CLINICAL SCIENCE

Rapid mutation detection by the Transgenomic wave analyser DHPLC identifies MYOC mutations in patients with ocular hypertension and/or open angle glaucoma

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Aims: To rapidly screen Scottish patients with a family history of open angle glaucoma (OAG) or ocular hypertension (OHT) for mutations in the myocilin gene (*MYOC*) and develop a new rapid screening method for *MYOC* mutation detection.

Methods: All three exons of the *MYOC* gene were amplified by PCR from genomic DNA and subjected to direct DNA sequencing. Mutation detection methodology was also developed based on denaturing high performance liquid chromatography (DHPLC). A recurrent mutation was investigated by analysis of microsatellite haplotypes at the *MYOC* gene locus.

Results: Mutations were identified by DNA sequencing in four families. *MYOC* mutation Q368X was found in three kindreds and the fourth family carried the mutation G367R. The Q368X mutation was found to be associated with the same haplotype for markers closely flanking the *MYOC* gene. The mutations were identified by direct sequencing and were also readily detected by DHPLC analysis of PCR fragments, demonstrating that this is a robust method for *MYOC* analysis in future.

Conclusions: Mutations in the *MYOC* gene were identified in patients presenting with highly variable phenotypes from normal through OHT to severe OAG. Haplotype analysis showed that mutation Q368X is likely to be an ancestral mutation in this population. DHPLC analysis is an accurate, rapid and cost effective method for *MYOC* mutation analysis in large population samples.

Open angle glaucoma (OAG) is a group of conditions characterised by progressive optic neuropathy. There are characteristic changes to the optic nerve head and retinal nerve fibre layer in the absence of other ocular pathologies or congenital anomalies.¹ The pathogenesis of this group of conditions has not been fully elucidated but there is an increased risk to first degree relatives of sufferers, strongly suggestive of a genetic component.^{2–4}

A significant amount of work has been done attempting to identify gene defects responsible for causing glaucoma. The juvenile onset variety is a good condition for such research because those suffering from the disease tend to have characteristic phenotypes and the age of onset is relatively young allowing for accurate pedigree charting. Much of the genetic research in glaucoma has been done looking at large families suffering from juvenile onset OAG. In 1993 Sheffield and colleagues mapped the first locus for this form of glaucoma to 1q21-q31.⁵ Subsequently, the *MYOC* gene which encodes myocilin, a protein component of the trabecular meshwork, was shown to reside within this chromosomal interval.⁶ *MYOC* was found to harbour mutations in many individuals affected by OAG.⁶ This gene has also been referred to as the *TIGR* gene in the literature.

Since 1996, we have been collecting sibling pairs with OAG/OHT (ocular hypertension) throughout Scotland with a view to performing linkage analysis and identification of further susceptibility loci. Before carrying out this analysis we screened our patient cohort to exclude those with *MYOC* mutations. Here we have identified two *MYOC* mutations segregating in four kindreds by direct DNA sequencing. Using these sequence verified mutations, we have developed a novel application of mutation detection methodology based on the transgenomic wave analyser for the detection of *MYOC* mutations.

PATIENTS

For full clinical details see Table 1. In summary, the first kindred comprises two siblings presenting in their 40s with OHT responding well to topical medication. The second pair comprised a brother with mild OHT and the sister who expressed the mutation but had no sign of either OHT or glaucoma. She is currently being followed up by her optometrist. The third sibling pair involves a patient with early onset, severe primary OAG and a sister with mild OHT diagnosed at a later age. These three sibling pairs all carry the Q368X mutation.

The final sibling pair carried the G367R mutation. They presented with remarkably similar phenotypes. They were both relatively young at diagnosis, they both had high initial pressures, and both had glaucomatous field loss at presentation. The sibling who presented later had significant field loss, as shown in Figure 1. Both have required surgical intervention to control the disease.

MATERIALS AND METHODS

Mutation detection by direct sequencing

Patient DNA was extracted from peripheral blood lymphocytes by standard methods. Individual exons of the *MYOC* gene were amplified by polymerase chain reaction (PCR) using the primer combinations outlined in Table 2. For mutation detection by sequencing, PCR was performed in a 50 µl volume containing Bioline PCR buffer with 1.5 mM MgCl₂ (Bioline, London, UK), 4% DMSO, and a final deoxynucleotide triphosphate (dNTP) concentration of 0.25 mM (each dNTP).

Abbreviations: OAG, open angle glaucoma; OHT, ocular hypertension; *MYOC*, myocilin gene; PCR, polymerase chain reaction; DHPLC, denaturing high performance liquid chromatography

Table 1 Clinical details of patients

Family/sib	Diagnosis	Age of onset	IOP (mm Hg)	Visual fields	Discs	Management	Mutation
A							
1	OHT	43	25, 25	N	0.6, 0.6	Betaxolol	Q368X
2	OHT	49	45, 36	N	0.8, 0.7	Triple therapy	Q368X
B							
1	OHT	51	27, 27	N	0.5, 0.5	Timolol	Q368X
2	Normal	NA	NA	NA	NA	NA	Q368X
C							
1	OHT	47	25, 25	N	0.5, 0.6	Timolol	Q368X
2	OAG	31	28, 32	A	0.8, 0.8	Bilateral trabs	Q368X
D							
1	OAG	21	43, 40	A	0.6, 0.7	Bilateral trabs	G367R
2	OAG	34	52, 52	A	0.9, 0.9	Bilateral trabs	G367R

A= glaucomatous change, N = no field defect, NA = not applicable.

Figure 1 Visual fields of patient D2 at presentation. This woman first presented 6 years after her younger brother was diagnosed with glaucoma. At the age of 34 years, she already had significant field loss.

Mineral oil was not used and PCR was carried out in an MBS Thermocycler with heated lid (Hybaid, Ashford, UK). The following PCR programme was used for amplification of all three exons: (94°C, 5 minutes) × 1; (94°C, 30 seconds, 55°C, 1 minute, 72°C, 1.5 minutes) × 35; (72°C, 5 minutes) × 1. PCR products were checked on 1.5% agarose/TBE gels. PCR products were purified using QIAquick PCR mini-columns (Qiagen, Crawley, UK) to remove unused primers and precursors and about 50 ng of this DNA template was directly sequenced with forward and reverse amplification primers on an ABI 377 automated sequencer using the BigDye chemistry, according to the manufacturer's recommended protocol (Applied Biosystems, Foster City, CA, USA).

Mutation detection by DHPLC

For denaturing high performance liquid chromatography (DHPLC) analysis of exon 3 of the *MYOC* gene, two overlapping PCR fragments were generated covering the 5' half (designated 3A, Table 2) and 3' half (3B, Table 2) of the exon. PCR conditions were as outlined above except that Perkin-Elmer PCR buffer (Applied Biosystems, Foster City, CA, USA) was used instead of Biorline buffer because the latter contains a high concentration

of detergent that can damage DHPLC columns. PCR programmes for these fragments were as detailed above except that the annealing temperature was raised to 60°C for the 3B fragment. Following PCR, reactions were denatured by heating to 94°C for 5 minutes, followed by cooling to 25°C over a 25 minute period to enhance heteroduplex formation. This stage was either added to the amplification programme or performed separately at a later time. The sequences of these fragments were analysed using the Wavemaker software package (Transgenomic, Crewe, UK), which predicted DHPLC temperatures of 61°C and 60°C for the 3A and 3B fragments, respectively. A volume of 8 µl was analysed by DHPLC,⁷ on the Transgenomic wave genetic analyser (Transgenomic, Crewe, UK).

MYOC microsatellite genotyping

The GT dinucleotide repeat polymorphisms immediately upstream and downstream of the *MYOC* coding sequences designated MY5 and MY3 respectively,⁸ were amplified using the primers listed in Table 2. The forward primer in each case was fluorescently labelled at the 5' end with the FAM fluorochrome (Applied Biosystems). PCR conditions used were as outlined for fragments 3A and 3B, above, with an annealing

Table 2 PCR primer sequences

Fragment	Forward primer (name, sequence 5'–3')	Reverse primer (name, sequence 5'–3')	Size
Exon 1	MYOCe1.L ATCCAGGCACCTCTCAGCAC	MYOCe1.R GCGCCTGTAGCAGGTCAC	733 bp
Exon 2	MYOCe2.L AACATAGTCAATCCTTGGGC	MYOCe2.R TTCTGTTCTCTCTCTCTCC	341 bp
Exon 3	MYOCe3.L TCACATCTACTGGCTCTGCC	MYOCe3.R CTGGCTGGCTGGCTCTCCCT	972 bp
Exon 3A	MYOCe3.L3 GTCCGCCAGGTTTTTGAGTA	MYOCe3.R3 GGTGCCACAGATGATGAAGG	423 bp
Exon 3B	MYOCe3.L4 GTGCCATTGTCCTCTCCAAA	MYOCe3.R4 CTCTCCCTTCAGCCTGCTC	395 bp
MY5	MY5.L TCTCTGTCCTGCTACGTCCT	MY5.R GCACCCATACCCCAATAATAG	~239 bp
MY3	MY3.L GTTGGGAGATGTGATTGCAG	MY3.R AGATGGAGGTGGGAAAGTGT	~145 bp

Figure 2 Excerpts of DNA sequencing traces corresponding to exon 3 of the *MYOC* gene, spanning codons 366–370 inclusive. (A) Sequence derived from a normal individual. (B) Sequence derived from the proband in family A, showing heterozygous transition mutation 1102C>T, which predicts the nonsense mutation Q368X. (C) Sequence derived from the proband in family D, showing heterozygous transition mutation 1099G>A, predicting the amino acid change G367R in the myocilin polypeptide.

temperature of 55°C in each case. PCR fragments generated from patient DNA were sized on an ABI 377 automated DNA sequencer using Genescan and Genotyper software (Applied Biosystems, Foster City, CA, USA).

RESULTS

Two MYOC mutations in Scottish families

Thirty four Scottish sib pairs diagnosed with OAG and/or OHT were analysed for mutations in the *MYOC* gene. Exons 1–3 of *MYOC* were amplified from genomic DNA derived from one individual from each sib pair. PCR products were directly sequenced and compared with both the published sequence and control sequences derived from a normal unrelated individual. All mutations detected were confirmed in the other affected family members available by direct sequencing. Three of the four kindreds (families A, B, and C) were found to carry heterozygous transition mutation 1102C>T in exon 3 of the *MYOC* gene, predicting the amino acid change Q368X (glutamine to stop at codon 368) (Fig 2). To check if the three families carrying this mutation might be very distantly related, we amplified two polymorphic dinucleotide repeat sequences located 313 bp upstream of the initiation codon (designated MY5) and 110 bp downstream of transcription termination site (designated MY3) of the *MYOC* gene.⁸ PCR products were fluorescently labelled and sized on a DNA sequencer. By this means, we found that all affected individuals in families A, B, and C share the same *MYOC* haplotype consisting of 146 bp for the MY5 marker and 241 bp for the MY3 marker (sizes using the primers listed in Table 2). Therefore, Q368X probably represents an ancestral mutation in this population.

The fourth sib pair was found to carry heterozygous transition mutation 1099G>A in exon 3 of the *MYOC* gene (Fig 2).

This mutation predicts the amino acid change glycine to arginine at codon 367 (designated G367R). Three other sequence changes were detected in a number of individuals: 227G>A, predicting amino acid change R76K in exon 1; silent polymorphism 366C>T (G122G) in exon 1; and 1041T>C, a silent mutation Y347Y in exon 3 (data not shown). These are known to be non-pathogenic polymorphisms found at low frequencies in the other normal populations.⁸

DHPLC detection of MYOC mutations

Using these known mutations as positive controls, we applied methodology based on DHPLC for rapid screening of *MYOC* mutations, using the Transgenomic wave analyser. Short PCR fragments covering all exons of the *MYOC* gene can be screened by this technique. Exons 1 and 2 can be scanned for mutations in a single fragment; however, the 972 bp PCR product encompassing all of exon 3 did not perform well in this assay (data not shown). Since exon 3 harbours the common *MYOC* mutations, including both of those found in this study, it was particularly important to develop an assay system for this exon. By using shorter overlapping PCR fragments, we were able to screen this exon and reliably detect the mutations found here, as shown in Figure 3. Using the fragments and conditions described here, the system was able to detect the polymorphisms R76K, G122G, and Y347Y (data not shown), suggesting that this system is a robust *MYOC* mutation detection method.

DISCUSSION

MYOC defects in Scottish families

We screened 34 Scottish sib pairs with OAG and/or OHT for defects in the *MYOC* gene. Two mutations were found in exon 3, Q368X in families A–C and G367R in family D, both of which had been reported previously in other OAG patients.^{6–8,11} The majority of *MYOC* screening studies focus on exon 3 where most reported mutations occur, especially the most commonly detected mutation Q368X. However, it is possible that mutations elsewhere in the gene might also be pathogenic. For example, a recessive mutation has been reported in exon 1.¹² Therefore, in our study, we chose to screen all exons for mutations for completeness. Two studies of more extensive Scottish kindreds have reported different *MYOC* mutations from those seen here, Q337R¹³ and G252R.¹⁴

Using a highly polymorphic microsatellite located in intron 3 of the keratin 12 gene, we have previously demonstrated an ancestral K12 mutation in a number of apparently unrelated German kindreds with Meesmann's corneal dystrophy.¹⁵ Here, we were able to show that the Q368X mutation is associated with a common haplotype for the microsatellite markers MY5 and MY3, which closely flank the 5' and 3' ends, respectively, of the *MYOC* transcription unit. The four families analysed originate in different parts of Scotland and there is no apparent relatedness. It has been shown that there is a shared ancestral haplotype for these markers in other populations but unfortunately, these authors did not report the allele sizes to allow comparisons to be made with subsequent studies.⁸ Here, we report the allele sizes observed in our three Scottish families to facilitate future studies of the genetic epidemiology of this *MYOC* mutation in this and other ethnic groups. Patients carrying the Q368X mutations from four different ethnic groups, including white people, have been shown to share the same *MYOC* haplotype⁸ and so it is likely that the haplotype seen here is not specific to the Scottish population but may be further evidence that this is an ancestral mutation in the global sense.

Rapid mutation screening by DHPLC

We report the first application of the recently developed DHPLC technology⁷ to the detection of *MYOC* mutations. We were able to show that this technique can readily detect the

Figure 3 DHPLC traces showing rapid detection of *MYOC* mutations. The x-axis shows DHPLC elution time in minutes and the y-axis shows DNA concentration as measured by optical density at 260 nm, in arbitrary units. (A) DHPLC trace representing analysis of *MYOC* exon 3 PCR product derived from the proband in family A, who carries the Q368X mutation. The double peak indicates the presence of a heterozygous sequence change. Identical traces were obtained for all patients in families A–C who carry this mutation. (B) DHPLC trace representing analysis of *MYOC* exon 3 PCR product derived from the proband in family D, who carries the G367R mutation. Again, the double peak indicates the presence of a heterozygous sequence change and identical traces were obtained for the sibling in family D who also carries this mutation. (C) and (D) DHPLC traces of the same PCR product analysed in (A) and (B) (above), derived from normal unrelated control individuals. Identical single peak traces such as these were observed for >10 normal controls.

common Q368X mutation in exon 3 of *MYOC*. Another mutation, G367R, and polymorphism Y347Y in this exon were also clearly detected. Therefore, this technique appears to be robust for a variety of sequence changes within a given fragment. Similarly, polymorphisms were detected in exon 1 of the gene. Although we found no positive control sample carrying a sequence change in exon 2 in this study, it seems likely that DHPLC will be able to detect mutations in this small exon.

This system is ideally suited to routine detection of mutations in *MYOC* since it is (a) rapid, taking about 5–7 minutes per sample; (b) accurate, as shown by our results here; and (c) economical, costing about £0.50 per sample. PCR products require no purification before DHPLC and the Transgenomic wave analyser incorporates robotic handling of samples in a 96 well format, so that user time is kept to a minimum. Typically, less than 10 µl amounts of PCR products were used for DHPLC analysis and therefore, the remaining PCR product from a 20–50 µl reaction can be used for sequencing or another confirmatory procedure. Overall, the system seems very suitable for mutation analysis in the context of a routine laboratory. As seen in family B here, *MYOC* mutations can be found in normal individuals before the onset of OHT or OAG and so routine screening is appropriate to identify presymptomatic at-risk individuals.

Genotype-phenotype correlation

Our group of patients in families A–C were observed to have a variety of phenotypes despite the fact that they express the

same Q368X mutation (Table 1). Of particular interest is the fact that the phenotypes of affected people in families A and B were fairly consistent in terms of age of onset and all had mildly raised IOPs that responded well to topical treatment. In contrast, there is marked intrafamilial phenotypic variation in family C. Since we completely sequenced the *MYOC* gene in all of these families, we know that there were no other sequence changes in the coding regions of this gene which might affect the phenotype. The intrafamilial and interfamilial phenotypic variation observed here and in previous studies^{9–10} suggests that it is not just the *MYOC* mutation responsible for the glaucoma phenotype but that there are unknown modifying factors, genetic and/or environmental, that modulate the development and clinical course of glaucoma in these patients. Since OAG is well known to be genetically heterogeneous and, indeed, a number of loci have been identified in addition to *MYOC*, it is conceivable that variations in these other loci contribute to phenotypic variation within and between families carrying *MYOC* mutations.

In family D, where the phenotype was most severe of the families studied, mutation G367R was detected.¹¹ In contrast with PTC mutation Q368X in the adjacent codon, this is a missense mutation. PTC mutations such as Q368X are known to cause acceleration of mRNA degradation through a mechanism known as nonsense mediated decay.^{16–17} In contrast, mutant alleles carrying missense mutations such as G367R are generally expressed at levels equal to that of the wild type allele in heterozygotes. Therefore, there is likely to be

a higher level of mutant mRNA and, consequently, more mutant protein expressed in patients carrying the G367R mutation. This offers a potential explanation for the more severe phenotype observed in family D.

In general, caution should be exercised in drawing firm conclusions about genotype-phenotype correlation based on small numbers of patients and so it will be of interest to compare the phenotypes of additional unrelated individuals carrying the G367R defect. This mutation has been observed once before in a Japanese kindred.¹¹ Unfortunately, the latter report contained little phenotype data to allow us to make comparisons with family D in the present study.

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