**MYOC Gene Sequencing Analysis in Primary Open-Angle Glaucoma Patients from the Centre Region of Portugal**

Análise por Sequenciação do Gene MYOC em Doentes com Glaucoma Primário de Ângulo Aberto da Região Centro de Portugal

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**ABSTRACT**

**Introduction:** Primary open-angle glaucoma is the most frequent subtype of glaucoma. Relatives of primary open-angle glaucoma patients have an increased risk of developing the disease, suggesting a genetic predisposition to the disease. MYOC was the first primary open-angle glaucoma-causing gene identified. This study aimed to identify sequence variations in the MYOC gene that may be responsible for the phenotype in a group of primary open-angle glaucoma patients from the Centre Region of Portugal.

**Material and Methods:** The three coding exons and the proximal splicing junctions of the MYOC gene were studied using a PCR-sequencing approach in a group of 99 primary open-angle glaucoma patients.

**Results:** The sequencing analysis enabled the identification of 20 variants, including four in the promoter region, seven in the introns and nine in exons one and three, of which four were missense variants.

**Discussion:** Initially, all four missense sequence variations identified were considered candidates to glaucoma causing disease mutations. However, after literature review, only variant c.1334C>T (Ala445Val) remained as likely responsible for mild late-onset normal tension glaucoma.

**Conclusion:** This is the first study performed in a group of primary open-angle glaucoma patients from the Centre Region of Portugal, contributing to the identification of one genetic variant in the MYOC gene and reinforcing the hypothesis that normal tension glaucoma could be also due to MYOC gene variations.

**Keywords:** Genetics; Glaucoma/diagnosis; Low Tension Glaucoma; Mutation, Missense

**INTRODUCTION**

Glaucoma is a group of optic neuropathies essentially characterized by a progressive degeneration of retinal ganglion cells (RGCs) and their axons, leading to excavation in the optic nerve head and, consequently, characteristic, progressive and irreversible visual field defects. At the beginning, the peripheral vision loss may not interfere with the daily routine and remains undetected. Therefore, until an advanced stage of the disease is reached, which usually consists on central vision loss, most patients are unaware that they have the disease and, consequently, remain undiagnosed and untreated.

This ocular disease is the second leading cause of blindness and the leading cause of irreversible blindness in...
the world, affecting 67 million people, of which 85% - 90% have primary open-angle glaucoma (POAG) in developed countries. It is estimated that the number of people with glaucoma in the world will increase to 76 million in 2020 and to 111.8 million in 2040, from which 52.7 million and 79.8 million, respectively, will be POAG patients.7 Although the pathogenesis of POAG has not been fully elucidated, there are known risk factors for the disease including elevated intraocular pressure (IOP), age, ethnicity, a positive family history, pseudoxfoliation, central corneal thickness, myopia and ocular perfusion pressure.4,6 And even though elevated IOP is a risk factor for POAG, it is not a necessary feature for diagnosis since an important subtype of POAG, known as normal tension glaucoma (NTG), occurs at low to normal IOP levels.6 NTG accounts for approximately 20% - 50% of all POAG cases.8

There is little doubt that a positive family history increases the risk of developing glaucoma. Relatives of POAG patients have been shown to have an eight-fold increase in the disease risk,4,6 exhibiting an autosomal dominant heredity.6 MYOC, a gene composed by three exons and encoding a protein with 504 amino acids,10 was the first POAG-causing gene identified11 and mutations in this gene are responsible for approximately 2% to 4% of the cases.12 Prior to the identification of this gene, Sunden et al mapped the GLC1A locus comprising an interval in the long arm of chromosome 1 (1q21-q31), that was associated with juvenile open-angle glaucoma (JOAG), a subtype of POAG with onset earlier than 35 years old (yo) and very high IOP.13 Afterwards, this region was limited to chromosome 1q23 - q25. The defective gene in this locus was finally identified as TIGR (Trabecular meshwork-Inducible Glucocorticoid Response).14 In the meantime, Japanese researchers mapped this gene by FISH within the chromosome 1q23 - 1q24 region, and due to its homologous regions with myosin, TIGR was named MYOC.14

The myocilin protein is ubiquitously expressed in normal tissues and organs,15 widely expressed in ocular tissues and highly expressed in the trabecular meshwork (TM), where it plays an important role in the regulation of IOP.16-18 Despite a number of studies over a 20 year period since its discovery in 1997, the physiological functions and biological activities of myocilin in the TM remain poorly understood. Aggregation of aberrant mutant myocilins is closely associated with glaucoma pathogenesis. The aggregation of misfolded/wild-type myocilins in the endoplasmic reticulum (ER) may be harmful for TM cells and lead to apoptosis.18 Previous results have suggested that the TM is several times thicker in patients with glaucoma harboring mutations compared with that in patients without myocilin mutations. Therefore, myocilin mutations appear to be involved in the morphological changes in the TM, which lead to cell apoptosis.19

The present study aimed to identify sequence variations in the MYOC gene that may be responsible for the phenotype in a group of POAG patients from the Centre Region of Portugal.

MATERIAL AND METHODS

Human subjects

A group of 99 Portuguese Caucasian POAG patients from the Centre Region of Portugal, consisting of 52 males and 47 females with an average age of 71.2 yo and ranging from 42 to 88 yo, and an average age at diagnosis of 61.7 yo and ranging from 23 to 82 yo, was recruited to this study and a clinical characterization was performed at the Ophthalmology Department of the Centro Hospitalar e Universitário de Coimbra. All patients underwent a detailed ophthalmologic examination to ensure the diagnosis of POAG including: 1) exclusion of secondary causes, 2) open drainage angles on gonioscopy (Shaffer’s grading III-IV), 3) presence of typical glaucomatous optic disc damage (excavation) and 4) visual field defects detected by automated perimetry (with Humphrey’s perimeter). The IOP was also evaluated since ocular tension enables the distinction of POAG subtypes. Accordingly, glaucoma patients with IOP equal or below 21 mmHg are diagnosed as NTG.

Prior to the identification of this gene, Sunden et al identified the MYOC gene mutation in a NTG patient, Acta Med Port 2021 34(AOP)

Sequence variations identification

The DNA was extracted from the peripheral blood of POAG patients using a standard phenol–chloroform method followed by ethanol precipitation.20

Individual exons and adjacent regions of the MYOC gene were amplified by polymerase chain reaction (PCR) using primers designed with Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). The primers nucleotide sequence will be made available upon request to the corresponding author. The PCR reactions were performed using 50 ng of genomic DNA mixed with the following reagents: 1X Taq Buffer 10X [with (NH₄)₂SO₄] (Fermentas), 0.2 µM of forward and reverse primers (Sigma-Aldrich), 1.5 mM MgCl₂ (Fermentas), 0.2 mM dNTPs (5PRIME), 1U Taq Polymerase (Fermentas) and RNase/DNase free Water (AccuGENE) to a final volume of 10 µl. The reaction mixtures were subjected to a specific PCR program with an initial denaturation step of five minutes (min) at 95°C followed by 35 cycles, each with denaturation at 95°C for 30 seconds (sec), annealing at 59°C - 63°C for 30 sec, and extension for one min at 72°C, with a final elongation step of 10 min at 72°C.

PCR products underwent an electrophoresis on an agarose gel containing 1% agarose SeaKem LE (Lonza) and 1%
The amplified PCR products were purified using 1 µl of ExoSAP-IT® and sequencing reactions were performed using BigDye® Terminator v3.1 according to manufacturer recommendations (Applied Biosystems) and the primers previously used for target DNA amplification. The sequencing reaction products were separated on a Genetic Analyzer 3130 (Applied Biosystems) equipment. DNA sequencing data obtained was analysed using Sequencing Analysis Software v.5.4® (Applied Biosystems).
and so it is still questionable if these variants may influence MYOC gene expression and lead to glaucoma. Also interesting for future line of research is the simultaneous identification of the promoter variant c.-83G>A and exon 1 missense alteration c.227G>A p.(Arg76Lys), even sharing the same genotype in every patient and suggesting a segregation in linkage disequilibrium as previously reported. If separately both are unanimously considered neutral polymorphisms for glaucoma, their impact in linkage remains elusive.

Personalized medicine using genetic information to anticipate disease onset and progression, and to implement preventive interventions for each patient is an evolving field. This is directly associated with the exponential drop in cost of high-throughput genome-wide genotyping platforms. Next generation, high-throughput DNA sequencing technology offers a powerful approach to identify causal genetic variants for many rare and common genetic disorders, including POAG. Genetic testing for POAG is clearly helpful in some specific situations, such as screening of family members in autosomal dominant POAG of early onset. Early identification of mutation carrying individuals creates the opportunity for early implementation of medical and surgical treatment alternatives for slowing down the progression or even preventing glaucoma from developing. But this is only possible if genetic testing is included in the diagnostic criteria for glaucoma. Recently, it was demonstrated that MYOC cascade genetic testing for POAG allows identification of at-risk individuals at an early stage or even before signs of glaucoma are present. This was only possible due to MYOC gene screening and identification of disease-causing mutations in POAG patients and further mutation screening in patients’ relatives. Without genetic testing as a diagnostic criterion there will be no mutation identification, no relatives tested, no early diagnosis achieved and no preventive therapies applied. Taking into consideration the present study, gathering of DNA samples for genetic testing from relatives of the patient with variant c.1334C>T p.(Ala445Val) is ongoing.

CONCLUSION

This is the first study performed in a group of POAG patients from the Centre Region of Portugal contributing to the identification of one genetic variant in the MYOC gene [c.1334C>T p.(Ala445Val)], probably responsible for a mild late onset glaucoma through a neurodegenerative mechanism that is independent of TM dysfunction. These findings will enable cascade genetic testing of patient’s relatives with the aim of identifying at-risk individuals and implementing therapeutic procedures to prevent the development of glaucoma. Accordingly, genetic testing should be included in the diagnostic approach for glaucoma.

PROTECTION OF HUMANS AND ANIMALS

The authors declare that the procedures were followed according to the regulations established by the Clinical Research and Ethics Committee and to the 2013 Helsinki Declaration of the World Medical Association.

DATA CONFIDENTIALITY

The authors declare having followed the protocols in use at their working center regarding patients’ data publication.

COMPETING INTERESTS

The authors have declared that no competing interests exist.

FUNDING SOURCES

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REFERENCES


**Figure 1** – Electropherogram of the genetic region surrounding variant c.1334C>T p.(Ala445Val) identified in a normal tension glaucoma patient. Heterozygous transition of a C to a T at nucleotide 1334, changing the codon GCA to GTA and causing an Alanine to Valine amino acid substitution at position 445. The nomenclature used in Fig. 1 for sequence variation is according to reference.45

**Table 1** – Sequence variations identified in MYOC gene of POAG patients

<table>
<thead>
<tr>
<th>Sequence variation</th>
<th>Amino acid change</th>
<th>Wild type homozygous</th>
<th>Genotype (n = 99)</th>
<th>Mutant homozygous</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.-224T&gt;C</td>
<td>-</td>
<td>54 TT</td>
<td>41 TC</td>
<td>4 CC</td>
</tr>
<tr>
<td>c.-190G&gt;T</td>
<td>-</td>
<td>98 GG</td>
<td>1 GT</td>
<td>-</td>
</tr>
<tr>
<td>c.-126T&gt;C</td>
<td>-</td>
<td>94 TT</td>
<td>5 TC</td>
<td>-</td>
</tr>
<tr>
<td>c.-83G&gt;A</td>
<td>-</td>
<td>83 GG</td>
<td>16 GA</td>
<td>-</td>
</tr>
<tr>
<td>c.39T&gt;G</td>
<td>p.(Pro13Pro)</td>
<td>96 TT</td>
<td>3 TG</td>
<td>-</td>
</tr>
<tr>
<td>c.141C&gt;T</td>
<td>p.(Cys47Cys)</td>
<td>97 CC</td>
<td>2 CT</td>
<td>-</td>
</tr>
<tr>
<td>c.227G&gt;A</td>
<td>p.(Arg76Lys)</td>
<td>83 GG</td>
<td>16 GA</td>
<td>-</td>
</tr>
<tr>
<td>c.477A&gt;G</td>
<td>p.(Leu159Leu)</td>
<td>96 AA</td>
<td>3 AG</td>
<td>-</td>
</tr>
<tr>
<td>c.604+50G&gt;A</td>
<td>-</td>
<td>97 GG</td>
<td>2 GA</td>
<td>-</td>
</tr>
<tr>
<td>c.605-332G&gt;A</td>
<td>-</td>
<td>71 GG</td>
<td>26 GA</td>
<td>2 AA</td>
</tr>
<tr>
<td>c.605-280G&gt;T</td>
<td>-</td>
<td>16 GG</td>
<td>57 GT</td>
<td>26 TT</td>
</tr>
<tr>
<td>c.605-210delT</td>
<td>-</td>
<td>97 TT</td>
<td>2 Tdel</td>
<td>-</td>
</tr>
<tr>
<td>c.730+35A&gt;G</td>
<td>-</td>
<td>45 AA</td>
<td>46 AG</td>
<td>8 GG</td>
</tr>
<tr>
<td>c.731-205A&gt;C</td>
<td>-</td>
<td>66 AA</td>
<td>32 AC</td>
<td>1 CC</td>
</tr>
<tr>
<td>c.731-73C&gt;T</td>
<td>-</td>
<td>95 CC</td>
<td>4 CT</td>
<td>-</td>
</tr>
<tr>
<td>c.878C&gt;A</td>
<td>p.(Thr293Lys)</td>
<td>98 CC</td>
<td>1 CA</td>
<td>-</td>
</tr>
<tr>
<td>c.975G&gt;A</td>
<td>p.(Thr325Thr)</td>
<td>96 GG</td>
<td>3 GA</td>
<td>-</td>
</tr>
<tr>
<td>c.1041T&gt;C</td>
<td>p.(Tyr347Tyr)</td>
<td>94 TT</td>
<td>5 TC</td>
<td>-</td>
</tr>
<tr>
<td>c.1193A&gt;G</td>
<td>p.(Lys398Arg)</td>
<td>97 AA</td>
<td>2 AG</td>
<td>-</td>
</tr>
<tr>
<td>c.1334C&gt;T</td>
<td>p.(Ala445Val)</td>
<td>98 CC</td>
<td>1 CT *</td>
<td>-</td>
</tr>
</tbody>
</table>

* Marks the individual with the variant responsible for late-onset NTG.

The nomenclature used in Table 1 for sequence variations is according to reference.45