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Common genetic variants associated with open-angle glaucoma

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Open-angle glaucoma (glaucoma) is a major eye disorder characterized by optic disc pathology. Recent genome-wide association studies identified new loci associated with clinically relevant optic disc parameters, such as the optic disc area and vertical cup–disc ratio (VCDR). We examined to what extent these loci are involved in glaucoma. The loci studied include *ATOH7, CDC7/TGFBR3* and *SALL1* for optic disc area, and *CDKN2B*, *SIX1*, *SCYL1/LTBP3*, *CHEK2*, *ATOH7* and *DCLK1* for VCDR. We performed a meta-analysis using data from six independent studies including: the Rotterdam Study ($n = 5736$), Genetic Research in Isolated Populations combined with Erasmus Rucphen Family study ($n = 1750$), Amsterdam Glaucoma Study ($n = 296$) and cohorts from Erlangen and Tübingen ($n = 1363$), Southampton ($n = 702$) and deCODE ($n = 36\,151$) resulting in a total of 3161 glaucoma cases and 42 837 controls. Of the eight loci, we found significant evidence ($P = 1.41 \times 10^{-8}$) for the association of *CDKN2B* with glaucoma [odds ratio (OR) for those homozygous for the risk allele: 0.76; 95% confidence interval (CI): 0.70–0.84], for the role of *ATOH7* (OR: 1.28; 95% CI: 1.12–1.47) and for *SIX1* (OR: 1.20; 95% CI: 1.10–1.31) when adjusting for the number of tested loci. Furthermore, there was a borderline significant association of *CDC7/TGFBR3*

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and *SALL1* (both $P = 0.04$) with glaucoma. In conclusion, we found consistent evidence for three common variants (*CDKN2B*, *ATOH7* and *SIX1*) significantly associated with glaucoma. These findings may shed new light on the pathophysiological protein pathways leading to glaucoma, and point to pathways involved in the growth and development of the optic nerve.

INTRODUCTION

Open-angle glaucoma (from here on called glaucoma) is a chronic neurodegenerative disease that leads to progressive damage to retinal ganglion cells and nerve fibers, resulting in visual field loss (1). Glaucoma is recognized as the commonest cause of irreversible blindness worldwide. However, the etiology of glaucoma remains obscure. Risk factors for glaucoma include old age, elevated intraocular pressure, myopia, African descent and positive family history (2,3). Only three causative genes have been established (*MYOC*, *OPTN* and *WDR36*) for late-onset glaucoma (4). High-risk variants in these genes are predominantly observed in familial cases of glaucoma, but their frequency in sporadic patients from the general population is low (3–5%) (4).

One of the first signs of glaucoma is damage to the optic disc (optic nerve head), visible as an increased excavation (cupping). The optic disc cupping occurs more in the vertical direction, which is commonly quantified as the vertical cup–disc ratio (VCDR) (1,5). An increased VCDR is a significant determinant of the risk of developing glaucoma (6–8). Recently, we identified new loci involved in the optic disc area as well as VCDR: *ATOH7, CDC7*/*TGFBR3* and *SALL1* for optic disc area, and *CDKN2B*, *SIX1*, *SCYL1*/*LTBP3*, *CHEK2*, and *DCLK1*, in addition to *ATOH7*, for VCDR (Table 1) (9). When considering the function of the proteins these genes encode for, two protein pathways emerge (Fig. 1) (9). To further elucidate the relation of genes implicated in the optic disc area and VCDR to glaucoma, we examined to what extent these loci are involved in glaucoma. For this purpose, we performed a meta-analysis using data from six independent studies comprising of Caucasian persons: the Rotterdam Study ($n = 5736$), Genetic Research in Isolated Populations combined with Erasmus Rucphen Family (ERF) study ($n = 1750$), Amsterdam Glaucoma Study (AGS) ($n = 296$), and cohorts from Erlangen and Tübingen ($n = 1363$), Southampton ($n = 702$) and deCODE ($n = 36\ 151$) resulting in a total of 3161 glaucoma cases and 42 837 controls.

RESULTS

Table 2 summarizes the general characteristics of the cases and controls for all cohorts. Cases of GRIP and Southampton were significantly older ($P < 0.001$) than their controls. As expected, in all studies, the intraocular pressure and intraocular pressure-lowering treatment were increased in glaucoma cases.

All studies showed marginal evidence for association ($P < 0.003$; adjusted for multiple testing) of rs1900004 (close to *ATOH7*), rs1063192 (*CDKN2B*) and rs10483727 (close to *SIX1*) with glaucoma for the homozygous effect (Fig. 2). For rs1900004 (*ATOH7*) as well as rs10483727 (*SIX1*), we found significant odds ratios (ORs) for glaucoma of 1.28 [95% confidence interval (CI): 1.12–1.47; $P = 2.49 \times 10^{-4}$] and 1.20 (95% CI: 1.10–1.31; $P = 7.65 \times 10^{-5}$), respectively, for those homozygous for the T-allele. For rs1063192 (*CDKN2B*), we found evidence for association with glaucoma in persons heterozygous and homozygous for the G-allele. The OR for the heterozygous ones was 0.85 (95% CI: 0.77–0.94; $P = 0.002$) and for the homozygous 0.76 (95% CI: 0.70–0.84; $P = 1.41 \times 10^{-8}$). The latter translates into a 1.32 increase in risk for the C-allele. Testing for heterogeneity showed no significant differences across the studies ($I^2 < 22.5\%$).

We could not find evidence for a significant association for the other loci with glaucoma when adjusting for multiple testing. Nonetheless, the associations of the homozygous effect for rs1192415 (*CDC7*/*TGFBR3*) and rs1362756 (close to *SALL1*) with glaucoma were borderline significant ($P = 0.044$ and $P = 0.040$, respectively). However, rs1192415 (*CDC7*/*TGFBR3*) is a rare single nucleotide polymorphism (SNP), which appeared to be monomorphic in the Southampton cohort. For this SNP, findings were inconsistent through the other studies. Finally, we evaluated whether the findings were robust when ignoring specific recessive effects for those hetero- and homozygous. The allelic effect showed significant evidence for rs1063192 (*CDKN2B*) and rs10483727 (*SIX1*; see Supplementary Material, Table S1) with glaucoma.

DISCUSSION

The present study yielded one significant gene (*CDKN2B*) involved in glaucoma in those heterozygous as well as those homozygous. The minor allele of the corresponding SNP (rs1063192) was genome-wide significantly associated with a lower VCDR and a reduced risk of glaucoma (9). In addition, there was also significant evidence for a role of *ATOH7* and *SIX1* in glaucoma when adjusting for multiple testing. For these genes, the effect appeared to be recessive, although the association remained significant when testing a multiplicative model for *SIX1*. Those homozygous for the minor allele had an increased risk of glaucoma. The three genes showed consistent evidence for a recessive effect through all cohorts. The other five regions (*CDC7*/*TGFBR3*, *SALL1*, *SCYL1*/*LTBP3*, *CHEK2* and *DCLK1*) that were previously reported to be associated with either the optic disc area or VCDR could not be significantly related to glaucoma. None of the genes was identified before in genome-wide association studies (GWAS) on glaucoma (10,11).

The region of *CDKN2B* has been implicated in other diseases (e.g. diabetes, myocardial infarction and gliomas) (12–14). Different variants have been associated with different disorders. The variant associated with glaucoma in our study was earlier implicated in glioma (14). Glioma and glaucoma appear to share the same risk allele. Most of the risk

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Table 1. Loci investigated in the current study

Most significant SNP	MA	MAF	Chromosome location	Position	Nearest gene (*symbol*; name)	Distance (b)	Quantitative trait associated with GWAS	Direction of effect size
rs1900004	T	0.22	10q21.3−q22.1	69 670 887	*ATOH7*; atonal homolog 7	9021	Optic disc area/ VCDR	−/−
rs1192415	G	0.22	1p22	91 849 685	*CDC7*/*TGFBR3*; cell division cycle 7 homolog/transforming growth factor, beta receptor III	116 719	Optic disc area	+
rs1362756	C	0.28	16q12.1	50 015 791	*SALL1*; sal-like 1	1 154 095	Optic disc area	+
rs1063192	G	0.44	9p21	21 993 367	*CDKN2B*; cyclin-dependent kinase inhibitor 2B	0	VCDR	−
rs10483727	T	0.44	14q22−23	60 142 628	*SIX1*; sine oculis-related homeobox 1 homolog	39 878	VCDR	+
rs17146964	G	0.20	11q13	65 005 721	*SCYL1*/*LTBP3*; SCY1-like 1/latent transforming growth factor beta binding protein 3	43 403	VCDR	−
rs1547014	T	0.26	22q12.1	27 430 711	*CHEK2*; CHK2 checkpoint homolog	0	VCDR	−
rs1926320	C	0.25	13q13	35 550 617	*DCLK1*; doublecortin-like kinase 1	0	VCDR	+

SNP, single nucleotide polymorphism; MA(F), minor allele (frequency); GWAS, genome-wide association study; VCDR, vertical cup−disc ratio.

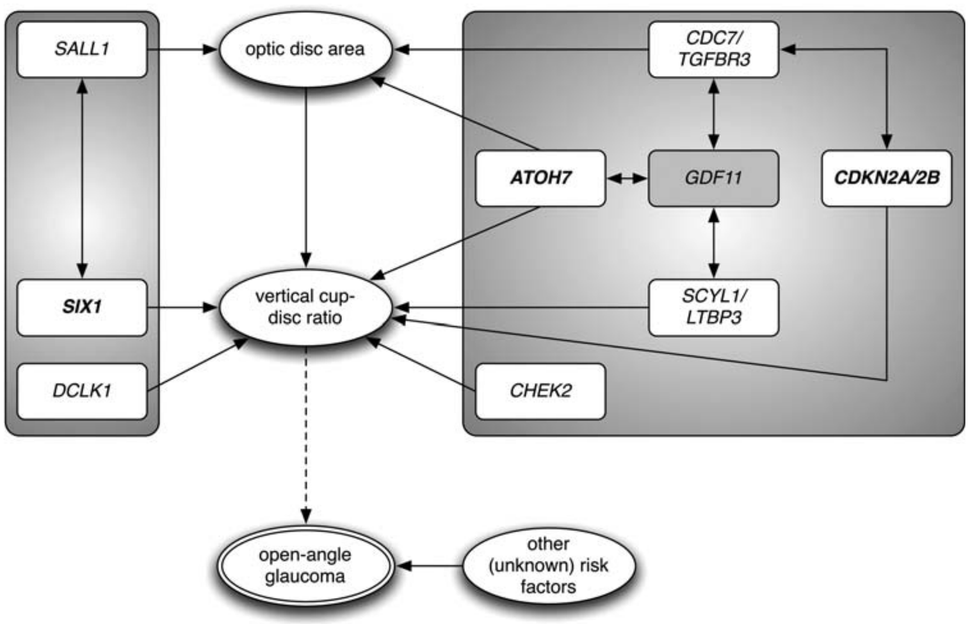

Figure 1. Overview of the biological interaction of the investigated genes in relation to open-angle glaucoma (left part: developmental pathway; right part: *TGFB*-signaling/growth pathway; genes associated with open-angle glaucoma in the present study are in bold).

variants at this locus are in non-coding regions. The consistent association with several diseases suggests these variants act by influencing the expression of nearby genes. The SNP associated with glaucoma in the current study is highly correlated with increased CDKN2B antisense RNA (*ANRIL*) expression. Thus, the SNP is involved in regulating *CDKN2B* levels in blood and other tissues, suggesting that modulation of *ANRIL* expression may mediate disease susceptibility (15). At present, little is known about the function of *ANRIL* in general and in neuronal tissue specifically.

ATOH7 has been implicated in eye development before and points to a role of early development of the optic nerve (see further). Recently, *ATOH7* has also been associated with optic nerve hypoplasia in humans (16). Deficiency of *ATOH7* in mice may result in a critical reduction in retinal ganglion cells (17).

SIX1 acts within a network of genes that trigger eye organogenesis (18). These findings combined with the current findings are of interest and may shed new light on the etiology of glaucoma.

Increased intraocular pressure is the predominant risk factor for glaucoma. About half of the glaucoma patients have a statistically normal intraocular pressure. Earlier, we showed that adjustment for intraocular pressure did not alter the findings for the investigated SNPs (Table 1), in that the association with the VCDR remained significant (9). This suggests that

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Table 2. Characteristics of the open-angle glaucoma cases presented as mean ± standard deviation (range) unless stated otherwise

	RS-I		ERF/GRIP		AGS		Erlangen and Tübingen		Southampton		deCODE	
	Cases ($n = 188$)	Controls ($n = 5548$)	Cases ($n = 104$)	Controls ($n = 1646$)	Cases ($n = 148$)	Controls ($n = 148$)	Cases ($n = 986$)	Controls ($n = 377$)	Cases ($n = 470$)	Controls ($n = 232$)	Cases ($n = 1265$)	Controls ($n = 34886$)
Age (years)	75.5 ± 7.4 (56–94)	74.5 ± 7.8 (55–105)	73.3 ± 9.2 (51–91)	46.8 ± 14.1 (18–84)	72.9 ± 11.1 (27–98)	72.3 ± 8.0 (55–92)	66.5 ± 14.1 (12–104)	73.9 ± 6.4 (34–97)	74.3 ± 10.5 (38–96)	69.1 ± 10.3 (50–91)	84.1 ± 11.8 (30–103)	61.6 ± 21.3 (5–106)
Gender women, *n* (%)	85 (45.2)	3289 (59.3)	55 (52.9)	942 (57.2)	68 (45.9)	84 (56.8)	600 (61.0)	101 (59.8)	232 (49.7)	119 (51.3)	682 (53.9)	19818 (56.8)
Intraocular pressure (mmHg)	18.2 ± 6.2 (6.0–54.6)	15.2 ± 3.5 (5.0–58.5)	25.9 ± 8.6 (12.0–62.0)	15.4 ± 3.0 (6.0–30.0)	26.9 ± 8.5 (13.0–54.0)	16.7 ± 3.4 (13.0–28.0)	27.8 ± 9.3 (11.0–65.0)	<21.0	26.0 ± 5.5 (14.0–54.0)	NA	NA	NA
Intraocular pressure treatment, *n* (%)	37 (19.7)	93 (1.7)	104 (100.0)	13 (0.8)	148 (100.0)	0 (0.0)	Almost 99%	0 (0.0)	470 (100.0)	0 (0.0)	NA	NA

RS, Rotterdam Study; ERF, Erasmus Rucphen Family; GRIP, Genetic Research in Isolated Populations; AGS, Amsterdam Glaucoma Study.

other mechanisms independent of intraocular pressure explain these associations. When we combine current findings with previous ones, two pathways emerge that may be relevant in addition to intraocular pressure (Fig. 1). One pathway related to growth, including *CDKN2B* and *ATOH7* involved through the *TGFB* pathway, which has been implicated before in the pathogenesis of glaucoma (19–21), and the other pathway related to development, including *SIX1*.

Only three out of the eight loci that were previously associated with the optic disc area and VCDR were significantly associated with glaucoma in the current study. It remains to be determined whether other genes associated with the VCDR will be relevant when studying larger samples. Only *ATOH7* was significantly associated with the optic disc area, but also had an independent effect on the VCDR (9).

So far, only one genome-wide significant gene has been identified and consistently replicated for glaucoma using GWAS (11). It is of interest that this gene (*CAV1*) interacts with *CDKN2A* through *CDKN1A* (www.ingenuity.com). Nevertheless, in the present study, the gene showing the strongest association in terms of *P*-values is *CDKN2B*. This gene reaches genome-wide significance ($P < 5 \times 10^{-8}$) in our study. At present, there is no interaction known of this gene with *CAV1*. Identification of the causal variant in the region is needed to increase our knowledge of the causal pathways.

Although this is one of the largest studies on the genetics of glaucoma, the power to detect genes with small effects is still limited. Furthermore, one of the major problems in glaucoma in general is the lack of standardized clinical criteria, which will remain a problem in future research. Despite this hampering our findings, the consistency in ORs suggests that this problem may have primarily affected the statistical power rather than heterogeneity of glaucoma cases.

In conclusion, the present study reveals three common variants implicated in glaucoma and supports the hypothesis of the involvement of the *TGFB* pathway in glaucoma. Further exploration of our findings may include expression and translational studies. The role of these genes in non-white populations (such as some African populations with a markedly higher prevalence of glaucoma) remains to be established. Nonetheless, we could relate three of the eight loci to glaucoma, opening new avenues to improve our understanding for this common form of sight-threatening disease.

MATERIALS AND METHODS

Study populations

The first cohort, the Rotterdam Study (RS-I), is a prospective population-based cohort study of 7983 residents aged 55 years and older living in Rotterdam, the Netherlands (22). RS-I was previously included in the gene discovery study (9). In this paper, we included cases and controls from RS-I. The second study included glaucoma cases from the Genetic Research in Isolated Populations (GRIP; $n = 104$) and controls from the ERF study. For GRIP, medical records in three local hospitals were assessed to identify patients with glaucoma. ERF is a family-based study in a genetically isolated population in the southwest of the Netherlands with over 3000 participants aged 18–86 years (23,24). Participants of GRIP were

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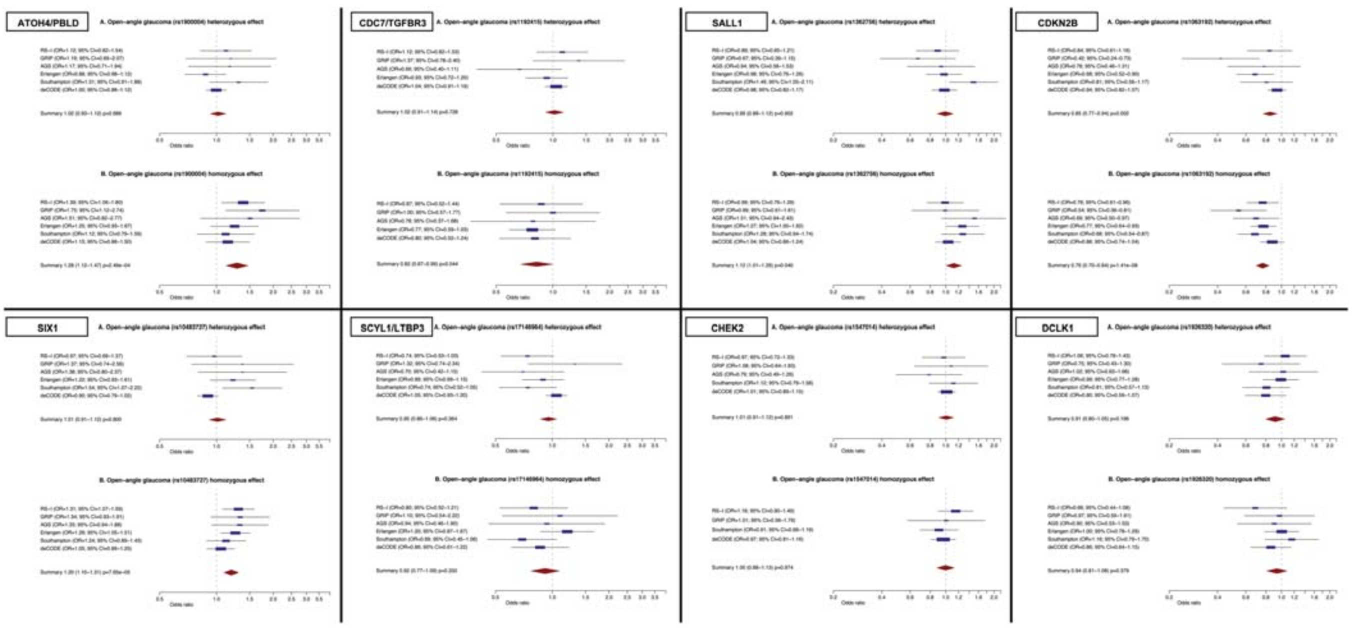

Figure 2. The heterozygous (**A**) and homozygous effect (**B**) of the top SNPs on open-angle glaucoma in RS-I, GRIP, AGS, Erlangen and Tübingen, Southampton, deCODE and the joint effect (OR, odds ratio; CI, confidence interval). Red diamonds indicate the overall OR. The size of the blue box is inversely proportional to the variance. Horizontal lines indicate 95% CI. The dashed vertical line in each panel shows the value for no effect (OR = 1.0). Genotyping of rs1547014 failed in Erlangen and Tübingen; no participant of AGS was homozygous for this SNP. The SNP rs1192415 was monomorphic in the Southampton cohort.

ascertained independently of ERF, but lived in the same region. The third study, the AGS, included 148 cases and 148 controls collected from eye clinics, meetings of the glaucoma patients' association, nursing homes and fairs for the elderly from all over the Netherlands. The fourth study included participants from Erlangen and Tübingen, Germany, comprising 986 glaucoma cases and 377 controls. Cases and controls were recruited from the same geographic regions. For the fifth study, from Southampton, glaucoma cases and controls ($n = 470$ and 232, respectively) were collected from specialist glaucoma and general clinics at the Southampton Eye Unit, UK. Finally, in deCODE, the sixth study, 1265 glaucoma cases were recruited from the Reykjavik Eye Study (25) and Icelandic glaucoma clinics. Controls ($n =$ 34 886) were selected among individuals who had participated in the various genetic programs at deCODE. The present study included a total of 3161 glaucoma cases and 42 837 controls, all of Caucasian ethnicity. All measurements in these studies were conducted after the respective relevant medical ethics committees had approved the study protocols, and all participants had given a written informed consent in accordance with the Declaration of Helsinki.

Ophthalmic examination

The ophthalmic assessment in RS-I included a medical history, autorefraction, keratometry, Goldmann applanation tonometry, visual field testing [Humphrey Field Analyzer II 740 (HFA; Carl Zeiss, Oberkochen, Germany) or Goldmann kinetic perimetry (Haag Streit, Bern, Switzerland)] and optic nerve head imaging with Topcon ImageNet System (Topcon Corporation, Tokyo, Japan) of both eyes after pharmacologic mydriasis. In GRIP, visual fields were tested with standard automated perimetry by means of the HFA 24-2 SITA Standard test program or the Octopus 101 (Haag Streit) G2 program with TOP strategy. The ophthalmic assessment in ERF was similar to RS-I, but no visual field testing was included, medical records were checked for any glaucoma pathology (L.M.E.v.K. and H.G.L.) and optic nerve head imaging was done with Heidelberg Retina Tomograph 2 (HRT; Heidelberg Engineering, Dossenheim, Germany). In AGS, all persons underwent ophthalmoscopy and biomicroscopy with a 90 diopter lens, and digital stereo images of the optic nerve head were taken after mydriatic drops. Participants from Erlangen and Tübingen underwent standardized clinical examinations for glaucoma at the Ophthalmology Department of the University of Erlangen-Nuremberg and at the University Eye Hospital in Würzburg and Tübingen, respectively. The examination included optic nerve head imaging (HRT 1 and 2; or biomicroscopy with Goldmann lens and a Haag-Streit slit lamp), and 24 h Goldmann applanation tonometry profile with five measurements (26,27). Patients from Southampton were examined by an experienced glaucoma specialist at the Southampton University Hospital Eye Unit. Biomicroscopy was performed and visual fields were measured using HFA 24-2 and HFA 30-2. Examination of participants from deCODE included biomicroscopy and visual field testing using the Octopus 123 perimeter (Haag-Streit, Köniz, Switzerland). Details have been described elsewhere (25).

Criteria for glaucoma

In RS-I, glaucoma diagnosis was primarily based on the presence of glaucomatous visual field loss, and not on the VCDR. The visual field of each eye was screened using a 52-point

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supra-threshold test that covered the central visual field with a radius of 24° (28,29), and tested the same locations as used in the Glaucoma Hemifield Test. In participants in whom visual field loss was reproducible on a second supra-threshold test, Goldmann kinetic perimetry or full-threshold HFA testing with 24-2 grid was performed on both eyes by a skilled perimetrist. Details about the classification process have been described before (8,28). Cases had to have an open anterior chamber angle and no history or signs of secondary glaucoma or manifest exfoliation were allowed.

In GRIP, the diagnosis of glaucoma was made by the ophthalmologist in attendance and verified by a glaucoma specialist (H.G.L.). The diagnosis was based on a glaucomatous appearance of the optic disc, combined with a matching glaucomatous visual field defect and open angles upon gonioscopy. Visual field test results had to be reliable and reproducible. Patients with any other disease that could cause visual field defects were excluded.

In AGS, glaucoma cases had to have glaucomatous optic neuropathy with corresponding glaucomatous visual field loss in at least one eye or a VCDR $\geq$ 0.8 when no visual field was available. In 84.5% of all cases, we had visual fields. In order to be eligible as a control person, one had to be aged 55 years or older, have a VCDR $\leq$ 0.6 on ophthalmoscopy and fundus photography.

In Erlangen and Tübingen, glaucoma was defined as the presence of glaucomatous optic disc damage (in at least one eye), visual field defects in at least one eye and intraocular pressure higher than 21 mmHg in one eye without therapy. Optic disc damage was classified according to Jonas *et al*. (30,31). A pathologic visual field was defined by a pathologic Bebié curve, three adjacent test points with more than 5 dB sensitivity loss, or at least one point with more than 15 dB sensitivity loss. In addition, controls were age and gender matched to the patients (26).

Details of the glaucoma cases from Southampton have been reported previously (32). In brief, diagnosis was made on the basis of characteristic glaucomatous visual field loss/glaucomatous optic disc damage/increased intraocular pressure. Patients presenting with narrow-angle, developmental or secondary glaucoma or any other known abnormalities of the anterior segment were excluded. Controls had no history of glaucoma and were not on any treatment to lower intraocular pressure.

Finally, in deCODE, glaucoma was based on glaucomatous optic neuropathy and glaucomatous visual field loss (11). Cases had to have an open anterior chamber angle on gonioscopy. Exfoliation syndrome was specifically looked for and if detected the participant was excluded. Controls with a reported history of glaucoma were excluded from the control group.

Laboratory analysis

In the RS-I, DNA was genotyped using the Illumina Infinium II HumanHap550chip v3.0® array according to the manufacturer's protocols (33,34). After exclusion of participants for reasons of low-quality DNA, a total of 5974 participants were available with genotyping data from RS-I, of whom 5736 had reliable optic disc measurements and visual fields. In ERF and GRIP, DNA was genotyped on four different platforms (Illumina 6k, Illumina 318K, Illumina 370K and Affymetrix 250K), which were then merged. A total of 2385 had genotyping data, of which 1646 from ERF had reliable optic disc data. For the AGS study, the SNPs were characterized by using TaqMan®. The Erlangen and Tübingen cohorts were genotyped using selected pre-developed TaqMan® Genotyping Assays (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. Genotyping of Southampton cases and controls was carried out using KASPar chemistry (KBioscience, Hoddesdon, UK). Finally, in deCODE, samples were assayed with the Illumina HumanHap300 or HumanHapCNV370 bead chips (Illumina, SanDiego, CA, USA).

Statistical analyses

Within each study logistic regression, analyses were used to examine the associations between the top SNPs (Table 1) and glaucoma adjusted for age and gender. With these logistic regression models, we calculated ORs with corresponding 95% CIs. The minor allele of the SNPs was considered the risk allele. Next, we performed meta-analyses using fixed-effects models to calculate the joint effect through the six independent cohorts for the heterozygous and homozygous effect of the SNPs. To adjust for multiple testing, we used Bonferroni's correction; a *P*-value of 0.003 [0.05/8 SNPs/2 (for hetero- and homozygous effect)] or smaller was considered statistically significant. Heterogeneity of the meta-analyses was measured by calculating I^2 (35). Finally, as a secondary analysis, we ran an allelic analysis assuming the risk associated with the genotype is multiplicative to the number of risk alleles. All statistical analyses were performed using SPSS version 15.0.0 for Windows (SPSS Inc., Chicago, IL, USA; 2006), and R statistical package version 2.11.1 for Mac (www.r-project.org).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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