RESEARCH REPORT

An rs9621532 Variant Near the TIMP3 Gene is not Associated with Neovascular Age-Related Macular Degeneration and Polypoidal Choroidal Vasculopathy in a Chinese Han Population

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ABSTRACT

Background: Recently, two genome-wide association studies with large cohorts both identified rs9621532, a new single nucleotide polymorphism (SNP) that is associated with advanced age-related macular degeneration (AMD) and located near the TIMP3 gene. Previous studies have demonstrated that AMD and polypoidal choroidal vasculopathy (PCV) share some common genetic background and that the incidence of PCV is higher in Asian populations than Caucasian populations. In this study, we aimed to investigate whether the rs9621532 SNP is associated with neovascular AMD (nAMD) and PCV in a Chinese Han population.

Methods: We performed a case-control study in a Chinese Han population. The rs9621532 SNP was genotyped in 136 patients with nAMD, 195 patients with PCV, and 181 control individuals using the Multiplex SNaPshot system and the direct DNA sequencing technique. Rs9621532 genotypes and allele frequencies in the nAMD, PCV and control groups were evaluated using PLINK software.

Results: In the nAMD, PCV, and control groups, the minor allele frequencies of the rs9621532 variant were 0.05147, 0.02564, and 0.03039, respectively. The rs9621532 SNP was not significantly associated with susceptibility to nAMD (p = 0.1773) or PCV (p = 0.6933). None of the p-values for the additive or dominant models were found to be statistically significant in the nAMD or PCV groups. No recessive homozygotes were genotyped in any of the three groups.

Conclusions: No evidence was found to support an association between the rs9621532 variant and susceptibility to either nAMD or PCV in a Chinese Han population.

KEYWORDS: Neovascular age-related macular degeneration, polypoidal choroidal vasculopathy, tissue inhibitor of metalloproteinase 3, single nucleotide polymorphism, Chinese Han population

INTRODUCTION

Age-related macular degeneration (AMD) is a common cause of blindness in the elderly population in both developed and developing countries. Neovascular AMD (nAMD) is typically characterized by choroidal neovascularization (CNV) in the area underlying the retina. Polypoidal choroidal vasculopathy (PCV) is a serosanguineous maculopathy with a higher incidence in Asian populations than Caucasian populations. It is characterized by branching choroidal vascular networks with polyp-like terminal aneurysmal dilations or scattered polypoidal dilations without an identifiable continuous branching vascular network on indocyanine green angiography (ICGA). Both nAMD and PCV can cause severe and rapid vision loss due to recurrent retinal exudation, subretinal haemorrhage, and serosanguineous detachments of the retinal pigment epithelium (RPE) and/or neurosensory retina. PCV has been recognized as a variant of CNV, and while its complications, treatment effects, and visual prognosis are somewhat different from nAMD, genetic studies have demonstrated that PCV and AMD share some common genetic background, including the CFH, ARMS2, and HTRA1 genes.
Recently, two genome-wide association studies (GWAS) with large cohorts both identified rs9621532, a new variant located near the TIMP3 gene, as a SNP associated with advanced AMD.\textsuperscript{18,19} However, neither GWAS included a Chinese Han population. Therefore, in the current study we aimed to determine whether the rs9621532 variant is associated with nAMD or PCV in a Chinese Han population.

**PATIENTS AND METHODS**

**Study Participants**

All study participants were Chinese Han individuals recruited from the Zhongshan Ophthalmic Center of Sun Yat-sen University. The study protocol was approved by the institutional review board at the Zhongshan Ophthalmic Center of Sun Yat-sen University and followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all study participants, who were fully informed about the purpose and procedures of this study.

All nAMD and PCV patients underwent ophthalmic examinations that included visual acuity measurements, slit-lamp biomicroscopy, ophthalmoscopic exams, color fundus photographs, fluorescein angiography (FFA), and ICGA. The nAMD patients were diagnosed by the identification of CNV on FFA or ICGA, and the diagnosis of PCV was based on the presence of characteristic polypoidal lesions with or without a continuous branching choroidal vascular network on ICGA. All PCV patients enrolled in this study met the criteria that were proposed by the Japanese PCV Study Group.\textsuperscript{20} All diagnoses were made by at least two of the authors. Cases diagnosed as probable or having both nAMD and PCV in the same eye\textsuperscript{21} were excluded. Patients with other neovascularized maculopathies, such as retinal angiomatic proliferation, idiopathic CNV, angioid streaks, pathological myopia, presumed ocular histoplasmosis, and other retinal or choroidal diseases that could account for CNV were also excluded.

All control subjects were unrelated to case subjects and were aged ≥50 years. They all underwent comprehensive ophthalmic examinations, and those with macular changes (such as drusen or pigment abnormalities), macular degeneration of any cause, or refracting media opacities preventing clear visualization of the fundus were excluded from recruitment.

**SNP Genotyping**

Genomic DNA was isolated from peripheral blood samples using the Nucleospin \textregistered Blood XL kit (Macherey-Nagel GmbH & Co., KG Düren, Germany) as previously described.\textsuperscript{22} The primer sequences used for fragment amplification (191bp) were: 5′-TTTCCCTTGTAGCTGGCTC-3′ (forward) and 5′-GAAAAACTGGGTACAGAGACG-3′ (reverse). The rs9621532 variant was genotyped using the Multiplex SNaPshot system and an ABI 3730XL Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequence of the extension primer was TTTTTTTTTT TTTTTTTTTTTTTCTGTGCTGTCTGACC. Genotypes of the SNP were determined using Genemapper software (Applied Biosystems, Foster City, CA). To confirm the accuracy of the Multiplex SNaPshot method, 10% of all samples (randomly selected) were analyzed by direct sequencing (Shanghai Generay Biotech Co., Ltd, China).

**Statistics**

Statistical analysis of the data was performed using SPSS software (version 16.0, SPSS Inc, Chicago, Illinois, USA). A p-value less than 0.05 was considered statistically significant. Age and gender differences between case and control subjects were assessed using the unpaired Student’s t-test for means and the chi-square test for proportions. Deviations from Hardy–Weinberg equilibrium were tested using the exact test implemented in the software package PLINK v1.07.\textsuperscript{23} Allele frequencies of the SNP in case and control subjects were determined using the chi-square test in PLINK. For calculations assuming the genotypic additive model, we used the logistic option in PLINK, which recommended a test based on logistic regression, and for calculations assuming the dominant model we used the model option in PLINK, which recommended a chi-square test. The odds ratio (OR) and corresponding 95% confidence interval (CI) were calculated relative to the minor allele and wild-type homozygote. Post-hoc power was calculated with G-Power 3.0 software\textsuperscript{24,25} using the following parameters: effect size = 0.20; α = 0.017; Degree of freedom (Df) = 1 for allelic frequencies, Df = 2 for genotype frequencies.

**RESULTS**

A total of 512 subjects consisting of 136 patients with nAMD, 195 patients with PCV, and 181 control individuals participated in this study. The baseline information (including gender and age) of case and control subjects is presented in Table 1. The gender distribution in the nAMD and PCV groups was not statistically different from the control group. The mean age of the PCV group (64 ± 8.75 years) was significantly lower than the control group (68 ± 9.18 years) (p < 0.001). Our data did not show any significant deviations from Hardy–Weinberg equilibrium in case or control subjects (Table 2). The minor allele frequencies of rs9621532 in each group are summarized in Table 2. Rs9621532 did not exhibit an association with nAMD (p = 0.1773, minor allele C: 5.147%...
TABLE 1 Demographic characteristics of the study population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>PCV</th>
<th>nAMD</th>
<th>Control</th>
<th>p-value*</th>
<th>p-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (n)</td>
<td>n = 195</td>
<td>n = 136</td>
<td>n = 181</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (female/male)</td>
<td>65/130</td>
<td>50/86</td>
<td>69/112</td>
<td>0.333</td>
<td>0.805</td>
</tr>
<tr>
<td>Mean age ± SD (years)</td>
<td>64 ± 8.75</td>
<td>67 ± 9.29</td>
<td>68 ± 9.18</td>
<td>&lt;0.001</td>
<td>0.223</td>
</tr>
<tr>
<td>Age range (years)</td>
<td>42–85</td>
<td>46–84</td>
<td>50–87</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: PCV, polypoidal choroidal vasculopathy; nAMD, neovascular age-related macular degeneration; SD, standard deviation.

p-value* PCV compared with control.
p-value† nAMD compared with control.

TABLE 2 Association test for the rs9621532 minor allele frequency in PCV, nAMD, and control subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>MAF*</th>
<th>HWE</th>
<th>OR (95%CI)</th>
<th>p</th>
<th>Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV</td>
<td>0.02564</td>
<td>1.000</td>
<td>0.8397(0.3523–2.001)</td>
<td>0.6933</td>
<td>0.9321</td>
</tr>
<tr>
<td>nAMD</td>
<td>0.05147</td>
<td>1.000</td>
<td>1.732(0.7735–3.876)</td>
<td>0.1773</td>
<td>0.8798</td>
</tr>
<tr>
<td>Control</td>
<td>0.03039</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: SNP, single nucleotide polymorphism; PCV, polypoidal choroidal vasculopathy; nAMD, neovascular age-related macular degeneration; MAF, minor allele frequency; HWE, p-value of Hardy-Weinberg equilibrium test; OR, odds ratio; 95%CI, 95% confidence interval.

*Minor allele was the C allele. MAF was calculated based on all case and control subjects.

in nAMD versus 3.039% in control) or PCV (p = 0.6933, minor allele C: 2.564% in PCV versus 3.039% in control). The genotype frequencies of rs9621532 in each group are summarized in Table 3. No recessive homozygotes were genotyped in the PCV, nAMD, or control groups. None of the p-values for the additive or dominant models were found to be statistically significant in nAMD or PCV (Table 3). The post-hoc power values for the allelic and genotype frequencies in the nAMD and PCV groups are presented in Tables 2 and 3.

**DISCUSSION**

While it is well known that environmental and genetic factors both contribute to the development and progression of AMD, the roles of genetic variants in AMD have only been described in detail in recent years. Most recently, the rs9621532 locus, which is located near the TIMP3 gene, was reported to be associated with AMD in two genome-wide association studies with large cohorts. TIMP3 is one of the inhibitors of the matrix metalloproteinases (MMPs), which play roles in degrading the extracellular matrix (ECM). The balance between MMP and TIMP3 is integral to ECM remodelling. Johnson reported that the TIMP3 gene protects tissues from irreversible destruction and suppresses vasculogenesis by inhibiting the function of MMP and controlling the balance between destruction and reformation of interstitial substances. Weber reported that the TIMP3 gene is mutated in Sorby’s fundus dystrophy (SFD), an inherited macular degenerative disease sharing clinical features with AMD but usually presenting before age 40. Histopathological study has shown that SFD patients have deposits of collagen, elastin, and glycoaminoglycans beneath the RPE and exhibit thickening of Bruch’s membrane, which results in RPE atrophy or CNV, processes implicated in the pathomechanism of AMD. The linkage of AMD to the TIMP3 region has previously been reported and indicates that TIMP3 plays an important role in the regulation of choroidal vascularization, as mutations in the gene have been shown to induce abnormal neovascularization. All of these data suggest that TIMP3 is an important candidate gene for choroidal neovascular disorders such as nAMD and PCV. The rs9621532 SNP is located slightly more than 100 kb upstream of the TIMP3 locus and may influence the function of the TIMP3 gene, resulting in an increased risk of nAMD and PCV.

However, the results of our case-control study in a Chinese Han population demonstrate that the rs9621532 SNP is not significantly associated with either nAMD or PCV, a finding that is inconsistent with previous studies in Caucasian populations but is consistent with the GWAS in the Japanese sample [OR = 1.38 (0.84, 2.28), p = 0.195]. The reason for the inconsistent outcomes of these studies lies primarily in the ethnic differences of the study populations. In our previous study,
we studied the serpin peptidase inhibitor, clade G, member 1 (SERPING1) gene in PCV. The association of the SERPING1 gene with AMD was initially reported in Caucasian populations, but we did not find any association between SERPING1 and PCV in a Chinese Han population, which is consistent with results that were found in a Japanese population. When compared with Caucasian AMD patient populations, the Asian AMD patient population is more predominantly male and has the distinguishing characteristics of unilateral presentation, a relatively low incidence of soft drusen, and a greater prevalence of nAMD and PCV. In this study, we classified AMD patients into separate nAMD and PCV groups and compared each of them with the control group. Our results demonstrate that neither nAMD nor PCV is associated with the rs9621532 variants found in Chinese Han populations. The discrepancy in results obtained from Caucasian and Chinese Han populations illustrates the importance of repeating trials in multiple ethnicities. Other reasons for the inconsistency of results include differences in case selection criteria (patients exhibiting geographic atrophy were excluded in this study) and differences in the genotyping equipment and technology used in these studies. In our study, the genotyping of all participants was performed at the same institution at the same time, minimizing any bias resulting from differences in genotyping conditions. Furthermore, in an effort to validate the results of genotyping, two genotyping methods (the Multiplex SNaPshot method and direct sequencing) were used in this study.

Calculations of our study’s statistical power revealed that our sample size was large enough to detect a gene-disease association with a power of more than 80%. If the individuals carrying rs9621532 variants in our study were at the same risk of developing AMD as individuals in previous studies, the statistical power of our study would have allowed us to detect such an association. The failure of this study to demonstrate a significant association between rs9621532 and AMD might not reflect type II error due to the sample size but rather might result from the low minor allele frequency in this population. Association analyses on SNPs with a low minor allele frequency are prone to false results, and the fact that the minor allele frequency of rs9621532 is less than 6% and none of the individuals in our study carried the rs9621532 homozygous CC genotype might explain our inability to detect an association. Although the current analyses have enough statistical power to assess the effect of rs962153 on the risk of nAMD and PCV (all statistical power in this study was greater than 80%), we suggest that future studies utilize larger sample sizes to effectively test the association of rs9621532 variants with nAMD and PCV.

There are some limitations to our study. Participants were only drawn from the Chinese Han population, and the PCV and control groups were not completely age matched. Due to the limitation of a relatively small sample size, our data cannot exclude the possibility that the rs9621532 polymorphism has an influence on susceptibility to nAMD and PCV. Larger cohorts will be needed to investigate the association between rs9621532 and nAMD or PCV.

In conclusion, we found no evidence to support a link between the rs9621532 variant and susceptibility to nAMD or PCV in a Chinese Han population. This suggests that the rs9621532 polymorphism does not play a significant role in the risk of developing nAMD or PCV in the Chinese Han population.

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REFERENCES