

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 mutations.

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

RESUMO

Introdução: O glaucoma primário de ângulo-aberto é o subtipo mais frequente de glaucoma. Os familiares de doentes com glaucoma primário de ângulo-aberto têm um risco maior de desenvolverem a doença, o que sugere uma predisposição genética para a doença. *MYOC* foi o primeiro gene causador de glaucoma primário de ângulo-aberto a ser identificado. Este estudo pretendeu identificar variações de sequência no gene *MYOC* que possam ser responsáveis pelo fenótipo num grupo de doentes com glaucoma primário de ângulo-aberto da Região Centro de Portugal.

Material e Métodos: Os três exões codificantes e as regiões adjacentes do gene *MYOC* foram estudados utilizando o método de PCR-sequenciação num grupo de 99 doentes com glaucoma primário de ângulo aberto.

Resultados: A análise de sequenciação permitiu identificar 20 variantes, incluindo quatro na região promotora, sete nos intrões e nove nos exões um e três, das quais quatro eram variantes missense.

Discussão: Inicialmente, todas as quatro variações de sequência missense identificadas foram consideradas candidatas a mutações causadoras de glaucoma. No entanto, após análise da literatura, somente a variante c.1334C>T (Ala445Val) permaneceu como provável responsável pelo glaucoma de pressão normal de início tardio.

Conclusão: Este é o primeiro estudo realizado num grupo de doentes com glaucoma primário de ângulo aberto da Região Centro de Portugal, contribuindo para a identificação de uma variante genética no gene *MYOC* e reforçando a hipótese de que o glaucoma de pressão normal também poderá ser causado por mutações no gene *MYOC*.

Palavras-chave: Genética; Glaucoma/diagnóstico; Glaucoma de Baixa Pressão; Mutação 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.¹ At the beginning, the peripheral vision loss may not interfere with the daily routine and remains undetected.² 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.³

This ocular disease is the second leading cause of blindness⁴ and the leading cause of irreversible blindness in the world, affecting 67 million people, of which 85% - 90%

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have primary open-angle glaucoma (POAG) in developed countries.⁵⁻⁶ 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, pseudoexfoliation, 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.⁹ *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)*.¹¹ 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.⁴ For the present study, 26 patients with high IOP and 73 with IOP equal or below 21 mmHg (NTG) were randomly recruited.

This study was approved by the Ethics Committee of the Faculty of Medicine, University of Coimbra, following the tenets of the Declaration of Helsinki 2013 and a written consent for genetic testing was obtained from adult probands.

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% ethidium bromide (Acros/Fisher bioreagents) in 1X Tris Borate EDTA (TBE) solution (National diagnostics).

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

four were found in intron one (c.604+50G>A, c.605-332G>A, c.605-280G>T and c.605-210delT) and three were found in intron two (c.730+35A>G, c.731-205A>C and c.731-73C>T) (Table 1). The missense variants c.878C>A p.(Thr293Lys) and c.1334C>T p.(Ala445Val) (Fig. 1) were identified in heterozygosity in 1 patient each, the c.1193A>G p.(Lys398Arg) in two patients and variant c.227G>A p.(Arg76Lys) was identified in 16 patients (Table 1). It is noticeable that the promoter variant c.-83G>A and the exon one missense alteration c.227G>A p.(Arg76Lys) were always found simultaneously in the same patients, even sharing the same genotype.

DISCUSSION

The sequencing analysis of the *MYOC* gene in 99 POAG patients allowed the identification of 20 variants including four missense alterations [c.227G>A p.(Arg76Lys), c.878C>A p.(Thr293Lys), c.1193A>G p.(Lys398Arg) and c.1334C>T p.(Ala445Val) (Fig. 1)] (Table 1). Initially, all four missense sequence variations were considered candidates to glaucoma causing disease mutations. However, after literature review, it was possible to determine that c.227G>A p.(Arg76Lys), c.878C>A p.(Thr293Lys) and c.1193A>G p.(Lys398Arg) variants were previously described in individuals without the glaucoma phenotype,²¹⁻²⁸ thus likely neutral polymorphisms. Nevertheless, the variant c.1334C>T p.(Ala445Val) (Fig. 1) was previously identified only in glaucoma patients and consequently reported as a glaucoma causing mutation.^{8,22,29-33}

With the aim of developing a biochemical assay to distinguish different forms of myocilin protein, a cellular assay with Triton X-100 detergent was applied to determine protein solubility of mutant and normal forms of the protein, taking into consideration that misfolded myocilin mutants aggregate in the ER and are insoluble. Variant c.1193A>G p.(Lys398Arg) was one of the studied variants and the assay established its solubility,³⁴ and consequently non-pathogenicity.

Upon crystal structure-based prediction, variant p.(Thr293Lys) is a remote surface exposed residue having wild-type-like stability, which makes it unlikely to promote misfolding of myocilin protein.³⁵ Considering this data and the identification of c.878C>A p.(Thr293Lys) in individuals without the glaucoma phenotype, this variant is most likely a neutral polymorphism.

Variant c.1334C>T p.(Ala445Val) (Fig. 1) is located in *MYOC* gene exon 3 and results from an alteration at the second nucleotide of codon 445, changing an amino acid alanine to a valine. Even if it is unlikely that this amino acid change causes alterations in the protein properties since both alanine and valine are non-polar and hydrophobic amino acids,²⁹ the strong former α alanine changes to a strong former β valine may possibly cause an increased preference for a β -sheet conformation³⁶ and a significant modification in the secondary structure of the myocilin protein. However, based on crystal structure-based prediction, p.(Ala445Val) is located on a remote surface

of olfactomedin (OLF) domain³⁵ and exhibits wild-type-like stability for OLF melting temperature,³⁷ suggesting that it is not prone to misfolding.³⁵ Nevertheless, using size exclusion chromatography, it was possible to determine that the p.(Ala445Val) OLF domain has a higher yield of aggregated species, allowing the identification of significant differences in the ratio of aggregate to monomer species when compared with wild type OLF domain, suggesting that p.(Ala445Val) is more similar to disease causing variants than to the wild-type.³⁷ Additionally, in the present study, the c.1334C>T p.(Ala445Val) (Fig. 1) variant was identified in a male NTG patient with 77 yo at diagnosis and an IOP of 17mm/Hg in both eyes. Taking into consideration that *MYOC* gene mutations are mainly associated with JOAG patients with an early onset before 35 yo and very high IOP, most likely caused by severe morphological changes in TM, our study suggests that the c.1334C>T p.(Ala445Val) variant may be responsible for a mild late onset form of glaucoma regardless of TM dysfunction and likely caused by a neurodegenerative mechanism affecting RGC, as previously proposed.³⁸ Finally, the present study supports the glaucoma causing mutation classification for the *MYOC* variant c.1334C>T p.(Ala445Val) based on its association with late onset NTG and high yield of aggregated species.

Additional results obtained in the present study include four non-coding sequence variations found in the *MYOC* gene promoter region (c.-224T>C, c.-190G>T, c.-126T>C and c.-83G>A) (Table 1). As much as it was possible to determine from the literature review, there is no association between the *MYOC* gene promoter region variants and any type of glaucoma, 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.^{32,39-42} 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.⁴³ This is directly associated with the exponential drop in cost of high-throughput genome-wide genotyping platforms.⁴⁴ 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.¹⁰ Genetic testing for POAG is clearly helpful in some specific situations, such as screening of family members in autosomal dominant POAG of early onset.⁴⁴ 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.⁴³ 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.⁴³ 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.

AUTHORS CONTRIBUTION

FS: Substantial contribution to the conception, design of the work, acquisition analysis and interpretation of data for the work. Drafting and final approval of the version to be published.

FF: Substantial contribution to the acquisition, analysis and interpretation of data for the work. Drafting the work and revising it critically for important intellectual content. Final approval of the version to be published.

PF: Substantial contribution to the conception, acquisition and interpretation of data for the work. Revising it critically for important intellectual content. Final approval of the version to be published.

IS: Substantial contribution to the acquisition of data for the work. Revising it critically for important intellectual content. Final approval of the version to be published.

MR: Substantial contribution to the acquisition and analysis of data for the work. Revising it critically for important

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JP, JFS: Substantial contribution to the acquisition of data for the work. Revising it critically for important intellectual content. Final approval of the version to be published.

MG, HG, PP: Substantial contribution to the conception of the work. Revising it critically for important intellectual content. Final approval of the version to be published.

JMP: Substantial contribution to the conception and acquisition of data for the work. Revising it critically for important intellectual content. Final approval of the version to be published.

All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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.

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