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Association between Primary Open-Angle Glaucoma and WDR36 sequence variance in Italian families affected by POAG

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Key Words: WDR36 gene, MYOC, primary open angle glaucoma, OPTN, normal tension
glaucoma

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Abstract

Background/aims: To assess the involvement of WDR36 sequence variance in primary open-angle glaucoma (POAG) in Italian patients.

Methods: A cohort of 34 Italian families affected by POAG has been analyzed by Denaturing High-Performance Liquid Chromatography for mutation in the WDR36 gene. Among the 34 families enrolled, 25 were affected by high tension glaucoma (HTG), 4 by juvenile open-angle glaucoma (JOAG) and 1 by normal tension glaucoma (NTG). In addition, 4 families presented within the same pedigree both JOAG and HTG-POAG patients.

Results: Four already known intronic polymorphisms (IVS5+30C>T; IVS12+90 G>T; IVS13+89G>A; IVS16-30A>G) and a novel one (IVS21-75G>A), have been identified. In addition, one proband was found to carry the D658G mutation reported, as the more recurrent disease-causing allele.

Conclusions: Our findings suggest that WDR36 sequence variance are only rare causes of glaucoma in Italian families with glaucoma. Clearly, investigation of additional families with extensive studies are request to clarify the role of WDR 36 in the pathophysiology of glaucoma.

1 Introduction

2   The term primary open-angle glaucoma (POAG) describes a heterogeneous group of optic
3   neuropathies that lead to optic nerve atrophy and permanent loss of vision.¹ It is the second most
4   prevalent cause of bilateral blindness in the Western world and affected about 67 million subjects in
5   the year 2000.^{2,3} POAG includes various clinical entities, such as ocular hypertension (OH),
6   glaucoma with elevated intraocular pressure (high tension glaucoma HTG-POAG) and normal
7   tension glaucoma (NTG), juvenile open angle glaucoma (JOAG).¹ Genetic factors play a major role
8   in the aetiology of HTG-POAG. Up to now 14 loci (GLC1A > GLC1N) have been associated with
9   POAG using linkage analysis.⁴ Some HTG-POAG families have mutations in the genes associated
10   to GLC1A, GLC1E and GLC1G loci. Mutations in the myocilin gene (MYOC) at locus GLC1A,
11   have been reported in 2-4% of HTG-POAG patients and in up to 33% of JOAG patients.^{5, 6} In
12   2002, the Optineurin gene (OPTN), associated to GLC1E locus was identified.⁷ In the 54 families
13   analysed with adult-onset HTG-POAG and in at least one member with NTG, OPTN mutations
14   have been found in 16.7% of cases.⁸ In these families individuals with autosomal dominant NTG
15   were present. In 2005 a new locus on chromosome 5q22 related to HTG-POAG and NTG was
16   identified.² The associated gene, WDR36, was also identified. Sequence variations in the WDR36
17   gene were identified in approximately 5% of POAG cases.² WDR36 is a member of the WD40
18   repeat protein family. WDR36 sequence variants can lead to an altered cellular phenotype
19   supporting the theory that WDR36 participate in polygenic form of glaucoma.⁹ Interestingly, the
20   T-cell mediated response has been in turn hypothesized to participate in optic nerve degeneration.¹⁰
21   The aim of this study was to define the frequency of WDR36 sequence variance in glaucoma
22   Italian families.

1   **Methods and Patients**

2

3   The study was performed in accordance with the tenets of the Declaration of Helsinki. The

4   local ethics committee approved the study. To record the clinical characteristics of the patient, a

5   standard form was provided and written informed consent was obtained from each participant. The

6   project structure provides that a continuous enrolment of glaucoma patients was made for two years

7   in 6 different Italian eye clinics (Siena, Torino, Milano, Cagliari, Roma, and Verona) in order to

8   obtain a consistent population. DNA samples of 34 patients (index cases or probands) and 107

9   relatives were collected from the 6 centers located throughout Italy. Patients included in the study

10   belonged to families in which at least two members were affected by POAG. The patients were

11   questioned and records kept to determine family and personal history, age, sex, race, age at

12   diagnosis, pedigree, medications used, prior laser and surgical procedures, co-existing systemic

13   diseases.

14   Each patient underwent a complete ophthalmic evaluation: anterior segment examination,

15   uncorrected and best-corrected visual acuity, gonioscopy with grading according to Van Herick-

16   Shaffers-Schwartz grading system,¹¹ fundus examination with indirect ophthalmoscopy,

17   biomicroscopic analysis of the optic nerve head (ONH) with a 78D lens and evaluation of the

18   cup/disk ratio, intraocular pressure (IOP) measurement with Goldmann tonometer and visual field

19   examination.

20   They were defined as "affected by glaucoma" if they fulfilled the following criteria: typical

21   glaucomatous visual field (VF) loss on either Octopus or Humphrey perimetry and glaucomatous

22   alterations of the ONH.¹²

23   A visual field was classified as glaucomatous when: 1) three adjacent points depressed by 5

24   dB, with one of the points depressed by at least 10 dB; 2) two adjacent points depressed by 10 dB;

25   or 3) a 10 dB difference across the nasal horizontal meridian in two adjacent points. None of the

26   points could be edge points unless immediately above or below the nasal horizontal meridian.¹² In

addition, visual field testing was considered reliable only when false-negative responses were less than 30% and fixation losses were less than 20%.¹² The perimetric defect type and stage evaluation were performed using the Brusini's glaucoma staging system.¹³

POAG is characterized by progressive narrowing of the neuroretinal rim. An optic nerve head was classified abnormal when it was present a ONH notch, or a concentric thinning of the neuroretinal rim, or both in combination.^{14,15}

At the end of the examination, glaucomatous patients were catalogued into three groups. Patients were classified as POAG when they were 35 years and older and either typical glaucomatous VF loss or glaucomatous alterations of the ONH. Based on the IOP values the POAG group was divided into two groups: HTG-POAG if an IOP $\geq$ 21 mmHg and NTG-POAG if IOP < 21 mmHg. If the patients were younger than 35 years and also an IOP > 21 mmHg and either typical glaucomatous VF loss or glaucomatous alterations of the ONH, they were classified as JOAG.¹

Molecular analysis

Blood samples were collected from affected and healthy family members (total number of collected samples was 141). Genomic DNA was isolated from the peripheral blood leukocytes by using the QIAamp DNA Blood Kit according to the manufacturer protocol (Qiagen, www.qiagen.com). We used the OD260/280 method on a photometer to determine the appropriate DNA concentration.¹⁶ All 23 coding exons and flanking introns of the WDR36 gene were amplified using primers designed on intronic sequences (Table 1). Mutation analysis was performed by Denaturing High Performance Liquid Chromatography (DHPLC) using the Transgenomic WAVE^{TM} 3500 HT (Transgenomic, San Jose, CA, USA; <http://www.transgenomic.com>) (Underhill PA et al Genome Res 7:996-1005, 1997). PCR product were denatured at 95°C, reannealed at 65°C for 10 minutes, and cooled to 4°C to generate heteroduplexes. The optimal column temperature for fragments analysis was calculated using the WaveMaker Software (Transgenomic, San Jose, CA,

USA). PCR and DHPLC analysis conditions are reported in Table 1. Direct sequencing of the purified PCR products was performed in both directions (PE Big dye terminator cycle sequencing kit) on an ABI310 Automated Sequencer and analysed with the Sequencher software .

Results

Thirty-four familial cases were enrolled in the study from the 6 eye centers involved in the project. Number of affected members per family varied from 2 to 6 individuals. Analysis of the pedigrees suggested an autosomal dominant inheritance in all families. A total number of 141 subjects underwent to accurate clinical examination. The clinical examination allowed us to define 69 affected individuals (37 females and 32 males). Among the 34 enrolled families, 25 were affected by HTG-POAG, 4 by JOAG and 1 by NTG-POAG. In addition, 4 families presented both JOAG patients and HTG-POAG patients within the same pedigree.

DHPLC mutation analysis of WDR36 gene was performed in the 34 probands of above reported families. In all families mutation in MYOC gene and in OPTN gene were previously excluded⁶ (and personal data). The following intronic variants have been identified: IVS12+190 G>T; IVS13+89G>A; IVS16-30A>G. None of these variants segregate with the disease in the families. The following four already known intronic polymorphisms were found: IVS5+30C>T; IVS12+90 G>T; IVS13+89G>A; IVS16-30A>G.² In addition, a novel polymorphism was found: IVS21-75G>A. This change was present in our population in 13% of patients and 14% of controls.

One proband was found to carry the D658G mutation reported as the more recurrent disease-causing allele.² The proband, aged 58 years, was affected by HTG-POAG, with an age at diagnosis of 45 years (Fig. 1). He had three sons, one female and two males, aged, 35, 32 and 28 years, respectively. In the sons, ocular hypertension started at 20, at 19 and 27 years, respectively and it was controlled by antihypertensive treatment. Segregation analysis showed that the mutation was inherited only by the youngest son (Fig. 1).

Discussion

The aim of this study was to define the involvement of WDR36 sequence variance in glaucoma Italian families. We previously demonstrated that in the Italian population, about 6% of the familial cases with primary open angle glaucoma has a mutation in MYOC gene.⁷ On the contrary, no mutations were found in OPTN gene.¹⁷ Since the WDR36 gene has been recently reported as the third glaucoma-causing gene, we decided to evaluate which fraction of Italian cases is due to this new gene. We did not find any pathogenic mutation in our cohort of patients. We identified only intronic polymorphisms. In one family we identified the D658G mutation, previously reported as the more recurrent disease-causing allele.¹⁷ Furthermore, in the four families in which we found JOAG and POAG, we can supposed that glaucoma onset was different among the family members, but the disease was likely the same. The different classification was due to the classification methods, that we use in clinical practice, indeed the main different between POAG and JOAG is the glaucoma onset.¹

Hauser et al analyzed a large cohort of patients with glaucoma for mutations in WDR36 gene. They found several nonsynonymous single-nucleotide polymorphisms, including those previously described as "disease-causing" and "disease susceptibility," in both patient and control subjects.¹⁰ They concluded that abnormalities in WDR36 were not sufficient alone to cause HTG-POAG.

In 2006, Pang et al mapped a new locus for juvenile open angle glaucoma in 5q22.1-q32.¹⁸ They described a region of maximum lod score located telomerically with respect to WDR36, by studying a family with 27 members, in which 9 were confirmed JOAG patients. The analysis of the WDR36 coding sequence did not reveal any pathogenic mutation and it definitively excluded the involvement of this gene in generating the phenotype.

In 2008, Pasutto et al recruited 399 patients with glaucoma and 376 healthy subjects and investigated the prevalence of WDR36 variants in German populations.¹⁹ A total of 44 WDR36 allelic variants were detected but the occurrence of several rare putative disease causing variants in

1 patients with glaucoma suggested to the authors that WDR36 may be a minor disease causing gene
2 in glaucoma at least in the German population.

3 In Hauser et al. HTG-POAG patients WDR36 sequence variations were associated with a
4 more severe disease phenotype than those without, suggesting that the sequence variants in WDR36
5 may play a role in disease susceptibility rather than causation.¹⁰

6 POAG is a disease with a complex inheritance that is likely to result from contributions of
7 multiple genes and possibly environmental conditions. Mendelian autosomal dominant and
8 recessive forms of glaucoma are caused by single gene defects but the genetic etiologies results
9 from contributions of multiple genetic factors that independently are able to cause the disease.
10 Genes that contribute to POAG may not cause clinical evidence of the disease unless they are
11 coupled with other genes or environmental factors. For this reason the identification of any one
12 disease-predisposing factor can be difficult when using traditional linkage approaches. Our
13 findings suggested that WDR36 sequence variance were only rare causes of glaucoma in Italian
14 families with glaucoma. Clearly, investigation of additional families with extensive studies are
15 request to clarify the role of WDR 36 in the pathophysiology of glaucoma. It is very important the
16 identification of susceptibility and glaucoma modifying genes for the complete genetic and
17 molecular understanding of POAG, for this reason the European Glaucoma Society has a project to
18 create an European genetic database to better study the glaucoma phenotypes.²⁰

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1 **Figure legends**

2 Figure1: Segregation analysis of D658G change.

3 The proband and the youngest son have the same mutation. On the contrary the other two sons do

4 not bear mutation. Symbols, white = unaffected subjects, black = glaucoma, grey = ocular

5 hypertension.

Tables

Table 1. Oligonucleotides primers and PCR/DHPLC conditions for WDR36 analysis.

Exon	Forward primer sequence Reverse primer sequence	PCR T _m	DHPLC T _m	DHPLC ³ % B
1	F 5'-TGGCCCTCGGAACCCAAGTC-3' R 5'-ttggcctctaactctgtcctg-3'	65°	61.4° 63.4°	59.8 ⁴
2	F 5'-ttcttatgaaggacagcatagc-3' R 5'-agggtgccaggataaatcatg-3'	62°	54°	56.9 ⁵
3	F 5'-aatgttatatgtatacttgagg-3' R 5'-tagtggagggaactttaatt-3'	56°	55.4° 57.4°	55
4	F 5'-agaagcatctcaggattagc-3' R 5'-aaacatctatgccagagattt-3'	52°	54.6°	57
5	F 5'-catttacaagttgcctctca-3' R 5'-cttaagtctcatttctccct-3'	56°	53°	59.2
6	F 5'-atacagctttcaactatttttc-3' R 5'-ctgaggaaatagtgttttggga-3'	52°	55°	54.5
7_8	F 5'-gcgtatcatcaggattatta-3' R 5'-tcctcctggctcatctaaact-3'	56°	53.2° 54.2°	62.3
9	F 5'-cttgcagatcagatggaaca-3' R 5'-cagcaagtattgagatgtgg-3'	58°	56.7°	54.1
10	F 5'-aatttgtgattcacatagtc-3' R 5'-gctggtcacgaaagaaac-3'	56°	55°	51.4
11	F 5'-aggattcatagctatgtatg-3' R 5'-taagcattcagaaggtaacc-3'	52°	55.1° 56.1°	55.1
12	F 5'-cattgtatccctagtgtctga-3' R 5'-gctcctgtgacttctaagag-3'	60°	54.1° 56.5°	59.3
13	F 5'-tgctttggaaagcataaaagt-3' R 5'-gatgggttataatcaaccct-3'	52°	54.8°	57.1
14_15	F 5'-ctgtgtgtattgttcagag-3' R 5'-acttgactcatttctacttt-3'	53°	55.5°	61.7
16	F 5'-aggcagcctgaatgtag-3' R 5'-cgatagttaatgggttatatg-3'	55°	50.5°	58
17	F 5'-ctgcatctcttatcccttat-3' R 5'-cttaattcactgtaagtctg-3'	55°	53.9° 55.9°	58.2
18	F 5'-caatctatctttgaggtatt-3' R 5'-tgtaggagatattgctacca-3'	54°	53.2°	56.5
19	F 5'-ttgatgtggtattgataagc-3' R 5'-gcattagaagagcaattaa-3'	52°	53.8°	60.5
20_21	F 5'-ggatttggtcagaagagatca-3' R 5'-ggaaatatcagcgttctcagt-3'	60°	53.5°	61.9
22	F 5'-cacaggaagaatttcctgta-3' R 5'-ccagcttgtcttctcttg-3'	57°	53.1°	58.1
23	F 5'-tcacctagtgtcttttctg-3' R 5'-CCAAGTGTCAATGTGAAAAG-3'	55°	53.8° 55.8°	57.7

Fig.1