In the current study, the PDT group showed no significant improvement in VA after initiation of treatment; mean VA was significantly decreased at 24 months. In the triple therapy group, however, VA was improved at 3 months after treatment and mean VA was improved by 0.11 (logMAR) at 6 months and by 0.09 (logMAR) at 12 months. In this triple therapy group, although improvement was not statistically significant, at least some improvement in VA was maintained throughout the 2-year follow-up period. At 24 months, VA improvement was achieved in only 12.5 % of eyes in the PDT group and in 41.7 % in the triple therapy group ($P0$ 0.044), so, based on our findings, triple therapy for PCV, compared to PDT alone, results in more rapid visual recovery and improved visual outcome at 24 months.

In our case series, both PDT alone and the triple therapy successfully reduced polypoidal lesions and exudative change, with complete disappearance of the polypoidal lesions confirmed at 24 months in 81.3 % of cases in the PDT group and in 91.7 % of cases in the triple therapy group. There were significant differences in the number of eyes with a recurrence and in the number of PDT treatments between the two groups. Furthermore, the retreatment-free period was significantly longer in the triple therapy group (20.6± 6.8 months) than in the PDT group (11.7±8.6 months).

Following treatment for PCV, one of the most vision-threatening complications of PDT is extensive hemorrhage. A previous report of PCV treated with PDT indicated that postoperative subretinal hemorrhage was seen in 28 of 91 eyes, and that bleeding resulted in a vitreous hemorrhage in six eyes [32]. In the current study, no eye in the triple therapy group developed a vitreous hemorrhage, although two eyes in the PDT group developed a vitreous hemorrhage. Recent reports by Gomi et al. [18] and by Sato et al. [19] suggested a lower incidence of subretinal hemorrhage after PDT when it was combined with bevacizumab, and it has been reported that the vasoconstrictive effect of bevacizumab may contribute to the suppression of postoperative hemorrhages [33].

Major limitations of the current study are its retrospective nature and its relatively small sample size. In addition, there were some statistical differences between the two groups, including baseline foveal thickness and the rate of serous retinal detachment, which may affect the response to treatment. Furthermore, this study was not a randomized, comparative trial. However, selection bias is small as both groups consisted of consecutive eyes that were treated at different time periods. Our findings suggest that intravitreal injection of bevacizumab and TA combined with PDT improves the 2-year visual outcome of PCV and may reduce postoperative hemorrhagic complications and the recurrence rate. However, because our findings are based on an observation period of only 24 months, it remains unclear whether triple therapy has a long-term effect.

Another limitation is that the safety and efficacy of the triple therapy were not compared with PDT combined with anti-VEGF therapy. Recently, the EVEREST study has shown the 6-month effects of PDT in combination with ranibizumab for PCV [34], in which the eyes treated with PDT combined with ranibizumab achieved the highest gains at 6 months. However, it remains unclear whether this combination therapy reduces the recurrence of polypoidal lesions after successful initial treatment. Further prospective, randomized, long-term studies are necessary to determine the efficacy and safety of triple therapy for PCV.

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ORIGINAL INVESTIGATION

Large scale international replication and meta-analysis study confirms association of the 15q14 locus with myopia. The CREAM consortium

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Abstract Myopia is a complex genetic disorder and a common cause of visual impairment among working age adults. Genome-wide association studies have identified susceptibility loci on chromosomes 15q14 and 15q25 in Caucasian populations of European ancestry. Here, we present a confirmation and meta-analysis study in which we assessed whether these two loci are also associated with myopia in other populations. The study population comprised 31 cohorts from the Consortium of Refractive Error and Myopia (CREAM) representing 4 different continents with 55,177 individuals; 42,845 Caucasians and 12,332 Asians. We performed a meta-analysis of 14 single nucleotide polymorphisms (SNPs) on 15q14 and 5 SNPs on 15q25 using linear regression analysis with spherical equivalent as a quantitative outcome, adjusted for age and

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sex. We calculated the odds ratio (OR) of myopia versus hyperopia for carriers of the top-SNP alleles using a fixed effects meta-analysis. At locus 15q14, all SNPs were significantly replicated, with the lowest P value 3.87×10^{-12} for SNP rs634990 in Caucasians, and 9.65×10^{-4} for rs8032019 in Asians. The overall meta-analysis provided P value 9.20×10^{-23} for the top SNP rs634990. The risk of myopia versus hyperopia was OR 1.88 (95 % CI 1.64, 2.16, $P < 0.001$) for homozygous carriers of the risk allele at the top SNP rs634990, and OR 1.33 (95 % CI 1.19, 1.49, $P < 0.001$) for heterozygous carriers. SNPs at locus 15q25 did not replicate significantly (P value 5.81×10^{-2} for top SNP rs939661). We conclude that common variants at chromosome 15q14 influence susceptibility for myopia in Caucasian and Asian populations world-wide.

Introduction

Refractive errors are common optical defects of the visual system. An important refractive error is myopia (near-sightedness), which occurs when the eye elongates beyond the focal plane. The prevalence of myopia is high, affecting about one-third of the world's population, and reaching over 70 % in certain Asian ethnic groups (He et al. 2004; Kempen et al. 2004; Lin et al. 2004; Vitale et al. 2008; Wu et al. 2001). High degrees of myopia are associated with pathologic ocular changes, such as myopic macular degeneration, retinal detachment, and glaucoma (Curtin and Karlin 1971; McBrien and Gentle 2003; Saw 2006; Saw et al. 2005; Tano 2002). Due to the limited treatment options, myopia is a common cause of visual impairment (Tano 2002; Young 2009).

Refractive errors, and myopia in particular, are complex genetic traits with a largely unknown etiology. Established environmental factors are education, early reading, and reduced outdoor exposure (Dirani et al. 2009; Ip et al. 2008; McBrien et al. 2008; Morgan and Rose 2005; Rose et al. 2008; Saw et al. 2001; Young 2009). Although heritability estimates are high [50–90 % (Young et al. 2007)], the search for myopia genes is still ongoing. Previous linkage and association studies have led to the identification of at least 18 myopia (MYP) loci, 10 additional chromosomal regions, and several candidate genes (Baird et al. 2010; Young 2009). Replication of these associations has been inconsistent, and their application to the general population is limited (Baird et al. 2010).

Recent genome-wide association studies (GWAS) reported several susceptibility loci for refractive error and

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myopia (Hysi et al. 2010; Li et al. 2011a, b; Nakanishi et al. 2009; Shi et al. 2011; Solouki et al. 2010). Solouki et al. (2010) and Hysi et al. (2010) were the first to perform a GWAS in a general Caucasian population, and identified susceptibility loci on chromosomes 15q14 and 15q25, respectively. In both studies, carriers of single nucleotide polymorphism (SNP) rs634990 at 15q14 (OR 1.83, 95 % CI 1.42–2.36) and of SNP rs8027411 at 15q25 (OR 1.16, 95 % CI 1.02–1.28) had a higher risk of myopia. Confirmation of these findings was obtained in various replication studies (Hayashi et al. 2011; Hysi et al. 2010; Solouki et al. 2010). However, these replication cohorts were relatively limited in size, increasing the chance of a type 1 error.

To address potential inaccuracies and to investigate generalizability, we investigated the associations between refractive error, and the 15q14 and 15q25 susceptibility loci in a large international replication and meta-analysis study (Consortium of Refractive Error and Myopia, CREAM) including 31 cohorts with various ethnicities from 4 different continents.

Results

Meta-analysis of allelic effects on spherical equivalent (SE)

Complete data on refractive error and genome-wide SNPs were available in all 29 population-based studies comprising 49,364 subjects: 42,224 Caucasians and 7,140 Asians (Table 1; Fig. 1, Supplementary Table 1). This includes the previously reported discovery set consisting of 15,608 (Solouki et al. 2010) and 17,608 subjects (Hysi et al. 2010), respectively.

Table 2 shows the results of the meta-analysis of the 14 SNPs (Hysi et al. 2010; Solouki et al. 2010) at locus 15q14 and 5 SNPs at locus 15q25. The frequency of the effect allele C for top SNP rs634990 at locus 15q14 ranged from 0.38 to 0.64, while frequency of the effect allele A for top SNP rs939661 at 15q25 showed a larger variation, ranging from 0.28 to 0.63 (Supplementary Figure 1). The sample size of each SNP per study is provided in Supplementary Table 1. For locus 15q14, the magnitude and direction of the effects were consistent in all cohorts except Croatia Vis and SIMES. For locus 15q25, there was less consistency; for top SNP rs939661 8 cohorts—both Caucasian and Asian (Australian Twins, Croatia Split, Croatia Vis, EGCUT, FITSA, GHS II, ORCADES, and SIMES)—had a regression beta coefficient in the opposite direction to that of the other studies.

For locus 15q14, the replication set, consisting of all studies except the ones previously used in the discovery analysis, showed a statistically significant association between SE and all SNPs with a best P value 4.53×10^{-14} for top SNP rs634990. Confirmation was achieved in 23 out of 25 Caucasian studies (overall P 3.87×10^{-12} for SNP rs634990), and in 3 out of 4 Asian studies (overall

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P 2.21 × 10^{-3} for SNP rs634990). Meta-analysis of the discovery and replication cohorts together provided P value 9.20 × 10^{-23} for SNP rs634990.

For locus 15q25, neither Caucasian nor Asian validation studies replicated the original association. Meta-analysis of the combined set of the 5 SNPs yielded a lowest P 1.22 × 10^{-4} for SNP rs939661. As a subsequent analysis, we investigated locus 15q25 in more detail, and tested another 26 SNPs in 26 out of 29 cohorts (no data available in ALSPAC, AREDS 1, and EGCUT). This set of SNPs was not replicated either, however, meta-analysis including the discovery cohort was still significant (best P 2.07 × 10^{-4} for SNP rs1915726; Supplementary Table 3).

Meta-analysis of risk of myopia for top SNP

Genotype distributions for rs634990 at locus 15q14 were available for 28 out of 31 studies (all but FITSA, Australian Twins, and SORBS). There was no evidence of heterogeneity in the analyses of homozygote carriers [v^2 21.35 (d.f. 26), P 0.724, I^2 0.0 %] or heterozygote carriers [v^2 24.22 (d.f. 26), P 0.564, I^2 0.0 %]. Therefore, only results from fixed effects meta-analysis were used. Figure 2 shows the forest plots for the risk of myopia for homozygous and heterozygous carriers of the top SNP rs634990. The OR of moderate to high myopia (SE ≤−3 D) versus moderate to high hyperopia (SE ≥+3 D) was 1.88 (95 % CI 1.64, 2.16, $P < 0.001$) for homozygous carriers of the risk allele at the top SNP rs634990, and 1.33 (95 % CI 1.19, 1.49, $P < 0.001$) for heterozygous carriers.

Discussion

Chromosome 15q was first implicated in refractive error and myopia by genome-wide analysis of two large studies located in Northern Europe (Hysi et al. 2010; Solouki et al. 2010). Here, in an international meta-analysis consisting of 31 independent studies from the CREAM consortium, we provide further support that the association with locus 15q14 is robust and present in both Caucasians and Asians. We combined the results with those of the initial study into a powerful meta-analysis of highly associated SNPs with a total study population of 55,177 participants. The combined results showed that all tested SNPs for locus 15q14 were associated with refractive errors, and that homozygous carriers of the top SNP rs634990 had approximately twice the risk of myopia. SNPs at the other locus, 15q25, could not be convincingly replicated.

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This study has strengths and limitations. Major strengths of the study include the sample size and the inclusion of different ethnicities. The CREAM consortium represents the largest study on refractive error known to date. Previous replication studies have not been large scaled and focused on populations of the same ancestry (Gao et al. 2012; Lu et al. 2011; Wang et al. 2011). Another advantage of our study is the incorporation of clinical relevant endpoints such as high myopia and high hyperopia. Among the limitations are differences in designs and methods of the studies. (1) Population-based as well as case control studies were incorporated. However, the latter were only two (Kyoto Study and SORBS) and both had results within the same range as the population-based studies. (2) Different types of equipment and measurement methods were used to detect refractive error. These differences are generally subtle, and are not likely to cause false findings. (3) Various methods of genotyping and imputation were used, and genotyping was not complete in all studies. All SNPs at 15q14 had similar effect; thus, we do not think this has influenced these associations. SNPs at 15q25 showed larger variation, and the incomplete genotyping may have underpowered this analysis.

Earlier replication of the 15q14 locus was reported by Hayashi et al. (2011) in a Japanese sample of high myopic probands and controls. In a comparison of 1,125 high myopes (axial length >26.1 mm) versus 1,295 controls, the risk of high myopia was increased for the carriers of the initial top SNP rs634990 [OR 1.84 in homozygotes (95 % CI 1.44–2.36)]. Taken together with the current findings, this suggests that 15q14 plays a role in both common and high myopia.

The 15q14 associated region contains two interesting genes that are both well expressed in the retina, *GJD2* and *ACTC1*. *GJD2* encodes the Connexin36 protein, which plays a crucial role in the transmission and processing of visual signals in the retina by enabling intercellular transport of small molecules and ions in photoreceptors, amacrine and bipolar cells (Deans et al. 2002; Guldenagel et al. 2001; Kihara et al. 2009; Striedinger et al. 2005). We speculated that the protein encoded by the other candidate gene, *ACTC1*, could play a role in scleral remodeling, given the fact that similar actin proteins have been shown to be increased in developing myopic tree shrew eyes (Jobling et al. 2009). Previous *GJD2* (Solouki et al. 2010) and *ACTC1* (unpublished data) direct sequencing experiments did not reveal a functional variant, but the 15q14 locus appeared to harbor regulatory elements which may influence transcription of these genes (Solouki et al. 2010).

The 15q25 region contains the interesting candidate gene *RASGRF1*, which is highly expressed in the retina and has previously been implicated in photoreception and visual sensory processes (Fernandez-Medarde et al. 2009; Jones and Moses 2004). The association with this locus and gene is not robust, since none of the initial SNPs replicated significantly, and determination of more SNPs did not increase significance. A type 1 error may explain the initial finding. Another potential cause for the non-replication is a large variation in allele frequencies. The range of allele frequencies at 15q25 (0.28–0.63) was only slightly larger

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Table 1 Descriptives of all study cohorts

Study	*n*	Mean age (SD)	Age range	Men (%)	Mean SE (SD)
1958 British Birth Cohort	1,658	42 (0.0)	40–50	54.2	−0.96 (2.00)
AGES Reykjavik	2,986	76.3 (5.4)	60–80+	35.3	1.22 (2.05)
ALSPAC	3,804	15.4 (0.3)	14.25–17.08	47.2	−0.38 (1.28)
AREDS 1	816	79.5 (5.1)	60–80+	43.5	0.68 (1.94)
AREDS 2	1,506	68.0 (4.7)	55–81	41.1	0.54 (2.25)
Australian Twins	1,819	22.2 (12.7)	5–90	44.0	−0.22 (1.28)
Blue Mountains Eye Study	1,574	64 (7.9)	50–80+	43.4	0.59 (1.96)
Croatia Split	366	49.8 (14.4)	18–85	46.0	−1.83 (1.83)
Croatia Vis Island	544	55.8 (14.0)	18–83	40.0	−0.16 (1.93)
Croatia Korcula Island	836	56.0 (13.8)	18–98	35.0	−0.25 (1.92)
ERF	2,032	48.5 (14.3)	18+	43.1	0.07 (2.13)
EGCUT	338	34.8 (15.2)	18–85	36.9	−2.60 (2.00)
Finnish Twin Study on Aging	127	68.2 (3.8)	63–76	0.0	1.68 (1.54)
Framingham Eye Study	1,500	55.5 (9.0)	20–80	42.5	−0.17 (2.40)
Gutenberg Health Study I	2,745	55.7 (11)	35–74	51.5	−0.38 (2.44)
Gutenberg Health Study II	1,142	55.0 (10.9)	35–74	49.8	−0.41 (2.58)
KORA	1,867	55.6 (11.7)	35–84	49.6	−0.29 (2.27)
MESA	1,462	62 (9.4)	46–86	49.5	−0.28 (2.62)
ORCADES	505	54.8 (13.7)	22–88.5	43.0	0.01 (2.14)
Rotterdam Study 1	5,328	68.5 (8.6)	55+	41.3	0.86 (2.45)
Rotterdam Study 2	2,009	64.2 (7.4)	55+	45.9	0.48 (2.51)
Rotterdam Study 3	1,970	56.0 (5.5)	45+	43.9	−0.35 (2.62)
OGP Talana	623	44.5 (21.1)	5–89	51.8	−0.15 (1.78)
SCORM	929	10.8 (0.8)	10–15	48.0	−2.02 (2.26)
SiMES	2,226	57.7 (10.8)	40–80	49.3	−0.08 (1.98)
SINDI	2,055	55.7 (8.7)	40–80+	51.2	0.01 (2.13)
SP2	1,930	47.5 (10.9)	20–80	45.4	−1.67 (2.89)
TwinsUK	4,270	55.0 (12.0)	20–82	7.4	−0.39 (2.73)
Young Finns	397	37.6 (5.2)	25–50	45.0	−1.20 (2.29)
Kyoto Study	5,192	na	na	na	na
Cases	1,143	58.4 (14.3)	20–91	33.3	−10.50 (6.44)
Controls 1	3,120	58.5 (13.6)	20–90	61.7	na
Controls 2	929	38.8 (11.8)	0–74	41.3	na
SORBS	621	na	na	na	na
Cases	100	45.4 (6.6)	18–40	36.4	na
Controls	521	28.3 (15.16)	18–80	45.0	na

than at 15q14 (0.38–0.64) in our consortium, making this an unlikely explanation (Supplementary Figure 1). Finally, population stratification within cohorts did not appear to play a major role, since only two cohorts had significant principal components, which were addressed in the analyses.

Other GWAS loci were only found for high myopia in Asian case control studies, and they were located on chromosomes 11q24.1 (Nakanishi et al. 2009), 5p15 (Li et al. 2011a), 4q25 (Li et al. 2011b), and 13q12.12 (Shi et al. 2011). The locus on chromosome 5p15 harbors the excellent candidate gene *CTNND2* which is involved in retinal morphogenesis, adhesion, retinal cell architecture integrity (Duparc et al. 2006; Paffenholz et al. 1999), and was replicated in subjects of the same ethnicity (Lu et al. 2011). Replication studies for the 4q25 (Gao et al. 2012) and 11q24.1 (Wang et al. 2011) loci were only successful in case of the 4q25 locus; these loci did not have prominent candidate genes.

What should be the next steps? For 15q14, comprehensive resequencing of the entire associated region and the flanking genes can reveal the responsible gene

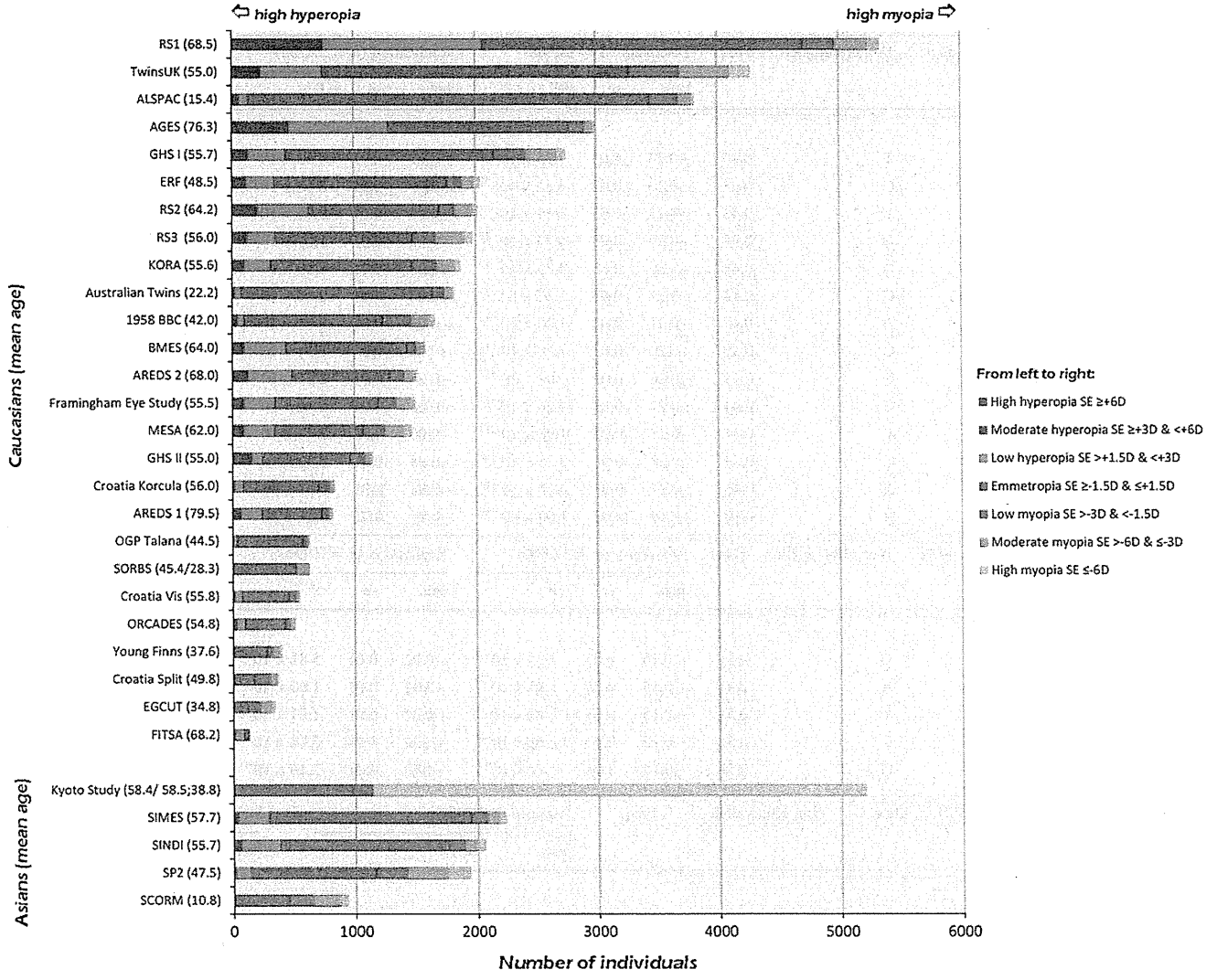

Fig. 1 Mean age and distribution of spherical equivalent in all study cohorts

defects which determine the association. Novel techniques such as next-generation sequencing are promising in this regard. Functional studies in knockout animals will shed light on potential protein effects. Finally, evaluation of gene-environment interactions may explain phenotypic variation and help identify high risk groups. For myopia genetics in general, performance of a genome-wide meta-analysis is a logical next step. The current CREAM collaboration is an excellent platform for this project.

In summary, we have convincingly demonstrated that common variants at chromosome 15q14 influence susceptibility for myopia in both Caucasian and Asian populations around the world. Identification of functional variants and responsible genes that explain this association will provide more insight in the complex etiology of myopia.

Materials and methods

Subjects and phenotyping

A total of 31 study cohorts from the Consortium of Refractive Error and Myopia (CREAM) participated in this meta-analysis. 29 population-based as well as 2 case–control studies were included. General methods, descriptives and phenotyping and genotyping methods of the study cohorts can be found in Table 1, the Supplementary Material and Supplementary Table 1, respectively. In short, 22 cohorts consisted of Caucasian, and 5 of Asian study subjects. All studies were performed with the approval of their local Medical Ethics Committee, and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Table 2 Meta-analysis of allelic effects on spherical equivalent at locus 15q14 and 15q25

SNP	Position	Effect allele	Non effect allele	Freq.	Discovery (n = 15,608)[a]			Replication (n = 33,755)[b]			Caucasian (n = 26,615)[c]		
					beta	se	P	beta	se	P	beta	se	P
Locus 15q14													
rs634990	32793365	C	T	0.49	−0.23	0.03	1.35×10^{-14}	−0.09	0.01	4.53×10^{-14}	−0.08	0.01	3.87×10^{-12}
rs560766	32788234	A	G	0.48	−0.20	0.03	4.82×10^{-12}	−0.09	0.01	3.53×10^{-14}	−0.08	0.01	3.91×10^{-12}
rs524952	32793178	A	T	0.48	−0.23	0.03	1.19×10^{-14}	−0.08	0.01	9.05×10^{-13}	−0.08	0.01	1.07×10^{-11}
rs688220	32786167	A	G	0.48	−0.20	0.03	4.43×10^{-12}	−0.08	0.01	1.01×10^{-13}	−0.08	0.01	1.38×10^{-11}
rs580839	32786121	A	G	0.48	−0.20	0.03	4.39×10^{-12}	−0.08	0.01	1.05×10^{-13}	−0.08	0.01	1.34×10^{-11}
rs11073060	32777143	A	C	0.48	−0.21	0.03	1.12×10^{-12}	−0.08	0.01	2.46×10^{-13}	−0.08	0.01	2.47×10^{-11}
rs4924134	32781857	G	A	0.45	−0.21	0.03	1.20×10^{-12}	−0.08	0.01	3.01×10^{-13}	−0.08	0.01	2.96×10^{-11}
rs7176510	32786771	T	C	0.45	−0.20	0.03	1.70×10^{-11}	−0.09	0.01	8.31×10^{-14}	−0.08	0.01	7.81×10^{-12}
rs619788	32782398	A	C	0.44	−0.20	0.03	3.94×10^{-12}	−0.08	0.01	2.21×10^{-13}	−0.08	0.01	2.29×10^{-11}
rs7163001	32777866	A	G	0.44	−0.21	0.03	1.26×10^{-12}	−0.08	0.01	6.28×10^{-13}	−0.08	0.01	4.16×10^{-11}
rs11073059	32776966	A	T	0.44	−0.21	0.03	1.98×10^{-12}	−0.08	0.01	8.78×10^{-13}	−0.08	0.01	4.85×10^{-11}
rs11073058	32776918	T	G	0.44	−0.20	0.03	2.23×10^{-12}	−0.08	0.01	8.52×10^{-13}	−0.08	0.01	4.84×10^{-11}
rs685352	32795627	G	A	0.46	−0.21	0.03	4.55×10^{-13}	−0.08	0.01	4.32×10^{-12}	−0.08	0.01	2.09×10^{-10}
rs8032019	32778782	G	A	0.40	−0.19	0.03	1.00×10^{-10}	−0.08	0.01	5.81×10^{-12}	−0.08	0.01	7.00×10^{-10}
SNP	Position	Effect allele	Non effect allele	Freq.	Discovery (n = 17,806)[a]			Replication (n = 31,557)[b]			Caucasian (n = 24,417)[c]		
					beta	se	P	beta	se	P	beta	se	P
Locus 15q25													
rs939661	77218118	A	G	0.51	−0.15	0.03	3.85×10^{-9}	−0.02	0.01	5.81×10^{-2}	−0.02	0.01	7.73×10^{-2}
rs939658	77238924	G	A	0.51	−0.15	0.03	1.85×10^{-9}	−0.02	0.01	1.60×10^{-1}	−0.02	0.01	2.16×10^{-1}
rs17175798	77251015	C	T	0.51	−0.15	0.03	1.99×10^{-9}	−0.02	0.01	1.81×10^{-1}	−0.01	0.01	2.38×10^{-1}
rs8033963	77242405	C	C	0.51	−0.15	0.03	1.86×10^{-9}	−0.01	0.01	2.18×10^{-1}	−0.02	0.01	2.20×10^{-1}
rs8027411	77248084	T	G	0.51	−0.15	0.03	2.07×10^{-9}	−0.01	0.01	2.49×10^{-1}	−0.02	0.01	2.16×10^{-1}

SNP	Position	Effect allele	Non effect allele	Freq.	Asian (n = 7,140)[d]			Meta-analysis (n = 49,363)[e]		
					beta	se	P	beta	se	P
Locus 15q14										
rs634990	32793365	C	T	0.49	−0.12	0.04	2.21×10^{-3}	−0.11	0.01	9.20×10^{-3}
rs560766	32788234	A	G	0.48	−0.12	0.04	1.47×10^{-3}	−0.10	0.01	1.03×10^{-21}
rs524952	32793178	A	T	0.48	−0.18	0.07	9.52×10^{-3}	−0.10	0.01	2.00×10^{-21}
rs688220	32786167	A	G	0.48	−0.12	0.04	9.80×10^{-4}	−0.10	0.01	3.44×10^{-21}
rs580839	32786121	A	G	0.48	−0.12	0.04	1.10×10^{-3}	−0.10	0.01	3.51×10^{-21}
rs11073060	32777143	A	C	0.48	−0.12	0.04	1.45×10^{-3}	−0.10	0.01	5.13×10^{-21}
rs4924134	32781857	G	A	0.45	−0.12	0.04	1.60×10^{-3}	−0.10	0.01	5.57×10^{-21}
rs7176510	32786771	T	C	0.45	−0.12	0.04	1.74×10^{-3}	−0.10	0.01	6.09×10^{-21}
rs619788	32782398	A	C	0.44	−0.12	0.04	1.54×10^{-3}	−0.10	0.01	6.97×10^{-21}
rs7163001	32777866	A	G	0.44	−0.11	0.04	2.81×10^{-3}	−0.10	0.01	1.41×10^{-20}
rs11073059	32776966	A	T	0.44	−0.11	0.04	3.64×10^{-3}	−0.10	0.01	2.63×10^{-20}
rs11073058	32776918	T	G	0.44	−0.11	0.04	3.50×10^{-3}	−0.10	0.01	2.68×10^{-20}
rs685352	32795627	G	A	0.46	−0.11	0.04	4.14×10^{-3}	−0.10	0.01	8.10×10^{-20}
rs8032019	32778782	G	A	0.40	−0.13	0.04	9.65×10^{-4}	−0.10	0.01	1.78×10^{-18}
Locus 15q25										
rs939661	77218118	A	G	0.51	−0.03	0.04	4.86×10^{-1}	−0.04	0.01	1.22×10^{-4}
rs939658	77238924	G	A	0.51	−0.04	0.05	3.94×10^{-1}	−0.04	0.01	4.32×10^{-4}
rs17175798	77251015	C	T	0.51	−0.05	0.06	3.70×10^{-1}	−0.04	0.01	6.12×10^{-4}

Table 2 continued

SNP	Position	Effect allele	Non effect allele	Freq.	Asian (n = 7,140)[d]			Meta-analysis (n = 49,363)[e]		
					beta	se	P	beta	se	P
rs8033963	77242405	C	C	0.51	−0.01	0.04	8.42×10^{-1}	−0.04	0.01	9.37×10^{-4}
rs8027411	77248084	T	G	0.51	0.00	0.04	9.12×10^{-1}	−0.03	0.01	1.14×10^{-3}

Freq average frequency

[a] For the 15q14 locus: RS1, RS2, RS3, ERF, TwinsUK; for the 15q25 locus: TwinsUK, RS1, RS2, RS3, ERF, 1958 British Birth Cohort, Australian Twins (adult samples only)

[b] For the 15q14 locus: 1958 British Birth Cohort, AGES, ALSPAC, AREDS 1, AREDS 2, Australian Twins, BMES, Croatia Split, Croatia Vis, Croatia Korcula, EGCUT, FITSA, Framingham, GHS I, GHS II, KORA, MESA, ORCADES, OGP Talana, SCORM, SiMES, SINDI, SP2, Young Finns; for the 15q25 locus: AGES, ALSPAC, AREDS 1, AREDS 2, BMES, Croatia Split, Croatia Vis, Croatia Korcula, EGCUT, FITSA, Framingham, GHS I, GHS II, KORA, MESA, ORCADES, OGP Talana, Young Finns, SCORM, SiMES, SINDI, SP2

[c] For the 15q14 locus: 1958 British Birth Cohort, AGES, ALSPAC, AREDS 1, AREDS 2, Australian Twins, BMES, Croatia Split, Croatia Vis, Croatia Korcula, EGCUT, FITSA, Framingham, GHS I, GHS II, KORA, MESA, ORCADES, OGP Talana, Young Finns; for 15q25 locus: AGES, ALSPAC, AREDS 1, AREDS 2, BMES, Croatia Split, Croatia Vis, Croatia Korcula, EGCUT, FITSA, Framingham, GHS I, GHS II, KORA, MESA, ORCADES, OGP Talana, Young Finns

[d] Asian replication: SP2, SIMES, SINDI, SCORM

[e] All studies

All studies used a similar protocol for phenotyping. Exclusion criteria were age ≤10 years, and bilateral cataract surgery, laser refractive procedures or other intra-ocular procedures which might alter refraction. Eligible participants underwent a complete ophthalmologic examination including a non-dilated measurement of refractive error (Table 1) of both eyes. Spherical equivalent was calculated according to the standard formula (SE = sphere + cylinder), and the mean of two eyes was used for analysis. When data from only one eye were available, the SE of this eye was used. SE was categorized into low (SE from −1.5 to −3 D), moderate (SE from −3 to −6 D) and high (SE of −6 D or lower) myopia; and also into low (SE from +1.5 to +3 D), moderate (SE from +3 to +6 D) and high (SE of +6 D or higher) hyperopia. Emmetropia was defined as SE equal to or between −1.5 and +1.5 D.

Genotyping and imputation

DNA was extracted according to standard procedures, and genotyping and imputation of SNPs across the entire genome was performed using various methods (Table 1). Samples with a low call rate, with excess autosomal heterozygosity, with sex-mismatch, or outliers identified by the identity-by-state clustering analysis were excluded.

Statistical analysis

Meta-analysis of allelic effects on spherical equivalent

We selected 19 SNPs within loci 15q14 (14 SNPs) and 15q25 (5 SNPs) with a P value of $<10^{-6}$ from two previous GWAS (Hysi et al. 2010; Solouki et al. 2010). Linear regression models with a 1 degree of freedom trend test were used to examine associations with SE as a quantitative trait outcome, adjusting for age and gender and significant principal components if applicable. From all population-based cohorts, we obtained effect allele, non effect allele, regression coefficient beta, standard error, P value, minor allele and minor allele frequency for each of these SNPs. METAL for Linux was used to perform a meta-analysis on betas and standard errors for all SNPs. First, discovery cohorts (Hysi et al. 2010; Solouki et al. 2010) and replication studies were analyzed separately, followed by a combined meta-analysis. As a second analysis, 26 additional SNPs within the same linkage disequilibrium (LD) block were selected and tested for association using the procedures mentioned above. For these analyses, Bonferroni corrected P values (0.05/number of tested SNPs) of 3.57×10^{-3} for 15q14, and 1.0×10^{-2} (5 SNPs, Table 2) or 1.92×10^{-3} (26 SNPs, Table 3 Supplementary Material) for 15q25 were considered statistically significant.

Meta-analysis of risk of myopia for top SNP

From all population-based and case control studies, we obtained genotype distributions of the replicated top SNPs. We calculated heterogeneity (v^2, I^2 calculated and corresponding P values) between studies, crude OR with corresponding 95 % CI and P value of moderate and high myopia versus moderate and high hyperopia with a random as well as fixed effects meta-analysis using Stata 11. When these analyses provided similar outcomes, data from fixed effect analysis were used. For studies without subjects with high or moderate hyperopia, emmetropia was used as a

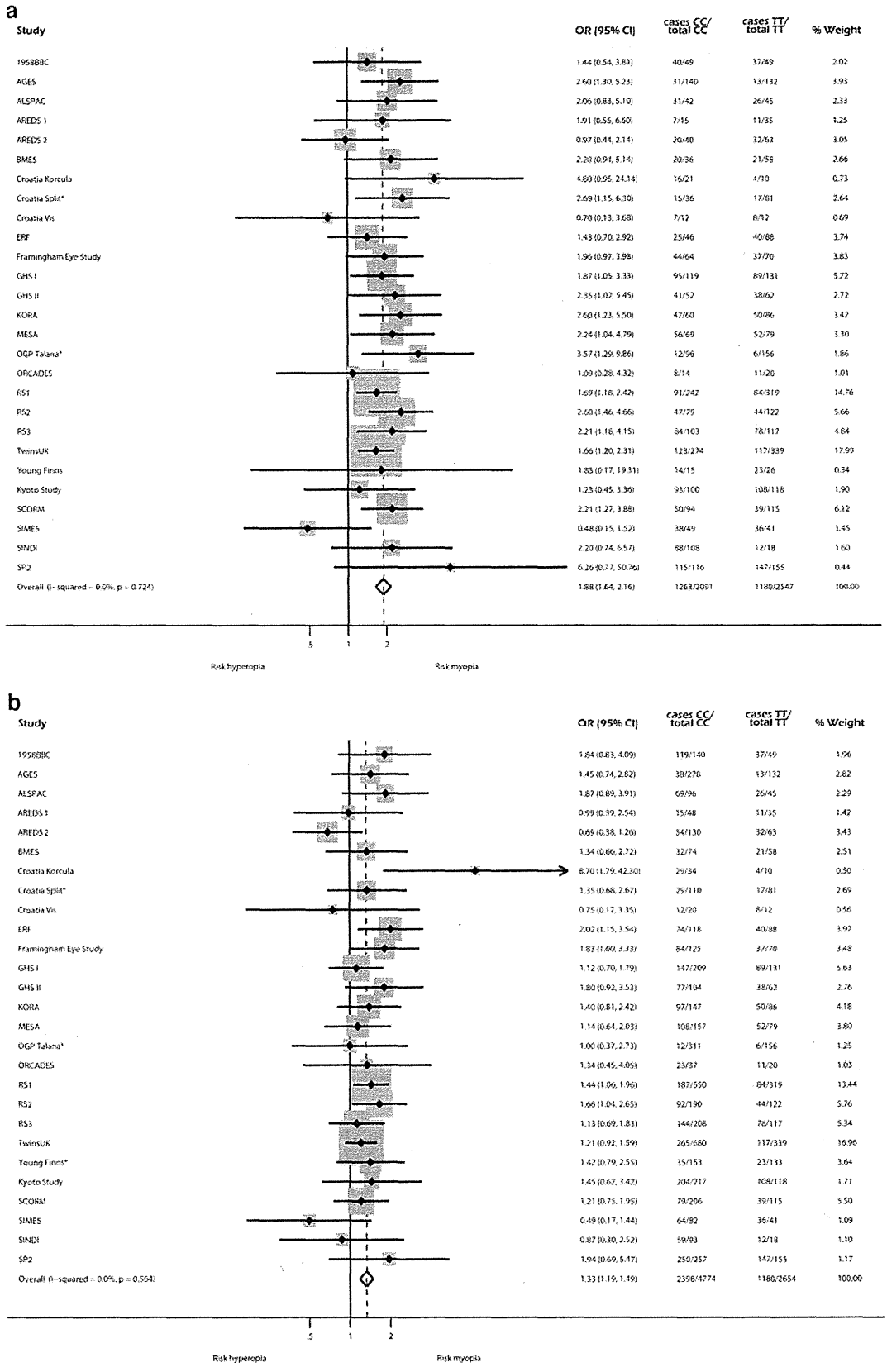

Fig. 2 Forest plots of odds ratios of myopia (spherical equivalent ≤ -3 diopters) versus hyperopia (spherical equivalent $\geq +3$ diopters) for top SNP rs634990. *For studies without subjects with high or moderate hyperopia, emmetropia was used as a reference group. **a** Homozygotes carriers of alleles TT versus CC for SNP rs634990. **b** Heterozygotes carriers of alleles TT versus TC for SNP rs634990

reference group. A standard P value of <0.05 was considered statistically significant.

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Vascular Endothelial Growth Factor Gene Polymorphisms and Choroidal Neovascularization in Highly Myopic Eyes

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Purpose. To investigate a potential association between VEGF gene polymorphisms and the occurrence and/or the size of choroidal neovascularization (CNV) in highly myopic eyes.

Methods. In the case-control study for CNV occurrence, 327 highly myopic Japanese patients were enrolled. One hundred and eighty-four patients had CNV in at least one eye, and 143 did not have CNV in either eye. Of the 184 patients with CNV, 83 patients were used to evaluate an association with CNV size, and an additional 76 patients with CNV were used to confirm the association. We genotyped four tag single nucleotide polymorphisms (SNPs) and four functional SNPs previously reported to be correlated with VEGF gene expression to evaluate the associations of these eight SNPs with CNV occurrence and size. To confirm the association between CNV size and VEGF gene polymorphism, the associated SNP was genotyped in 76 additional patients with myopic CNV.

Results. There was no significant association between the occurrence of myopic CNV and the SNPs in the VEGF gene ($P > 0.16$). Of the eight SNPs evaluated, however, rs2010963 showed significant association with CNV area ($P = 0.0047$). This association was successfully replicated in the additional 76 eyes with myopic CNV, and pooled analysis revealed significant association of rs2010963 with CNV size ($P = 0.00078$).

Conclusions. VEGF gene polymorphisms were not associated with CNV occurrence in highly myopic eyes but were significantly associated with the size of CNV, suggesting roles in the growth rather than the emergence of CNV. (*Invest Ophthalmol Vis Sci.* 2012;53:2349–2353) DOI:10.1167/iovs.11-9405

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Myopia is one of the most common ocular disorders worldwide. The prevalence of myopia is much higher in Asian populations, with a reported incidence of roughly 40% in the Japanese and Chinese population and 25% in Caucasians.[1–3] Pathological myopia, also called high myopia, is defined as a spherical equivalent refractive error of at least −6 diopters or an axial length ≥26.5 mm. Myopic axial length elongation can lead to chorioretinal atrophy and choroidal neovascularization (CNV), which is the most vision-threatening complication in highly myopic eyes.[4] Since the long-term visual outcomes of myopic CNV are extremely poor,[5] it is critical to determine in which highly myopic patients CNV will occur. CNV usually occurs in young adults with high myopia in the fourth and fifth decades of life. However, many eyes with high myopia do not have CNV even after 60 years of age. Furthermore, the size of the CNV seriously affects the visual prognosis because it determines the size of the scotoma, and some smaller CNVs can regress without treatment.[6] Since it is difficult to prevent the development of myopia, it is important to investigate the mechanisms underlying CNV occurrence and growth in myopic eyes; this may lead to the prevention of CNV development and the subsequent visual disturbance.

Genetic backgrounds may affect the development of high myopia; recently, we have determined a susceptible locus for pathological myopia using a genome-wide association study (GWAS).[7] Furthermore, recent GWASs reveal that myopia susceptibility loci exist in chromosome 15.[8–10] The occurrence of CNV in highly myopic eyes might also depend on genetic variations. Thus far, however, few studies have investigated the genetic background of patients with CNV in highly myopic eyes.

Since anti-VEGF treatment has been developed for neovascular AMD, it has become a popular treatment for ocular neovascularization. Anti-VEGF drugs have been shown to be effective in treating CNV secondary to high myopia.[11–13] In contrast to neovascular AMD, myopic CNV is easily inactivated with anti-VEGF treatment. In this study, we evaluated the associations between VEGF gene polymorphisms and CNV development in highly myopic eyes in Japanese patients.

Methods

This study was performed in accordance with the tenets of the Declaration of Helsinki. The Institutional Review Board/Ethics Committee of each institution approved the study protocols. All patients were fully informed of the study purpose and procedures, and written consent was obtained from each patient. For the case-control study of CNV occurrence, 327 highly myopic, unrelated Japanese patients with axial lengths of >26.0 mm in both eyes and who were ≥60 years of age were recruited from Kyoto University Hospital and Tokyo Medical and

TABLE 1. Characteristics of the Study Population

	With CNV	Without CNV	P Value
Number	184	143	
Mean age ± SD (years)	69.97 ± 6.35	69.23 ± 6.74	0.52*
Axial length ± SD (mm)			
Right	28.97 ± 1.72	29.11 ± 1.72	0.49*
Left	28.75 ± 1.72	28.84 ± 1.86	0.68*
Sex (male/female)	32/152	58/85	3.27×10^{-6}†

* Unpaired *t*-test.
† χ^2 test.

Dental University Hospital. The number of patients with macular CNV in at least one eye was 184, and the number of patients without macular CNV in either eye was 143 (Table 1). All patients underwent detailed ophthalmologic examinations, including dilated indirect and contact lens slit-lamp biomicroscopy, automatic objective refraction, measurement of the axial length by A-scan ultrasound (UD-6000; Tomey, Nagoya, Japan) or partial coherence interferometry (IOLMaster; Carl Zeiss Meditec, Dublin, CA), color fundus photography, optical coherence tomography, and fluorescein angiography. Individuals with a history of ocular surgery, with the exception of cataract surgery, were excluded from the study. Patients with secondary choroidal neovascular diseases, such as angioid streaks, presumed ocular histoplasmosis syndrome, and ocular trauma, were also excluded.

Of the 184 patients with myopic CNV, 83 patients underwent angiography with HRA2 (Heidelberg Engineering, Heidelberg, Germany) in Kyoto University Hospital. To evaluate the association between VEGF gene polymorphisms and CNV size, the area of CNV (mm^2) in these 83 patients was measured with the HRA-2 software. An additional 76 patients with myopic CNV were enrolled from Kyoto University Hospital to confirm the aforementioned associations. The average age of these patients was 63.8 ± 12.6 years, and the average axial length was 30.1 ± 1.1 mm.

For selecting tag single nucleotide polymorphisms (SNPs), we used the public dbSNP database build 126 (NCBI build 36.1) and HapMap database phase 2, release 22,[35] to extract the relevant sequencing information for the *VEGFA* gene and the genotyping information for the SNPs. A set of four tagging VEGF SNPs were selected for investigation: two SNPs on the promoter region, named rs699946 and rs699947, and two intronic SNPs, rs3025033 and rs3025035. This set of four tagging SNPs provided 100% coverage for all 14 common HapMap SNPs within a 26.3 kb region (16.3 kb gene length; 10 kb upstream) spanning the VEGF gene on chromosome 6 (r^2 threshold of 0.95). Furthermore, we evaluated four functional SNPs (rs1570360, rs2010963, rs833061, and rs3025039). Since these SNPs have been shown to affect VEGF expression,[14–17] many studies have evaluated the association of these SNPs with various diseases such as AMD, diabetic retinopathy, Behçet's disease, Alzheimer's disease, and diabetes.[18–26]

Genomic DNA was prepared from peripheral blood by a DNA extraction kit (QuickGene-610L; Fujifilm, Minato, Tokyo, Japan). VEGF-tagged SNPs (rs699946, rs699947, rs3025033, and rs3025035) and functional SNPs (rs1570360, rs2010963, rs833061, and rs3025039) were genotyped by a Taqman SNP assay with the ABI PRISM 7700 system (Applied Biosystems, Foster, CA). Deviations in genotype distributions from the Hardy-Weinberg equilibrium (HWE) were assessed with the HWE exact test. A χ^2 test for trend or its exact counterpart was used to compare the genotype distributions of the two groups. To adjust for age and sex, we performed logistic regression analysis. Mean age and axial length were compared using unpaired *t*-test or ANOVA, and sex ratio was compared with the χ^2 test. The associations between genotype and CNV size were evaluated using the Jonckheere-Terpstra trend test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

The demographics of the study population are shown in Table 1; there was no significant difference between patients with CNV and patients without CNV with respect to either age or axial length. The mean age of each group was 70.0 ± 6.4 years and 69.2 ± 6.7 years, respectively ($P = 0.52$). However, CNV is more predominant in women compared with men ($P = 3.27 \times 10^{-6}$) with an odds ratio (OR) of 3.24 (95% confidence interval [CI] = 2.27–4.64).

The genotype counts, associations, and ORs for the eight SNPs are shown in Table 2. The genotype distributions were not significantly different between patients with CNV and patients without CNV (nominal $P > 0.16$). Evaluation of the associations in a recessive model and a dominant model also showed no associations ($P > 0.10$). Even when adjusted for age and sex, the genotype distributions were not significantly different ($P > 0.10$).

In addition, we performed subset analysis for patients aged 70 years or older. In our cohort, 86 patients with CNV and 63 patients without CNV were ≥70 years of age. Associations between the eight SNPs with the occurrence of CNV were not statistically significant ($P > 0.17$).

Of the 184 patients with myopic CNV, the area of CNV was measured in 83 patients who underwent angiography with HRA2 in Kyoto University Hospital. The genotype distribution of rs2010963 was significantly correlated with CNV area ($P = 0.0047$), while the other seven SNPs did not show significant associations with CNV area (Fig. 1). The size of CNV was largest (1.71 ± 1.29 mm^2) in patients with a CC genotype of rs2010963, intermediate (0.98 ± 0.84 mm^2) with a CG genotype, and smallest (0.78 ± 0.78 mm^2) with a GG genotype. There was no significant difference in axial length, age of patients, or male/female ratio among the three

TABLE 2. Genotype Counts, Associations, and Odds Ratios for VEGF SNPs

		CNV (+)			CNV (−)				Age- and Sex-Adjusted	
SNP	Genotype	Genotype Count	MAF	HWE *P*	Genotype Count	MAF	HWE *P*	Nominal *P*	*P*	OR (95% CI)
rs699946	AA/AG/GG	64/82/33	G, 0.41	0.399	40/73/23	G, 0.44	0.250	0.543	0.10	0.80 (0.62–1.04)
rs699947	AA/AC/CC	22/77/85	A, 0.33	0.477	17/60/63	A, 0.34	0.626	0.856	0.68	0.93 (0.66–1.31)
rs3025033	AA/AG/GG	125/53/4	G, 0.17	0.286	90/44/8	G, 0.21	0.151	0.160	0.60	0.94 (0.73–1.20)
rs3025035	CC/CT/TT	90/71/17	T, 0.29	0.391	79/49/12	T, 0.26	0.200	0.355	0.34	1.13 (0.88–1.44)
rs1570360	AA/AG/GG	11/42/130	A, 0.17	0.005	8/32/102	A, 0.17	0.020	0.858	0.79	0.94 (0.60–1.47)
rs2010963	CC/GC/GG	34/84/62	C, 0.42	0.547	23/73/42	C, 0.43	0.348	0.820	0.42	0.88 (0.65–1.20)
rs833061	CC/CT/TT	22/75/82	C, 0.33	0.451	17/60/66	C, 0.33	0.554	0.922	0.69	0.93 (0.66–1.31)
rs3025039	CC/CT/TT	116/56/5	T, 0.19	0.402	87/45/8	T, 0.22	0.298	0.328	0.81	0.97 (0.76–1.24)

MAF, minor allele frequency.

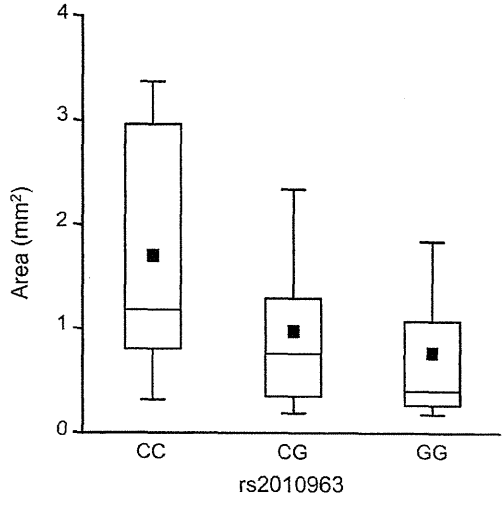

FIGURE 1. The area (mm²) of choroidal neovascularization among the three genotypes of rs2010963 in 83 patients. The area was significantly associated with the genotype ($P = 0.0047$).

genotypes of rs2010963 ($P = 0.54$, 0.98, and 0.69, respectively). To confirm the aforementioned association between rs2010963 and CNV size, we genotyped rs2010963 in an additional 76 patients with myopic CNV (20 male and 56 female). The genotype distribution of rs2010963 was significantly correlated with the CNV area ($P = 0.032$), while there was no significant difference in the axial length, age of patients, or male/female ratio among the three genotypes of rs2010963 ($P = 0.91$, 0.15, and 0.20, respectively). When these two cohorts were pooled for further evaluation of this association, the genotype distribution of rs2010963 was significantly correlated with the CNV area (Fig. 2, $P = 0.00078$).

DISCUSSION

In the present study, we found no association between VEGF gene polymorphisms and the occurrence of CNV in highly myopic eyes in Japanese patients, although rs2010963 was

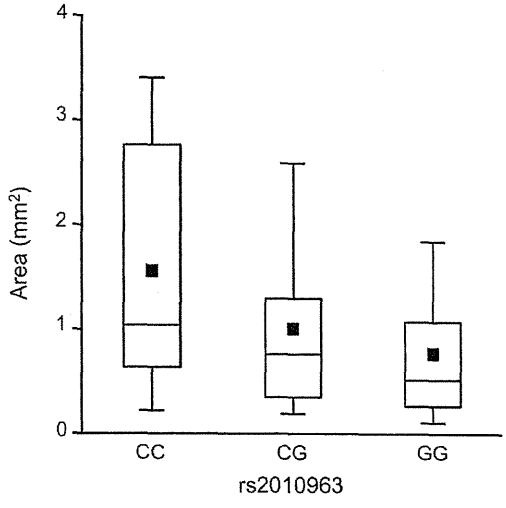

FIGURE 2. The area (mm²) of choroidal neovascularization among the three genotypes of rs2010963 in 159 patients. The area was significantly associated with the genotype ($P = 0.00078$).

significantly associated with the size of CNV. To evaluate factors associated with CNV occurrence in highly myopic eyes, the age of the cohort is of critical importance. Therefore, when a younger cohort is used, some patients assigned to the group without CNV may eventually develop CNV, which can obscure potential differences between the two groups. Fernandez-Robredo et al. have evaluated the association of CFH Y402H and ARMS2 A69S polymorphisms with myopic CNV using 196 myopic patients who were aged ≥30 years.[27] We have previously evaluated the same association using 353 myopic patients who were ≥50 years of age,[28] and the present study consisted of 327 myopic patients who were aged ≥60 years. However, the association of VEGF gene polymorphism with CNV occurrence was not statistically significant. Furthermore, we evaluated the association using a cohort of patients older than 70 years, but statistical significance was still not found.

Genetic associations with myopia have been investigated for several decades. Linkage studies have identified 18 possible loci for myopia (MYP1-18). Numerous candidate genes have been evaluated, and we have recently completed a GWAS study.[7] Furthermore, recent GWAS studies have revealed myopia susceptibility loci on chromosome 15, and we have successfully reproduced the association of these susceptibility loci with high myopia.[8-10] However, susceptibility genes for myopia have not been revealed; this makes it difficult to determine how to prevent myopia. Compared with the prevention of myopia, prevention and/or control of CNV occurrence and growth in highly myopic eyes might be a more practical approach. Since CNV is one of the most vision-threatening complications in highly myopic eyes, it is of great value to investigate the mechanism underlying CNV development in these eyes.

Although anti-VEGF treatments have been developed for the management of neovascular AMD, they are also substantially effective in treating myopic CNV.[11-13] Considering the effectiveness of these anti-VEGF treatments, we had hypothesized that VEGF is associated with the occurrence of CNV in highly myopic eyes. The present study, however, suggests that VEGF gene variations do not affect the occurrence of CNV in these eyes. In contrast with CNV occurrence, VEGF gene polymorphism rs2010963 was significantly associated with CNV size. Thus, it appears that VEGF contributes to CNV growth rather than CNV occurrence in highly myopic eyes. Experimental studies have shown that inhibition of VEGF leads to smaller CNV in laser-induced CNV models.[29-31] However, inhibition of VEGF does not always completely suppress CNV occurrence after laser photocoagulation to disrupt Bruch's membrane. This evidence suggests that VEGF only affects CNV size/growth, and other factors are responsible for triggering CNV occurrence, partly by interacting with Bruch's membrane.

The size of CNV is critical for visual prognosis in highly myopic eyes. Smaller CNVs can lead to smaller scotomas and spare the visual functions of the surrounding retina. Furthermore, very small CNVs can disappear completely after treatment.[6] Our findings suggest that development of larger CNVs in highly myopic eyes can be prevented by targeting VEGF, while prevention of CNV occurrence might be accomplished by targeting other factors.

Watson et al. reported that the amount of lipopolysaccharide-induced VEGF production from peripheral blood mononuclear cells (PBMNCs) is highest in individuals with a GG genotype of rs2010963, intermediate with a CG genotype, and lowest with a CC genotype.[17] In contrast to the findings of this study, we discovered that the size of CNV was largest in patients with a CC genotype, intermediate with a CG genotype, and smallest with a GG genotype. Considering that VEGF is a pro-angiogenic factor, these two findings seem contradictory. However, an evaluation of PBMNC function in in-vitro studies

does not always reflect their function in in-vivo situations. Furthermore, PBMNCs include several cell types such as lymphocytes, monocytes, and macrophages, and we have performed in vivo experiments that show that PBMNCs induce endothelium apoptosis[32] and that lymphocytes are negative regulators of pathological neovascularization, while monocytes are positive regulators in an ischemic retinopathy model.[33] Further studies are required to evaluate the roles of VEGF produced individually by monocytes or lymphocytes during myopic CNV development. In addition to VEGF produced from PBMNCs, VEGF produced from the RPE could also affect the growth of CNV in highly myopic eyes. Although we cannot evaluate the VEGF-producing ability of the RPE in an in-vivo situation, elucidation of the roles of the RPE in myopic CNV development might lead to better control of CNV size. It is also important to consider that VEGF can have several isoforms with different properties; we have demonstrated that VEGF165 is associated with pathological neovascularization, while VEGF121 is associated with physiological neovascularization.[33] Furthermore, recent studies have shown that some isoforms of VEGF are anti-angiogenic.[34] Additional studies on the role of different VEGF isoforms in myopic CNV development may lead to prevention of larger CNV secondary to high myopia.

Limitations of the present study include the age of the cohort and the small sample size. Although we used a cohort older than 60 years of age and performed a subanalysis using samples with patients older than 70 years, some participants included in the group without CNV might develop CNV in the future. Furthermore, our study is retrospective in nature, and the associations discovered herein need to be evaluated in prospective studies.

In conclusion, we have shown that VEGF gene polymorphisms have no association with the occurrence of CNV in highly myopic eyes in Japanese individuals; however, VEGF rs2010963 affects the size of CNV. Treatments that target VEGF may prevent large CNV formation in highly myopic eyes and help achieve better visual prognosis. To prevent CNV occurrence, further studies are needed to clarify the mechanism and/or background causes of CNV occurrence in highly myopic eyes.

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