Novel Genetic Loci Associated With Retinal Microvascular Diameter

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Background—There is increasing evidence that retinal microvascular diameters are associated with cardiovascular and cerebrovascular conditions. The shared genetic effects of these associations are currently unknown. The aim of this study was to increase our understanding of the genetic factors that mediate retinal vessel size.

Methods and Results—This study extends previous genome-wide association study results using 24000+ multiethnic participants from 7 discovery cohorts and 5000+ subjects of European ancestry from 2 replication cohorts. Using the Illumina HumanExome BeadChip, we investigate the association of single-nucleotide polymorphisms and variants collectively across genes with summary measures of retinal vessel diameters, referred to as the central retinal venule equivalent and the central retinal arteriole equivalent. We report 4 new loci associated with central retinal venule equivalent, one of which is also associated with central retinal arteriole equivalent. The 4 single-nucleotide polymorphisms are rs7926971 in TEAD1 ($P=3.1\times10^{-11}$; minor allele frequency=0.43), rs201259422 in TSPAN10 ($P=4.4\times10^{-9}$; minor allele frequency=0.27), rs5442 in GNB3 ($P=7.0\times10^{-10}$; minor allele frequency=0.05), and rs1800407 in OCA2 ($P=3.4\times10^{-8}$; minor allele frequency=0.05). The latter single-nucleotide polymorphism, rs1800407, was also associated with central retinal arteriole equivalent ($P=6.5\times10^{-12}$). Results from the gene-based burden tests were null. In phenotype look-ups, single-nucleotide polymorphism rs201255422 was associated with both systolic ($P=0.001$) and diastolic blood pressures ($P=8.3\times10^{-04}$).

Conclusions—Our study expands the understanding of genetic factors influencing the size of the retinal microvasculature. These findings may also provide insight into the relationship between retinal and systemic microvascular disease. (Circ Cardiovasc Genet. 2016;9:45-54. DOI: 10.1161/CIRCGENETICS.115.001142.)

Key Words: exome ◼ genetics ◼ meta-analysis ◼ microcirculation ◼ retina

Microvascular disease, affecting blood vessels 100–300 µm in size, plays a significant role in many conditions, such as diabetes mellitus, stroke, hypertension, coronary artery disease, and cognitive decline. It is difficult to study the microvasculature in organs, such as the heart and brain, and it requires invasive methods. However, retinal venules and arterioles can be photographed noninvasively, and their characteristics can be quantified using computer software. In addition, the physiology and embryology of the brain and retinal microvasculature are similar. Because evidence suggests that changes in retinal vessels may provide an indirect indicator of similar changes in the brain, heart, and kidneys, a better understanding of the genes associated the retinal blood vessels may provide insight into microvascular morbidity elsewhere in the body.

Clinical Perspective on p 54

Current evidence suggests that retinal vessel diameters are associated with a wide array of diseases, reflecting the role and contribution of microvascular disease processes in these conditions. For instance, narrower retinal arterioles is a sign of hypertension, and remarkably, the association between...
blood pressure and smaller retinal arteriole diameter can be seen with current, past, and incident hypertension.

On the basis of previous studies, retinal venule diameter is associated with different pathophysiologic processes compared with retinal arterioles. Wider retinal venules are associated with hyperglycemia, dyslipidemia, measures of inflammation, obesity, stroke, subclinical cerebrovascular disease, coronary heart disease, cardiovascular mortality in younger participants, cognitive impairment, and renal dysfunction in diabetics.

Even as the evidence that retinal microvascular diameter is a marker of more generalized microvascular changes in other organs increases, our understanding of the genetic factors that control retinal vessel size is limited. Previous studies have shown that retinal vessel diameters are more highly correlated among related than unrelated individuals. These values range from 0.23 (95% confidence interval, 0.16–0.31) in retinal venule diameter and 0.20 (95% confidence interval, 0.12–0.28) in retinal arteriole diameter of siblings compared with 0.03 and 0.04 in spouses. In a twin study, the heritability of retinal vessels diameters was estimated at 83% (95% confidence interval, 73–89) for retinal venule diameter and 70% (95% confidence interval, 54–80) for retinal arteriole diameter. A genome-wide linkage study estimated the heritability at 48% to 51% for retinal vessel diameter and showed evidence that linkage regions overlapped with regions previously associated with hypertension, the endothelial nitric oxide synthase–related pathway, coronary heart disease, and vasculogenesis. Results from our first genome-wide association studies (GWAS) found evidence for 4 novel loci associated with retinal venule diameter and 1 associated with retinal arteriole diameter. These studies only included whites and were both smaller with 15358 and 18722 participants, respectively.

Nevertheless, much of the heritability of retinal vessel diameters remains to be explained. It is theorized that rare single-nucleotide polymorphisms (SNPs), those with a minor allele frequency (MAF) of <1%, account for some of this unexplained heritability. Recently, the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium cohorts were genotyped with the Illumina HumanExome BeadChip. In this study, we combine 24000+ participants from 4 ethnic groups (whites, blacks, Asians, and Hispanics) to investigate the association of single-variant SNPs and SNP variants collectively across genes with summary measures of retinal venule and arteriole diameters, referred to as the central retinal venule equivalent (CRVE) and the central retinal arteriole equivalent (CRAE).

**Methods**

**Participants**

The discovery phase included 4 members of the CHARGE Consortium: the Age, Gene, Environment, Susceptibility Study–Reykjavik (AGES), the Atherosclerosis Risk in Communities (ARIC) study, the Cardiovascular Health Study (CHS), and the Multi-Ethnic Study of Atherosclerosis (MESA). Three additional Asian cohorts also contributed data: the Singapore Chinese Eye Study (SCES), the Singapore Malay Eye Study (SiMES), and the Singapore Indian Eye Study (SINDI). The overall mix of ethnic backgrounds included European ancestry (60%), blacks (14%), Asians (21%), and Hispanics (4%) Table 1.

The replication cohorts included the Rotterdam Study (RS) and a subset of the Blue Mountain Eye Study (BMES) that had exome chip data. Additional details of each participating study are provided in the Data Supplement. All participating cohorts secured approval from their respective institutional review boards, and all participants provided written informed consent in accordance with the Declaration of Helsinki.

**Retinal Phenotypes**

The CRVE and CRAE are summary measures of the 6 largest retinal microvascular diameters. Standardized protocols and software were developed at the University of Wisconsin. Retinal photographs were centered on the optic nerve. Photographs were obtained through pharmacologically dilated (AGES, BMES, and RS) or undilated pupils of one (ARIC and CHS) or both eyes. Photographs were digitized using a high-resolution scanner (ARIC, CHS, and RS) or digitally captured. All digital retinal images were analyzed with a semiautomated measurement system, and the calibers of all retinal venules and arterioles were measured in the area located between half and 1 disc diameter from the optic disc margin. Trunk vessels were measured using the Parr–Hubbard–Knudtson formulas, and measures are expressed in micrometers (µm).

**Exome Chip**

Cohort participants (AGES, ARIC, CHS, MESA, RS, SCES, and SINDI) were genotyped with the Illumina HumanExome BeadChip version 1.0 (Illumina Inc, San Diego, CA) and version 1.1 for SiMES. It covers putative functional exonic variants selected from >12000 individual exome and whole-genome sequences. It consists of 247870 markers comprised primarily of 21962 nonsynonymous SNPs representing European, African, Chinese, and Hispanic populations. Additional markers include splice-site variation, stop codons, promoter region SNPs, GWAS tag markers, ancestry informative markers, X/Y mitochondrial variants, SNPs in the extended major histocompatibility complex region, human leukocyte antigen tag variants, identical by descent markers, and insertion–deletion (exome-array design: http://genome.sph.umich.edu/wiki/Exome_Chip_Design).

**Genotyping and Quality Control**

Approximately 62000 ethnically diverse samples from the CHARGE Consortium were genotyped with the Illumina HumanExome BeadChip. The raw data files for the samples were assembled into a single project for joint calling and are described in detail elsewhere. Briefly, Illumina’s GenTrain version 2.0 clustering algorithm in GenomeStudio or zCall was used for genotype calling. A total of 8997 variants failed quality control measures and were eliminated from all analyses. Additional details on genotyping and quality control performed centrally and by each study are summarized in the Data Supplement. Annotation of the version 1.0 exome chip variants are expressed with dbNSFP version 2.0.

**Statistical Analysis**

The discovery phase included single-variant tests and gene-based association analyses in which CRVE and CRAE were analyzed separately. Where applicable, each of the 7 cohorts, (AGES, ARIC, CHS, MESA, SCES, SiMES, and SINDI) analyzed their participants by ethnicity, white, black, Asian, or Hispanic. This resulted in 12 ethnic specific groups that were combined for meta-analysis to elucidate any ethnic specific differences in the results. For the single-variant tests, genome-wide significance was set at $P=2.0\times10^{-7}$ (0.05/250000 variants). Significance for the gene-based tests was set at $2.5\times10^{-4}$ (0.05/20000 genes). To validate findings from the discovery process, a replication phase was performed using 2 independent cohorts (BMES and RS). Replication was considered significant at $P<0.05$, and the direction of the effect was consistent with discovery.
Association analyses, including the Sequence Kernel Association Test (SKAT) formed by ethnic group for single-variant tests and gene-based association plots, and details of the in silico algorithms used to predict whether SNPs would be damaging are provided in the Data Supplement.

RS did not genotype all of their subjects using the exome chip. For those that were not genotyped, they imputed the single-nucleotide polymorphism for the remaining subjects. For clarity, we analyzed the 2 groups separately.

Diabetes mellitus was defined as fasting blood glucose of ≥126 mg/dL (7.0 mmol/L), current use of insulin, or other hypoglycemic agents. If fasting blood glucose was not available, then a random glucose of >198 mg/dL (11.0 mmol/L) was used. Hypertension was defined as current use of blood pressure medication or systolic blood pressure (SBP)/diastolic blood pressure of ≥140/90 mm Hg.

Residuals were analyzed because results using untransformed residuals for replication, 3 CRVE SNPs (rs7926971 in OCA2, rs201259422 in TSPAN10, and rs2306765 in GSG1) reached exome-wide significance, rs7926971 (P = 7.0×10^{−9}) in OCA2 reached exome-wide significance, rs201259422 (P = 8.0×10^{−9}) in TSPAN10, and rs2306765 (P = 5.8×10^{−10}) in GNG3. Two other SNPs of interest reached nominal significance (<0.05) or better. SNPs rs201259422 and rs2306765 in GNG3. One CRAE SNP reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide significance, rs1800407 (P = 6.9×10^{−6}) in OCA2 reached exome-wide signifi

### Results

Table 1 displays the demographic and covariate characteristics stratified by the ethnic group within participating cohorts where available. A total of 24,342 participants had measures of CRVE, and 24,345 had measures of CRAE. After marker-level and sample-level quality control, 22,566 variants were available for analyses of CRVE and 24,0931 for CRAE.

Table 2 contains the results from the discovery phase of our meta-analysis for the single-variant tests. Three CRVE SNPs reached exome-wide significance, rs7926971 (P = 7.0×10^{−9}) in TEAD1, rs201259422 (P = 8.0×10^{−9}) in TSPAN10, and rs5442 (P = 5.8×10^{−10}) in GNG3. Two other SNPs of interest were carried forward for replication, rs1800407 (P = 2.1×10^{−7}) in OCA2 and rs2306765 (P = 6.9×10^{−6}) in GSG1. One CRAE SNP reached exome-wide significance, rs1800407 (P = 4.5×10^{−10}) in OCA2. During replication, 3 CRVE SNPs (rs7926971 in TEAD1, rs5442 in GNG3, and rs1800407 in OCA2) and 1 CRAE SNP (rs1800407 in OCA2) all reached nominal significance (P < 0.05) or better. SNPs rs201259422...
in TSPAN10 and rs2306765 in GSG1 did not replicate, but the direction and effect size for rs201259422 in TSPAN10 were consistent in the replication sample and overall increased the statistical significance in the combined sample. Figures 1 to 6 show the regional association plots for each of the SNPs carried forward for replication. No additional ancestry specific SNPs reached exome-wide significance for CRVE or CRAE.

Table I in the Data Supplement contains a more detailed summary of the top SNPs associated with CRVE, including the 4 SNPs we have previously reported\textsuperscript{23} and the other SNPs in high linkage disequilibrium with those 4 SNPs. Similarly, Table II in the Data Supplement contains a more detailed summary of the top SNPs associated with CRAE, including an SNP in high linkage disequilibrium with the SNP we have previously reported.\textsuperscript{24} All of the previously reported GWAS findings reached exome-wide significance in the present study. These tables include the annotation of the version 1.0 exome chip variants.

Results from the gene-based T1 burden tests were null for both CRVE and CRAE. In the Sequence Kernel Association Test, GSG1 was associated with CRVE ($P=1.3\times10^{-6}$) and OCA2 was associated with CRAE ($P=2.1\times10^{-8}$). In GSG1, there were 9 SNPs with cumulative MAF of 0.06. We repeated gene-based testing by excluding rs2306765 after which the gene was not associated with CRVE (cumulative MAF of 0.01; $P=0.01$). For CRAE in OCA2, there were 38 SNPs included with cumulative MAF of 0.09. Similarly, the gene was not associated with CRAE after excluding rs1800407 (cumulative MAF of 0.04; $P=0.03$). Neither gene, GSG1 for CRVE or OCA2 in CRAE, reached nominal significance in replication.

Figures III to VIII in the Data Supplement show the direction of effects across all studies. These figures show no overall heterogeneity in the meta-analysis results in any of the SNPs.
There was evidence that the effect size in MESA Hispanics for rs7926971 (TEAD1) differed from other ethnic groups (P=0.01; Figure III in the Data Supplement) and that the effect size in MESA Asians for rs201259422 (TSPAN10) differed from other Asian cohorts (P=0.02; Figure IV in the Data Supplement).

A look-up of the 4 newly and 4 previously identified SNPs associated with CRVE or CRAE was performed by the CHARGE Exome Chip Blood Pressure Consortium (Table III in the Data Supplement). Of the 8 SNPs, 5 SNPs were either associated with SBP, diastolic blood pressure, hypertension, or a combination of the 3 phenotypes. However, after correction for multiple testing (P=0.05/24=0.002), only 2 SNPs remained associated with blood pressure phenotypes, rs201259422 in TSPAN10 and rs10774625 in ATXN2.

Discussion

Our collaboration has identified 4 new SNPs associated with retinal arteriole diameter and 1 new SNP associated with retinal venule diameter bringing the total to 8 SNPs now associated with venule diameter and 2 with arteriole diameter. After adjusting for age, sex, body mass index, diabetes mellitus (yes/no), hypertension (yes/no), and SBP, the new variants explained 1% of the total variability in CRVE (4 SNPs) and 1½% for CRAE (1 SNP). This increased to 2% for CRVE (8 SNPs) and ½% for CRAE (2 SNPs) with all known variants.

Four SNPs identified in our discovery cohort showed evidence of replication in our study; 3 SNPs for CRVE in TEAD1, GNB3, and OCA2 and 1 SNP for CRAE in OCA2. The SNP in TSPAN10 for CRVE did not replicate, but the direction of the association and the combined discovery and replication results provide some support for its association with CRVE. Our inability to replicate outright may have been due in part to the small size of the replication cohort and smaller effect sizes compared with those seen in some of the other SNPs. The SNP in GSG1 did show a significant P value in replication, but the association was in the opposite direction from what was observed in discovery. It is of note that none of the new findings were low-frequency SNPs. The SNPs in TSPAN10 and OCA2 were not genotyped or imputed in our previous GWAS. The SNP in TEAD1 was included as an imputed SNP. The SNP in GNB3 was genotyped, but results did not pass quality control. It is unclear whether genotyping, covariate adjustment, smaller effect sizes, or a combination of these factors resulted in the conflicting results in our GWAS and the present study.

SNP rs5442 in GNB3 is a predicted damaging nonsynonymous SNP on chromosome 12. Each copy of the minor allele decreased CRVE, 2.3±0.4 µm (this decrease and the changes cited below are the observed effects on CRVE and CRAE in the untransformed analyses, not the β and SEs from the inverse-quantile normalized traits). Heterotrimeric guanine nucleotide–binding proteins (G proteins), integrate signals between receptors and effector proteins, are composed of an α, a β, and a γ subunit and are expressed in all tissues.40 A separate SNP (C825T) in this gene is associated with essential hypertension, obesity, and dyspepsia.40,41

SNP rs1800407 in OCA2 is a predicted damaging nonsynonymous SNP on chromosome 15. Each copy of the minor allele decreased CRAE and CRVE, 1.7 ± 0.3 and 2.2 ± 0.4 µm, respectively. OCA2 encodes the human homolog of the mouse p (pink-eyed dilution) gene.42 The encoded protein is believed to be an integral membrane protein involved in small molecule transport, specifically tyrosine, which is a precursor to melanin synthesis. It is involved in mammalian pigmentation, where it may control skin color variation and act as a determinant of brown or blue eye color. Mutations in this gene result in type 2 oculocutaneous albinism.43

Figure 2. Central retinal venule equivalent (CRVE) regional association plot for single-nucleotide polymorphism (SNP) rs201259422 in TSPAN10.
SNP rs7926971 in **TEAD1** is an intronic SNP on chromosome 11. Each copy of the minor allele increased CRVE 0.5 ± 0.1 µm. **TEAD1** encodes a ubiquitous transcriptional enhancer factor that is a member of the TEA (transcriptional enhancer activator)/ATTS (AbaA, TEC1 p, TEF-1 Sequence) domain family. This protein directs the transactivation of a wide variety of genes and, in placental cells, also acts as a transcriptional repressor. Mutations in this gene cause Sveinsson chorioretinal atrophy.44 The expression of **TEAD1** is significantly induced during smooth muscle cell phenotypic modulation and negatively correlates with smooth muscle–specific gene expression. Mechanistically, **TEAD1** competes with myocardin for binding to serum response factor, resulting in disruption of myocardin and serum response factor interactions and thereby attenuating expression of smooth muscle–specific genes.45

SNP rs201259422 in **TSPAN10** (tetraspanin 10) is a predicted damaging SNP on chromosome 17. Each copy of the
minor allele decreased CRVE, 1.0 ± 0.2 µm. \textit{TSPAN10} encodes a transcript that is expressed in human RPE and choroid.\textsuperscript{46} Tetraspanins are widespread, numerous, and largely mysterious in function but are involved in diverse processes, such as cell activation and proliferation, adhesion and motility, differentiation, and cancer.\textsuperscript{47} Two related tetraspan proteins of the retina, \textit{ROM1} and \textit{RDS}, are both involved in retinal degenerations.\textsuperscript{48}

The SNP in \textit{OCA2} and the previously reported SNP rs17421627 in \textit{LINC00461} adjacent to \textit{MEF2C}\textsuperscript{23,24} represent 2 SNPs now that are associated with both CRAE and CRVE. None of the other previously reported SNPs associated with CRVE were associated with CRAE in our data, nor was the SNP in \textit{TEAD1} (Table I in the Data Supplement). The associations between CRAE and the SNPs in \textit{TSPAN10} and \textit{GNB3} are weaker than for CRVE but not trivial. Despite the strong correlation between CRVE and CRAE, it seems that not only retinal venule diameter is associated with different pathophysiologic processes compared with retinal arterioles but that the
It is unknown why genetic loci have been identified more for CRVE than for CRAE. CRVE has slightly less measurement error than CRAE, but is not likely the major reason. Second, hypertension and medications to treat hypertension influence retinal arterioles (CRAE) more than venules (CRVE). Consequently, we adjusted for both SBP and medication use in our analyses. Finally, there are anatomical differences in the 2 types of vessels. Unlike retinal venules, retinal arterioles autoregulate blood flow in response to changes in perfusion pressure, and like the cerebral vasculature, they are largely independent of extrinsic neurogenic factors. This may make genetic effects on CRAE smaller than the corresponding effects on CRVE and thus may take larger sample sizes to detect, for instance see rs201259422 (TNSPAN10; Table III in the Data Supplement).

Two of the 8 SNPs associated with CRVE or CRAE were associated with blood pressure phenotypes, rs201259422 on TNSPAN10 and rs10774325 on ATXN2. SNP rs10774625 (ATXN2) was previously reported to be associated with blood pressure. However, to our knowledge, this is the first reported association of rs201259422 (TNSPAN10) with blood pressure phenotypes. Current thinking is that wider retinal venules and narrower retinal arterioles are associated with increased risk of hypertension, but that relationship does not hold for rs201259422 (TNSPAN10) and rs10774625 (ATXN2) in our analyses (Table III in the Data Supplement). Other SNPs, like rs1800407 (OCA2) and rs17421627 (MEF2C), had relatively large effects on retinal vessel caliber. However, there was no obvious relationship with blood pressure phenotypes. These findings may reflect developmental differences independent of cumulative or secondary effects occurring over an individual’s lifetime. SNPs rs7926971 (TEAD1) and rs2287921 (RASIP1) show an association with CRVE but not CRAE or blood pressure phenotypes. The significance of these finding remains to be determined.

Strengths of this study include a large sample size, an ethnically diverse population-based sample, quantitative phenotypes assessed in a similar manner, and genotyping performed on the exome array enriched for coding variants with many cohorts involved in joint calling. Weaknesses include a small replication cohort, cross-sectional measures, and a phenotype that is a summary measure of the 6 largest retinal arteriole and venule diameters.

In summary, this study increases our understanding about genes associated with retinal venule and arteriole diameter. However, considerable work remains to be done to determine how these genes act: whether their effects vary over time, whether these genes interact or are subject to epigenetic factors, and ultimately, whether these genes contribute to the risk of microvascular disease in other organs or are associated with major conditions, such as diabetes mellitus, stroke, hypertension, coronary disease, and cognitive decline.

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Disclosures

None.

Appendix

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**CLINICAL PERSPECTIVE**

Microvascular disease, affecting blood vessels 100–300 µm in size, plays a significant role in many conditions, such as diabetes mellitus, stroke, hypertension, coronary artery disease, and cognitive decline. It is difficult to study the microvasculature in organs, such as the heart and brain, and it requires invasive methods. However, retinal vessels and arterioles can be photographed noninvasively, and their characteristics can be quantified using computer software. In addition, the physiology and embryology of the brain and retinal microvasculature are similar. Because evidence suggests that changes in retinal vessels may provide an indirect indicator of similar changes in the brain, heart, and kidneys, a better understanding of the genes associated with the retinal blood vessels may provide insight into microvascular morbidity elsewhere in the body.