

Ordered subset analysis supports a glaucoma locus at GLC1I on chromosome 15 in families with earlier adult age at diagnosis

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Abstract

Open angle glaucoma (OAG) is a complex disorder with varying etiologies due to multiple genes and environmental effects. This genetic heterogeneity can confound efforts to map loci. Increased homogeneity in a sample can be achieved using either ordered subset analysis (OSA) which groups families, or individual OSA (IOSA), which groups individuals based on disease related covariates. Recently, GLC1I was mapped to 15q11–13 in families with early adult onset of OAG. We tested for linkage to GLC1I in an independent sample of 167 individuals in 25 multiplex OAG families of European descent. We carried out nonparametric linkage analysis on the complete set of 25 families and obtained a maximum LOD score of 1.00 at 9.0 cM. Using mean age at diagnosis (AAD) across the affected individuals within each family to order the families as a proxy for age at onset, we found a maximum OSA LOD score of 2.09 ($p=0.021$) at 26.1 cM. The mean (\pm s.d.) AAD across the 14 earlier AAD families that contributed to the OSA LOD score was 50.6 years (± 5.38); the mean AAD for the other 1210 later AAD families that did not contribute to the OSA LOD score (the high-AAD) was 61.7 years (± 3.50). We also ran IOSA on our families using AAD as our covariate on which to subset affected individuals. The maximum LOD score was 1.01 at 14.3 cM when ordering subjects from early to late AAD. Ordered subset analysis of this sample has provided evidence of linkage close to the previously identified GLC1I glaucoma locus on 15q11–13 in families with middle-aged mean age at diagnosis.

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1. Introduction

Glaucoma is the second leading cause of blindness in the world affecting over 60 million people (Quigley, 1996). The most common form of glaucoma in individuals of European

ancestry is primary open angle glaucoma (POAG) in which intraocular pressure (IOP) is often elevated without a known underlying condition. Family history is a significant predictor of glaucoma (Wilson et al., 1987). Pedigrees demonstrating different apparent modes of inheritance were available in the literature long before the first glaucoma gene was mapped (Bell, 1932; Berg, 1932; Stokes, 1940; Beiguelman and Prado, 1963; Combie and Cullen, 1964; Teikari, 1990).

Elevated IOP and older age are also important risk factors for both occurrence and progression of glaucoma (Wilson, 1990; Klein et al., 1992; Nouri-Mahdavi et al., 2004). Although elevated IOP is a risk factor, not all patients with ocular hypertension (OHT) progress to glaucoma (Quigley et al., 1994). Furthermore, about 30% of patients with OAG have

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normal tension glaucoma (NTG) in which IOP has been documented not to be elevated (Bonomi et al., 1998). Although most cases of OAG develop later in life, onset before 35 years of age can be seen in individuals with juvenile open angle glaucoma (JOAG).

The genetic relationship of POAG, NTG, and JOAG is not yet clear since any given OAG family may show one predominant diagnosis while some family members may have one or both of the other diagnoses (Morissette et al., 1995; Richards et al., 1996; Shimizu et al., 2000; Monemi et al., 2005). Genotype–phenotype studies among glaucoma genes identified so far indicate that in many families, cases of POAG may be identical by descent with the predominant JOAG or NTG cases in those families (Morissette et al., 1995; Shimizu et al., 2000; Monemi et al., 2005).

To date, it is appreciated that OAG is highly heterogeneous and it is likely that many more glaucoma loci will be mapped. This large number of loci is not unusual for eye disorders (Vincent et al., 2003; Hejtmancik and Kantorow, 2004; Tuo et al., 2004). An especially dramatic example is retinitis pigmentosa, for which more than 133 loci have been mapped, with at least 75 genes now identified (RetNet; Rivolta et al., 2002; Daiger, 2004). OAG loci mapped so far include GLC1A (1q23–q24, MIM137750) (Sheffield et al., 1993), GLC1B (2cen–q13, MIM606689) (Stoilova et al., 1996), GLC1C (3q21–q24, MIM601682) (Wirtz et al., 1997), GLC1D (8q23, MIM602429) (Trifan et al., 1998), GLC1E (10p14–p15, MIM602432) (Sarfarazi et al., 1998), GLC1F (7q35–q36, MIM603383) (Wirtz et al., 1999), GLC1G (5q22.1) (Monemi et al., 2005), GLC1H (14q11–q13) (Genatlas; Wiggs et al., 2000), GLC1I (15q11–13) (Allingham et al., 2005), GLC1J (9q22, also called JOAG2, MIM608695) (Wiggs et al., 2004), and GLC1K (20p12, also called JOAG3, MIM608696) (Wiggs et al., 2004).

Studies have indicated further heterogeneity in hereditary glaucoma. A genome-wide linkage scan was carried out on 182 affected sibling pairs that identified five additional regions of interest (Wiggs et al., 2000) and additional regions of the genome showed moderate evidence for linkage to OAG in a genome scan of participants in the Barbados Eye Study (Nemesure et al., 1996). Recently, eight Finnish families with POAG were genotyped at glaucoma loci GLC1A–GLC1F and eight other candidate gene regions. Evidence for linkage was not found in any of the tested regions (Lemmela et al., 2004).

Three of the mapped OAG genes have so far been identified: myocilin (MYOC, MIM601652) at the GLC1A locus (Stone et al., 1997; Nguyen et al., 1998), optineurin (OPTN, MIM602432) at the GLC1E locus (Rezaie et al., 2002), and WD-repeat36 (WDR36) at GLC1G (Monemi et al., 2005). MYOC mutations account for about 3% of OAG, with the Gln368Stop MYOC nonsense variant accounting for many, but not all, of the adult onset OAG cases caused by mutations at the GLC1A locus (Shimizu et al., 2000; Alward et al., 2002). Most of the other MYOC variants are missense mutations usually associated with a JOAG phenotype. A small number of mutations in OPTN are found in families in which most affected individuals have NTG (Rezaie et al., 2002). Mutations in WDR36 are the first to be found segregating through families in

which the more prevalent adult onset glaucoma involving elevation of IOP is predominant (Monemi et al., 2005). Much remains to be understood about the underlying mechanisms by which mutations in these genes predispose to glaucoma and most cases cannot be accounted for by these three genes.

Genetic heterogeneity can confound mapping efforts. That populations of African ancestry show differences in prevalence, average age at diagnosis, and response to therapies suggests that separate evaluation of European and African populations in glaucoma mapping studies might assist in reducing the complex etiology of the disease (Lotufo et al., 1989; Tielsch et al., 1991; Ederer et al., 2004). Increased homogeneity in a sample can also be achieved by grouping families or individuals based on covariates, such as age at diagnosis or maximum pre-treatment IOP. Where a clinically significant cutoff value for a covariate has not been identified, it may be unclear which families and/or subjects to include in an analysis.

In ordered subset analysis (OSA), pre-specified cutoff values are not necessary. Families are ranked by an ordinal or continuous covariate and nonparametric linkage analysis is performed on the first family. At each subsequent round of analysis a family, or families, is added based on their covariate ranking and the linkage analysis is repeated. The analysis continues until all families have been added. The LOD scores at each step are compared and a maximum score is obtained for a subset of the families (Hauser et al., 2004). Individual OSA (IOSA) performs a similar analysis except that affected individuals, rather than entire families, are added sequentially based on their covariate values. Both methods attempt to create a more homogeneous sample. In OSA, using a summary statistic across all affected family members may remove some of the within-family variability due to environmental and/or genetic interactions. IOSA may account for phenocopies as the presentation of glaucoma, and thus covariate values, in these sporadic cases may differ from that in familial cases in the same families. In identifying the GLC1I locus, Allingham and colleagues performed OSA by sorting the families based on age at diagnosis (AAD), a proxy for age of onset. The families with an earlier mean AAD (44.1 years \pm 9.1) were linked to GLC1I, while families with a later mean AAD (61.3 years \pm 10.4) were not (Allingham et al., 2005).

In this study we tested for a locus on 15q11–13 by subsetting our sample based on AAD. We compared the results obtained via three statistical approaches: OSA, IOSA, and nonparametric linkage analysis of the whole family set. Consistent with the results of Allingham et al. (2005), we report modest evidence for a locus on chromosome 15, with a maximum LOD score of 1.00 at the GLC1I locus, and increased evidence for linkage when we focused on early AAD families identified using OSA, with a maximum OSA LOD score of 2.09 17 cM from the GLC1I locus.

2. Methods

2.1. Subjects

Subjects provided informed consent and blood samples according to protocols approved by the Institutional review

board for human subject research of the University of Michigan medical school. For purposes of this study, the narrow definition of affected POAG status was based on open angles, glaucomatous optic neuropathy, and visual field defects consistent with glaucoma. Glaucomatous optic neuropathy was defined as a narrowed neuroretinal rim, notching of the neuroretinal rim, and/or marked asymmetry in the cup to disc ratio. Glaucomatous visual field defects were based on the Glaucoma Hemifield Test and clinician interpretation. In addition, we considered a broad definition of the term affected that included individuals for whom visual field tests were not available but who presented with open angles and glaucomatous optic neuropathy when diagnosed with POAG according to an ophthalmologist's exam. Those individuals with the above characteristics, but who were reported to have pigment dispersion syndrome or pseudoexfoliation, were considered to have an indeterminate phenotype and not classified as either affected or unaffected in the analysis. We also screened all probands for mutations in MYOC and OPTN and found no causative mutations.

2.2. Markers

We carried out initial genotyping using microsatellite markers from the Applied Biosystems MD-10 panel set (Applied Biosystems, Foster City, CA) as well as markers from the Marshfield Clinic map (<http://research.marshfieldclinic.org/genetics>) which were produced by Invitrogen (Carlsbad, CA). After evaluation of marker quality and coverage, four chromosome 15 markers from the MD-10 panel set were used in the analysis. To complete coverage and to improve data quality, four markers from the Marshfield map were added to replace poorly performing markers and to map more finely the region reported to contain GLC1I. The final marker coverage in this region on chromosome 15 gave an average intermarker distance of 4.3 cM. The eight markers used in this analysis were: D15S128, D15S822, D15S1002, D15S1048, D15S1007, D15S1040, D15S1042, and D15S994.

2.3. Marker amplification

We amplified markers using the TrueAllele PCR Premix from Applied Biosystems according to the manufacturer's instructions. To improve throughput with simultaneous amplification of multiple markers, we also used the Qiagen Multiplex PCR Kit according to manufacturer's instructions. We amplified up to six markers in one reaction using the Perkin-Elmer 9700 or 9600 thermocycler according to manufacturer's instructions.

2.4. Genotyping

Allele sizes were read on either an ABI 377 Automated Sequencer or an ABI 3100 Automated Sequencer from Applied Biosystems, with multiplex loading of one panel of markers per sample per run. Allele sizes were called using Genotyper from Applied Biosystems version 3.7 for data generated on the ABI

3100 or version 3.0 for data generated on the ABI 377. Allele sizes were adjusted to reference CEPH individual 1347-02 and entered into a database through the use of the Cicada web interface at <http://eyegene.ophty.med.umich.edu>. Markers were evaluated for the appropriate bin definition using the histogram editor in Cicada. Data were extracted from the database using Cicada and formatted for use in analysis software.

2.5. Error checking

We included an internal duplicate control and a negative control for each marker. We used Pedstats (Wigginton and Abecasis, 2005) to check the data for Mendelian errors and Merlin (Abecasis et al., 2002) to identify possible double recombination events involving closely neighboring markers. We also checked family relationships using GRR (Abecasis et al., 2001). We identified an affected individual who appeared to be a half sibling. As we did not have parental genotypes, we could not determine which parent was incorrectly specified and we removed that subject from the analysis. Based on genotypes from one set of monozygotic twins and our internal control, we estimated an initial genotyping error rate of 1.3% for a larger data set of 382 markers that includes these eight markers from chromosome 15. To reduce the error rate in this chromosome 15 data set, a second researcher re-genotyped the eight chromosome 15 markers listed above. The two researchers resolved any discrepancy in allele calls. Each genotype was called independently in the absence of information on individual identities, affected status, or family relationships.

2.6. Statistical analysis

We performed nonparametric analyses on the chromosome 15 markers for our complete set of families using Merlin. To identify a potentially more homogeneous subset of families, we subsequently ran OSA and IOSA using AAD as our covariate. The analysis was performed with ascending and descending AAD rankings. To adjust for multiple testing, we calculated an empirical p -value by permuting the covariate values across families (Hauser et al., 2004). We further corrected our significance level by dividing 0.05 by two for testing both late-to-early and early-to-late ordering, thus giving a p -value cutoff of 0.025.

3. Results

To evaluate whether the GLC1I locus could be detected in a second independent sample, we genotyped markers on chromosome 15 from 167 individuals in 25 multiplex OAG families of European ancestry. Under the narrow affection criteria, 90 people were considered affected; under the broad criteria 107 people were considered affected. For nineteen of the families, we genotyped were affected individuals in two or more generations. There were eight cases of JOAG among six families and eighteen cases of NTG among twelve families. When considering the narrow definition of affected status,

AAD ranged across subjects from 16 to 86 years, with a median age of 57.5 and a mean age (\pm s.d.) of 55.6 (\pm 14.5) years. The maximum detected pre-treatment IOP ranged from 14.0 to 51 mmHg with a mean of 26.6 (\pm 6.2) mmHg. When considering the broad definition, the AAD ranged from 16 to 86, years and the median age was 55.3 (\pm 14.2) years. Maximum IOP ranged from 14.0 to 51.0 mmHg with a mean of 26.3 (\pm 6.6) mmHg. The correlation between AAD and maximum IOP across individuals was not significant for either the narrow ($r = -0.08$, $p = 0.46$) or broad criteria ($r = -0.11$, $p = 0.29$). Familial mean AAD and mean maximum IOP were not correlated across families for the narrow criteria ($r = -0.36$, $p = 0.09$), but were negatively correlated for the broad ($r = -0.41$, $p = 0.048$). Family characteristics are in Table 1.

We carried out nonparametric linkage analysis on the complete set of 25 families. The maximum LOD scores obtained were 1.00 at 9.1 cM for the broad definition of affected status and 0.51 at 26.1 cM for the narrow definition of affected status (Fig. 1(A)). One family was not analysed using the broad definition and a second family was not analysed using the narrow definition.

To identify a more homogeneous subset, we used OSA with mean AAD as the covariate on which the families were ordered. The maximum LOD score obtained from this analysis was 2.09 at 26.1 cM, using mean AAD across affected members of a family as the summary statistic (Fig. 1(A)).

Table 1
Family-specific characteristics

| Family | AAD | IOP | Cumulative LOD ^a |
|--------|------|------|-----------------------------|
| F0190 | 35.0 | 29.5 | 0.00 |
| F0034 | 46.3 | 25.5 | 0.01 |
| F0046 | 47.0 | 31.0 | n/a |
| F0300 | 48.3 | 28.0 | 0.32 |
| F0201 | 49.0 | 26.0 | 0.66 |
| F0096 | 49.3 | 28.3 | 0.53 |
| F0001 | 51.0 | 28.8 | 0.88 |
| F0003 | 51.0 | 28.8 | – |
| F0081 | 51.7 | 26.4 | 0.79 |
| F0255 | 52.6 | 27.8 | 1.09 |
| F0060 | 52.7 | 30.0 | 1.33 |
| F0058 | 53.7 | 26.3 | 1.43 |
| F0082 | 54.0 | 30.0 | 1.55 |
| F0029 | 56.7 | 29.0 | 2.06 |
| F0055 | 56.7 | 25.3 | – |
| F0048 | 57.7 | 25.3 | 2.07 |
| F0334 | 58.0 | 21.3 | 1.88 |
| F0056 | 58.5 | 22.3 | 1.78 |
| F0333 | 59.3 | 26.0 | 1.45 |
| F0320 | 59.7 | 23.7 | 0.80 |
| F0373 | 60.8 | 28.5 | 0.61 |
| F0070 | 62.0 | 26.7 | 0.72 |
| F0024 | 65.7 | 23.0 | 0.58 |
| F0240 | 66.3 | 21.2 | 0.56 |
| F0094 | 67.3 | 30.3 | 0.5 |

AAD, age at diagnosis; IOP, intraocular pressure; Cumulative LOD, cumulative LOD score generated by including families with AAD less than or equal to family value in linkage analysis.

^a Family F0046 was not included in analysis for cumulative LOD.

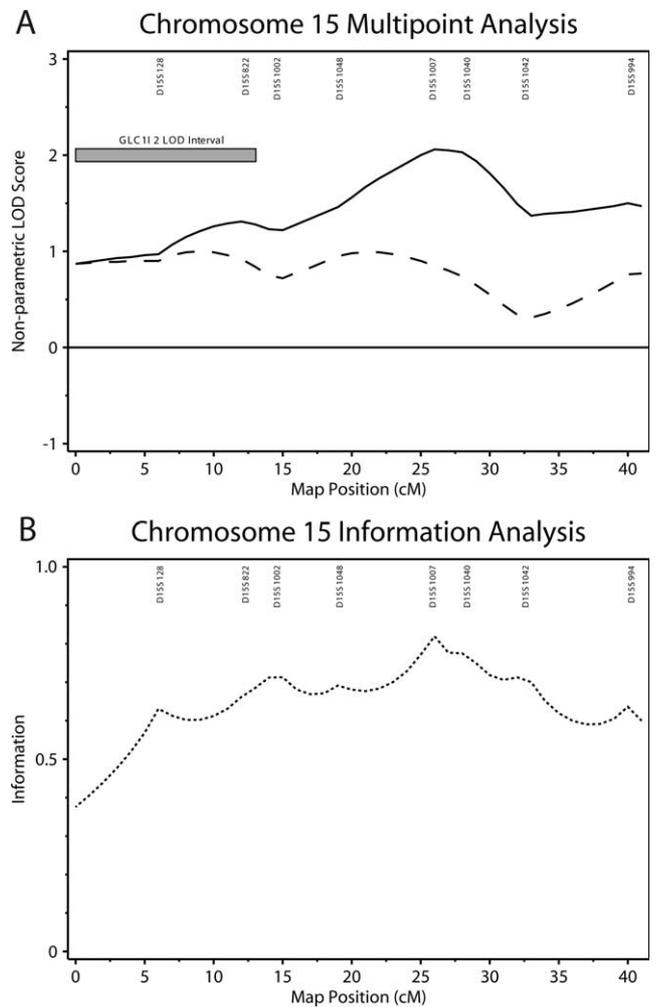


Fig. 1. (A) Nonparametric LOD scores for the OSA subset (earlier AAD) (solid line) and LOD scores before subsetting (dashed line). (B) Information plot for genotypes for families in the maximum OSA LOD score subset.

This LOD score was obtained when the families were ordered from early to late, using the broad affection criteria. The empirical p -value for observing this large an increase in LOD score across chromosome 15 was 0.021. The informativeness of our marker data peaked at 25.9 cM and was lowest at 8.1 cM (Fig. 1(B)). The mean AAD across the 14 (58%) earlier AAD families that contributed to the maximum OSA LOD score was 51.0 years (\pm 5.50); the mean for the other 10 later AAD families (the high-AAD) was 62.0 years (\pm 3.60).

We also ran IOSA on our subjects using AAD as our covariate on which to subset affected individuals. Using the broad affection criteria, the maximum LOD score was 1.01 at 14.3 cM when ordering subjects from early to late AAD and was 1.05 at 14.3 cM for late to early.

4. Discussion

The purpose of this study was to explore the role of the recently reported GLC11 OAG locus on chromosome 15 (Allingham et al., 2005) in a second independent sample.

In addition to evaluating this locus in our set of families, we were interested in comparing the results obtained from non-subsetted multipoint linkage analysis to the results from two different subsetting approaches: OSA which subsets families based on the family's summary covariate and IOSA, which subsets individuals based on the subject's covariate, independent of family.

4.1. Use of ordered subset analysis to detect OAG linkage to 15p

Although standard nonparametric multipoint linkage analysis did not identify this region of chromosome 15 as providing compelling evidence of linkage, use of OSA to subset families based on AAD identified a broad region of interest close to the GLC1I locus (LOD=2.09, $p=0.021$). Thus our data provide confirmatory evidence for linkage to the GLC1I locus in an independent study cohort, but our results are interesting only in context of the previous report by Allingham and colleagues (2005) and would not be considered adequate to constitute initial identification of a locus.

Our data are consistent with a replication of the GLC1I locus despite a 17 cM separation of the maximum OSA peak LOD score in our study from the peak LOD score in the Allingham study, when positions of markers from both studies are evaluated on the same map. Our strongest evidence for linkage was found at 26 cM on chromosome 15 on the Marshfield map (<http://research.marshfieldclinic.org/genetics>), with evidence for linkage across a broad region including GLC1I (Fig. 1(A)), while Allingham and colleagues reported their maximum LOD score at a marker that sits at approximately 9 cM on the Marshfield map. It has been shown that there can be large variation in the particular location identified for a given locus for complex disorders like OAG. Simulations showed that this effect is accentuated as the number of families considered becomes smaller, and that evidence for a location as far as 20–30 cM from a given locus could indicate confirmation (Roberts et al., 1999). We have used a relatively small number of families, so the distance of our linkage peak from GLC1I does not exceed reasonable expectations as a confirmation of GLC1I on 15q11–13, and suggests but certainly does not prove that the same locus may be relevant in both sets of families.

It is worth noting that before subsetting, our greatest evidence for linkage is at 9 cM, in the vicinity of GABRB3, which is where Allingham et al., find their peak OSA score (Allingham et al., 2005); however, this LOD score is low enough that this location would not normally have been selected in the course of a full genome scan. It is interesting that the LOD increases slightly using OSA, even though it does not end up providing the maximum LOD score. The genotypes in our family subset are less informative near the GLC1I peak at 9 cM than around 26 cM, the location of our peak near D15S1007 (Fig. 1(B)). Clearly, additional studies with more informative markers will be needed to optimize localization of GLC1I and to confirm that the two studies are identifying the same locus.

4.2. GLC1I and earlier age at diagnosis

Our finding of linkage to the GLC1I region used the same covariate as Allingham and colleagues (2005), earlier AAD. It should be noted that mean family AAD is strongly negatively correlated with mean family maximum IOP in our study. The mean IOP in the high-AAD families is 24.8 mmHg (± 3.07) and is 27.8 mmHg (± 1.65) in the low-AAD families. The mean IOPs of the two subsets are significantly different ($p=0.005$). Thus we have to consider that earlier family mean AAD might be a proxy measurement for high family mean IOP, and that the appropriate covariate to consider is in fact higher IOP. This becomes relevant for evaluation of this locus, and should be considered when choosing individuals for further study.

Using OSA, we were able to subset our data without a priori decisions about regarding which families to classify as earlier or later AAD. A subset of 14 families within our set were identified that, when considered as a group, more than doubled the nonparametric LOD score that was calculated on the complete sample. The simulations performed by OSA demonstrate that if we have randomly selected families to include in the linkage analysis, we would not expect a LOD score as high as the one we observed at this location. Thus, OSA has identified an earlier AAD subset that is significantly younger than the other families.

It is prudent to exercise due caution when interpreting results obtained using OSA, as these results could be due to chance. It is important to carefully consider results obtained using OSA in the context of the family data and in the context of data from other researchers. We have tested the specific hypothesis that a previously identified locus on 15q11–13 is influencing risk of glaucoma, however our sample had only 25 families and the evidence for linkage in both studies is modest. Therefore, it remains possible that this is a false positive finding.

4.3. Individual ordered subset analysis of the data

OSA attempts to create an etiologically homogeneous sample of families, but there is also within family variation in a complex disease like OAG. Subsetting individuals within families may serve to create more etiologically homogeneous families for analysis, which is what Individual OSA (IOSA) attempts to accomplish. However, IOSA did not provide further information, since subsetting family members on AAD did not substantially improve our scores. It is interesting that although both this study and the Allingham study identified optimal subsets at this location by considering the families with the earliest average AAD within an adult onset set of OAG families, we did not gain the same effect by considering only the youngest affected individuals in a given family.

In summary, this study has provided evidence to confirm the previously identified GLC1I glaucoma locus through use of OSA to detect linkage of OAG to markers located on 15q11–13. Both studies detected linkage to this region through use of AAD as the covariate for ordering the families; therefore,

future studies should be conducted using OAG families with earlier adult age at diagnosis and/or higher IOP. However, comparison of the two studies suggests that the locus as currently defined covers a broad region near the centromere on 15q. Thus, further work is needed to validate and refine the region around GLC1I.

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