susceptibility gene \textit{HTRA1} \textsuperscript{39} is also associated with an increased risk of PCV.\textsuperscript{42,43} However, the variant showed a much stronger association with neovascular AMD than with PCV. The odds ratio for neovascular AMD was more than twice as high as that for PCV,\textsuperscript{44} suggesting the presence of additional susceptibility genes for PCV.

For this initial screen of ELN, we applied the robust tag SNP approach to capture most of the common sequence variation, with little loss of power.\textsuperscript{24,45,46} Although none of the SNPs genotyped are known to be functional, and the mechanistic basis for the association between the common ELN variant and PCV is not known, our study provides evidence that ELN is a new candidate gene for PCV and is worthy of more detailed examination. Identification of a causative ELN variant and its functional consequence will clarify the exact role of ELN in susceptibility to PCV.

The negative result for the ELN gene and neovascular AMD suggests that it does not significantly contribute to AMD pathogenesis. However, we cannot completely exclude the possibility that it may have a weak association that is undetectable due to the limitation of statistical power (rs2856728, allelic \textit{P} = 0.19; odds ratio = 0.71; 95% CI: 0.42–1.19). For example, the power to detect the association of rs2856728 with neovascular AMD was only 26%.

Histopathologic analysis has shown that the integrity of the elastic layer in the Bruch’s membrane is lower in neovascular AMD than PCV.\textsuperscript{7,15} It is possible that the detected genetic diversity in ELN is a contributing factor to the histopathologic differences observed between neovascular AMD and PCV. Taken together, neovascular AMD and PCV are likely to share a common pathologic process that is linked to the \textit{HTRA1} variant, and additional genetic factors, such as the ELN polymorphisms, may be required for progression to the different phenotypes.

In conclusion, our findings implicate ELN as a susceptibility gene for PCV, and suggest that different pathogenic processes are involved in the phenotypic expression of neovascular AMD and PCV. Further work is necessary to identify causal DNA changes and to elucidate the mechanism by which this region modulates the risk of PCV, thereby facilitating the understanding of PCV pathogenesis and differences in the pathogenesis of neovascular AMD and PCV.

**Acknowledgments**

The authors thank all who participated in the study.

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**Table 5.** Pair-wise LD Coefficients (upper; \( r^2 \), lower; \(|D'| \)) among All ELN SNPs Genotyped

<table>
<thead>
<tr>
<th></th>
<th>rs868005</th>
<th>rs884843</th>
<th>rs2301995</th>
<th>rs13239097</th>
<th>rs2856728</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs868005</td>
<td>1</td>
<td>0.41</td>
<td>0.07</td>
<td>0.17</td>
<td>0.09</td>
</tr>
<tr>
<td>rs884843</td>
<td>1</td>
<td>1</td>
<td>0.50</td>
<td>0.35</td>
<td>0.13</td>
</tr>
<tr>
<td>rs2301995</td>
<td></td>
<td>1</td>
<td>0.98</td>
<td>0.13</td>
<td>0.65 ( r^2 )</td>
</tr>
<tr>
<td>rs13239097</td>
<td></td>
<td></td>
<td>1</td>
<td>0.18 (0.37)</td>
<td>0.092 (0.015)</td>
</tr>
<tr>
<td>rs2856728</td>
<td></td>
<td></td>
<td></td>
<td>0.97</td>
<td>0.97</td>
</tr>
</tbody>
</table>

LD was measured with use of data from all subjects in the present study. Each column shows pair-wise LD values: \( r^2 \) (upper right triangle) and \(|D'| \) (lower left triangle).

**Table 6.** Inferred Haplotype Frequencies and Haplotype-Based Association Study

<table>
<thead>
<tr>
<th>Haplotype*</th>
<th>Neovascular AMD</th>
<th>PCV</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAG</td>
<td>0.60</td>
<td>0.53</td>
<td>0.60</td>
</tr>
<tr>
<td>GGC</td>
<td>0.26</td>
<td>0.21</td>
<td>0.23</td>
</tr>
<tr>
<td>AGT</td>
<td>0.14</td>
<td>0.25</td>
<td>0.15</td>
</tr>
<tr>
<td>AGC</td>
<td>0.0064</td>
<td>0.0049</td>
<td>0.019</td>
</tr>
</tbody>
</table>

All haplotypes with frequency >1% in the combined samples from neovascular AMD patients, PCV patients, and controls are shown.

* Haplotypes were defined by the following three contiguous SNPs: rs868005, rs884843, and rs2301995.
References


