Original Article
A functional polymorphism in the promoter of αA-crystallin increases the risk of nAMD

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Abstract: Objective: To analyze the association between the promoter of αA-crystallin (CRYAA) variants with neovascular age-related macular degeneration (nAMD) and polypoidal choroidal vasculopathy (PCV) in a northern Chinese population. Methods: We performed a case-control study in a group of Chinese patients with nAMD (n = 345) or PCV (n = 371) and contrasted the results against an independent control group comprising 514 mild cataract patients without any evidence of age-related maculopathy. An association analysis of allele frequencies was performed for 6 single-nucleotide polymorphisms (SNPs) at the CRYAA locus (rs3761381, rs3761382, rs7954582, rs13053109, rs7278468, and rs117396767). Differences in the observed genotypic distributions between the cases and controls were tested using chi-square tests, and logistic regression models were used to calculate the odds ratio (OR) and 95% confidence interval (CI) of nAMD or PCV. Results: The CRYAA rs7278468 variant was significantly associated with neovascular age-related macular degeneration (OR = 1.253, 95% CI 1.018-1.542, P = 0.033). No association was detected between the other five SNPs and nAMD (P > 0.05). No association was detected between these six SNPs and PCV (P > 0.05). Conclusions: Our data suggest CRYAA rs7278468 increases the risk of nAMD. The data might provide crucial information for future clinical studies on the mechanisms of nAMD and may require larger studies to accurately dissect.

Keywords: αA-crystallin, single-nucleotide polymorphism, neovascular age-related macular disease, polypoidal choroidal vasculopathy

Introduction
Age-related macular degeneration (AMD) constitutes a major part of visual loss in aging populations in many countries [1]. Neovascular age-related macular degeneration (nAMD or wet) characterized choroidal neovascularization (CNV) and geographic atrophy (late dry) are two forms of late stage AMD, which causes chronic, irreversible blindness [2]. Polypoidal choroidal vasculopathy (PCV) is a macular disease, and patients with PCV tend to be younger than those with AMD. Recurrent hemorrhagic or serous detachments of the RPE are often observed [3]. Imaging features in indocyanine green angiography (ICGA) are characterized by an abnormal branching vascular network, and the dilated terminals are referred to as polyps [4-7]. Previous studies mainly analyzed the characteristics of PCV as a separate clinical entity differing from AMD and divided it into pachychoroid spectrum of conditions. However, it remains controversial whether PCV represents a sub-type of nAMD [8].

Recently, numerous studies done by direct examinations of single nucleotide polymorphisms (SNP) in chromosomal regions identified by GWAS (genome-wide association studies) have presented several genetic markers shared with these two diseases, including complement factor H, ARMS2 and HTRA1 genes [9, 10]. The association between AMD, PCV and three SNPs in these gene regions, namely rs1061170 (CFH), rs10490924 (ARMS2), and rs11200638 (HTRA1), were verified by a num-
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αA-crystallin (CRYAA) is known to protect cells from stress-induced apoptosis, regulate cell growth, and enhance genomic stability. It was recently shown that CRYAA expression is unregulated in DR [13], inflammatory eye diseases [14, 15], and tumor angiogenesis [16]. As has been proven, CRYAA may protect against geographic atrophy in AMD [17]. In our previous studies, we found that CRYAA is related to the formation of neovascularization in oxygen stress-induced retinopathy and laser-induced choroid neovascularization in a CRYAA gene knockout animal model [18]. However, it remains uncertain whether the CRYAA SNPs play a role in nAMD and PCV and whether there is a lack of sufficient evidence to interpret the difference between the two diseases. We attempted to investigate the relationship between these CRYAA SNPs and nAMD and PCV and tried to illustrate its relationship with the pathogenesis of nAMD and whether it is a common genetic risk factor shared with PCV.

Patients and methods

Subjects

The 1230 unrelated Chinese subjects were grouped as having PCV, having nAMD, or serving as a control for 6 SNPs. A total of 345 patients had nAMD, and 371 patients had PCV; 514 individuals without age related maculopathy (ARM) were studied as controls. The cases were patients at the outpatient clinic of the Department of Ophthalmology in the Peking University People’s Hospital, and the study was approved by the Ethical Committee of Peking University People’s Hospital. An informed consent was obtained from all patients following the guidelines of the Helsinki Declaration. All subjects received a comprehensive ophthalmologic examination, indocyanine green angiograms (ICGA) with HRA2 (Heidelberg Engineering, Heidelberg, Germany) and optic coherence tomography (OCT). PCV was diagnosed from the ICGA results, which showed a complex network of branching vessels that is characteristic of saccular terminated polypoidal lesions. The exclusion criteria included any eye with any other macular abnormalities. The control cases had no abnormalities in the ophthalmologic examinations except mild age-related cataracts, but not severe cataracts, and had normal findings on the fundus photographs, without any signs of PCV or CNV secondary to AMD in either eye.

Genetic analysis

Five milliliter samples of peripheral venous blood from all the subjects were collected in ethylene diamine tetraacetic acid tubes and stored at -80°C. Genomic DNA was isolated from venous blood leukocytes using a TIANamp Blood DNA Midi Kit (Tiangen Biotech Co., Beijing, China) following the manufacturer’s protocol, and six nucleotide polymorphisms in the CRYAA gene including rs3761381, rs-3761382, rs79545821, rs13053109, rs7278468 and rs117396767 were genotyped by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) as previously described [19]. All DNA samples were sent to the company (BGI, The Beijing Genomics Institute, Beijing) for gene detection. More specifically, approximately 30 ng DNA samples were amplified (The primer sequences for 6 SNPs of CRYAA gene are shown in Table 1), and then the PCR products were used for locus-specific single-base extension reactions and the following products were desalted and transferred to a 384 SpectroCHIP array (Sequenom, San Diego, CA, USA). Allele detection was performed using MALDI-TOF-MS. A MassARRAY Typer software version 4.0 (Sequenom, San Diego, CA, USA) was applied to analyze the mass spectrograms.

Table 1. The primer sequence

<table>
<thead>
<tr>
<th>SNP_ID</th>
<th>2nd-PCR</th>
<th>1st-PCR</th>
<th>UEP_SEQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3761381</td>
<td>ACGTTGGATGGGATAACAGGATCATGTGGG</td>
<td>ACGTTGGATGGAGTGGCTACGCTCTGTTGGGTC</td>
<td>TGGTTGGGTGTTGGGTC</td>
</tr>
<tr>
<td>rs3761382</td>
<td>ACGTTGGATGAGCGCTGAGGGTCATAGTGGG</td>
<td>ACGTTGGATGAGACAGGACCACCGTGTCGTTGGG</td>
<td>GGGTTACATCGAGGGGA</td>
</tr>
<tr>
<td>rs79545821</td>
<td>ACGTTGGATGATCCCTGTAACCCGTTGGG</td>
<td>ACGTTGGATGATACCGACGACCTACGCCTTCGTTGGG</td>
<td>TCCGTACACTCACCACCTAA</td>
</tr>
<tr>
<td>rs13053109</td>
<td>ACGTTGGATGATGCCCTGAACTTGTTGTTGCT</td>
<td>ACGTTGGATGAGACAGGACCACCGTGTCGTTGGG</td>
<td>GGGTTACATCGAGGGGA</td>
</tr>
<tr>
<td>rs7278468</td>
<td>ACGTTGGATGCTGCTGAGGCTACCTTCGTTGCT</td>
<td>ACGTTGGATGAGACAGGACCACCGTGTCGTTGGG</td>
<td>GGGTTACATCGAGGGGA</td>
</tr>
<tr>
<td>rs117396767</td>
<td>ACGTTGGATGTCCTGGATGCTCCTGGCTTGGG</td>
<td>ACGTTGGATGAGACAGGACCACCGTGTCGTTGGG</td>
<td>GGGTTACATCGAGGGGA</td>
</tr>
</tbody>
</table>
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Statistical analysis

The statistical analysis was performed using SPSS for Windows version 20.0. The Hardy-Weinberg equilibrium was performed with an $\chi^2$ analysis. The demographic data were analyzed with an $\chi^2$ analysis or with Fisher’s exact tests. Differences in the observed genotypic distributions between the case and control groups were tested using logistic regression analysis with age and gender adjusted. Values of $P < 0.05$ were considered statistically significant. The odds ratios (OR) and their 95% confidence intervals (CI) were calculated to estimate the strength of the association.

Results

The Hardy-Weinberg equilibrium test of CRYAA polymorphisms

The case-control cohort consisted of 1230 subjects. All six SNPs were in HWE ($P > 0.05$). The data are shown in Table 2.

Characteristics of study participants

The characteristics of the study population at baseline are described in Table 3. There were no statistically significant differences in the mean ages, and males are prone to suffer nAMD or PCV ($P = 0.000$).

The association between allele distributions, nAMD and PCV

Six polymorphisms were selected in the promoter region of CRYAA: rs3761381, rs3761382, rs79545821, rs13053109, rs7278468, and rs117396767. The allele distributions for each identified SNP are shown in Tables 4 and 5. Only one SNP (CRYAA rs7278468) showed a significant association with nAMD in the analysis of the genotype frequencies of the patients and control subjects. The G allele may increase the risk of nAMD (OR = 1.253, 95% CI 1.018-1.542, $P = 0.033$). Also, none of these SNPs showed a significant relationship with PCV in the analysis of the allele frequencies. Moreover, we noted no evidence of heterogeneity between the nAMD and PCV groups for these SNPs.

Discussion

We first evaluated 6 SNPs of the promoter region of CRYAA in both nAMD and PCV. The correlation between CRYAA and nAMD was verified at the gene level, which is confirmed by our earlier studies.

CRYAA is composed of 173 amino acids encoded by the CRYAA gene on chromosome 21. It is one of the heat shock proteins and is the major components of the lens protein. However, αA-crystallin can be expressed in ganglion cells, photoreceptors, and other tissues [20]. It plays an important role in protecting the functions of retinal nerve cells [21].

Some studies have evaluated the possible association between the SNPs in the promoter region of CRYAA and age-related cataracts [22, 23]. Previous studies on the SNP of the CRYAA promoter and age-related cataracts identified 6 SNPs in the CRYAA promoter region: rs3761381, rs3761382, rs79545821, rs13053109, rs7278468, and rs117396767. Ma [24] has reported that the polymorphism of the rs7278468 resulted in the downregulation of CRYAA expression, which is associated with age-related cataracts.

A meta-analysis shows that cataracts are associated with an increased risk of geographic atrophy. Further experiments found that CRYAA
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Nskata et al. indicated that CRYAA accumulates in Bruch’s membrane and choroidal connective tissues to a greater degree in AMD than in normal aging [26]. Recent studies have shown that CRYAA is also associated with amyloid fibrils, a common electron microscopic manifestation of drusen [27]. At the same time, the formation of AMD is associated with oxidative stress. In this process, CRYAA may be produced in retinal pigment epithelial cells to combat oxidative stress [28]. Therefore, CRYAA may play an important role in protecting the retina from oxidative damage, and there may be a relationship between CRYAA and AMD at the genetic level.

An increasing number of researchers hope to investigate the pathogenesis of nAMD and PCV, but the existing evidence regarding the genetic risk factors is inconsistent. In recent years, some studies have been reported on AMD and PCV related genes and their polymorphic loci, including rs1061170 (CFH), rs10490924 (ARMS2), and rs11200638 (HTRA1) [29, 30]. Evidence regarding whether the single nucleotide polymorphisms of the CRYAA genes are related to AMD has not been found. In this study, we found the SNP of CRYAA rs7278468 in the northern Chinese population was significantly correlated with nAMD, and G allele carriers had an increased risk of AMD (OR = 1.253, P = 0.033). The genetic locus in this promoter might have a certain effect on the occurrence of nAMD by regulating the expression of CRYAA. The other 5 SNPs: rs3761381, rs3761382, rs79545821, rs13053109 and rs117396767 are not related to nAMD. This suggests that the genetic polymorphism of CRYAA might be associated with the occurrence of AMD. However, there was no significant difference between the PCV group and the control group or between the PCV group and the nAMD group, which indicates that the genetic factors of nAMD and PCV are not identical. A previous study suggests that the T allele of rs7278468 in the CRYAA promoter is associated with age-related cataracts by increasing the binding of KLF-10 and thus decreasing CRYAA transcription [31]. Whether the difference is related to ethnicity and sample selection remains to be further studied.

In conclusion, we observed that CRYAA rs7278468 is a susceptibility gene for nAMD, but not PCV. This finding sheds some light on the differential pathogenesis of these two diseases. However, further studies with larger cohorts for more accurate results are needed to illustrate the mechanisms of CRYAA in the development of nAMD and PCV.

Acknowledgements

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Table 3. Demographic distribution of the study subjects

<table>
<thead>
<tr>
<th></th>
<th>nAMD n = 345</th>
<th>PCV n = 371</th>
<th>Control n = 514</th>
<th>nAMD-control</th>
<th>PCV-control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females, n</td>
<td>126 (36.5%)</td>
<td>144 (38.8%)</td>
<td>287 (55.8%)</td>
<td>P = 0.000</td>
<td>P = 0.000</td>
</tr>
<tr>
<td>Males, n</td>
<td>219 (63.5%)</td>
<td>227 (61.2%)</td>
<td>227 (44.2%)</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Age, mean ± SD, y</td>
<td>69.2 ± 8.7</td>
<td>66.6 ± 9.6</td>
<td>67.2 ± 9.6</td>
<td>P = 0.624</td>
<td>P = 0.844</td>
</tr>
</tbody>
</table>

Table 4. Allele frequencies of the 6 CRYAA SNPs

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Allele</th>
<th>nAMD n (%)</th>
<th>PCV n (%)</th>
<th>Control n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3761381</td>
<td>C</td>
<td>585 (84.8%)</td>
<td>622 (84.1%)</td>
<td>877 (85.6%)</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>105 (15.2%)</td>
<td>118 (15.9%)</td>
<td>147 (14.4%)</td>
</tr>
<tr>
<td>rs3761382</td>
<td>C</td>
<td>660 (95.9%)</td>
<td>712 (96.2%)</td>
<td>968 (94.3%)</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>28 (4.1%)</td>
<td>28 (3.8%)</td>
<td>58 (5.7%)</td>
</tr>
<tr>
<td>rs79545821</td>
<td>G</td>
<td>582 (84.6%)</td>
<td>623 (84.0%)</td>
<td>876 (85.4%)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>106 (15.4%)</td>
<td>119 (16.0%)</td>
<td>150 (14.6%)</td>
</tr>
<tr>
<td>rs13053109</td>
<td>G</td>
<td>661 (95.8%)</td>
<td>713 (96.1%)</td>
<td>970 (94.4%)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>29 (4.2%)</td>
<td>29 (3.9%)</td>
<td>58 (5.6%)</td>
</tr>
<tr>
<td>rs7278468</td>
<td>G</td>
<td>478 (70.0%)</td>
<td>482 (65.1%)</td>
<td>665 (64.9%)</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>206 (30.0%)</td>
<td>258 (34.9%)</td>
<td>359 (35.1%)</td>
</tr>
<tr>
<td>rs117396767</td>
<td>C</td>
<td>655 (94.9%)</td>
<td>700 (94.3%)</td>
<td>980 (95.3%)</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>35 (5.1%)</td>
<td>42 (5.7%)</td>
<td>48 (4.7%)</td>
</tr>
</tbody>
</table>
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Disclosure of conflict of interest

None.

Abbreviations

nAMD, neovascular age-related macular degeneration; CNV, choroidal neovascularization; PCV, polypoidal choroidal vasculopathy; ICGA, indocyanine green angiograms; SNPs, single-nucleotide polymorphisms; GWAS, genome-wide association studies; CRYAA, αA-crystallin; OCT, optic coherence tomography; ARM, age related maculopathy; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; OR, odds ratio; CI, confidence interval.

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References


Table 5. Association between allele distributions, nAMD and PC

<table>
<thead>
<tr>
<th>SNP</th>
<th>nAMD-Control</th>
<th>P value</th>
<th>OR (95% CI)</th>
<th>nAMD-PCV</th>
<th>P value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3761381</td>
<td>0.621</td>
<td>0.934 (0.712-1.225)</td>
<td>0.704</td>
<td>1.057 (0.794-1.407)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs3761382</td>
<td>0.141</td>
<td>1.412 (0.890-2.241)</td>
<td>0.781</td>
<td>0.927 (0.543-1.582)</td>
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<td></td>
</tr>
<tr>
<td>rs79545821</td>
<td>0.654</td>
<td>0.940 (0.718-1.231)</td>
<td>0.743</td>
<td>1.049 (0.789-1.395)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs13053109</td>
<td>0.182</td>
<td>1.363 (0.863-2.152)</td>
<td>0.778</td>
<td>0.927 (0.548-1.568)</td>
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<tr>
<td>rs7278468</td>
<td>0.033</td>
<td>1.253 (1.018-1.542)</td>
<td>0.056</td>
<td>1.242 (0.994-1.552)</td>
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<tr>
<td>rs117396767</td>
<td>0.702</td>
<td>0.917 (0.586-1.433)</td>
<td>0.622</td>
<td>1.123 (0.708-1.781)</td>
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</tr>
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</table>

*P < 0.05.
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