Polymorphism analysis of miR182 and CDKN2B genes in Greek patients with primary open angle glaucoma

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Abstract

Glaucoma is a progressive optic neuropathy resulting from retinal ganglion cells death; it represents one of the leading causes of irreversible blindness worldwide. Although, primary open angle glaucoma (POAG) is the most common type of the disease, the pathogenesis of POAG and the genetic factors contributing to disease development remain poorly understood. The aim of this study was to investigate whether the polymorphisms rs76481776 in miR182 gene and rs3217992 in cyclin-dependent kinase inhibitor-2B (CDKN2B) gene are risk factors for POAG in a series of patients of Greek origin. A case-control study was conducted including 120 patients with POAG and 113 unaffected healthy controls of Greek origin, surveyed for polymorphisms with potential correlation to POAG. DNA from each individual was tested for the miR182 rs76481776 and CDKN2B rs3217992 polymorphisms. Regarding the miR182 rs76481776 polymorphism, the T allele occurred with significantly higher frequency in POAG patients compared to controls (OR: 2.62, 95% CI: 1.56–4.39; p = 0.0002). The CDKN2B rs3217992 A allele frequency was found significantly increased in POAG patients compared to healthy individuals (OR: 1.72, 95% CI: 1.18–2.49; p = 0.005). Therefore, both rs76481776 polymorphism in miR182 gene and rs3217992 polymorphism in CDKN2B gene seem to be associated with the development of POAG in a Greek population. The carriers of the T allele of rs76481776 in miR182 and the carriers of the A allele of rs3217992 in CDKN2B have an increased risk of developing POAG.

Introduction

Glaucoma represents one of the leading causes of irreversible blindness worldwide. [1] Epidemiological studies suggest that the prevalence of the disease is estimated to have reached 76
Glaucoma is characterized by progressive optic neuropathy resulting from retinal ganglion cells death. Intraocular pressure (IOP) elevation, myopia, vascular factors, aging and positive family history are considered major risk factors for glaucoma. Although, primary open angle glaucoma (POAG) represents the most common type of the disease, the pathogenesis of POAG and factors contributing to disease progression remain poorly understood.

Genetic studies have established three POAG susceptibility genes: myocilin (MYOC), optineurin (OPTN) and WD repeat domain 36 (WDR36). However, these genes are responsible for less than 10% of sporadic POAG cases. Genome-wide association studies (GWAS) have revealed several single nucleotide polymorphisms (SNPs) at different loci associated with glaucoma, including caveolin 1 (CAV1), caveolin 2 (CAV2), cyclin-dependent kinase inhibitor 2B-antisense noncoding RNA (CDKN2B-AS1), neurotrophin-4 (NTF4), transmembrane and coiled-coil domains 1 (TMCO1) and more recently, thioredoxin reductase 2 (TXNRD2), ataxin 2 (ATXN2), forkhead box C1 (FOXC1), SIX6, GLI-similar 3 (GLIS3), fibronectin type III domain containing 3B (FNDC3B), LIM homeobox transcription factor 1B (LMX1B), phospholysine phosphohistidine inorganic pyrophosphate phosphatase (LHPP), myeloid ecotropic insertion site 2 (MEIS2) and lysyl oxidase like 1 (LOXL1).

MicroRNAs (miRNAs) are small, non-coding RNAs consisting of about 22 nucleotides that regulate gene expression at the post-transcriptional level, affecting a variety of biological processes. Growing evidence suggests that miRNAs play an important role in the pathogenesis of POAG. Specifically, miR-93 induces apoptosis in trabecular meshwork cells, miR-187 inhibits the oxidative stress-induced retinal ganglion cells apoptosis and promotes cell proliferation and miR-183 targets integrin-β1 and affects trabecular meshwork physiology. miR-29b, regulated by TGF-β2, modulates the expression of extracellular matrix genes, which function in the aqueous outflow pathway.

Previous studies have shown that sequence alterations in MIR genes encoding miRNAs have profound effects on miRNA biogenesis and function and contribute to the development risk of various diseases. For example, hsa-miR-568 polymorphism has been identified in sporadic keratoconus and a mutation altering the miR-184 seed region has been reported to cause keratoconus with congenital cataract. Genetic variants of miR-146a are associated with pediatric uveitis and mutations in miR-182 predispose to Behcet’s disease and Vogt-Koyanagi-Harada syndrome. Variants in several MIR genes have been also associated with POAG. Recently, variants in the miR-612 precursor and in the miR-4707 seed region were found to be significantly associated with vertical cup-to-disc ratio and cup area. In addition, polymorphisms in miR-3196 and miR-182 have been associated with POAG.

The CDKN2B gene encodes a cyclin-dependent kinase inhibitor, which plays a pivotal role in the maintenance of cell cycle progression. Animal studies have shown that elevated IOP is associated with overexpression of CDKN2B, leading to disruption in cell cycle and abnormal cell proliferation. Additionally, chromosome 9p21, where the CDKN2B gene is located, has been identified as an important susceptibility locus for glaucoma with various SNPs having positive associations with POAG in different ethnic populations. Both rs3217992 and rs1063192 polymorphisms in CDKN2B gene are miRNA-binding-site variants, with the association of rs1063192 with POAG being widely studied in various ethnic groups. However, the possible association between CDKN2B rs3217992 polymorphism and glaucoma risk has not been studied in any population.

The aim of our study was to investigate the possible association between miR182 rs76481776 and CDKN2B gene rs3217992 polymorphisms and POAG susceptibility in a well-defined Greek cohort. To our knowledge, this is the first study in the literature to conduct such genotyping in Greek patients with POAG.
Materials and methods

Study population

Greek patients with POAG (n = 120) and 113 unaffected healthy controls with no history of any ophthalmologic or other systematic disease were recruited in the study at Glaucoma Unit of the First Department of Ophthalmology, National and Kapodistrian University of Athens, Athens, Greece. The diagnosis of POAG was clinically confirmed at least 10 years before recruitment in the study. All participants signed an informed consent and had a blood sample taken for genetic analysis. The study was approved by the Ethics committee of “Evangelismos” General Hospital of Athens (Director Mr Lamprinakis Ioannis, approval number 19877).

All participants were selected after ophthalmological evaluation and clinical data collection. Ophthalmological evaluation included slit lamp examination with gonioscopy, fundus examination for cup/disc ratio evaluation of the optic disc and IOP measurement with Goldmann applanation tonometer (Haag Streit AG, Bern, Switzerland). Automated perimetry with a Humphrey Automated Field Analyzer (Humphrey Inc., San Leandro, CA, USA) using 30–2 SITA protocol was performed at both cases and controls.

Inclusion criteria for the POAG patient group were the following: glaucomatous optic neuropathy in at least one eye, defined as a cup/disc ratio \( \geq 0.6 \) on fundoscopy and repeatable glaucomatous visual field defect on automated perimetry, a Schaffer III-IV angle on gonioscopy and initial IOP \( \geq 21 \text{mmHg} \) (high tension POAG). The glaucomatous visual field defects included a Bjerrum, altitudinal or nasal step scotoma. The control group had no family history of glaucoma, normal IOP values (<21mmHg) and no glaucomatous defects were detected at the ophthalmological examination and automated perimetry.

Genotyping

Genomic DNA was isolated from peripheral blood leukocytes using a commercial kit (Nucleospin Blood, Macherey-Nagel, Germany) according to the manufacturer’s instructions. The rs76481776 polymorphism was analyzed using PCR followed by restriction analysis, as previously described. [28] Regarding the rs3731249, the genotyping analyses were also performed by using PCR followed by restriction analysis, as previously described. [38]

Statistical analysis. Genotype frequencies were analyzed with the \( \chi^2 \) test with Yate’s correction using S-Plus (v.6.2 Insightful, Seattle, WA, United States) software. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated with GraphPad (v.300, GraphPad Software, San Diego, CA, United States). Hardy-Weinberg Equilibrium (HWE) deviation was tested by Pearson’s \( \chi^2 \) test. All p values are two-sided. A p value <0.05 was considered significant.

Results

The demographic and clinical characteristics of the study population are shown in Table 1. Among POAG patients, 48 (40%) were male and 72 (60%) were female, with a mean age of 73,

Table 1. Demographics and clinical characteristics of the study population.

| Variables                  | Controls (n = 113) | POAG (n = 120) | P value |
|----------------------------|-------------------|----------------|---------|
| Sex (male/female)          | 58/55             | 48/72          | 0.08    |
| Age (mean±SD), years       | 59.3±18.6         | 73.4±11.5      | <0.005  |
| IOP (mean±SD), mmHg        | 14.3±2            | 17.2±4         | <0.001  |
| Duration of therapy (mean±SD), years | -              | 7.2±6.7       |         |

POAG, primary open-angle glaucoma; IOP, intraocular pressure

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4±11.5 years. Among controls, 58 (51%) were male and 55 (49%) were female, with a mean age of 59, 3±18.6 years. Controls were statistically significant younger than POAG patients (p<0.005). POAG patients had a mean IOP of 17.2±mmHg, all were receiving anti-glaucoma medications and had a mean therapy duration of 7, 2±6.7 years.

The genotype and allele frequency distribution of the rs76481776 miR182 gene and rs3217992 CDKN2B gene across controls and POAG patients is shown in Table 2. The miR182 rs76481776 CT genotype frequency was significantly higher in patients with POAG than in controls (30.83% and 15.93% respectively, OR: 2.59, 95% CI: 1.36–4.92; p = 0.005), as well as the TT genotype was significantly overrepresented in POAG patients compared to controls (8.33% and 2.65% respectively, OR: 4.2, 95% CI: 1.11–15.83; p = 0.04). The T allele of rs76481776 occurred with significantly higher frequency in POAG patients (OR: 2.62, 95% CI: 1.56–4.39; p = 0.002).

Concerning the CDKN2B rs3217992 polymorphism, the GA genotype was significantly increased in POAG patients compared to controls (58.33% and 44.25% respectively, OR: 2.45, 95% CI: 1.36–4.42; p = 0.003). The AA genotype frequency was also significantly higher in patients with POAG than in controls (18.33% and 12.39%, respectively, OR: 2.75, 95% CI: 1.22–6.21; p = 0.016). The minor A allele frequency was higher in POAG patients (OR: 1.72, 95% CI: 1.18–2.49; p = 0.005).

### Discussion

POAG is a progressive neurodegenerative disorder induced by a combination of multiple genetic and environmental factors. [39] This study aimed to investigate the possible association of miR182 gene rs76481776 and CDKN2B gene rs3217992 polymorphisms with POAG susceptibility. To our knowledge, the genetic contribution of variants at the miR182 and CDKN2B loci among Greek POAG patients is not known. Our results demonstrated a significant association of the T allele of the rs76481776 miR182 gene with POAG, suggesting that the carriers of the T allele have an increased risk of developing POAG. Moreover, we showed an increased frequency of the CDKN2B rs3217992 A allele in POAG patients compared to controls, indicating that individuals carrying the A allele of the rs3217992 polymorphism are associated with the development of POAG in our population.

Concerning the miR182 gene, our results are in agreement with a previous study, [31] which identified a significant association of rs76481776 in miR182 gene with POAG in subjects with European ancestry from the United States. The study also reported an elevated

| Polymorphism     | Genotype | Controls (n = 113) | POAG (n = 120) | OR (95% CI) | P value |
|------------------|----------|-------------------|----------------|-------------|---------|
| MIR182 rs76481776 | CC       | 92 (81.42%)       | 73 (60.83%)    | 1.00        |         |
|                  | CT       | 18 (15.93%)       | 37 (30.83%)    | 2.59 (1.36–4.92) | 0.005   |
|                  | TT       | 3 (2.65%)         | 10 (8.33%)     | 4.2 (1.11–15.83) | 0.04    |
|                  | C allele | 202 (89.38%)      | 183 (73.25%)   | 1.00        |         |
|                  | T allele | 24 (10.62%)       | 57 (23.75%)    | 2.62 (1.56–4.39) | 0.0002  |
| CDKN2B rs3217992 | GG       | 49 (43.36%)       | 28 (23.33%)    | 1.00        |         |
|                  | GA       | 50 (44.25%)       | 70 (58.33%)    | 2.45 (1.36–4.42) | 0.003   |
|                  | AA       | 14 (12.39%)       | 22 (18.33%)    | 2.75 (1.22–6.21) | 0.016   |
|                  | G allele | 148 (65.49%)      | 126 (52.5%)    | 1.00        |         |
|                  | A allele | 78 (34.51%)       | 114 (47.5%)    | 1.72 (1.18–2.49) |         |

POAG, primary open-angle glaucoma; OR, odds ratio; 95% CI, 95% confidence interval, p value ≤0.05
expression of miR182 in ciliary body, trabecular meshwork and aqueous humor samples from patients with high-tension glaucoma compared to those from unaffected controls. The authors suggested a potential contribution of miR182 in POAG pathogenesis, probably through regulation of aqueous humor dynamics and IOP. Another research from Li and colleagues revealed a 7- to 9-fold upregulation of miR182 expression in primary cultures of human trabecular meshwork cells during stress-induced premature senescence, which may contribute to the phenotypic alterations of senescent cells. [40] It is hypothesized that miR182 overexpression in trabecular meshwork cells may lead to potential cellular dysfunction, such as cell contractility and phagocytosis ability [31] and may underlie, as a consequence, the increase in glaucoma risk. Interestingly, the minor T allele of the rs76481776, which is overrepresented in our POAG patients, has been previously reported to increase the expression of mature miR-182 in vitro. [25] However, there is still little information regarding the involvement of miR182 and its polymorphisms in the pathophysiology of glaucoma and more research is needed for a better understanding of their role in glaucoma development.

CDKN2B is a tumor suppressor gene involved in cell signaling pathways and polymorphisms in this gene have been associated with various diseases, including multiple cancers, gestational diabetes mellitus, [42] familial combined hyperlipidemia [43] and glaucoma. [44–49] The gene is located at the chromosome 9q21, which is considered a strong candidate for POAG risk. [36] Several studies have investigated the association of SNP rs1063192, a common variant near CDKN2B, with POAG in different ethnic groups but with conflicting findings. [9, 35, 36] The small sample sizes, the clinical heterogeneity and the different ethnic populations seem to be the main factors contributing to the inconclusive findings of the studies. [37] A recent meta-analysis of Hu and He [37] found that rs1063192 polymorphism decreases the risk of glaucoma among Caucasians, Asians and Africans. However, the SNP of CDKN2B rs3217992 in patients with glaucoma has not been genotyped until now. Our study demonstrated an association between the CDKN2B rs3217996 polymorphism and the development of POAG in a Greek population. Both rs3217992 and rs1063192 polymorphisms are considered miRNA-binding-site variants that has been shown to affect the miRNA-mediated regulation of the CDKN2B gene in vitro. [43, 50] It has been shown that rs3217992 and rs1063192 affect miR-138-3p- and miR-323b-5p-mediated regulation of CDKN2B respectively and an allelic-specific regulation in these miRNA-binding sites is considered a potential mechanism, at least in part, to explain the association between the CDKN2B polymorphisms and POAG. [30, 50]

Limitations of our study include the small sample size, the small number of SNPs and the difference concerning the age between the two groups of the study.

In conclusion, our study demonstrated that rs76481776 in miR182 gene and rs3217992 in CDKN2B gene are associated with the development of POAG in a Greek population. Specifically, the carriers of the T allele of rs76481776 in miR182 and the carriers of the A allele of rs3217992 in CDKN2B have an increased risk of developing POAG in our population. Taking into consideration the complexity of the disease pathogenesis and the variety of the genetic and environmental factors involved, future studies of populations with different ethnicities are needed to investigate the association of these polymorphisms with POAG risk.

Supporting information
S1 Data. (XLSX)
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