Effects of adiponectin polymorphisms on the risk of advanced age-related macular degeneration

Guiqun Cao1*,#, Yulong Chen1*, Jinlong Zhang1, Yulan Liu1, Ming Zhang2, Kang Zhang1,2, and Zhiguang Su1

1Molecular Medicine Research Center, State Key Laboratory of Biotherapy and 2Department of Ophthalmology, West China Hospital, Sichuan University, Chengdu, China

Abstract

Objective: To determine the relationships between variants in adiponectin gene (ADIPOQ) with advanced forms of age-related macular degeneration (AMD) susceptibility.

Methods: A total of 189 advanced AMD patients and 168 controls were recruited. Seven tagging single-nucleotide polymorphisms in ADIPOQ were genotyped by the SNaPshot method.

Results: Alleles or genotypes of rs822396 distributed significantly differently in advanced AMD patients and controls. The minor allele G at rs822396 was associated with an increased risk of advanced AMD in a dominant model. Furthermore, haplotype analysis revealed that haplotypes AGGACCT and TGACCCC were significantly increased the advanced AMD susceptibility, whereas haplotypes AGAACGC, TGAACGT and TGACAGC had protective effects.

Conclusion: ADIPOQ genetic variant rs822396 might affect an individual’s susceptibility to AMD, making it efficient genetic biomarkers for early detection of AMD.

Keywords
Adiponectin, age-related macular degeneration, haplotype, SNPs

Introduction

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in developed countries, affecting 9 million people in USA (Friedman et al., 2004). Nearly 7% of the Chinese population suffers from AMD (You et al., 2012), and the prevalence of the disease is expected to continue rising due to increased life expectancy. AMD poses a heavy disease burden to society as well as the patients themselves. Although the pathophysiology of AMD has yet to be fully elucidated, numerous previous studies have revealed that genetic predispositions play an important role in AMD pathogenesis. Multiple studies have repeatedly shown significant associations between AMD and polymorphisms in landmark genes such as complement factor H (CFH) (Edwards et al., 2005; Haines et al., 2005; Klein et al., 2005) and high-temperature requirement factor A-1 (HTRA1)/LOC387715 (Dewan et al., 2006; Yang et al., 2006). In Chinese cohorts, polymorphisms in CHF and HTRA1 have also demonstrated increased risk of AMD (Wang et al., 2014; Zhou et al., 2014).

Accumulating evidence indicates that chronic retinal oxidative stress and inflammation are strongly linked to AMD pathogenesis and progression (Seddon et al., 2004; Shaw et al., 2012). Overweight/obesity causes chronic inflammation and oxidative damage. Adipose tissues from obese subjects have increased infiltration of immune cells including macrophages and lymphocytes, and excess inflammatory responses (Lumeng et al., 2007). Adiponectin is an adipocytokine abundantly expressed in adipose tissue. Its plasma concentration is negatively correlated with body mass index and visceral fat accumulation. Adiponectin attenuates chronic inflammation through modulating the function and phenotype of macrophages (Ohashi et al., 2010, 2012). A number of experimental studies with genetic manipulation of mice have exhibited that adiponectin deficiency contributes to the development of obesity-related diseases including insulin resistance, hypertension and atherosclerosis (Maeda et al., 2002; Mori et al., 2014), atherosclerosis is also shown to be a risk factor for AMD (Klein et al., 2013).

Genetic factor accounts for ~40–70% of the variation in plasma adiponectin levels ranging 3–30 μg/ml (Guo et al., 2006). Genetic variants in the gene encoding adiponectin (ADIPOQ) have been reported to be associated with adiponectin level in several genome-wide linkage and association studies (Guo et al., 2006; Heid et al., 2006, 2010). We hypothesized that polymorphisms in the ADIPOQ gene might modulate susceptibility to AMD. To test this hypothesis, we investigated the association of common genetic variants in the ADIPOQ gene with the risk of AMD in a Chinese Han population.
**Materials and methods**

**Subjects and phenotypes**

A total of 189 Chinese patients with advanced AMD and 168 healthy controls were recruited from the West China Hospital. All the patients and control subjects were unrelated native Chinese. As described previously (Wang et al., 2014; Zhou et al., 2014), participants underwent a standard ophthalmologic examination, which included visual acuity measurements, applanation tonometry, dilated slit lamp biomicroscopy and indirect ophthalmoscopy. Imaging studies including stereoscopic color fundus photography, optical coherence tomography and fluorescein fundus angiography were performed as needed. Diagnosis of advanced AMD was based on the presence of geographic atrophy or choroidal neovascularization (CNV). Determination of unilateral or bilateral AMD was made at the final visit. Control subjects were defined as being >60 years old, having fewer than five small drusen of which none exceeded 63 μm in diameter, and not exhibiting retinal pigment epithelium abnormalities.

The use of human tissue and the protocol in this study adhered strictly to the principles of the Declaration of Helsinki and were approved by the Ethical Committee of the West China Hospital, Sichuan University. Written informed consent was obtained from all the subjects before their participation in the study.

**Single-nucleotide polymorphism selection and genotyping**

Genotype data of the Chinese population for the ADIPOQ region were obtained from the HapMap website (http://www.hapmap.org/), and seven tagging single-nucleotide polymorphisms (SNPs) (rs710445, rs16861205, rs822396, rs7627128, rs1501299, rs3821799 and rs1063537) were selected using the Tagger software implemented in the Haploview software (Broad Institute of MIT and Harvard, MA), with an $r^2$ threshold of 0.8 and minor allele frequencies (MAFs) of 0.1.

Blood samples were collected and stored at ~80°C before DNA extraction. Genomic DNA was extracted from peripheral blood leukocytes according to established protocols. Genotyping was performed with polymerase chain reaction (PCR) followed by SNaPshot as described previously (Xu et al., 2012; Yuan et al., 2012). Briefly, DNA fragments containing SNPs were amplified by PCR using primers in Supplementary Table S1. The PCR products were then purified by shrimp alkaline phosphatase (SAP) and exonuclease I. The purified PCR product and the SNaPshot primers (Supplementary Table S1) were then used to perform a single base-pair extension with the SNaPshot multiplex mix (Applied Biosystems Inc., Foster City, CA). After an additional purification step using SAP, the product was analyzed on an ABI 3130xl genetic analyzer (Applied Biosystem Inc.), and the genotyping results were obtained directly.

**Statistical analysis**

The distributions of socio-demographic characteristics between cases and controls were tested using the Student’s $t$-test for the continuous variables and Pearson’s $\chi^2$ test for the categorical variables. A two-sided significance level of $z < 0.05$ was used for all the significant tests. Statistical analyses were performed in SPSS version 17.0 (IBM Corp., Armonk, NY) and Microsoft Excel (Microsoft Corp., Redmond, WA).

Deviation from the Hardy–Weinberg equilibrium (HWE) was tested with the standard $\chi^2$ test. Differences in the distribution of genotypes or alleles under different genetic models (including dominant, recessive and additive models) between the AMD patients and the controls were estimated by using the $\chi^2$ test, and the best genetic model for each SNP was determined using Akaike’s information criterion (AIC). Odds ratio (OR) and 95% confidence interval (CI) were calculated by unconditional logistic regression analyses.

Haplotype reconstruction was performed using SHEsis (http://analysis.bio-x.cn). For haplotype analysis, only haplotypes with a frequency >3% in at least one group were tested.

**Results**

**General characteristics of the subjects**

We studied a total of 357 unrelated Chinese individuals including 189 cases with advanced AMD and 168 controls. The mean ages of patients and controls were 64.7 and 65.3, respectively. There were a higher number of females in both groups. We did not find significant differences in age and gender between patients and control subjects ($p = 0.63$ and 0.25, respectively) (Table 1).

**Distribution of the SNPs in ADIPOQ between AMD patients and controls**

Seven SNPs in ADIPOQ, including rs710445, rs16861205, rs822396, rs7627128, rs1501299, rs3821799 and rs1063537 were screened in all 189 patients with AMD and 168 controls using the SNaPshot method. The genotype and allele frequencies of each SNP in both AMD patients and controls are presented in Table 2. All of the tested SNPs did not significantly deviate from that expected for a Hardy–Weinberg equilibrium (HWE) in the AMD patients and controls (Table 2, all $p$ values were >0.05), illustrating that our subjects presented the source population well.

We compared the differences in frequency distributions of genotypes or alleles of every SNP between AMD patients and controls by $\chi^2$ test. As shown in Table 2, significant differences in allele or genotype frequencies were observed between AMD patients and controls at rs822396 (allele: $p = 0.007$, OR = 1.65 and 95% CI = 1.15–2.38; genotype: $p = 0.031$).

**Association of genotypes with AMD under different genetic models**

For each SNP, if one allele frequency is relatively lower compared to another one, it is recognized as the minor

| Table 1. Characteristics of the study cohorts with or without AMD$^a$. |
|-----------------|-----------------|-----------------|-----------------|
| Characteristics | Cases ($n = 189$) | Controls ($n = 168$) | $p$ Value |
| Age (years)     | 64.7 ± 7.8      | 65.3 ± 6.4      | 0.63          |
| Gender, male n (%) | 88 (46.6%)     | 68 (40.5%)      | 0.25          |

$^a$The data are presented as mean ± SEM. $p$ Values are calculated from t-test or chi-squared test.
We assumed that the minor allele of each SNP was a risk allele compared to the wild type allele. We compared the genotype frequencies of every polymorphism between groups under the dominant, recessive and additive genetic models, respectively. As shown in Table 3, the minor allele G at rs822396 was observed to be associated with an increased AMD risk using a dominant model as determined by AIC (AG + GG versus AA: \( p = 0.016 \), OR: 1.70, 95% CI: [1.10–2.62]).

Among the 189 cases with AMD, 118 (62.4%) had unilateral and 71 (37.6%) had bilateral advanced AMD. As shown in Table 4, the distributions of rs822396 were not statistically different between unilateral and bilateral advanced AMD \( (p > 0.05) \). When compared with control subjects, rs822396 were only shown to be significantly associated with an increased risk of bilateral AMD \( (OR = 2.11, 95\% \ CI = 1.97–3.72, \ p = 0.009) \).

### Table 2. Distributions of the ADIPOQ SNPs in AMD patients and controls.

| SNP     | Genotype | HWE | Allele |
|---------|----------|-----|--------|
|         | Group    | \( p \) | Number (freq.) | OR [95% CI] |
| rs710445| Control  | 0.967 | 43 (25.6) 89 (53.0) 36 (21.4) | 1.01 |
|         | AMD      | 0.597 | 49 (26.0) 98 (51.8) 42 (22.2) | 0.951 |
| rs16861205| Control | 0.943 | 105 (62.5) 57 (33.9) 6 (3.6) | 1.01 |
|         | AMD      | 0.840 | 121 (64.0) 61 (32.3) 7 (3.7) | 0.951 |
| rs822396| Control  | 0.031 | 115 (68.5) 48 (28.6) 5 (3.0) | 1.65 |
|         | AMD      | 0.097 | 106 (56.1) 69 (36.5) 14 (7.4) | 1.01 |
| rs7627128| Control | 0.922 | 75 (44.6) 77 (45.8) 16 (9.5) | 1.01 |
|         | AMD      | 0.997 | 86 (45.5) 83 (43.9) 20 (10.6) | 1.01 |
| rs1501299| Control | 0.967 | 73 (43.5) 79 (47.0) 16 (9.5) | 1.01 |
|         | AMD      | 0.415 | 84 (44.8) 86 (44.6) 17 (9.0) | 1.01 |
| rs3821799| Control | 0.943 | 65 (38.7) 79 (47.0) 24 (14.3) | 1.05 |
|         | AMD      | 0.885 | 70 (37.0) 91 (48.2) 28 (14.8) | 1.05 |
| rs1063537| Control | 0.836 | 86 (51.2) 69 (41.1) 13 (7.7) | 1.05 |
|         | AMD      | 0.218 | 95 (50.3) 82 (43.4) 12 (6.3) | 1.05 |

The bold values indicate \( p < 0.05 \).

HWE, Hardy–Weinberg equilibrium; OR, odd ratio; CI, confidence interval.

### Table 3. Association between ADIPOQ SNPs and the risk of AMD under different genetic models.

| SNP     | Genetic modela | \( p \) | OR [95% CI]b |
|---------|----------------|-------|--------------|
| rs710445| recessive GG + AG versus (GG + AG) | 0.939 | 1.02 [0.56–1.88] |
| rs16861205| dominant (AG + AA) versus GG | 0.766 | 0.94 [0.61–1.44] |
| rs822396| dominant (AG + GG) versus AA | 0.016 | 1.70 [1.10–2.62] |
| rs7627128| additive AC versus CC | 0.782 | 0.94 [0.61–1.46] |
| rs1501299| recessive AA versus (CC + AC) | 0.816 | 1.09 [0.53–2.25] |
| rs3821799| dominant TT versus CC | 0.748 | 1.07 [0.70–1.65] |
| rs1063537| additive TT versus CC | 0.877 | 1.20 [0.36–1.93] |

aOnly the estimates for the best genetic model for each SNP, as determined by AIC, are provided.
bOR: odd ratio; CI: confidence interval. The ORs and CIs that are statistically significant are bolded, along with the rs number of the corresponding SNP.

### Table 4. Risk of rs822396 for unilateral and bilateral advanced AMD.

| Genotype | Unilateral AMD number (freq.) | Bilateral AMD number (freq.) | Control number (freq.) |
|----------|-------------------------------|-----------------------------|------------------------|
| AA       | 70 (59.3)                     | 36 (50.7)                   | 115 (68.5)             |
| AG       | 39 (33.1)                     | 30 (42.3)                   | 48 (28.6)              |
| GG       | 9 (7.6)                       | 5 (7.0)                     | 5 (3.0)                |

The bold values indicate \( p < 0.05 \).

**ADIPOQ haplotype and AMD**

We estimated the frequencies of haplotypes constructed from phased multi-locus genotypes in ADIPOQ. The haplotypes
with a frequency >3% in at least one group were involved in the haplotype analysis (Table 5). Global haplotype association analyses showed that haplotypes AGGACCT (OR = 2.89, 95% CI = 1.01–8.28, p = 0.039) and TGACCCCC (OR = 2.47, 95% CI = 1.12–5.44, p = 0.021) were significantly associated with the increased risk of AMD. In addition, three protective haplotypes AGAACGC (OR = 0.69 [0.27–1.77]), AGGCCGG (OR = 0.38 [0.19–0.75]) and AGAACCT (OR = 0.27 [0.08–1.00]) were significantly associated with a decreased risk of AMD. The overall frequency distribution of haplotype composed of all seven SNPs was significantly different between cases and controls (total global $\chi^2 = 64.88$ while df = 7, $p = 1.85 \times 10^{-8}$).

### Discussion

Our current findings suggested that rs822396 is associated with the risk of advanced AMD. In comparison with allele A at rs822396, the allele G could increase the risk of AMD under a dominant genetic model. In addition to the genotype analysis, our study also adopted a haplotype-based approach, in which several SNPs within the same gene are evaluated simultaneously. Using this approach, we provided strong support that **ADIPOQ** variations contributed to the susceptibility to AMD. Some haplotypes with low frequency were found to affect the risk of AMD dramatically, indicating the complexity of **ADIPOQ** gene in the development of AMD.

The significant **ADIPOQ** variants associated with AMD in this unrelated case–control cohort occur within the intron region of the **ADIPOQ** that is usually removed during the gene-splicing process. Although there is no apparent functional change, intrinsic SNPs may modify gene function by affecting the regulation of gene expression (Korb et al., 1993). In addition, the associated SNP with the statistical signal might just play a role as a surrogate marker for the causal functional SNP or SNPs. Therefore, rs822396 in **ADIPOQ** gene could be in LD with another polymorphism of the gene that may impact the **ADIPOQ** expression level. However, it is also likely that the causal sequence change(s) in this region has yet to be identified as suggested by analysis of the significant haplotypes. For example, SNP rs710445 was significantly associated with AMD risk as part of a haplotype but not individually (Table 5). We selected SNPs with MAFs of >10% in the Han Chinese population using HapMap project data, but this is not suited for situations where genetic architecture is such that multiple rare disease-causing variants contribute significantly to disease risk. Recent studies demonstrate that identification of rare variants may lead to critically important insights about disease etiology through implication of new genes and/or pathways (Nelson et al., 2012; Schaibley et al., 2013). The rare variants in the **ADIPOQ** gene should be investigated to clarify their susceptibility to the development of AMD.

Emerging evidence suggests that adiponectin is associated with several retinal diseases (Bora et al., 2007; Higuchi et al., 2010). Adipose tissue is an important endocrine organ that secretes a number of factors, adipocytokines or adipokines (Yu et al., 2011). Several of these factors, such as adiponectin, IL-1β, IL-6 and TNF-α increased the risk of obesity that is estimated to be a moderate risk factor for AMD (Maralani et al., 2015; Seddon, 2013; Yu et al., 2011). Adiponectin is inversely related to both adiposity and many chronic inflammatory diseases including atherosclerosis (Hao et al., 2013). In addition to obesity, atherosclerosis is also shown to be a risk factor for AMD (Klein et al., 2013). Adiponectin mediates its cellular message via binding and activation of its specific receptors (**ADIPOR**), both adiponectin and **ADIPOR** are present in the eye (Bora et al., 2007), and the **ADIPOR** variant might be a candidate for AMD genetic risk factor in Finnish population (Kaarniranta et al., 2012). Moreover, recombinant adiponectin or peptide generated from adiponectin may have therapeutic potential in CNV treatment associated with wet AMD (Bora et al., 2007; Lyzogubov et al., 2009, 2012). Given the number of processes/pathways in which adiponectin functions and the significance of association between **ADIPOQ** and AMD, it is unlikely that **ADIPOQ** alone contributes to the sequelae of events in AMD but rather that

### Table 5. Frequencies of pairwise haplotype constructed by SNPs in **ADIPOQ**.

| Haplotype | Freq (case) | Freq (control) | $\chi^2$ | Fisher’s $p$ | OR [95% CI]$^b$ |
|-----------|-------------|----------------|---------|-------------|-----------------|
| AGAAACCC  | 0.013       | 0.034          | 3.08    | 0.079       | 0.39 [0.13–1.16]|
| AGGAAGC   | 0.030       | 0.065          | 4.00    | 0.046       | 0.48 [0.23–1.00]|
| AGGCCGG   | 0.167       | 0.154          | 0.82    | 0.366       | 1.21 [0.80–1.84]|
| AGGCCGG   | 0.037       | 0.014          | 4.26    | 0.039       | 2.89 [1.01–8.28]|
| AGGCCGG   | 0.063       | 0.035          | 3.80    | 0.051       | 2.03 [0.99–4.17]|
| TAACAGC   | 0.102       | 0.081          | 1.75    | 0.186       | 1.42 [0.84–2.40]|
| TAACCCT   | 0.057       | 0.041          | 1.40    | 0.237       | 1.522 [0.76–3.06]|
| TGACAGT   | 0.008       | 0.030          | 4.37    | 0.037       | 0.27 [0.08–1.00]|
| TGAACCT   | 0.019       | 0.036          | 1.47    | 0.226       | 0.57 [0.22–1.44]|
| TGACAGT   | 0.034       | 0.088          | 8.19    | 0.004       | 0.38 [0.19–0.75]|
| TGACAGT   | 0.059       | 0.027          | 5.33    | 0.021       | 2.47 [1.12–5.44]|
| TAACCC    | 0.000       | 0.049          | 17.98   | 2.26 $\times 10^{-5}$ | – |
| AGAACCT   | 0.030       | 0.000          | 11.12   | 8.58 $\times 10^{-4}$ | – |
| Global    |             |                | 64.88   | 1.85 $\times 10^{-8}$ | – |

The bold values indicate $p < 0.05$.

$^a$The order of SNPs from left to right is rs710445, rs16861205, rs822396, rs7627128, rs1501299, rs3821799 and rs1063537. Only haplotypes with a frequency >3% in at least one group were listed.

$^b$OR: odds ratio; CI: confidence interval. The OR could not be calculated for the haplotypes TAACCCC and AGAACGT, because of the zero value in the population.
genes regulated by \textit{ADIPOQ}, or those that regulate \textit{ADIPOQ}, may influence the disease. Therefore, the investigation of adiponectin-related pathways and gene networks may lead to a better understanding of the pathophysiology of AMD.

In conclusion, our comprehensive analysis of SNPs in the \textit{ADIPOQ} gene suggested that \textit{ADIPOQ} genotypes and haplotypes are associated with AMD risk. However, additional studies are required to confirm our findings, and functional studies are required to reveal the potential mechanisms accounting for adiponectin and AMD.

\textbf{Declaration of interest}

The authors report no declarations of interest. This study was supported by the National High Technology Research and Development Program of China (863 project) (No. 2014AA021604), Sichuan Province Science and Technology Support Program (No. 2015SZ0140) and the Program for New Century Excellent Talents in University (No. NCET-10-0600).

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