A Genetic Epidemiologic Study of Candidate Genes Involved in the Optic Nerve Head Morphology

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Purpose. The size of the optic nerve head, referred to as disc area (DA), and the vertical cup-disc ratio (VCDR), are clinically relevant parameters for glaucomatous optic neuropathy. Although these measures have a high heritability, little is known about the underlying genes. Previously, the genes *SALL1* and *SIX1* were found to be genome-wide significantly associated with DA and VCDR. The purpose of the present study was to investigate whether genes encoding protein known to interact with protein encoded by *SALL1* and *SIX1* are also associated with either DA or VCDR.

Methods. A total of 38 candidate genes were chosen covering all known proteins interacting with *SALL1* and *SIX1*. These were initially studied in the Rotterdam Study (RS)-I, including 5312 Caucasian subjects characterized for DA and VCDR. Positive findings were further investigated in two independent cohorts (RS-II and RS-III) and finally replicated in a fourth population (ERF). Bonferroni correction was applied to the meta-analyses.

Results. Three loci were found to be associated with DA. The only locus significant after correcting for multiple testing is located on chromosome 11p13. Three single nucleotide polymorphisms (SNPs) in *ELP4*, a gene which neighbors and plays a crucial role in the expression of *PAX6*, show association in meta-analysis of the four cohorts yielding P values of respectively 4.79×10^{-6}, 3.92×10^{-6}, and 4.88×10^{-6} which is below the threshold dictated by the most conservative Bonferroni correction ($P = 5.2 \times 10^{-6}$).

Conclusions. This study suggests that the *ELP4-PAX6* region plays a role in the DA. Further research to confirm this finding is needed. (*Invest Ophthalmol Vis Sci.* 2012;53:1485–1491) DOI:10.1167/iovs.11-7384

One of the major clinical markers associated with the development of glaucomatous optic neuropathy is the vertical cup-disc ratio (VCDR), which is a quantitative measure for the size of the optic cup relative to the optic disc size (disc area; DA).[1] Enlargement of the optic cup is a diagnostic sign of primary open-angle glaucoma (POAG), a leading cause of visual field loss and blindness worldwide.[2] VCDR has been shown to be the most useful clinical sign in the diagnosis of glaucoma; the cup most commonly enlarges vertically in POAG.[1] While the nature of the relationship of DA to POAG has been a subject of debate, VCDR is associated with DA. In that realm, DA has been studied as an endophenotype for POAG and other disorders.[3] Both DA and VCDR are known to be highly heritable (52%–59%),[4,5] and the genes involved in DA are just beginning to be discovered. Previously, we performed a genome-wide association study and found several genes associated with optic disc parameters, including sal-like 1 (*SALL1*; chromosome 16q12.1) with DA and sine oculis homeobox homolog 1 (*SIX1*; chromosome 14q22–23) with VCDR.[3] Recently, the latter gene has also been associated with open-angle glaucoma.[6] *SALL1* encodes a zinc finger transcriptional repressor involved in organogenesis and may be linked to POAG through its interaction with *SIX1*.[7] *SIX1* is a transcription factor known to activate *SALL1* in kidney development.[7] Transcription from the SALL1 promoter has been shown to be strikingly activated by the SIX1 protein.[7] While the Drosophila *Six* homolog is known to interact within a network of genes including *eyeless*, *eyes absent*, and *dachshund* to induce compound eye organogenesis, *Six1* is not expressed in the developing mouse eye.[8] However, the *optix* subclass of *Six* genes, comprising *Six3* and *Six6*, may play a role in the vertebrate eye.[8] *SIX1* and *SALL1* operate within a regulatory complex. Investigations of genes that operate within this complex may reveal the connections between the protein pathways implicated in DA, VCDR, and glaucomatous pathology.

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In the present study we aimed to investigate whether the genes that encode proteins known to interact with the Sall1 and Six1 proteins are associated with either DA or VCDR to further elucidate the operational pathways. For this purpose we tested 38 candidate genes encoding proteins interacting with the proteins encoded by *SIX1* and *SALL1* and assessed their association with DA and/or VCDR in the Rotterdam Study-I. We replicated our findings in three independent cohorts.

METHODS

Study Populations

This study was conducted in four cohorts from the Netherlands, the Rotterdam Study (RS)-I, RS-II, RS-III, and the Erasmus Rucphen family study (ERF). RS-I is the discovery cohort and a prospective population-based cohort study of 7983 residents aged 55 years and older living in Ommoord, a district of Rotterdam, the Netherlands.[9] Baseline examination for the ophthalmic part took place between 1991 and 1993; follow-up examinations were performed from 1997 to 1999 and from 2002 to 2006.

The RS-II prospective population-based cohort study comprised 3011 residents aged 55 years and older from the same district of Rotterdam while similarly the RS-III study included 3932 residents, but aged 45 years and older. The rationale and study designs of these cohorts were similar to that of the RS-I.[9] The baseline examination of RS-II took place between 2000 and 2002; follow-up examination was performed from 2004 to 2005. Baseline examination of RS-III took place between 2006 and 2009.

In RS-I, of 5974 participants who were genotyped, 5107 had reliable baseline optic disc data and another 205 had reliable follow-up disc data resulting in a total of 5312 included participants. From RS-II a total of 2048 out of 2157 genotyped participants were included in the study, of which 90 were based on follow-up data. From RS-III a total of 1966 genotyped participants were included, resulting in a total of 9326 participants.

Finally, the fourth cohort was ERF, now consisting of 1919 genotyped participants. This is a family-based study of several thousand individuals from a genetic isolate in the southwest of the Netherlands, founded in the mid-18th century by some 150 individuals.[10] Data collection began in June 2002 and finished in February 2005.

All measurements in these studies were conducted after the Medical Ethics Committee of the Erasmus University Rotterdam had approved the study protocols and all participants had given their written informed consent in accordance with the Declaration of Helsinki.

Ophthalmic Examination

In RS-I and RS-II digitized stereoscopic imaging of the optic disc was used for the assessment of the optic nerve head (Topcon ImageNet System; Topcon Corporation, Tokyo, Japan), while RS-III and ERF used confocal scanning laser ophthalmoscopy (Heidelberg Retina Tomograph [HRT]; Heidelberg Engineering, Dossenheim, Germany).[3] It has been shown that both instruments are comparable and that there is high correlation for all stereometric parameters.[11]

To determine DA and VCDR with the stereoscopic imaging system (ImageNet System) used for the RS-I and RS-II populations, two trained technicians marked points on the disc margin and near the retinal blood vessels which were used by the stereoscopic imaging system (ImageNet System) to outline the disc margin. The stereoscopic imaging system (ImageNet System) systematically measures a larger DA than HRT. In brief, the mean difference of agreement between HRT and the stereoscopic imaging system (ImageNet System) is −0.05; 95% confidence interval (CI) of the difference is −0.07 to −0.03; limits of agreements −0.25 to 0.16. When measured with both methods there is a systematic difference in DA.[11]

Genotyping and Imputations

Genomic DNA was extracted from whole blood samples using standard methods.[12] Genome-wide single nucleotide polymorphism (SNP) genotyping was performed using an assay (Infinium II; Illumina, Inc., San Diego, CA) on a microarray (HumanHap 550 Genotyping Bead Chips; Illumina Inc). Approximately 2 million SNPs were imputed using release 22 HapMap CEU population (www.hapmap.org) as reference. The imputations were performed using MACH software (http://www.sph.umich.edu/csg/abecasis/MACH/). The quality of imputations was checked by contracting imputed and actual genotypes at 78,844 SNPs not present on an array (Illumina 550K; Illumina, Inc.) in 437 individuals for whom these SNPs were directly typed using an array set (Affymetrix 500K; SeqWright, Inc., Houston, TX). Using the "best guess" genotype for imputed SNPs the concordance rate was 99% for SNPs with R^2 (ratio of the variance of imputed genotypes to the binomial variance) quality measure greater than 0.9; concordance was still high (94%) when R^2 was between 0.5 and 0.9. For imputed SNPs we chose an R^2 value of 0.85 as the minimum for consideration.

Choice of Candidate Genes

We constructed a list of interacting proteins for *SALL1* and *SIX1* using internet databases (GeneCards; www.genecards.org). Because *SIX3* and *SIX6* are expressed during development of the human eye and *SIX4* and *SIX5* also play a role in the eye,[8] we decided to include all the *SIX* genes in our analysis. Table 1 provides an overview of the candidate genes investigated in this study. Of note is the overlap of the regions enframing *SIX3* and *SIX2* on chromosome 2 and *SIX6*, *SIX1* and *SIX4* on chromosome 14.

To determine gene positions we used NCBI build version 36.3 (ftp://ftp.ncbi.nih.gov/genomes/H_sapiens/ARCHIVE/BUILD.36.3/mapview/seq_gene.md.gz). We extracted SNPs within a region of interest comprising 100 kb on each side of the 38 candidate genes to be certain we included the promoter regions. Only SNPs with $P \geq 0.0001$ for Hardy-Weinberg equilibrium test and with genotype call rate $\geq$ 95% were included. A total of 9611 SNPs met these criteria and were selected for the association test.

Statistical Analysis

All analyses were performed using R statistical package (version 2.10.0 for Linux; www.r-project.org). Allele-based linear regression methods were used to test for association between a single SNP and the traits using ProbABEL (version 0.2.0-beta).[13] Betas and their standard errors for each SNP were derived adjusting for age and sex in the analyses of DA, and additionally adjusted for DA in the analyses of VCDR. All genes with nominal significant results in RS-I were followed up in the RS-II and RS-III cohorts in a meta-analysis using MetABEL R package (version 0.0-3).[14] For each candidate gene we calculated the P value significance threshold (p-sig) according to Bonferroni by adjusting for the number of SNPs tested within the respective region. After the meta-analysis in the three Rotterdam Study cohorts, the top SNPs were then followed up in another independent cohort, ERF. Finally we performed a meta-analysis in all four cohorts using the MetABEL R package. We used two different cutoffs for significance: Bonferroni adjustment for the number of SNPs sent for replication ($P \leq 0.05/6 = 8.33 \times 10^{-3}$); and the more conservative Bonferroni adjustment on all SNPs tested in the discovery cohort ($P \leq 0.05/9611 = 5.20 \times 10^{-6}$).

RESULTS

The general characteristics of the study populations are shown in Table 2. Participants of RS-III and ERF were younger compared with the other two cohorts. Slightly > 50% of participants were female in all populations. Of note is that in RS-III and ERF the mean DA and VCDR were smaller than in the other two populations. This is due to the difference in equipment[11]

TABLE 1. The 38 Genes/35 ROIs for Association with the Traits DA and VCDR

Gene	Chr	Start Gene*	End Gene*	Start Region	End Region	ROI	SNPs (*n*)	DA†	VCDR†
EYA3	1	28300819	28415131	28200819	28515131	*EYA3*	150		
CTBS	1	85018804	85040163	84918804	85140163	*CTBS*	273	Y	Y
MYOG	1	203052257	203055377	202952257	203155377	*MYOG*	230		Y
SIX3	2	45169037	45172390	45069037	45272390	*SIX32*	358	Y	
SIX2	2	45232324	45236542	45132324	45336542	*SIX32*			
HOXD13	2	176957532	176960666	176857532	177060666	*HOXD13*	134		
EPHA4	2	222282747	222437010	222182747	222537010	*EPHA4*	281		
PAX3	2	223064607	223163700	222964607	223263700	*PAX3*	282		Y
EPHA3	3	89156674	89531284	89056674	89631284	*EPHA3*	457	Y	
MDFI	6	41606195	41621982	41506195	41721982	*MDFI*	150	Y	
TAF8	6	42018251	42048644	41918251	42148644	*TAF8*	138	Y	
EYA4	6	133562495	133853258	133462495	133953258	*EYA4*	586		Y
EZR	6	159186773	159240456	159086773	159340456	*EZR*	262		
HOXA13	7	27236499	27239725	27136499	27339725	*HOXA13*	326		
SHH	7	155595558	155604967	155495558	155704967	*SHH*	299		Y
MNX1	7	156797547	156803347	156697547	156903347	*MNX1*	151		Y
EYA1	8	72109668	72274467	72009668	72374467	*EYA1*	500	Y	Y
TERF1	8	73921097	73959987	73821097	74059987	*TERF1*	382		
TG	8	133879205	134147143	133779205	134247143	*TG*	746	Y	Y
PAX2	10	102505468	102589695	102405468	102689695	*PAX2*	231		Y
LBX1	10	102986733	102988717	102886733	103088717	*LBX1*	169		
FGF8	10	103529887	103535827	103429887	103635827	*FGF8*	43		
MYOD1	11	17741110	17743678	17641110	17843678	*MYOD1*	174		
PAX6	11	31806340	31839509	31706340	31939509	*PAX6*	151	Y	
MYF5	12	81110708	81113447	81010708	81213447	*MYF5*	135		
TBX5	12	114791735	114846247	114691735	114946247	*TBX5*	281	Y	Y
DACH1	13	72012098	72441330	71912098	72541330	*DACH1*	641	Y	Y
SLC22A17	14	23815527	23822080	23715527	23922080	*SLC22A17*	182	Y	Y
COCH	14	31343741	31359822	31243741	31459822	*COCH*	256	Y	Y
OTX2	14	57267425	57277184	57167425	57377184	*OTX2*	246	Y	
SIX6	14	60975938	60978525	60875938	61078525	*SIX614*	531		Y
SIX1	14	61111417	61116155	61011417	61216155	*SIX614*			
SIX4	14	61176256	61190792	61076256	61290792	*SIX614*			
SALL1	16	51169886	51185183	51069886	51285183	*SALL1*	207		Y
GOSR2	17	45000486	45018733	44900486	45118733	*GOSR2*	128	Y	
SIX5	19	46268044	46272312	46168044	46372312	*SIX5*	173		
PAX1	20	21686297	21696620	21586297	21796620	*PAX1*	151	Y	
EYA2	20	45523509	45817492	45423509	45917492	*EYA2*	413		Y
Total	38					35	9817	15	16

Chr, chromosome; ROI, region of interest.

* Using NCBI build 36.3.

† Y = nominally significant for trait (for 1 or more SNPs $P < 0.05$) in the RS-I (our discovery cohort).

SALLI and *SIX1* previously found to be genome-wide significantly associated with DA and VCDR.

and does not affect the results[3] because all cohorts were analyzed separately and later meta-analyzed.

Of the 38 investigated genes in our initial analysis in RS-I we found 15 regions to be nominally significant ($P < 0.05$) for DA and 16 for VCDR (Table 1). Next we performed a meta-analysis for these candidate genes including all three Rotterdam Study cohorts. Four genes reached statistical significance for DA after adjusting for multiple testing (based on the number of SNPs in the gene). These were paired box 6 (*PAX6*), paired box 1 (*PAX1*), ephrin receptor tyrosine kinase A3 (*EPHA3*), and sine oculis homeobox homolog 2 (*SIX2*) (data not shown). A single SNP in the gene paired box 2 (*PAX2*) was significant for VCDR (rs11190730). However, the SNP was discarded due to its low imputation quality ($R^2 = 0.50$).

To corroborate the association with DA found in the Rotterdam Study cohorts, we tested the six top SNPs—three from *PAX6* and one each from *PAX1*, *EPHA3*, and *SIX2* each in a fourth cohort, ERF. These data were combined with those of the other three cohorts in a final meta-analysis (Table 3). When adjusting for the number of SNPs selected for replication, five of the six SNPs remained significant (rs953476 from gene *EPHA3* with a *P*-value of 0.02 was discarded), but when per-

TABLE 2. General Characteristics of the Four Study Populations

	RS-I	RS-II	RS-III	ERF
Total sample size (*N*)	5312	2048	1966	1919
Age, y	68.0 ± 8.4 (55–99)	64.3 ± 7.8 (55–98)	55.6 ± 5.5 (45–89)	47.0 ± 14.0 (18–85)
Gender, female, *n* (%)	3099 (58.3)	1109 (54.2)	1102 (56.1)	1081 (56.3)
Disc area, mm^2*	2.42 ± 0.48 (0.58–5.44)	2.32 ± 0.48 (1.06–6.20)	1.92 ± 0.45 (0.70–7.20)	1.91 ± 0.35 (1.07–4.33)
VCDR*	0.50 ± 0.14 (0.00–0.89)	0.50 ± 0.14 (0.00–0.87)	0.42 ± 0.17 (0.00–1.00)	0.46 ± 0.15 (0.00–0.84)

Data are presented as mean ± standard deviation (range) unless stated otherwise.

* In RS-I and RS-II measured with stereoscopic images; in RS-III and ERF with confocal scanning laser ophthalmoscopy.

Table 3. Results of SNPs Sent for Replication on Disc Area in the Four Meta-analyzed Cohorts

Gene	SNP	Chr	Position	Distance	Effallele	EAF	RS-I			RS-II			RS-III			ERF			Meta-analysis			
							β	SE	*P*	*β*	SE	*P*	*β*	SE	*P*	*β*	SE	*P*	*β*	SE	*P*	*P*-sig
PAX6	rs7126851	11	31710522	96	C	0.75	−0.04	0.01	7.27×10^{-4}	−0.02	0.02	0.21	−0.07	0.02	1.68×10^{-4}	−0.01	0.01	0.46	−0.03	0.01	4.79×10^{-6}	0.0083
PAX6	rs7104512	11	31725460	81	C	0.24	0.04	0.01	4.94×10^{-4}	0.02	0.02	0.24	0.07	0.02	1.39×10^{-4}	0.01	0.01	0.50	0.03	0.01	3.92×10^{-6}	0.0083
PAX6	rs10835818	11	31749627	57	G	0.24	0.04	0.01	7.19×10^{-4}	0.03	0.02	0.14	0.06	0.02	2.74×10^{-4}	0.01	0.01	0.47	0.03	0.01	4.88×10^{-6}	0.0083
PAX1	rs2064773	20	21781737	85	G	0.67	−0.03	0.01	1.65×10^{-3}	−0.04	0.02	0.01	−0.02	0.02	0.16	−0.02	0.01	0.21	−0.03	0.01	1.58×10^{-5}	0.0083
EPHA3	rs953476	3	89095996	61	G	0.63	0.03	0.01	8.84×10^{-3}	0.04	0.02	0.01	0.02	0.01	0.24	−0.02	0.01	0.10	0.01	0.01	0.02	0.0083
SIX2	rs2280220	2	45245405	13	G	0.17	0.04	0.01	2.84×10^{-3}	0.02	0.02	0.34	0.01	0.02	0.44	0.00	0.02	0.97	0.02	0.01	4.70×10^{-3}	0.0083

Distance, distance to gene (kb); EAF, effective allele frequency; Effallele, effective allele.

forming a more stringent multiple testing adjustment only the *PAX6* region remains significant (p-sig $< 5.20 \times 10^{-6}$). Though the SNPs for *PAX6* were not significant in ERF, the direction of the effect was the same as in the other three cohorts.

Figure 1 shows regional plots of the three candidate genes using the *P* values of RS-I only, as this cohort is the only one in which we investigated all SNPs. The three top SNPs for *PAX6* (rs7126851, rs7104512, and rs10835818) were located 57 kb to 96 kb 3′ of *PAX6* in its neighboring gene *ELP4* (Fig. 1A). The top SNP (rs2064773) for *PAX1* was located 85 kb from the 3′ end of the gene with no genes in between the top SNP and the gene of interest (Fig. 1B). Also for *SIX2* (Fig. 1C) no genes could be found between the top SNP and the gene of interest. The top SNP (rs2280220) for *SIX2* was located only 13 kb from the 5′ end of the gene.

Discussion

We found three genes to be associated with DA: *PAX6*, *PAX1*, and *SIX2.* When adjusting for the number of SNPs sent for replication, one gene (*PAX6*) survived the most stringent Bonferroni correction on all SNPs tested in this study. None of the selected candidate genes could be associated with VCDR.

The protein products of *PAX6* and *PAX1* are among 25 proteins listed in the internet databases (GeneCards; www.genecards.org) which interact with *SIX1*. The PAX family of genes is a group of highly conserved developmental control genes encoding nuclear transcription factors, regulating the expression of other genes, which have been shown to play a role in organogenesis. Our only significant finding after adjustment for multiple testing concerns *PAX6* (three SNPs with *P* values to the order of 10^{-6}). *Pax6* has been shown to function in the genetic control of eye development in organisms ranging from planarians to humans.[15] In both insects and vertebrates, *Pax6* is expressed in the embryo just before and during formation of the eye in the region of its development.[16] For eye morphogenesis in insects and vertebrates, the *Pax6* paired domain seems to be paramount.[17] The Drosophila *Pax6* homologue is the *eyeless* gene (*ey*).[18] In mice *Pax6* causes *Small eye (sey)*; in humans *PAX6* is associated with aniridia, Peter's anomaly and other types of anterior segment dysgenesis.[19–21] Clinically, aniridia has frequently been associated with glaucoma; more than half of aniridic patients will develop glaucoma.[22,23] A mutation in one allele is sufficient to cause ocular defects through haploinsufficiency, while compound heterozygosity is usually lethal and includes defects in the brain and other organs.[21,24] *PAX6* has tissue maintenance functions and continues to be expressed in the adult eye, leading investigators to speculate that *PAX6* may possibly play a role in adult-onset POAG and/or age-related macular degeneration.[5,24] The three SNPs that were found to be significantly associated with DA in this study were located downstream of the *PAX6* gene within an intronic region of neighboring *ELP4*. *PAX6* is one of many genes listed in the internet databases (GeneCards; www.genecards.org) which interact with *ELP4.* Possibly *ELP4* has a role in regulating *PAX6*.[25,26] The *ELP4* gene encodes a component of the six subunit elongator complex, a histone acetyltransferase complex that associates directly with the RNA polymerase II (Pol II) holoenzyme, and is involved in transcriptional elongation. Elongator may play a role in chromatin remodeling and is involved in acetylation of histones H3 and probably H4. Some familial aniridia cases have an undamaged *PAX6* gene but a deletion in the region 3′ to it at the level of the *ELP4* gene; microdeletions 3′ of *PAX6* have been known to suppress expression of *PAX6* and cause aniridia.[25–27] Perhaps new techniques like Next Generation Sequencing will be able to discover which of the two genes is truly associated with DA.

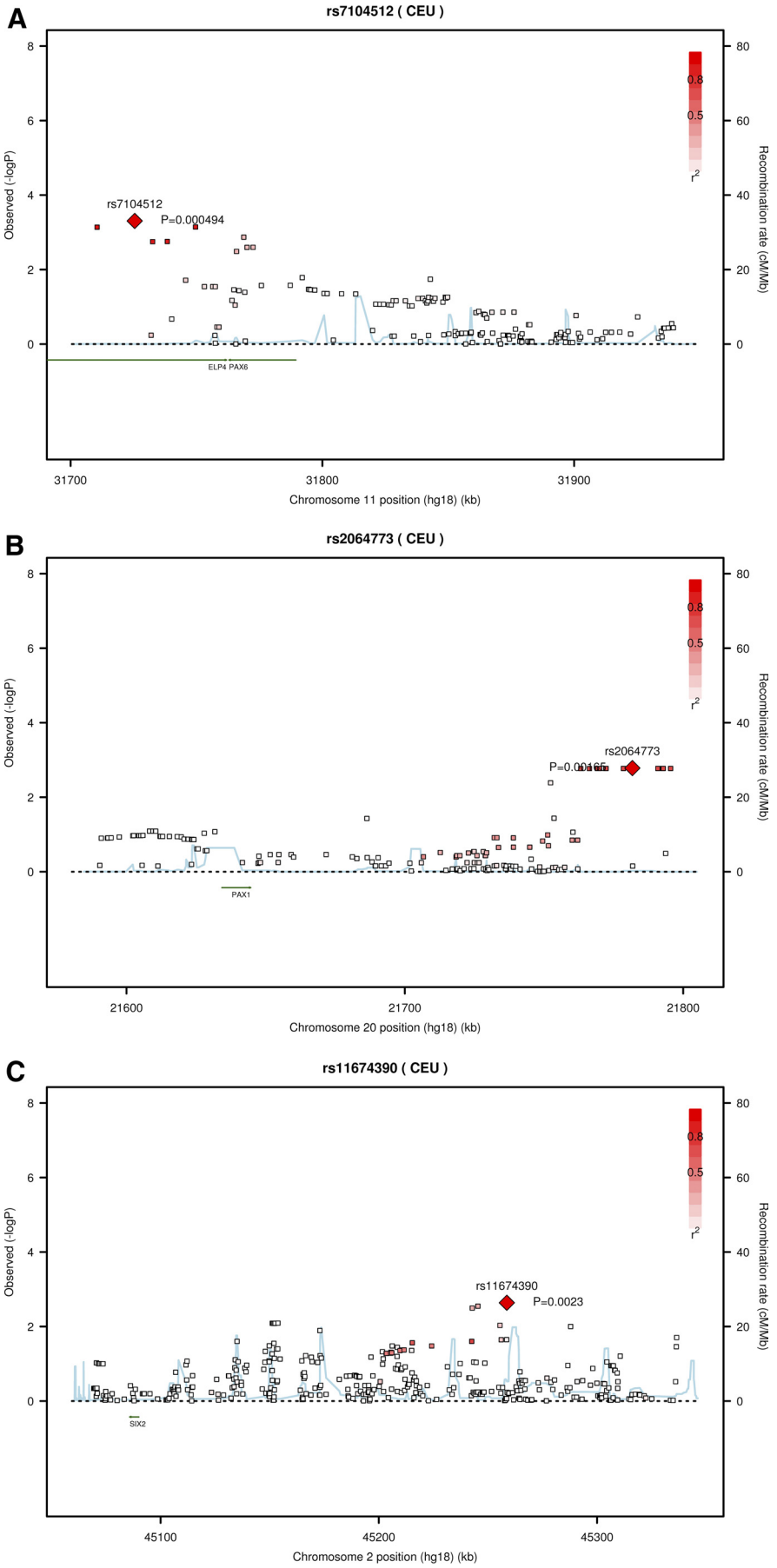

FIGURE 1. Regional plots of the three loci found to be associated with optic disc area in the meta-analysis with our four cohorts for *PAX6* (**A**), *PAX1* (**B**) and *SIX2* (**C**).

Although not significant after adjusting for multiple testing like *PAX6*, *PAX1* also plays a role in the development of the central nervous system. In mice *Pax1* is known to be expressed in segmented structures during embryogenesis. The mouse *Pax1* mutant phenotype *undulated*, caused by a missense mutation in the paired box of *Pax1*, shows segmentation anomalies along the entire axial skeleton and defects in the pectoral girdle.[28] By phenotypic similarity, human *PAX1* has been postulated as a candidate gene for Klippel-Feil syndrome, characterized by failed segmentation of the cervical vertebrae and other vertebral anomalies.[28] However, this association is inconclusive.[28,29] Interesting to note is that in human *PAX1* the protein product contains additional amino acids upstream of the paired box, while in *PAX6* the protein sequences are identical between mice and humans.[24,28] As of yet, the literature on *PAX1* is limited. As pointed out by several authors, many members of the eye developmental cascade of transcription factors, including *PAX6*, also play a role in the genesis of other tissues.[17,24] Further research may reveal more about the nature of the association between *PAX1* and DA.

SIX2, which interacts with *SALL1,* is a member of the Drosophila sine oculis homeobox homologues and encodes a transcription factor associated with organ development. Also this finding is not significant after adjusting for multiple testing but is of interest. The Six proteins are part of the Pax/Eya/Six/Dach retinal determination cascade involved in embryonic cell fate determination.[30] As are a number of other genes in the Pax/Eya/Six network, *Six2* is expressed in the mesenchyme and plays an important role in early kidney development as well as in eye and muscle formation in mammals.[31] In mammals and humans, *Six2* has been associated with renal hypoplasia,[32] suggesting a role in regulating mesenchymal progenitor cells,[33] frontonasal dysplasia[34] and anterior cranial base defects.[35] In mouse studies, *Six2* transcripts have been found in a variety of nonneuronal connective tissues, including the eye, during organogenesis.[36]

Other candidate genes in the network, for example *SIX3* and *PAX2*, did not interact with *PAX6*[17]. This may in part be explained by a lack of statistical power. Larger numbers will be needed to determine whether these genes are associated with DA or not. More generally, one would expect that more of the genes that interact in the pathways of the PAX regulatory network, such as the SIX and EYA (eyes absent homolog) families, known to play a role in oculogenesis in Drosophila, would be significantly associated with either DA or VCDR. Obviously, these transcription factors play multiple roles in complex pathways involved not specifically or exclusively in the eye but more broadly in the central nervous system and in sensory neurogenesis, affecting the development of multiple organ systems. Many of these genes are highly conserved and serve multiple functions within a complex network of interdependencies and feedback loops. The individual effect of a gene on human optic nerve tissue may be subtle, absent, or subject to dosage effects. We may not have sufficient statistical power to detect these by association with either DA or VCDR in our populations. Further research is needed to tease out any of the possible roles these genes may play in the formation of the optic nerve head or optic disc cupping, or more generally in ocular pathology; at present these roles remain somewhat speculative.

While none of our SNPs reached genome-wide significance, the *PAX6/ELP4* region remained significant after adjusting for multiple testing. When we attempted to corroborate the associations we found with DA in the ERF cohort we found that, although not significant, the direction of the effect for the SNPs in *PAX6* was the same as in the other three cohorts. Further research in a larger sample is required to validate the association between *PAX6/ELP4* and DA.

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