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Identification and functional characterization of a novel *MYOC* mutation in two primary open angle glaucoma families from The Netherlands

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Purpose: Glaucoma is the second most prevalent cause of blindness worldwide, projected to affect more than 60 million people by 2010, 75% of which represents primary open angle glaucoma (POAG). Of the three genes, namely, *Myocilin* (*MYOC*), *Optineurin* (*OPTN*), and WD repeat-containing protein 36 (*WDR36*), which have been shown to cause POAG when defective, *MYOC* is the most frequently mutated gene, accounting for 3%-4% of all POAG cases. The purpose of this study was identification and functional characterization of *MYOC* mutations in adult-onset, high-pressure POAG patients from The Netherlands.

Methods: The following criteria were required for study participants to be included: have at least two affected family members, an age of diagnosis of more than 35 years, intraocular pressure (IOP) of more than 22 mmHg, glaucomatous optic neuropathy in both eyes, visual field loss consistent with assessed optic neuropathy in at least one eye, and an open anterior chamber angle without morphological abnormalities by gonioscopy. Sequence analysis was performed in genomic DNA of 30 probands for the protein coding region of the *MYOC* gene. A Chinese hamster ovarian cell line (CHO-K1) was used to express wild type and mutant MYOC protein. Detergent solubility of MYOC was assayed and its secretory property was analyzed by immunoprecipitation.

Results: We recruited 250 individuals from 30 families (120 affected and 130 unaffected family members) with a positive history of POAG. We identified a novel mutation c.1288T>C (p.Phe430Leu) in exon 3 of *MYOC* in two unrelated families showing the same haplotype around the mutant allele. The novel mutation segregated completely with the disease in these families and was absent in 250 ethnically matched controls. All patients harboring this mutation showed severe glaucomatous damage, pointing to the deleterious effect of this mutation. Compared to the wild type, the mutant protein was less soluble when extracted with Triton X-100 and was secretion-defective.

Conclusions: The novel MYOC mutation, p.Phe430Leu, has the same origin in both POAG families from The Netherlands. The pathogenic nature of this mutation is suggested by the severe phenotype of mutant patients and mistrafficking of mutant protein as observed for other severe disease-causing mutations of *MYOC*.

Glaucoma is an anterior optic neuropathy with characteristic changes of the optic disc due to loss of retinal ganglion cells. The major risk factor for the disease is elevated intraocular pressure (IOP). Glaucoma is the second leading cause of blindness worldwide, projected to affect more than 60 million people by 2010 [1], and with the increasing average human life expectancy, the prevalence is rising. Primary open angle glaucoma (POAG, OMIM 137760) is the major subtype, and to date three genes, namely, *Myocilin* (*MYOC*), *Optineurin* (*OPTN*), and WD repeat-containing protein 36 (*WDR36*), have been reported to be primarily responsible for POAG [2-4]. POAG shows autosomal dominant inheritance with variable penetrance. The presence of many sporadic patients points to a complex genetic etiology. Myocilin (*MYOC*, OMIM 601652) is the most frequently mutated gene in POAG, accounting for 3%-4% of all the cases. To date, more than 70 missense variants in *MYOC* have been reported to be causal for POAG.

To investigate the role of *MYOC* in POAG patients from The Netherlands, we analyzed 30 families and described a novel mutation in exon 3 of *MYOC* that segregated with the disease in two different families from The Netherlands and was found to have the same disease-associated haplotype. Functional characterization strongly suggested it to be a pathologic variant.

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METHODS

Selection of subjects: We conducted a cross-sectional study on native Dutch patients with inherited POAG. The study was done in accordance with the principles of the Declaration of Helsinki and with the approval of the Ethics Committee. The broad purpose of the entire program is to identify novel genes causing a familial form of high-tension POAG. In the first phase of the program, we analyzed the occurrence of *MYOC* mutations. We included subjects with an age of diagnosis of more than 35 years, IOP of more than 22 mmHg measured by applanation tonometry in both eyes on two glaucoma medications, glaucomatous optic neuropathy in both eyes at funduscopy, visual field loss consistent with assessed optic neuropathy in at least one eye, an open anterior chamber angle without morphological abnormalities by gonioscopy, and at least two other affected first-degree family members.

Clinical examination: We recorded the ophthalmic and general medical history, and evaluated the best-corrected visual acuity (BCVA) with use of Snellen charts. The clinical examination included slit-lamp inspection of the anterior eye, IOP measurement by Goldmann applanation tonometry, anterior chamber angle evaluation by gonioscopy, corneal thickness calculation by ultrasound pachymetry, and fundus examination including optic disc assessment.

All study participants underwent static automated white-on-white Swedish Interactive Thresholding Algorithm (SITA) perimetry (SITA fast strategy, program 30-2, Humphrey perimeter; Carl Zeiss Meditec, Dublin, CA). In addition, a morphometric analysis of the optic disc was performed by the Heidelberg Retina Tomograph II (HRT II; Heidelberg Engineering, Heidelberg, Germany) as described elsewhere [5]. An ophthalmic photographer masked to the results of the previous tests conducted the examination. A mean topography of three images was generated in a 15°x15° frame, and the contour line was drawn on the mean image. Disc and cup areas were analyzed directly by custom software (version 2.01b, Heidelberg Engineering, Heidelberg, Germany) using the standard reference plane. The HRT Moorfields regression analysis was used for classification of the optic disc [6].

We have included subjects who were at least 35 years old when POAG was diagnosed. Diagnosis of POAG was given when IOP was more than 22 mmHg, which was measured by applanation tonometry in both eyes on two glaucoma medications, and glaucomatous optic neuropathy was present in both eyes at funduscopy as well as when visual field loss was consistent with assessed optic neuropathy in at least one eye.

Genomic DNA isolation, polymerase chain reaction, and sequencing analysis: EDTA-anticoagulated venous blood was collected after obtaining the informed consent from the participating individuals. Genomic DNA was isolated from EDTA blood using standard procedures [7]. A panel of 250 anonymous healthy Dutch control subjects was tested for the presence of the novel variant identified in this study. In the initial phase of the program, we routinely screened the open reading frame of *MYOC* to exclude the families with any mutation in this gene. For the results reported in this study, 30 probands (one patient per family) were screened for mutation in *MYOC*. The primers for amplification and sequencing of the open reading frame and splice junctions of *MYOC* are given in Table 1. The chromatograms were analyzed for the presence of variant alleles with the help of Vector NTI software (Invitrogen, Breda, The Netherlands).

Microsatellite marker analysis: A panel of five microsatellite markers were selected around *MYOC* spanning about 2.7 Mb of genomic DNA. The genomic location and the primer sequences are given in Table 2. After amplifying each locus with fluorescent-labeled primers, the products were run in an ABI 3700 sequencing machine and the genotypes were scored with Genemapper (Applied Biosystems, Ijssel, The Netherlands). Haplotypes were constructed manually.

Site-directed mutagenesis on MYOC *cDNA and cell culture:* Human full-length *MYOC* cDNA was cloned into pcDNA3.1 vector (Invitrogen). Mutations in *MYOC* cDNA were introduced by a polymerase chain reaction (PCR)-based method using a QuickChange II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) and specific primers. The sense primer sequence with the altered base in red for p.Phe430Leu MYOC is 5'-GCA GTC AGT CGC CAA TGC CCT CAT CAT CTG TGG CAC CTT G-3'. Primers for p.Cys245Tyr and p.Tyr437His are the same as reported elsewhere [8]. All constructs were verified by direct sequencing.

The same number of CHO-K1 cells (American Tissue Culture Collection, Manassas, VA) were transfected with plasmids containing wild type or different mutant *MYOC* cDNAs by FUGENE 6 according to manufacturer's protocol (Roche Diagnostics, Rotkreuz, Switzerland) with a ratio of 3 µl transfection reagent per µg DNA. Cells were used within three days after transfection.

Monitoring Myocilin secretion: Cells were incubated with media containing 20 µCi/ml (^{35}S) cysteine and (^{35}S) methionine (Anawa, Wangen, Switzerland) for 24 h. Culture media were collected and centrifuged to remove cell debris. The supernatant was immunoprecipitated (IP) with rabbit polyclonal anti-human MYOC peptide (amino acids 188-204) antibody [9] bound to protein A Dynabeads® (Invitrogen). IP proteins were denatured in SDS sample buffer containing 20% β-mercaptoethanol and resolved by 10% SDS-PAGE. The gel was treated with EN^{3}hance (Perkin Elmer, Walthum, MA) and radioactivity was detected by Bio-imaging Analyzer (BAS-1800II, Fujifilm, Dielsdorf, Switzerland).

Monitoring Triton X-100 solubility of intracellular Myocilin: Cells were washed twice with ice-cold PBS and lysed in buffer containing 100 mM Tris-HCl (pH 7.4), 3 mM EGTA, 5 mM $MgCl_2$, 0.5% Triton X-100 (Tx, Sigma, Basel, Switzerland), protease inhibitor cocktail (Roche, Rotkreuz, Switzerland), and 1 mM Phenylmethyl sulfonylfluoride (PMSF, Sigma) for two min on ice. The concentration was $5x10^6$ cells per ml lysis buffer. After centrifugation, the supernatant containing Tx-soluble proteins was immunoprecipitated for MYOC or GAPDH (Ambion, Rotkreuz, Switzerland). The cell remnants were washed twice with ice-cold PBS, scrapped in 300 µl PBS containing protease-inhibitor cocktail and 1 mM PMSF, and centrifuged. The pellet was sonicated and denatured in 50 µl SDS sample buffer containing 9 M urea. Tx-

insoluble proteins from samples equivalent to $2x10^5$ cells were analyzed by 10% SDS-PAGE and western blot, using antibodies against MYOC (1:800 dilution) or β-actin (Sigma, 1:3000 dilution) and appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Bioscience, Piscataway, NJ). The signals were detected by enhanced chemiluminescence (Amersham Bioscience).

RESULTS

Two hundred and fifty individuals, representing 30 unrelated families with a positive history of POAG (120 affected and 130 unaffected family members), were enrolled in our present study. All affected study participants had an IOP of more than 22 mmHg and an age of diagnosis of more than 35 years. The mean age of diagnosis was 52.7±14.8 years. Ophthalmic and medical history did not reveal any other ocular or systemic disorder. For sequencing the open reading frame of *MYOC*, the proband from each family was used.

Identification of a novel variant in MYOC*:* A novel variant (c.1288T>C) that would result in a nonconservative amino acid change (p.Phe430Leu) was discovered in exon 3 of *MYOC* in two "unrelated" families from The Netherlands (Figure 1A). All the affected members were heterozygous for the change. The variant segregated completely with the disease in both families (Figure 1B,C) and was absent in 250 ethnically matched controls selected from the general population without any history of glaucoma. The phenylalanine at this position was conserved in mammals. As reported by almost every study on *MYOC*, the two polymorphisms (-83G>A and c.227G>A) in the promoter and exon 1 of *MYOC*, respectively, were found in complete linkage disequilibrium in the patients (data not shown).

c.1288T>C is part of the same haplotype in two primary open angle glaucoma families: Five microsatellite markers near *MYOC* were selected from 1q24 to perform the haplotype analysis in the two affected families (Figure 1B,C and Table 2). The marker panel covered 2.7 Mbp of genomic DNA. Four individuals from family W05-125 and three from family W05-295 were haplotyped for these markers. All the members carrying the mutant allele showed the same haplotype, suggesting that the two families are ancestrally related.

p.Phe430Leu Myocilin mutant individuals show severe glaucoma phenotype: The detailed phenotypic characterization of the probands with the novel *MYOC* variant is described in this section.

In Family W05-125, a 76-year-old female Caucasian patient (individual II-4 in Figure 1B), was diagnosed to have POAG at the age of 49 years. Despite laser trabeculoplasty of both eyes and treatment with bimatoprost and dorzolamide/timolol eyedrops in both eyes, she had an IOP of 23 mmHg and 30 mmHg in the right and left eye, respectively, and worsening visual fields. When she was included in the study, she had glaucomatous-affected visual fields and optic discs where both eyes showed a superior peripheral nasal step in the visual fields (caused by loss of corresponding bundles of peripheral arcuate nerve fibers) and the optic discs of the right and left eye are "outside normal limits" and "borderline", respectively, as scored by the HRT Moorfields regression analy-

Table 1. Primer sequences and polymerase chain reaction conditions for amplifying three exons of *MYOC*

Exon	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature (°C)	Mg^{2+} (mM)	Amplicon size (bp)
1	1F-GGCTGGCTCCCCAGTATATA	6R-CTGCTGAACTCAGAGTCCCC	58	2.0	782
2	7F-AACATAGTCAATCCTTGGGCC	7R-GAATAAAGACCACGTGGGCAC	52	2.0	237
3	8F-TTATGGATTAAGTGGTGCTTCG	13R-AGCATCTCCTTCTGCCATTG	58	2.5	872

For sequencing of exon 1, two nested primers were used: 3F (5'-AGT GGC CGA TGC CAG TAT AC-3') and 3R (5'-CTG GTC CAA GGT CAA TTG GT-3'). A similar method was used to sequence exon 3, the two nester primers were: 10F (5'- ATA CTG CCT AGG CCA CTG GA-3') and 10R (5'- CAA TGT CCG TGT AGC CAC C-3'). Exon 2 was sequenced with the same primers used for PCR. All the primers were obtained from the literature [37].

Table 2. Microsatellite markers used for haplotyping the *MYOC* variant p.Phe430Leu

Marker name	Forward primer (5'-3')	Reverse primer (5'-3')	Genomic location (1q24)	Amplicon size range (bp)
D1S2851	TCTTCCCACCACTGCC	TTTATATTTGTATTCCTACTGCCCA	168.6 Mbp	169-199
D1S452	TAATGGGTTCAGTGGACCTT	TGCAGTTCCATATTCCAGGT	168.8 Mbp	218-230
MY-3	GTTGGGAGATGTGATTGCAG	AGATGGAGGTGGGAAAGTGT	169.87 Mbp	150-160
MYOC	Not applicable	Not applicable	169.87 Mbp	NA
MY-5	TCTCTGTCCCTGCTACGTCTT	GCACCCATACCCCAATAATAG	169.88 Mbp	244-254
D1S2790	AAAATGCTCATTAGTCCAGAAAG	TGGCTATGTTTTACTAGCTCAAG	171.3 Mbp	240-260

The genomic locations are obtained from the UCSC genome browser. The location of *MYOC* is shown in the table. Mbp, Mega base pairs. The genomic location is approximate and measured from the telomere of 1p. In the "Amplicon size range" column, NA indicates not applicable.

sis 4 (Figure 2A). Gonioscopy revealed an open angle (grade III) in both eyes. Trabeculectomy was done in both eyes and IOP was reduced to less than 15 mmHg.

The other affected siblings (Figure 1B) were treated in different clinics and were reported to have comparable severity of the disease. Individual II-2 also underwent a trabeculectomy in both eyes.

In Family W05-295, a 40-year-old male Caucasian patient (individual II-1 in Figure 1C) showed glaucomatous affected visual fields and optic discs in both eyes (Figure 2B). His initial IOP was 36 mmHg in both eyes. Gonioscopy revealed an open anterior chamber angle (Shaffer's grade III) in both eyes. Treatment with latanoprost/timolol and brinzolamide eye drops only reduced IOP to 26 mmHg and 24

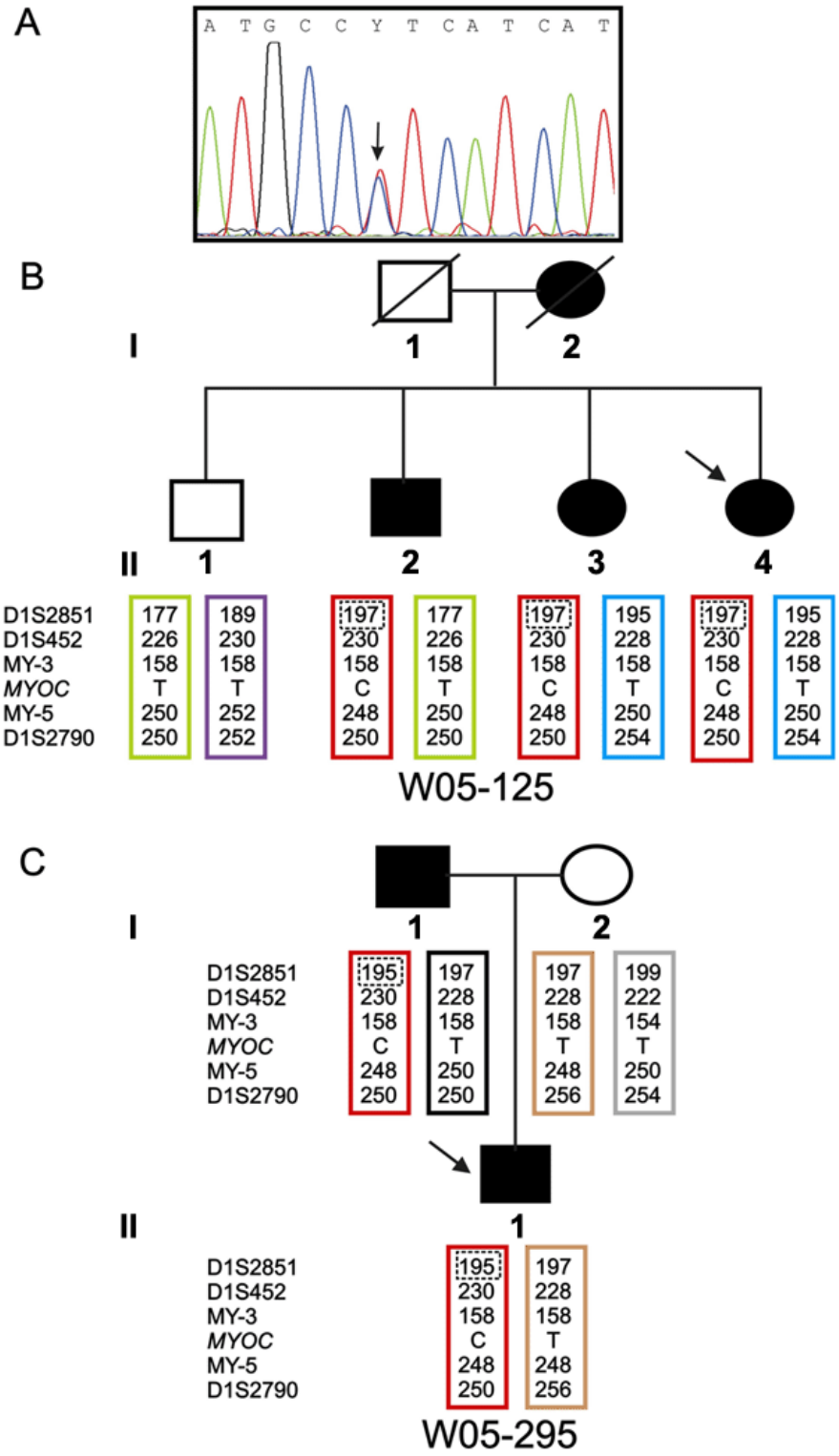

Figure 1. The p.Phe430Leu mutation in the Myocilin gene and a common disease haplotype. **A**: The chromatogram shows the heterozygous mutation (c.1288T>C; p.Phe430Leu; arrow) in exon 3 of *MYOC*. **B**: Individual II-4 (arrow) in family W05-125 is the proband. The haplotypes for microsatellite markers are shown. The different alleles are given by the amplicon lengths. The "red haplotype" contains the mutant allele; **C**: Similar representation as **B** of the mutant (red) haplotype is shown but for family W05-295. Individual II-1 (arrow) is the proband. The dotted line around the alleles at D1S2851 (**B**,**C**) denotes the difference in allele sharing. Note that the mutant (red) haplotype is the same between the two families with the same mutation (except at D1S2851).

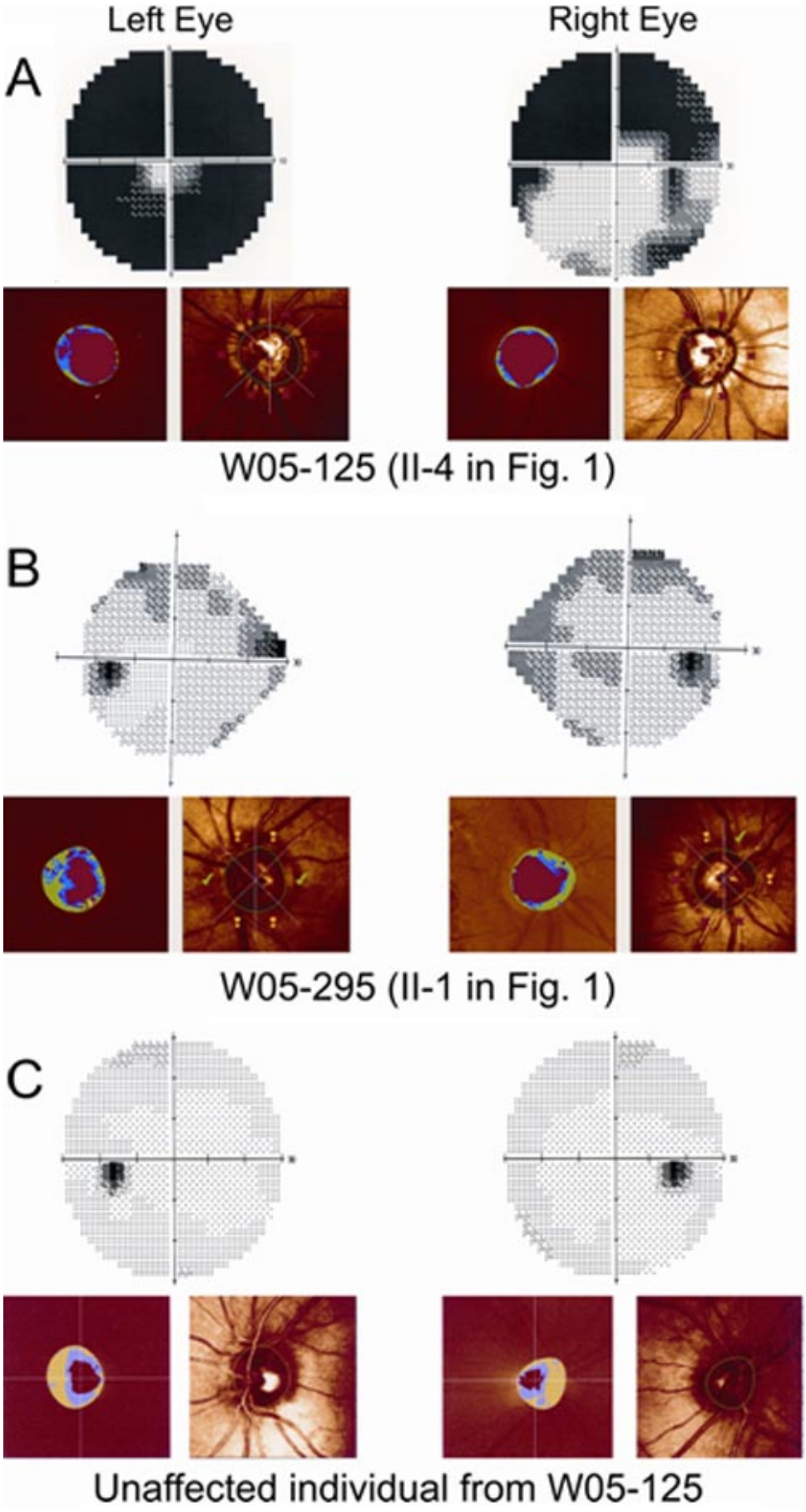

Figure 2. Phenotypic characterization of the probands with p.Phe430Leu mutation in the *MYOC* gene. Each panel has two parts; the upper part depicts the visual field printouts of the Humphrey Field Analyzer (HFA) and the lower part shows screenshots from Heidelberg Retina Tomograph II (HRT) analysis to detect loss of the papillary neuro-retinal rim. The right and left columns correspond to the right and left eyes of the probands, respectively. **A**: HFA and HRT results for proband of W05-125 (II-4 in Figure 1B) is shown. The large dark areas in the HFA results correspond to glaucomatous scotomas due to nerve fiber layer defects. HRT scans demonstrate glaucomatous increased optic disc cupping (red areas) and suspect (yellow exclamation marks) or manifest pathological (red crosses) neuro-retinal rim measures (according to the Moorfield's regression analysis) within the different quadrants of the optic disc. **B**: A similar representation as **A** is shown but for proband of W05-295 (II-1 in Figure 1C). The results are less severe than in proband W05-125, however, a nasal step characteristic for glaucoma can be observed in HFA, and a thinned neuro-retinal rim (blue-green area) can be seen by HRT. **C**: HFA and HRT results for a 55-year-old, unaffected individual from W05-125 are shown for comparison. HFA results indicate that there is no darkening due to glaucomatous defects on HFA. The small temporal black area corresponds to the physiological blind spot. HRT results show no thinning of the neuro-retinal rim is visible.

mmHg in the patient's right and left eye, respectively. For better IOP management, trabeculectomy was done in both eyes, which resulted in IOP being less than 15 mmHg in both eyes. The patient's father (individual I-1 in Figure 1C) and paternal grandmother also had POAG.

For comparison, results of the visual field and HRT analysis of a 55-year-old, unaffected female from W05-125 is given in Figure 2C. The person is not depicted in the pedigree. She tested negative for the p.Phe430Leu mutation. She does not show any (glaucomatous) visual field loss (Figure 2C, upper part) as present in the two probands described above.

p.Phe430Leu mutant Myocilin protein is secretion-defective and becomes detergent insoluble: Myocilin is a secreted protein. Reduced secretion of mutant MYOC is reported to be a major determinant for pathologic variants in MYOC [8]. When expressed in CHO-K1 cells in contrast to wild type MYOC (Figure 3A, lane 2), the mutant (p.Phe430Leu) protein showed much less secretion (Figure 3A, lane 4). Only a minor band at about the 55 kDa position was observed. For comparison, two reported mutations in MYOC, namely p.Cys245Tyr [10] and p.Tyr437His [2], were used in the same assay. These mutant proteins also showed reduced secretion (Figure 3A, lanes 3 and 5). This experiment strengthened our assumption that the missense change (p.Phe430Leu) identified in this study was pathologic.

In the intracellular fraction, the deleterious effect of p.Phe430Leu MYOC was tested by its solubility in Triton X-100. It was reported that as the severity of the mutation increases, the Tx solubility of the mutant protein decreases [11]. When expressed in CHO-K1 cells, a major fraction of wild type MYOC were found in Tx soluble fraction (Figure 3B, lane 2) whereas the p.Phe430Leu mutant protein was absent (Figure 3B, lane 4). As mentioned in the previous paragraph, the two reported mutations also showed poor Tx solubility comparable to p.Phe430Leu (Figure 3B, lanes 3 and 5).

Western blotting performed with the Tx insoluble fraction showed that practically the entire amount of the p.Phe430Leu mutant protein remained in this fraction, proving the reduced solubility of the mutant protein in Tx (Figure 3C, lane 4). In comparison, the two reported mutations also showed similar effect albeit with a higher quantity (Figure 3C, lanes 3 and 5). This could be due to the variable transfection efficiencies among different mutants. Also, in the Tx insoluble fraction for the wild type MYOC (Figure 3C, lane 2),

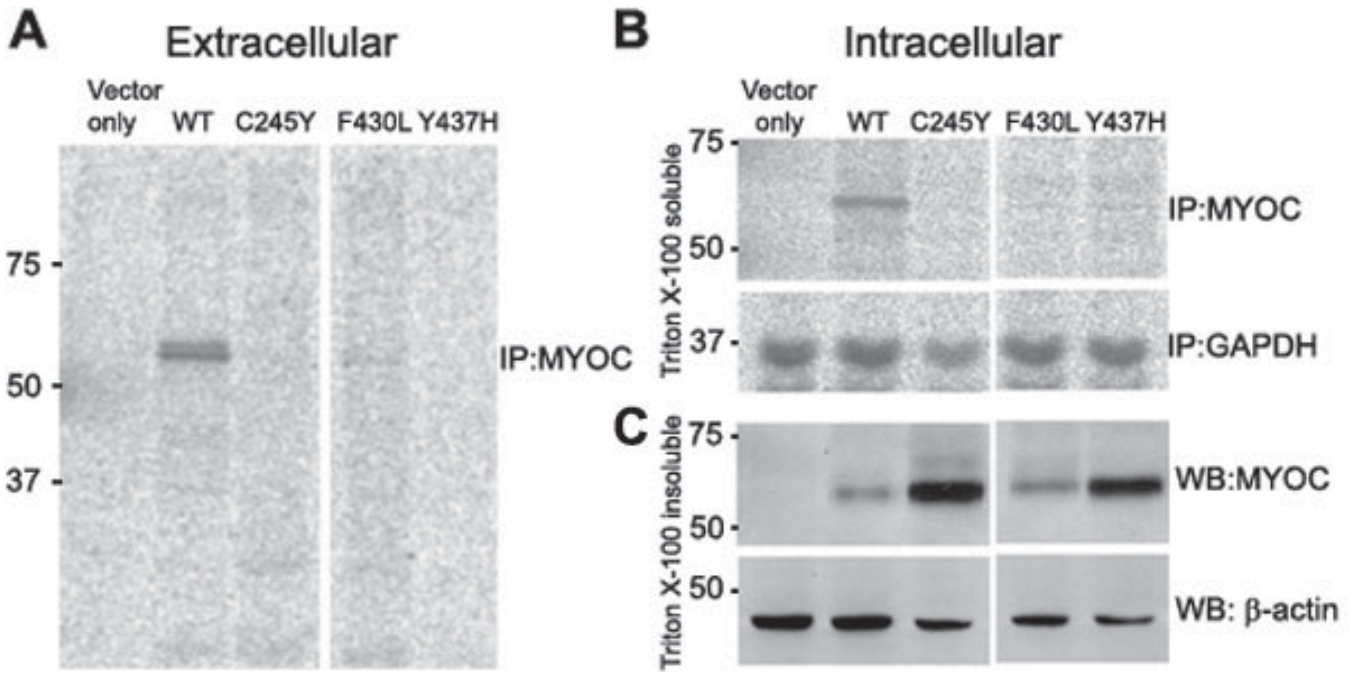

Figure 3. Functional characterization of the p.Phe430Leu mutation in Myocilin. **A**: Immunoprecipitation assay is used to monitor secretory properties of Myocilin. Lane 2 is the wild type protein and lane 4 is the mutant protein for p.Phe430Leu. Lanes 3 and 5 contain two reported mutations (p.Cys245Tyr and p.Tyr437His, respectively) that are used for comparison. Note the lack of secretion for all three mutant proteins compared to the wild type. **B**: Immunoprecipitation for the Triton X-100 soluble fraction of intracellular Myocilin is illustrated. Note the absence of the mutant proteins in this fraction compared to the wild type. GAPDH is the housekeeping protein for loading reference. **C**: Western blotting was used to detect Myocilin in the Triton X-100 insoluble fraction. Note the abundance of all three mutant proteins compared to the wild type. β-Actin is the housekeeping protein for loading reference. CHO-K1 cells were used to express wild type and mutant MYOC proteins. The loading order of the gel was identical in all three panels: lane 1, vector only; lane 2, wild type Myocilin protein; lane 3, p.Cys245Tyr mutant Myocilin; lane 4, p.Phe430Leu mutant Myocilin (this study); and lane 5, p.Tyr437His mutant Myocilin. The single letter amino-acid codes are used in the figure due to lack of space. The numbers on the left-hand side of each panel denote the molecular weight range. This figure shows that the p.Phe430Leu mutation diminishes the secretory properties of Myocilin and makes it highly insoluble in Triton X-100.

a band was observed which was comparable to that of the mutant, p.Phe430Leu. This was due to the overexpression of wild type MYOC in the transfection where the cells could not handle the large excess of protein properly, resulting in an imperfectly processed insoluble fraction. To assess the effect of the mutations on the protein, the distribution of total protein into the Tx soluble and insoluble part should be taken into account. The results clearly showed that almost the entire amount of the mutant protein for p.Phe430Leu remained in the insoluble fraction whereas the wild type MYOC was distributed in approximately equimolar amounts.

DISCUSSION

Glaucoma is a progressive optic neuropathy responsible for 12.3% of the total blind population in the world. POAG is the most common form of glaucoma. It accounts for more than 75% of primary glaucoma except in people of eastern Asian (Mongoloid) descent where angle closure glaucoma is more prevalent [12]. In POAG, early identification of affected individuals can help to prevent visual damage by early treatment of the disease [13]. Blindness due to POAG occurs in an estimated 4% of cases in a white population but is more common (8%) and occurs at an earlier age in a nonwhite population [14]. Understanding the genetic background of POAG will facilitate identification of high-risk individuals as well as estimation of the severity of the glaucoma.

In this article we report a novel missense mutation (p.Phe430Leu) in exon 3 of *MYOC*. The two Dutch POAG families described here showed segregation of the disease with the mutation (p.Phe430Leu), which might be an indication for the deleterious effect of the nonsynonymous substitution. However, due to lack of family members from the younger generation, we are unable to study the penetrance of the mutation. Haplotype analyses revealed that both families inherited the mutation through the same ancestral chromosome. A different allele of the first marker, D1S2851 (Figure 1B,C; alleles with a stippled line), suggested two possibilities: (1) a historical recombination at that locus or (2) an effect of a replication slippage since the difference is only two nucleotides i.e. one repeat unit. The average recombination frequency around this marker is higher than that of the downstream region (1.7 cM/Mb compared to less than 1.0 cM/Mb; data from the UCSC genome browser), which made recombination a likely event at D1S2851.

The pathogenesis in POAG caused by *MYOC* defects is yet unknown. However, approximately 97% of the mutations are in exon 3 of the gene coding for an olfactomedin-like domain, which points to its crucial function. The p.Phe430Leu mutation is also in this domain and is conserved among all other mammalian Myocilins, which is indicative of its importance. While some *MYOC* mutations like p.Pro370Leu are found in almost every population studied [15], others (for example, p.Gln48His in India) are present only in specific populations [16,17]. Thus, the novel mutation p.Phe430Leu, reported in this study, could be a prevalent mutation in the Dutch population. We plan to screen a large cohort of POAG patients from The Netherlands to determine the prevalence of this mutation.

A search for reported *MYOC* mutations in the human gene mutation database [18] revealed 64 published missense mutations. Two different blocks of 20 codons, 361-380 and 420-440, contain 11 and 9 mutations, respectively, which account for more than 30% of the mutations (regions A and B in Figure 4). Interestingly, codon 430 is in the middle of the second region (codons 420-440, region B in Figure 4). Both of these regions are in the olfactomedin domain of the protein. Although we cannot accurately predict the exact function of these regions in the olfactomedin domain, the aromatic residue Phe at codon 430 might be crucial for the correct spatial orientation of the cysteine residue at codon 433 involved in disulfide bonds

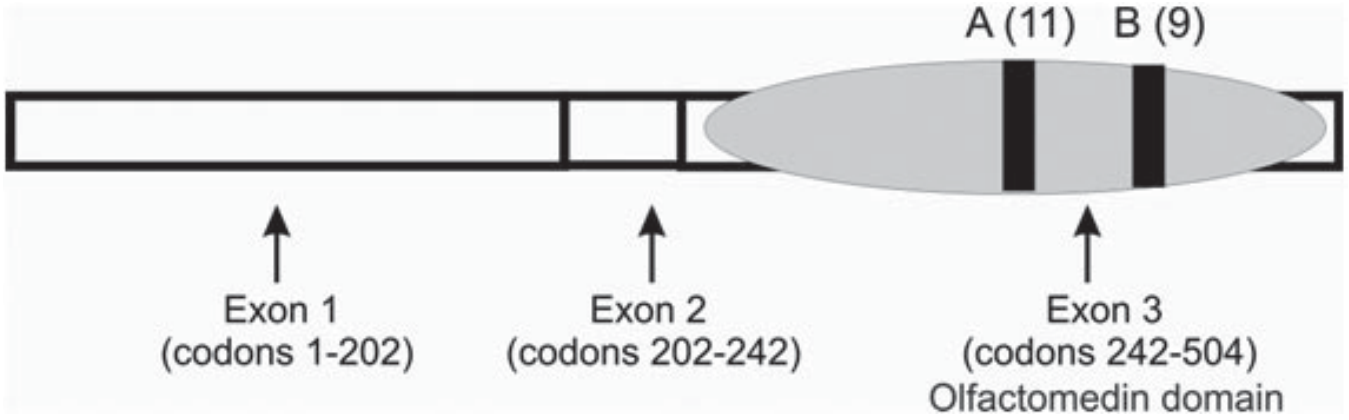

Figure 4. Distribution of the missense mutations in the Myocilin gene. The diagram illustrates *MYOC* and its protein. The boxes represent the encoded regions by each exon (exon and codon numbers given below the diagram). The gray oval shows the conserved olfactomedin domain, and the location of the two highest mutable regions are shown by black bars (marked regions, A and B). The codons 361-380 (A) contain 11 mutations and codons while 421-440 (B) contain nine mutations. Mutations reported from regions A and B together account for more than 30% of the total missense mutations in *MYOC* reported to date (20 out of 64). Note that the novel mutation p.Phe430Leu is located in the middle of region B. The reported mutations are retrieved from the human gene mutation database.

[19,20]. Fautsch et al. [19] described that the mutation at codon 433 (Cys to Arg) abolishes the secretory properties of the protein similar to what we observed in this study for p.Phe430Leu (Figure 3).

The *MYOC* gene encodes a 504-aa polypeptide with a theoretical molecular mass of 56.9 kDa. In ocular tissues, the MYOC protein is mainly localized within the trabecular meshwork, the Schlemm's canal, the sclera, the ciliary body, the retina, and the optic nerve [21,22]. The function of the polypeptide is still unknown, but its colocalization with extracellular matrix proteins such as fibronectin, laminin, or type IV collagen [23,24] supports that at least part of its role is performed in the extracellular environment. As expected, Myocilin is observed to be secreted in the aqueous humor [25-27]. In contrast, the glaucoma-causing Myocilin mutations that have been tested prevent the mutant polypeptides from being secreted [26,28] and decrease the expressed protein's solubility in Triton X-100 [11]. In our opinion, the defective secretion could be due to mistrafficking caused by misfolding of the mutant protein and possibly by the formation of insoluble aggregates. This experiment strongly suggests that p.Phe430Leu is a pathologic variant.

Gobeil et al. [8] showed that wild type and mutant MYOC proteins interact, and these heterodimeric complexes are not secreted. This proved the hypothesis of several reports from genetic [29-32] and biochemical [26,33-35] viewpoints that the *MYOC* mutants that cause autosomal dominant POAG probably act through a dominant negative mechanism caused by the intracellular accumulation of mutant proteins. In our study, the mutant MYOC protein for p.Phe430Leu was always found in heterozygously affected individuals, and it was retained inside the cell, implicating that this mutation also acts through the same dominant negative mechanism.

Phenotypic characterization revealed severe high-pressure glaucomatous visual field damage, and HRT analysis showed significant thinning of the papillary neuroretinal rim, which is in contrast to an unaffected control subject with wild type *MYOC* sequence (Figure 2). Carriers of the novel mutation had a high risk to undergo filtering glaucoma surgery. In our present study, three out of five patients underwent trabeculectomy, which contrasts the results of a large study in France where only 10% of high-tension POAG patients underwent trabeculectomy [36]. These clinical data support the genetic and biochemical findings pointing to the deleterious effect of Phe to Leu substitution at codon 430 of the Myocilin protein.

The novel missense change (c.1288T>C; p.Phe430Leu) in *MYOC* exon 3 was found to segregate with POAG in two families from the Netherlands and was absent in 500 chromosomes from control individuals. The affected individuals represent severe glaucomatous damage, which had to be treated surgically; the mutant protein was secretion-defective and not soluble in Triton X-100. Our data strongly suggest that p.Phe430Leu is a novel pathologic *MYOC* mutation causing high-pressure POAG.

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