Fine mapping of new glaucoma locus GLC1M and exclusion of neuregulin 2 as the causative gene

Bao Jian Fan,1 Wendy Charles Ko,1 Dan Yi Wang,1 Oscar Canlas,2 Robert Ritch,3 Dennis S. C. Lam,1 Chi Pui Pang2

(The first two authors contributed equally to this publication)

1Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Hong Kong, China; 2Jose B. Lingad Memorial Regional Hospital, City of San Fernando, Philippines; 3Departments of Ophthalmology, the New York Eye and Ear Infirmary, New York, and the New York Medical College, Valhalla, NY

Purpose: We recently identified a novel glaucoma locus on 5q22.1-q32, designated as GLC1M, in a family from the Philippines with autosomal dominant juvenile-onset primary open angle glaucoma (JOAG). No mutations in myocilin (MYOC), optineurin (OPTN), and WD-repeat protein 36 (WDR36) were found. Neuregulin 2 (NRG2) is an excellent potential functional as well as positional candidate at GLC1M. The goal of the present study was to evaluate the role of the NRG2 gene in this JOAG family and unrelated JOAG patients and to refine the critical interval for GLC1M.

Methods: Genomic DNA was obtained from 27 family members. All coding exons and splicing sites of NRG2 were screened for sequence alterations by polymerase chain reaction (PCR) and DNA sequencing. A cohort of 92 unrelated JOAG patients and 92 control subjects were genotyped for the three single nucleotide polymorphisms (SNPs) of NRG2 by PCR and DNA sequencing. Haplotype and segregation analyses were performed in the family. Fisher’s exact test was used to compare the frequencies of the NRG2 polymorphisms between affected and unaffected subjects in the family and between unrelated JOAG patients and control subjects.

Results: Three SNPs were identified: c.98G>A (S33N), IVS3+13A>G (rs889022), and c.1976A>G (G659G). None of them segregated with the JOAG phenotype in this family. No association was found between NRG2 and JOAG in the case-control study (p>0.12). However, further inspection of the haplotypes in the family localized the NRG2 gene telomeric to the disease locus. The critical interval of GLC1M was therefore refined to a region of 28 Mb between D5S2051 and NRG2.

Conclusions: The linkage interval for GLC1M was refined to a smaller region. The NRG2 gene was excluded as the causative gene for JOAG.

Primary open angle glaucoma (POAG; OMIM 137760) is the most common form of glaucoma, affecting over 33 million people worldwide. It is a progressive optic neuropathy characterized by a specific pattern of cupping of the optic disc with correspondent visual field loss and is potentially blinding [1]. There are two types of POAG: juvenile-onset POAG (JOAG) and adult-onset POAG. By definition, JOAG develops before 35 years of age [2,3] and is typically inherited as an autosomal-dominant trait, whereas adult-onset POAG is inherited as a complex trait [4]. Elevated intraocular pressure (IOP; greater than or equal to 22 mmHg) is the most common known risk factor for POAG [5]. However, approximately 25% of patients have an IOP level lower than this reference level and are considered to have normal-tension glaucoma (NTG) [6] or low-tension glaucoma [7].

POAG is genetically heterogeneous, with links to at least 22 genetic loci [8,9]. Among them, 14 loci designated GLC1A to GLC1N have been defined for POAG using family-based linkage studies. So far, three genes have been identified for POAG from the reported genetic loci: myocilin (MYOC, OMIM 601652) [10,11], optineurin (OPTN, OMIM 602432) [12], and WD repeat domain 36 (WDR36, OMIM 609669) [13]. Only MYOC has been established as directly causative of glaucoma [14-19], while the role of OPTN is still unclear due to conflicting evidence [19-23] and WDR36 is considered to be a modifier gene for glaucoma [24-27]. Mutations in these three genes account for no more than 10% of POAG cases [8]. Moreover, at least 16 POAG-associated genes have been reported from association studies [8]. There is discrepancy in the reported roles of these genes in the etiology of POAG. It is therefore evident that additional loci or genes are involved in the development of POAG.

Recently, we mapped a novel JOAG locus to 5q22.1-q32 in a large autosomal-dominant JOAG family from the Philippines [24]. This five-generation family had a total of 95 members, 22 of whom were affected with JOAG. Complete oph-
thalamic examination was given to 27 family members, in which nine were confirmed JOAG patients [28]. After exclusion of MYOC and OPTN as disease-causing genes in this family, a genome-wide search was carried out using 382 microsatellite markers with average spacing of 10 cM. Fine mapping and haplotype analysis identified a new JOAG locus at 5q22.1-q32 within a region of 36 Mb flanked by D5S2051 and D5S2090, designated as GLC1M (OMIM 610535) by the HUGO gene nomenclature committee. This JOAG locus did not overlap with the GLC1G minimal interval between D5S1466 and D5S2051 [13]. However, discrepancy between the genetic and physical maps may still position the WDR36 gene, located at GLC1G, within our disease interval. We therefore screened WDR36 for mutations in affected family members. No sequence variations in the coding exons or splicing junctions of WDR36 were found to be associated with glaucoma. Although we could not rule out possible variations within the introns or the promoter of WDR36, our data strongly suggested the presence of an independent JOAG gene on 5q.

Further search for the causative gene at GLC1M is warranted.

A candidate gene, neuregulin 2 (NRG2, OMIM 603818), located at 5q22-q33, was within the critical region of GLC1M [29]. NRG2 is a member of neuregulins that are a family of growth and differentiation factors related to epidermal growth factor. Through interaction with the ErbB receptors, neuregulins induce the growth and differentiation of epithelial, neuronal, glial, and other types of cells [30]. In particular, it has been demonstrated that neuregulin-ErbB signaling pathways play crucial roles in regulating the proliferation and differentiation of Schwann cells, which are the myelin-forming cells in the peripheral nervous system. NRG2 has been identified as a factor capable of promoting the subventricular zone proliferation, leading to the formation of new neurons [31]. NRG2 promotes GFAP+ cell proliferation and polysialylated neural cell adhesion molecule (PSA-NCAM+) neuroblast generation [31]. Although the fundamental pathophysiology of glaucoma is largely unknown, it is believed that retinal ganglion cell death is the ultimate pathway. Glaucoma is thus considered a disorder of optic nerve degeneration. NRG2 is therefore an excellent potential functional as well as positional candidate gene for glaucoma at GLC1M. In the present study, we evaluated the role of the NRG2 gene in the JOAG family from whom the GLC1M locus was originally identified and unrelated JOAG patients, and further refined the linkage interval for GLC1M.

METHODS

Description of family with JOAG: As previously reported, a large family was recruited from the Ibanez region of the Philippines [28]. Two ophthalmologists (Drs. Canlas O. and Ritch R.) examined the family members and evaluated the whole family. The study protocol was approved by the Ethics Committee for Human Research, the Chinese University of Hong Kong. In accordance with the tenets of the Declaration of Helsinki, informed consent was obtained from all participants after explanation of the nature and possible consequences of the study. This five-generation family had a total of 95 members, in which 22 were affected with JOAG. Complete ophthalmic examination was given to 27 family members, nine of whom were confirmed JOAG patients. Peripheral venous whole blood from these subjects was collected for genomic DNA extraction. The other family members did not agree to participate in this study. Their clinical information was obtained through previous medical records. A definition of JOAG was based on the following criteria: exclusion of secondary causes (e.g., trauma, uveitis, or steroid-induced glaucoma), Shaffer grade III or IV open iridocorneal angle on gonioscopy, IOP greater than or equal to 22 mmHg in both eyes by applanation tonometry, characteristic optic disc damage or typical visual field loss by Humphrey automated perimeter with the Glaucoma Hemifield test, and diagnosis before age 35. For the affected subjects, age at diagnosis ranged from 12 to 33 years (mean ± SD: 19.4 ± 2.2 years), the highest IOP from 24 to 44 mmHg (mean ± SD: 32.5 ± 6.3 mm Hg), vertical cup-disc ratio from 0.7 to 0.9 (mean ± SD: 0.8 ± 0.04), and visual field loss was compatible with glaucoma in two consecutive Humphrey testing. For the unaffected subjects, age at inclusion ranged from 3 to 73 years (mean ± SD: 25.0 ± 19.9 years), IOP < 22 mmHg, vertical cup-disc ratio from 0.2 to 0.5 (mean ± SD: 0.3 ± 0.06), and visual field was in normal range.

Unrelated juvenile open angle glaucoma patients and controls: A cohort of 92 unrelated patients with JOAG and 92 unrelated control subjects without glaucoma were genotyped for the 3 NRG2 polymorphisms identified from the JOAG family. The unrelated JOAG group was comprised of 54 males and 40 females. Their age at diagnosis ranged 8-34 years (mean ± SD: 25 ± 5.4 years), the highest IOP was 23-50 mmHg (mean ± SD: 29.5 ± 5.9 mm Hg), vertical cup-disc ratio was 0.7-0.9 (mean ± SD: 0.8 ± 0.05), and their visual field loss was compatible with glaucoma in two consecutive Humphrey testing. The control group had 51 males and 43 females, whose age at inclusion ranged 60-83 years (mean ± SD: 73 ± 3.8 years), IOP < 22 mmHg, and whose vertical cup-disc ratio was 0.1-0.5 (mean ± SD: 0.3 ± 0.07), visual fields within normal range, and had no family history of glaucoma.

Mutation screening: Genomic DNA was extracted from 200 µl of blood using a commercial kit (Qiagen Blood Kit; Qiagen, Hilden, Germany). Quantification of extracted DNA was performed using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The coding exons and splicing sites of NRG2 were amplified by polymerase chain reaction (PCR), followed by DNA sequencing. Primers used to obtain the initial amplicons are given in Table 1. Initial PCRs were performed on a thermal cycler (model 9700; Applied Biosystems [ABI], Foster City, CA) in a total volume of 25 µl containing 200 ng of genomic DNA, 0.4 µM of each primer, 200 µM dNTPs, 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 to 3.0 mM MgCl2, and 1 U of Taq DNA polymerase (AmpliTag Gold; ABI). Cycling conditions were as follows: first denaturation step of 12 min at 94 °C, 35 cycles of denaturation (94 °C for 40 s), annealing (primer-specific annealing temperature for 60 s), elongation (72 °C for 40 s), and a final single elongation step of 7 min. The PCR products were electrophoresed on 2% agarose gel and visualized using a video...
gel documentation system (Gel-Doc 2000; BioRad Laboratories, Hercules, CA) to check for the quality. The PCR products were then purified with ExoI-SAP kit (USB Corp., Cleveland, OH) to remove unconsumed dNTPs and primers. A second PCR was performed using the sequencing primers as described in Table 1 on a thermal cycler (model 9700; ABI) to incorporate the sequencing dyes (BigDye® Terminator v3.1 cycle sequencing kit; ABI) using a protocol of 25 cycles of denaturation (96 °C for 10 s), annealing (50 °C for 5 s), and elongation (60 °C for 4 min). Sequence data were then aligned using Sequence Navigator analysis software (version 1.0.1: ABI) and compared with the published NRG2 gene sequence (GenBank AH009107).

Statistical analyses were performed using SAS statistical software (version 9.1.3; SAS Institute, Cary, NC). Fisher’s exact test was used to compare the frequencies of the NRG2 polymorphisms between affected and unaffected subjects in the family and between unrelated JOAG patients and controls.

**RESULTS**

**Evaluation of NRG2 as a candidate gene in GLC1M for juvenile open angle glaucoma:** As previously reported [28], the five generations of vertical inheritance of the JOAG phenotype based on the published pedigree structure [24], only seven family members who are informative for refinement of the critical region of GLC1M were included in this figure. Squares denote male family members while circles indicate females. Shaded shapes are family members with juvenile open angle glaucoma. Markers S33N, rs889022, and G659G are single nucleotide polymorphisms of NRG2. A rectangle encases segregating haplotypes. The haplotype for subject III:9 was inferred by using known genotypes from her offspring and husband.

Table 2. NRG2 polymorphisms identified in a family with juvenile open angle glaucoma

| Sequence change | Codon change | Location | Minor Allele frequency | Genotype frequency |
|----------------|--------------|----------|------------------------|--------------------|
| c.98C>G       | S33N         | Exon 1   | 5 (0.28)               | 2 (0.06)           | 0/5/4               |
| G659G         | rs889022     | Intron 3 | 6 (0.33)               | 7 (0.19)           | 0/6/3*              |
| rs889022      | G659G        | Exon 10  | 0 (0)                  | 3 (0.08)           | 0/0/9               |

Fisher’s exact test was used to compare the frequencies of the NRG2 polymorphisms between affected and unaffected subjects. The asterisk indicates a p<0.05. However, no polymorphism was segregated with the juvenile open angle glaucoma phenotype in the family.

Figure 1. Haplotypes of markers flanking GLC1M. Based on the published pedigree structure [24], only seven family members who are informative for refinement of the critical region of GLC1M were included in this figure. Squares denote male family members while circles indicate females. Shaded shapes are family members with juvenile open angle glaucoma. Markers S33N, rs889022, and G659G are single nucleotide polymorphisms of NRG2. A rectangle encases segregating haplotypes. The haplotype for subject III:9 was inferred by using known genotypes from her offspring and husband.

### Table 1. Polymerase chain reaction primers and conditions for NRG2 mutation screening

| Primer | Primer sequence | Amplicon size (bp) | Mg+ concentration (mM) | Annealing temperature (°C) |
|--------|-----------------|--------------------|------------------------|---------------------------|
| 1AF    | TTTGCGGTTCAGCAAGG | 408                | 3.0                    | 60                        |
| 1AR    | CTGTCGAGTCCGTCATCC | 783                | 1.5                    | 64                        |
| 1AF    | TTTGCGGTTCAGCAAGG | 450                | 3.0                    | 62                        |
| 1BF    | CCTGACTCCTCAACTACCTGA | 284              | 1.5                    | 60                        |
| 2F     | CGTGGAGGGAAACTCCCTTCG | 188              | 1.5                    | 60                        |
| 3F     | GAGATTTCGATGGGAAGG | 231                | 1.5                    | 60                        |
| 4F     | GAGATTTCGATGGGAAGG | 128                | 1.5                    | 60                        |
| 5F     | GAGATTTCGATGGGAAGG | 310                | 1.5                    | 60                        |
| 6F     | GAGATTTCGATGGGAAGG | 186                | 2.0                    | 58                        |
| 7F     | GAGATTTCGATGGGAAGG | 233                | 1.5                    | 58                        |
| 8F     | GAGATTTCGATGGGAAGG | 251                | 1.5                    | 58                        |
| 9F     | GAGATTTCGATGGGAAGG | 222                | 1.5                    | 58                        |
| 10F    | GAGATTTCGATGGGAAGG | 393                | 3.0                    | 58                        |

Primers used to obtain the initial PCR amplicons and for subsequent sequencing of the NRG2 gene are listed in this table. Exon 1 was split into three amplicons for PCR and sequencing. Exon 12 was initially amplified by PCR using primers 12AF and 12AR and subsequently sequenced using primers 12AF and 12BF.
type displayed a direct male-to-male transmission with similar numbers of affected males and females. It was consistent with an autosomal-dominant pattern of inheritance. We screened a total of 27 subjects (nine with JOAG) for sequence alterations in the coding regions and splicing sites of NRG2. No disease-causing mutation was identified in NRG2 in the JOAG family. Instead, three single nucleotide polymorphisms (SNPs) were found: one nonsynonymous SNP c.98G>A (S33N), one noncoding SNP IVS3+13A>G (rs889022), and one synonymous SNP c.1976A>G (G659G). S33N was found in 25.9% (7/27) of the subjects, and G659G in 37.0% (10/27) of the subjects, and G659G in 11.1% (3/27) of the subjects (Table 2). However, none of these SNPs segregated with the JOAG phenotype in this family (Figure 1).

To evaluate the role of NRG2 on unrelated patients with JOAG, we genotyped the three SNPs (S33N, rs889022, and G659G) in a cohort of 92 unrelated patients with JOAG and 92 unrelated control subjects without glaucoma. S33N and G659G were found to be wild-type in all subjects. rs889022 was found in 13.0% (12/92) of the JOAG patients and in 22.8% (21/92) of the control subjects (p=0.12). No association was found between rs889022 and the JOAG phenotype in this family (Figure 1).

Refinement of the GLC1M locus using intronic polymorphisms of NRG2: Haplotype analysis of the three SNPs of NRG2 in the family with JOAG confirmed the recombination event in two affected individuals (IV:10 and IV:11, Figure 1). The NRG2 gene was therefore placed telomeric to the disease locus. The critical interval of GLC1M was further refined to a region of 28 Mb between D5S2051 and NRG2 (Figure 1 and Figure 2).

**DISCUSSION**

In the present study, we identified three SNPs in NRG2. The SNP rs889022 has been previously reported as a common polymorphism [29], while S33N and G659G are novel. None of these SNPs segregated with the JOAG phenotype in the family. No association was found between NRG2 and JOAG in the case-control association study involving unrelated JOAG patients and controls. The NRG2 gene was therefore excluded as the causative gene for JOAG. It indicates that an unidentified gene is associated with glaucoma in this family. Further inspection of the haplotypes of these SNPs in the family localized the NRG2 gene telomeric to the disease locus. When we reanalyzed the original genotype data, we clarified the genotypes with respect to two markers, D5S2011 and D5S638, for individuals IV:10 and IV:11 (Figure 1). With the correct haplotypes of these two markers together with the haplotypes of 3 SNPs in NRG2, we redefined the critical region of GLC1M between D5S2051 and NRG2 (Figure 2). The linkage interval of GLC1M was therefore refined to a smaller region of 28 Mb compared to the originally reported interval of 36 Mb [24].

As the revised candidate interval of GLC1M still covers a large distance of 28 Mb, the region can be further refined by recruiting more family members and genotyping more genetic markers. This, in turn, will be helpful in discovering the disease-causative gene. Besides NRG2, we also screened the secreted protein acidic and rich in cysteine gene (SPARC, OMIM 182120) but found no mutations in this JOAG family (data not shown). Although both genes are considered excellent potential functional as well as positional candidates at GLC1M, our work demonstrated that the candidate gene screening process is inefficient and is limited in its ability to identify disease-causative genes. We therefore attempted an alternative approach to better identify the disease-causative genes in linkage loci. We learned the principle from genome-wide association study [32], although our intention was not to investigate the whole genome, but instead a limited region of the genome, e.g., 15q22-q24 (GLC1N), where another JOAG locus was recently mapped within a genetic distance of 16.6 Mb [9]. To do that, we selected more than 100 gene-based SNPs within GLC1N, roughly one SNP for one gene. We genotyped these

| Sequence change | Codon change | Location | Minor allele frequency | Genotype frequency |
|-----------------|--------------|----------|-----------------------|-------------------|
| IVS3+13A>G      | S33N         | Exon 1   | 14 (0.08)             | 23 (0.13)         |
| rs889022        | T           | Inttron 3| 0 (0)                 | 0/0/92            |
| c.1976A>G       | G659G        | Exon 10  | 14 (0.08)             | 23 (0.13)         |

Fisher’s exact test was used to compare the frequencies of the NRG2 polymorphisms between patients with juvenile open angle glaucoma (JOAG) and controls. No association was found between NRG2 and JOAG. A p>0.12 was obtained for all three polymorphisms.
SNPs in a cohort of 100 unrelated JOAG patients and 100 control subjects. Several genes demonstrated significant association with JOAG in this case-control regional association study. These genes will be a priority in the search for the disease causative gene at GLC1N [33]. This new approach should enable us to exhaustively search for disease-associated genes in genetic loci.

ACKNOWLEDGEMENTS

We thank the family who participated in this study. This study was supported in part by a block grant from the Chinese University of Hong Kong and a direct grant (2040997) from the Medical Panel, the Chinese University of Hong Kong.

REFERENCES

1. Tielsch JM, Sommer A, Katz J, Royall RM, Quigley HA, Javit J. Racial variations in the prevalence of primary open-angle glaucoma. The Baltimore Eye Survey. JAMA 1991; 266:369-74.
2. Johnson AT, Richards JE, Boehnke M, Stringham HM, Herman SB, Wong DJ, Lichter PR. Clinical phenotype of juvenile-onset primary open-angle glaucoma linked to chromosome 1q. Ophthalmology 1996; 103:808-14.
3. Wiggs JL, Del Bono EA, Schuman JS, Hutchinson BT, Walton DS. Clinical features of five pedigrees genetically linked to the juvenile glaucoma locus on chromosome 1q21-q31. Ophthalmology 1995; 102:1782-9.
4. Wiggs JL, Damji KF, Haines JL, Pericak-Vance MA, Allingham RR. The distinction between juvenile and adult-onset primary open-angle glaucoma. Am J Hum Genet 1996; 58:243-4.
5. Wilson R, Martone J. Epidemiology of chronic open-angle glaucoma. In: Ritch R, Shields MB, Krupin T, editors. The Glaucomas. Vol. II. Mosby; St. Louis; 1996. p. 753-68.
6. Werner EB. Normal-tension glaucoma. In: Ritch R, Shields MB, Krupin T, editors. The glaucomas. Vol. II. St Louis; Mosby; 1996. p. 768-97.
7. Hitchings RA. Low tension glaucoma—its place in modern glaucoma practice. Br J Ophthalmol 1992; 76:494-6.
8. Fan BJ, Wang DY, Lam DS, Pang CP. Gene mapping for primary open angle glaucoma. Clin Biochem 2006; 39:249-58.
9. Wang DY, Fan BJ, Chua JK, Tam PO, Leung CK, Lam DS, Pang CP. A genome-wide scan maps a novel juvenile-onset primary open-angle glaucoma locus to 1q5. Invest Ophthalmol Vis Sci 2006; 47:5315-21.
10. Stone EM, Fingert JH, Alward WL, Nguyen TD, Polansky JR, Sunden SL, Nishimura D, Clark AF, Nystuenn A, Nichols BE, Mackey DA, Ritch R, Kalenak JW, Craven ER, Sheffield VC. Identification of a gene that causes primary open angle glaucoma. Science 1997; 275:668-70.
11. Kubota R, Soda S, Wang Y, Minoshima S, Asakawa S, Kudoh J, Mashima Y, Oguchi Y, Shimizu N. A novel myosin-like protein (myocilin) expressed in the connecting cilium of the photoreceptor: molecular cloning, tissue expression, and chromosomal mapping. Genomics 1997; 41:360-9.
12. Rezaie T, Child A, Hitchings R, Brice G, Miller L, Coca-Prados M, Heon E, Krupin T, Ritch R, Kreutzer D, Crick RP, Sarfarazi M. Adult-onset primary open-angle glaucoma caused by mutations in optineurin. Science 2002; 295:1077-9.
13. Monemi S, Speath G, DaSilva A, Popinchalk S, Ilitchew E, Liebmann J, Ritch R, Heon E, Crick RP, Child A, Sarfarazi M. Identification of a novel adult-onset primary open-angle glaucoma (POAG) gene on 5q22.1. Hum Mol Genet 2005; 14:725-33.
region. Arch Ophthalmol 2006; 124:1328-31.
28. Wang DY, Fan BJ, Canlas O, Tam PO, Ritch R, Lam DS, Fan DS, Pang CP. Absence of myocilin and optineurin mutations in a large Philippine family with juvenile onset primary open angle glaucoma. Mol Vis 2004; 10:851-6.
29. Ring HZ, Chang H, Guilbot A, Brice A, LeGuern E, Francke U. The human neuregulin-2 (NRG2) gene: cloning, mapping and evaluation as a candidate for the autosomal recessive form of Charcot-Marie-Tooth disease linked to 5q. Hum Genet 1999; 104:326-32.
30. Burden S, Yarden Y. Neuregulins and their receptors: a versatile signaling module in organogenesis and oncogenesis. Neuron 1997; 18:847-55.
31. Ghashghaei HT, Weber J, Pevny L, Schmid R, Schwab MH, Lloyd KC, Eisenstat DD, Lai C, Anton ES. The role of neuregulin-ErbB4 interactions on the proliferation and organization of cells in the subventricular zone. Proc Natl Acad Sci U S A 2006; 103:1930-5.
32. Hirschhorn JN, Daly MJ. Genome-wide association studies for common diseases and complex traits. Nat Rev Genet 2005; 6:95-108.
33. Wang DY, Fan BJ, Leung DY, Tham CC, Tam PO, Lam DS, Pang CP. Strong association of SLC24A1 at GLC1N with susceptibility to primary open angle glaucoma. ARVO Annual Meeting; 2007 May 6-10; Fort Lauderdale (FL).