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Identification of myopia-associated WNT7B polymorphisms provides insights into the mechanism underlying the development of myopia

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Myopia can cause severe visual impairment. Here, we report a two-stage genome-wide association study for three myopia-related traits in 9,804 Japanese individuals, which was extended with trans-ethnic replication in 2,674 Chinese and 2,690 Caucasian individuals. We identify *WNT7B* as a novel susceptibility gene for axial length ($rs10453441$, $P_{\text{meta}} = 3.9 \times 10^{-13}$) and corneal curvature ($P_{\text{meta}} = 2.9 \times 10^{-40}$) and confirm the previously reported association between *GJD2* and myopia. *WNT7B* significantly associates with extreme myopia in a case-control study with 1,478 Asian patients and 4,689 controls (odds ratio (OR)_{meta} = 1.13, $P_{\text{meta}} = 0.011$). We also find in a mouse model of myopia downregulation of *WNT7B* expression in the cornea and upregulation in the retina, suggesting its possible role in the development of myopia.

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High myopia increases the risk of other pathological ocular complications¹, owing to which myopia is emerging as a major public health concern in many parts of the world. The prevalence of high myopia and associated visual complications is increasing in many Asian and Caucasian populations, and this trend is particularly notable in urbanized areas^{2–4}. The World Health Organization recognizes myopia as a major cause of visual impairment⁵.

Emmetropia, the absence of refractive error, is determined by the precise coordination of ocular parameters, such as axial length, corneal curvature, lens thickness and anterior chamber depth. Disruption of this coordination causes refractive errors, such as myopia and hyperopia^{6,7}. Of these components, axial length plays a major role in refraction; therefore, in clinical settings, the degree of myopia is predominantly determined by axial length^{8–10}. A recent large-scale, genome-wide meta-analysis by the Consortium for Refractive Error and Myopia (CREAM) reported that 23 of 29 loci associated with refraction were also associated with axial length^{11,12}. However, genes associated with axial length are not necessarily associated with refractive error¹². This can be explained on the basis of the compensatory effects of other ocular biometric components, such as corneal curvature that influence the effect of an increased axial length through a flatter cornea⁶. Indeed, in genome-wide association studies (GWASs), some genes found to be associated with corneal curvature were also recently reported to be associated with axial length, but not with refractive error^{7,13}.

On the other hand, the retina is thought to play a crucial role in the development of myopia¹⁴. For example, it is well known that central hyperopic defocus can cause myopia in chicks¹⁵ and mice models¹⁶. In addition, experiments conducted by Smith *et al.*^{17–19} have shown that peripheral hyperopic defocus can cause myopic shift in monkeys. This is a herald of ‘peripheral defocus theory’ that implicates defocus at the peripheral retina as the trigger for myopia. These reports suggest that the retina can perceive the defocus and evoke signals to accelerate the progression of myopia. This hypothesis is supported by animal experiments that show amacrine cells and their transcription factor, ZENK, have important roles in the perception of retinal defocus^{20,21}. Nevertheless, the mechanisms by which these signals are transduced are not yet known.

In the current study, we conduct a two-stage GWAS on three myopia-related traits—axial length, spherical error and corneal curvature—within the Nagahama Prospective Genome Cohort for Comprehensive Human Bioscience (the Nagahama Study), which involves 9,804 healthy Japanese individuals. The analysis of an additional 2,674 Chinese individuals across two independent cohorts and 2,690 Caucasian individuals across five independent cohorts confirms the trans-ethnic genetic associations. In addition, we evaluate the associations with extreme myopia in Asian individuals.

Results

Two-stage GWAS within the Nagahama study. The cohort demographics used in this study are shown in Supplementary Table 1. After stringent quality control, a total of 1,773,334 single nucleotide polymorphisms (SNPs) were included in the analyses of the Nagahama cohort. Axial length, spherical equivalent and corneal curvature were used as the dependent variables for three genome-wide quantitative trait loci (QTL) analyses. We included age, sex and height as covariates, but did not include principal components in these analyses. However, inflation factors (λ_{GC}) of 1.031 (axial length), 1.024 (spherical equivalent) and 1.016 (corneal curvature) indicated excellent control of population substructure. The QQ plots are shown in the Supplementary Fig. 1.

During the first stage, we identified five loci that showed a suggestive association with a P value $<1.0 \times 10^{-6}$ by linear regression; one of these SNPs was *WNT7B* rs200329677, which exceeded genome-wide significance with a P value of 1.13×10^{-9} in the association with corneal curvature (Fig. 1 and Table 1). In the following replication stage, we further genotyped 3,460 Japanese individuals for three SNPs that showed suggestive association with a P value $<1.0 \times 10^{-6}$ and had a minor allele frequency (MAF) of at least 5% in the discovery stage. We investigated the associations of each of the three traits with each SNP and evaluated the results of the first and second stage in a meta-analysis (Table 2). This analysis revealed associations with two traits for the two SNPs in *GJD2* and *WNT7B*. First, we successfully replicated the previously reported association between *GJD2* rs11073058 and axial length ($P_{\text{combined}} = 2.4 \times 10^{-8}$; inverse variance meta-analysis of discovery and replication

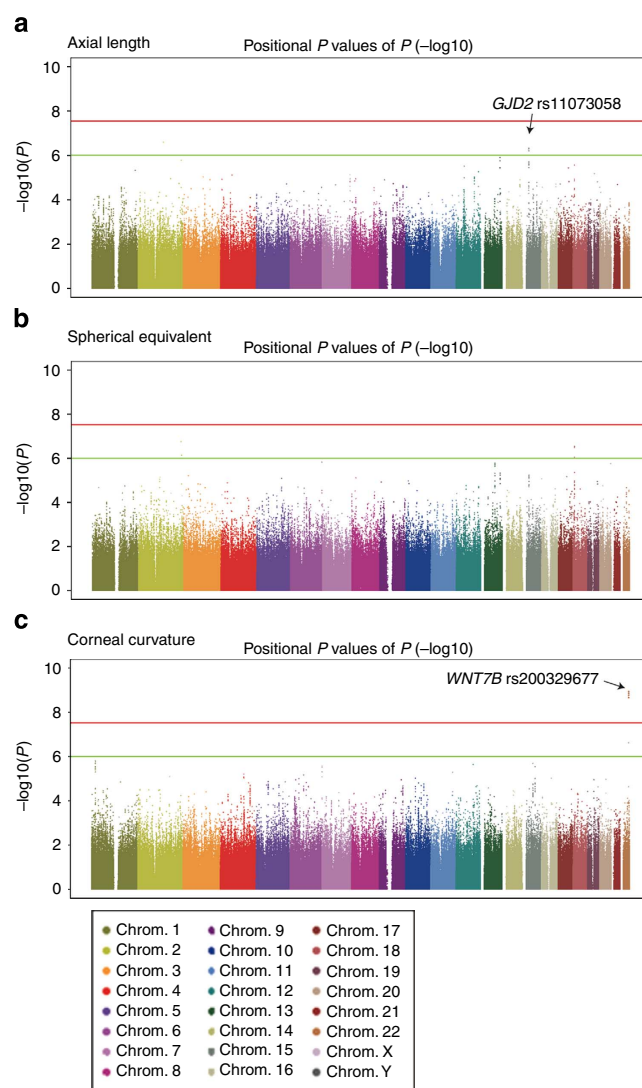


Figure 1 | Manhattan plots of the first stage GWAS for three myopia-related traits. Each plot shows $-\log_{10}$ -transformed P values for all SNPs. The upper horizontal line represents the genome-wide significance threshold of $P < 2.82 \times 10^{-8}$, and the lower line represents the suggestive threshold of $P < 1.00 \times 10^{-6}$. Nine SNPs from five genes surpassed the suggestive P value, including previously reported *GJD2* ($P = 4.72 \times 10^{-7}$ by linear regression using 2,991 individuals) for axial length and the novel genome-wide hit *WNT7B* for corneal curvature ($P_{\text{top}} = 1.13 \times 10^{-9}$ by linear regression using 2,747).

Table 1 | Results of the discovery stage.

	Nearby genes		rs number	CHR	Position	n	Effect allele	β	Standard error	MAF	P value
Axial length	<i>THSD7B</i>	Not in gene	rs78534307	2	1,368,233,336	2,991	G	0.83	0.16	0.01	2.52×10^{-7}
Spherical equivalent	<i>GJD2</i>	In gene	rs11073058	15	34,697,425	2,991	T	0.17	0.03	0.46	4.72×10^{-7}
	<i>LOC101928675</i>	Not in gene	rs6732520	2	228,821,808	2,730	C	-1.85	0.35	0.01	1.73×10^{-7}
	<i>RPL6P27</i>	Not in gene	rs4798444	18	6,455,641	2,730	A	0.36	0.07	0.50	2.86×10^{-7}
Corneal curvature	<i>WNT7B</i>	In gene	rs200329677	22	45,973,898	2,747	C	0.047	0.0077	0.28	1.13×10^{-9}

CHR, chromosome.

Regions with a P value (linear regression) less than 1.0×10^{-6} are shown.**Table 2 | Results of the replication stage within the Nagahama Study (n = 3,460).**

	Discovery trait	Axial length	Spherical equivalent	Corneal curvature
Replication trait	rs number	rs11073058	rs4798444	rs200329677
	Nearby gene	<i>GJD2</i>	<i>RPL6P27</i>	<i>WNT7B</i>
	Effect allele	T	A	C
	EAF	0.473	0.497	0.306
Axial length	β	0.09	0.05	0.13
	SE	0.03	0.03	0.03
	P value	2.6×10^{-3}	0.07	6.4×10^{-5}
	Meta P value	2.4×10^{-8}	2.1×10^{-5}	1.0×10^{-8}
Spherical equivalent	β	-0.25	-0.01	-0.01
	SE	0.06	0.06	0.07
	P value	3.6×10^{-5}	0.92	0.91
	Meta P value	2.7×10^{-9}	6.2×10^{-4}	0.68
Corneal curvature	β	-0.010	0.015	0.054
	SE	0.006	0.006	0.006
	P value	0.08	0.01	4.6×10^{-17}
	Meta P value	0.40	0.14	2.5×10^{-25}

Associations that reached genome-wide significance in the meta-analysis are described in bold.

EAF, effect allele frequency.

P values derived using linear regression.

results from Japanese cohort) as well as spherical equivalent ($P_{\text{combined}} = 2.7 \times 10^{-9}$). We did not observe any association between *GJD2* rs11073058 and corneal curvature. Second, we confirmed the novel associations of *WNT7B* rs200329677 with corneal curvature ($P_{\text{combined}} = 2.5 \times 10^{-25}$) and axial length ($P_{\text{combined}} = 1.0 \times 10^{-8}$). This SNP did not show association with the spherical equivalent.

Replication in other ethnicities and meta-analysis. Of the current commercially available DNA microarrays, the *WNT7B* rs200329677 was included only on the Illumina HumanOmni 2.5 and 5M chips. This SNP is not available in the 1000 Genomes Project or in the HapMap Project; thus, imputation to these reference genomes did not allow us to directly assess rs200329677 for association. Association with a trait was sought with rs10453441, which was in very high linkage disequilibrium with

rs200329677 ($R^2 = 0.967$ in the Nagahama Study data set), and strong evidence was found for association to corneal curvature ($P_{\text{discovery}} = 2.16 \times 10^{-9}$ and $P_{\text{replication}} = 4.6 \times 10^{-17}$ in the Nagahama data sets).

We conducted replication of both SNPs in an additional 2,674 Chinese individuals across two independent collections, as well as across 2,835 Caucasian individuals across five collections (Supplementary Methods). The SNP rs10453441 showed significant evidence of replication (Table 3) for axial length and corneal curvature both in Asian ($P_{\text{AL}} = 7.1 \times 10^{-6}$, $P_{\text{CC}} = 3.0 \times 10^{-14}$) and, to a lesser extent, in Caucasian ($P_{\text{AL}} = 2.0 \times 10^{-3}$, $P_{\text{CC}} = 4.1 \times 10^{-2}$) groups, with no heterogeneity across the two collections of Asian individuals ($I^2_{\text{AL}} = 0$, $I^2_{\text{CC}} = 0$) and moderate-to-high heterogeneity across the five collections of Caucasian individuals ($I^2_{\text{AL}} = 0.38$, $I^2_{\text{CC}} = 0.89$). Meta-analysis of the data from all the Asian collections showed genome-wide significant associations with both axial length ($P_{\text{meta-Asian}} = 1.20 \times 10^{-9}$, $\beta = 0.129$ mm) and corneal curvature ($P_{\text{meta-Asian}} = 9.00 \times 10^{-39}$, $\beta = 0.053$ mm), with no evidence of heterogeneity.

Finally, meta-analysis of the Asian and Caucasian data also revealed strong associations between *WNT7B* rs10453441 and axial length ($P_{\text{meta-ALL}} = 3.9 \times 10^{-9}$, $\beta = 0.121 \pm 0.017$ mm), as well as corneal curvature ($P_{\text{meta-ALL}} = 2.7 \times 10^{-40}$, $\beta = 0.051 \pm 0.004$ mm).

Only in the Genes in Myopia cohort²² could we obtain Caucasian genotypes for both rs10453441 and rs200329677, so that we could assess the linkage disequilibrium (LD) between these two SNPs in Caucasians. They were in moderate LD ($R^2 = 0.499$, $D' = 0.943$).

Possible interactive effect of *WNT7B* and *GJD2*. *GJD2* is an established susceptibility gene for increased axial length and myopic refraction^{11,23}. In the current study, these associations with *GJD2* were replicated in our independent Japanese sample collections. To further our understanding of the role of *GJD2* and *WNT7B*, we investigated the potential interactive effect of these two genes on myopia susceptibility by the directly genotyped cohorts: Nagahama replication samples, SCES, SP2, Raine, Croatia-split, ORCADES and Genes in Myopia. Figure 2 shows the effect of the *GJD2* rs11073058 T allele on spherical equivalent, stratifying by the genotypes of *WNT7B* rs10453441. As described in Fig. 2, in the Asian populations, the effects of *GJD2* rs11073058 on spherical equivalent were the highest when individuals were homozygous (AA genotype) at *WNT7B* rs10453441 ($\beta = -0.54$ D; 95% CI: -0.82 to -0.27 D) compared with that in the individuals with the AG genotype ($\beta = -0.24$ D;

Table 3 Replication of the association of <i>WNT7B</i> SNPs with three traits.														
SNP	Effect allele	Cohort*	Ethnicity	n	EAF	Axial length			Corneal curvature			Spherical equivalent		
						Beta	SE	P value	Beta	SE	P value	Beta	SE	P value
rs200329677	C	Nagahama	Japanese	3,306	0.31	0.13	0.03	6.4×10^{-5}	0.054	0.006	4.6×10^{-17}	−0.08	0.07	0.91
		SCES	Chinese	1,771	0.30	0.19	0.05	1.2×10^{-4}	0.060	0.009	207×10^{-10}	−0.03	0.10	0.80
rs10453441	A	GEM	Caucasian	534	0.41	0.08	0.10	0.42	0.008	0.066	0.91	−0.24	0.19	0.21
		Nagahama	Japanese	3,460	0.31	0.11	0.03	7.0×10^{-4}	0.055	0.006	2.5×10^{-18}	0.04	0.06	0.52
		SCES	Chinese	1,923	0.33	0.14	0.05	3.1×10^{-3}	0.054	0.009	1.2×10^{-9}	0.03	0.10	0.74
		SP2†	Chinese	851	0.33	—	—	—	0.066	0.014	2.3×10^{-6}	0.05	0.14	0.74
		Meta	—	5,383,	—	0.12	0.03	7.1×10^{-6}	0.058	0.008	3.0×10^{-14}	0.04	0.05	0.43
		Asian replication	—	6,234	—	—	—	—	—	—	—	—	—	—
		Meta Asian	—	8,374, 8,981	—	0.13	0.02	1.2×10^{-9}	0.053	0.004	9.0×10^{-39}	0.02	0.04	0.58
		ORCADES‡	Caucasian	677	0.55	0.19	0.05	7.4×10^{-4}	—	—	—	0.07	0.11	0.54
		GEM	Caucasian	551	0.56	0.05	0.10	0.60	0.084	0.064	0.19	0.08	0.06	0.19
		CROATIA-Korcula	Caucasian	909,	0.52	0.15	0.07	5.0×10^{-2}	0.029	0.021	0.17	−0.13	0.12	0.28
		CROATIA-Split	Caucasian	926	—	—	—	—	—	—	—	—	—	—
		RAINE	Caucasian	445,	0.59	0.01	0.06	0.94	0.023	0.019	0.22	0.09	0.12	0.43
		Meta Caucasian	—	422	—	—	—	—	—	—	—	—	—	—
		Meta ALL§	—	108	0.56	−0.02	0.12	0.85	−0.032	0.228	0.89	0.07	0.25	0.78

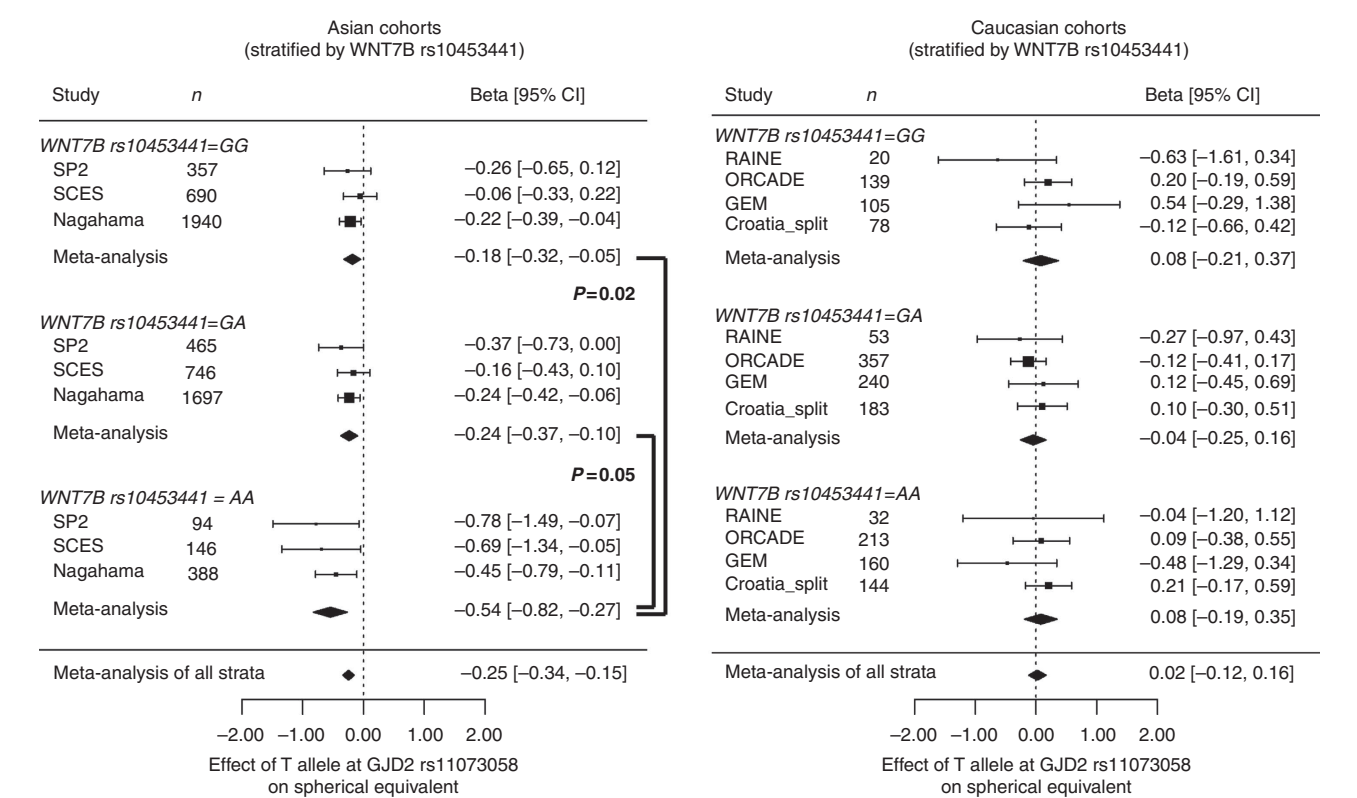


Figure 2 | Assessment of interactive effect between WNT7B and GJD2 and association with the spherical equivalent. In Asian cohorts, the effects of GJD2 rs11073058 on the spherical equivalent were highest when the individual had the homozygous variant (AA genotype) at WNT7B rs10453441 ($\beta = -0.54$ D; 95% CI = -0.82 to -0.27 D), compared with individuals with the AG genotype ($\beta = -0.24$ D; 95% CI = -0.37 to -0.10 D; $P = 0.05$ by z-test) or those with the GG genotype ($\beta = -0.18$ D; 95% CI = -0.32 to -0.05 D; $P = 0.02$ by z-test). This trend was not observed in Caucasian cohorts.

95% CI: -0.37 to -0.10 D) or in those with the GG genotype ($\beta = -0.18$ D; 95% CI: -0.32 to -0.05 D). The genotypic model revealed marginal effect-measure modification ($P_{\text{genotypic (analysis of variance)}} = 0.07$), with *post hoc* P values of 0.02 (AA versus GG), 0.05 (AA versus AG) and 0.60 (AG versus GG) based on z -test. On the other hand, the recessive model of effect-measure modification was statistically significant ($P_{\text{recessive}} = 0.024$) as the effect of *GJD2* rs11073058 T allele on spherical equivalent was -0.21 D (95% CI: -0.11 to -0.31 D) in individuals with non-AA genotype at *WNT7B* rs10453441.

Clinical relevance of *WNT7B* to extreme myopia. To evaluate the possible association between *WNT7B* and extreme myopia, we performed a case-control study. We found significant association between *WNT7B* rs10453441 and extreme myopia (patients with average axial length of ≥ 28 mm) in the Japanese cohort (odds ratio = 1.14 (95% CI = 1.02–1.27), $P_{\text{meta-Japanese}} = 0.018$). Although the association in Chinese cohorts was not statistically significant ($P_{\text{meta-Chinese}} = 0.30$), the effect size and its direction were similar to those in Japanese cohort (odds ratio = 1.10 (95% CI = 0.92–1.30)). The lack of association might be due to the limited sample size of extreme myopia individuals (1,064 Japanese and 414 Chinese) as these results were consistent ($I^2 = 0\%$, $P_{\text{heterogeneity}} = 0.84$). Indeed, overall meta-analysis revealed more significant association (odds ratio = 1.13 (95% CI = 1.03–1.24), $P_{\text{meta-all}} = 0.011$). These results are summarized in Table 4 and Supplementary Fig. 2.

The expression of *WNT7B* in experimental myopia mice. Our immunohistochemical study of the mouse cornea showed that *WNT7B* was predominantly expressed in the endothelial cell layer (Fig. 3a). In contrast to corneal studies, the role of *WNT* signaling in the posterior pole has been examined in various parts of the tissue, such as the neural retina, retinal vasculature and retinal pigment epithelium^{24–28}. To evaluate the expression pattern of *WNT7B* in the posterior pole of eyes, we examined the localization of *WNT7B* in the mouse eye. In the retina of a 21-day-old male mouse, *WNT7B* expression was observed predominantly in ganglion cells (Fig. 3b). Ganglion cells were also stained with the ganglion cell marker Brn-3a. Not all ganglion cells expressed Brn3a, and hence we could not confirm expression of *WNT7B* in

all ganglion cells, but we could confirm that all ganglion cells expressing Brn-3a also co-expressed *WNT7B*.

To explore the roles of *WNT7B* in myopia development, we evaluated the expression of the *WNT7B* gene in the cornea and the neural retina of myopia-induced mice (Fig. 4). *WNT7B* mRNA levels were significantly higher in the retinas of myopic eyes than those of corresponding control eyes ($P = 0.016$). In

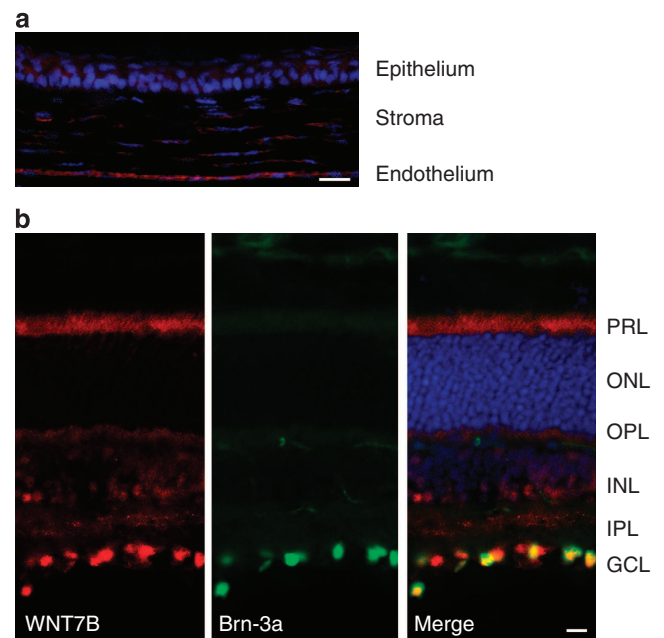


Figure 3 | Expression of *WNT7B* in retinal ganglion cells of C57BL/6 mice. (a) Mouse corneal sections were immunostained with antibodies against *WNT7B* (red). The upper part of the panel shows the corneal epithelium. (b) Mouse retinal sections were immunostained with antibodies against *WNT7B* (red) and Brn-3a (green). The nuclei are counterstained with 4',6-diamidino-2-phenylindole (blue) in the merged image. The upper part of the panel shows the retinal pigment epithelium side, while the lower part shows the vitreous side. *WNT7B* expression was observed in ganglion cells stained with Brn-3a. Scale bar, 10 μ m. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; PRL, photoreceptor layer.

Table 4 | Association of *WNT7B* rs10453441 with extreme myopia.

Ethnicity	Collection	Control [*]							Extreme myopia [†]							Odds ratio (95% CI) [‡]	P value [‡]
		n	SE [D] [§] median (first, third quartile)	rs10453441			A allele freq.	n	SE [D] [§] median (first, third quartile)	rs10453441			A allele freq.				
				GG	GA	AA				GG	GA	AA					
Japanese	Nagahama/ Kyoto	1,902	0.03 (−0.69, 0.81)	952	781	169	0.29	938	−13.50 (−16.50, −11.25)	449	376	113	0.32	1.13 (1.00–1.28)	0.043		
	Yokohama	395	0.375 (0.125, 0.875)	173	178	44	0.34	109	−11.62 (−13.38, −10.00)	43	48	18	0.38	1.23 (0.90–1.68)	0.18		
	Meta	2,297	—	—	—	—	1,064	—	—	—	—	—	—	1.14 (1.02–1.27)	0.018		
Chinese	SCES	1,493	0.44 (−0.31, 1.31)	668	679	146	0.32	22	−11.73 (−13.56, −8.06)	6	14	2	0.40	1.44 (0.74–2.74)	0.20		
	Hong Kong	422	0.19 (−0.13, 0.50)	186	186	50	0.33	257	−11.19 (−12.95, −9.86)	114	106	37	0.35	1.05 (0.71–1.55)	0.68		
	Sichuan	477	0.0 (−0.63, 0.50)	224	211	42	0.30	135	−13.00 (−17.00, −10.00)	57	67	11	0.32	1.10 (0.68–1.68)	0.51		
	Meta	2,392	—	—	—	—	414	—	—	—	—	—	—	1.10 (0.92–1.30)	0.30		
Meta	5 Collections	4,689	—	—	—	—	—	1,478	—	—	—	—	—	1.13 (1.03–1.24)	0.011		

CI, confidence interval; D, diopter; SE, spherical equivalent.
P values derived using chi-squared test for trend and inverse variance meta-analysis.
*Controls: 22 mm $\leq$ axial length $\leq$ 24 mm except for Sichuan; -1 D $\leq$ spherical equivalent $\leq$ 1 D for Sichuan.
†Extreme myopia: axial length ≥ 28 mm.
‡Based on the logistic regression analysis.
§Only phakic samples whose refraction were available.
||Controls from Nagahama study and cases from Kyoto high myopia cohort.

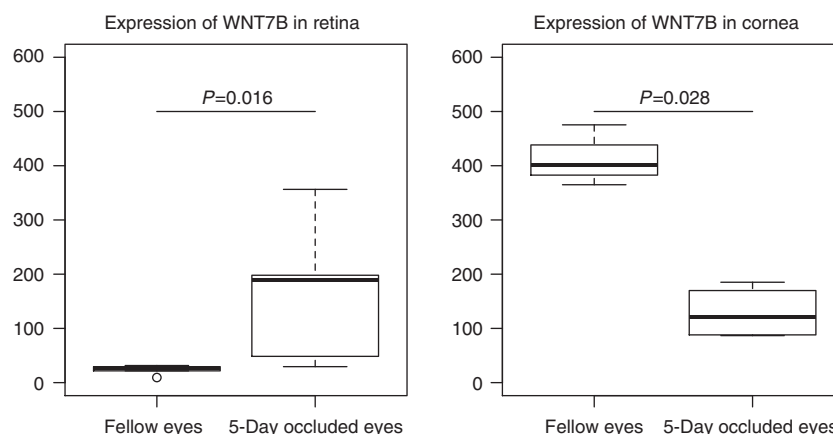


Figure 4 | Differential expression of WNT7B between treated eyes and fellow eyes in experimental myopia mice ($n = 5$ in each arm). The vertical axis indicates WNT7B expression normalized to β -actin expression. WNT7B expression in the retina was significantly upregulated in the experimental myopia eyes ($P = 0.016$ by Wilcoxon test), but was significantly downregulated in the cornea ($P = 0.028$ by Wilcoxon test).

contrast, *WNT7B* mRNA levels were significantly reduced in the corneas of myopia eyes ($P = 0.029$).

Discussion

We performed a GWAS in a large Japanese cohort and found that *WNT7B* was significantly associated with axial length and corneal curvature. The associations with axial length and corneal curvature were successfully replicated in both Chinese and Caucasian cohorts. In addition, the *WNT7B* genotype strengthened the effects of *GJD2*, a widely replicated myopia susceptibility gene^{11,23}. Furthermore, the *WNT7B* polymorphism rs10453441 was also significantly associated with extreme myopia. Our findings that *WNT7B* expression was downregulated in the cornea and upregulated in retinal ganglion cells in experimental myopia suggested that *WNT7B* plays a key role in controlling myopia development through compensatory mechanisms.

Although many candidate gene studies^{29–33} and GWASs^{10–12,34–39} have been conducted to investigate myopia-associated traits, little is known about the pivotal pathways involved in the development of myopia. The largest genome-wide meta-analysis conducted by CREAM¹¹ on refractive error and a recent large GWAS conducted by 23andMe³⁹ on age of myopia onset reported a total of 42 loci associated with myopia. Many of them were associated with signaling cascades in the neural retina, retinal pigment epithelium, choroid and sclera. However, not all the susceptibility genes were detected by previous GWASs because it is difficult to detect SNPs with low minor-allele frequencies or small effect sizes and because SNPs that were not included in the commercially available assay could not be evaluated. Even though a genomic imputation technique was used, the imputation quality was not necessarily high as it is dependent on whether imputed SNPs are in high LD with directly genotyped SNPs. To overcome this problem, we applied the dense 2.5 M chip for 53.7% of the samples and a 610 K chip for 49.3% of the samples, which were imputed to the intra-cohort reference panel (296 samples from the Nagahama Study cohort genotyped using all platforms). This strategy provided a dense, high-quality data set and enabled us to find a novel susceptibility gene for corneal curvature and axial length. The novel susceptibility gene, *WNT7B*, had stronger effects on axial length ($\beta = 0.13$) than the established susceptibility gene, *GJD2* ($\beta = 0.09$ in the current study and $\beta = 0.06–0.07$ in a previous report¹²).

Although the genotypes of the SNPs within *WNT7B* were not directly associated with refractive error in the Nagahama Study,

which only included a small number of subjects with extreme myopia (49 individuals in first and second stage), SNPs within *WNT7B* displayed significant associations with extreme myopia in our case-control studies. These data suggest that the effects of *WNT7B* might become apparent when the compensatory mechanisms of eye components to control refractive error are disrupted, resulting in extreme myopia. Therefore, we investigated the interactive effect of *WNT7B* on *GJD2* for refractive error. The effects of the T-risk allele of rs11073058 in *GJD2* on myopia were the largest when the genotype of *WNT7B* rs10453441 was AA. As the reported effect size of *GJD2* on refraction ranged from -0.10 D to -0.12 D (refs 12,40), the acceleration of myopia by *WNT7B* up to -0.54 D per copy of the T allele at *GJD2* rs11073058 in Asians is of note, which is statistically significant in the recessive model. Interestingly, this interactive effect was not apparent in Caucasian samples. This might be due to the limited sample size of the Caucasian cohorts, allelic differences between Asians and Caucasians, or it might, in part, explain the higher prevalence of myopia in Asians.

In a mouse model, *WNT7B* was downregulated in the cornea and upregulated in the retina of experimental myopic eyes. Considering that the effect of *WNT7B* rs10453441 on myopia through axial length is in the opposite direction to that through corneal curvature, *WNT7B* may be under strict control to correlate the anterior and posterior segments in the emmetropization of the eye. To date, accumulating evidence suggests that retinal image defocus is the trigger for myopia, and the role of amacrine cells in delivering an image defocus signal has been intensively investigated in the context of myopia development^{20,21,41}. Nevertheless, the precise mechanisms mediating the transduction of this defocus signal leading to myopia are still unclear. *GJD2* is reportedly localized at the synapses between alpha-ganglion cells, AII amacrine cells, cone bipolar cells and photoreceptor cells^{42–51}. Of all the interactions between these cells, the coupling between the amacrine cells and ganglion cells has been reported to be dramatically reduced in *GJD2* knockout mice⁵². Together with our findings indicating that *WNT7B* was localized to retinal ganglion cells and that its expression was significantly upregulated in experimental myopic eyes, these data support the hypothesis that *WNT7B* and *GJD2* co-operatively regulate the normal growth of the eye through interactions between the alpha-ganglion cells and AII amacrine cells. Interestingly, alpha-ganglion cells are predominantly observed in the peripheral retina and have large dendritic regions to receive inputs from amacrine cells^{47,53–57}. The hypothesis that alpha-

ganglion cells and AII amacrine cells are implicated in emmetropization is consistent with the peripheral defocus theory, a theory of myopia development that has been supported by clinical evidence^{58–61}.

Although the current study revealed for the first time the possible involvement of *WNT7B* in myopia development, the involvement of Wnt signaling itself in this process has been suggested previously. For example, we showed that polymorphisms in *PAX6* and δ -catenin (*CTNND2*) are associated with high-grade myopia^{29,32,33,35}. *PAX6* can directly control both *CTNND2* and *WNT7B* expression^{62,63}, and both molecular pathways can result in increased β -catenin nuclear translocation to activate Wnt signaling^{64,65}. Independently, the CREAM consortium found that polymorphisms in *RSPO1* and *ZNRF3*, which regulate Wnt signaling by controlling the turnover of Wnt receptors, were significantly associated with axial length^{12,66}. The present study suggests that *WNT7B* can be involved in the development of myopia. Further studies are needed to investigate interactions between genes in the same or different cascades of Wnt-signaling pathways. Better understanding of the pathophysiology underlying myopia will guide interventions for prevention and treatment.

In summary, we have demonstrated significant association of *WNT7B* with corneal curvature and axial length in three different ethnic groups and found that *WNT7B* could facilitate the development of myopia through cooperation with *GJD2*. Furthermore, the *WNT7B* polymorphism rs10453441 was significantly associated with extreme myopia in patients with Asian ancestry. In the retina, *WNT7B* was localized to ganglion cells, and its expression was significantly increased in mice with experimental myopia, whereas in the cornea its expression was localized to the endothelium and its expression downregulated in experimental myopia. We, therefore, propose that Wnt-signaling pathways that are vital for coordinating vision and disruption in these pathways leads to myopia.

Methods

Patient enrollment. There were 9,804 Japanese participants in the Nagahama Prospective Genome Cohort for Comprehensive Human Bioscience (the Nagahama Study). The Nagahama Study cohort was recruited from 2008 to 2010 from the general population living in Nagahama City, Shiga Prefecture, Japan^{67,68}. All ophthalmological measurements and blood sampling were performed at the time of enrollment. Genomic DNA was extracted from peripheral blood samples by the phenol-chloroform method. All study procedures were approved by the Ethics Committee of Kyoto University Graduate School of Medicine.

For the replication stage, we also included seven other cohorts that consisted of five Caucasian cohorts and two Singaporean Chinese cohorts. Detailed information and sample collections for these cohorts can be found in the Supplementary Methods. All patients were enrolled in the study after giving informed consent, ethical approval was granted by the SingHealth Centralized Institutional Review Board (cohorts SP2 and SCES), the Ethics Committee of the Medical School, University of Split and the NHS Lothian South East Scotland Research Ethics Committee (CROATIA-Split and CROATIA-Korcula) and the NHS Orkney Research Ethics Committee and the North of Scotland Research Ethics Committee (ORCADES). The Human Research and Ethics Committee of the Royal Victorian Eye and Ear Hospital, Melbourne (GEM) and the King Edward Memorial Hospital and Princess Margaret Hospital for Children ethics boards (RAINE). Genomic DNA was extracted from peripheral blood samples according to standard laboratory procedures.

Individuals who had undergone ocular surgery (with the exception of cataract surgery) and who had undergone ocular laser treatment were excluded from the analysis of all three traits. In addition, individuals who had undergone cataract surgery were excluded from analyses of the spherical equivalent and corneal curvature.

Evaluation of ophthalmological measurements. All the participants in the Nagahama Study had their axial length (millimeter (mm); IOL Master, Carl Zeiss Meditec, Dublin, CA, USA), spherical equivalent (diopter (D); ARK-530A, Nidek, Aichi, Japan) and corneal curvature (mm; ARK-530A, Nidek) measured for both eyes. Colour fundus photographs were also obtained from all the participants (CR-DG10, Canon, Tokyo, Japan). History of cataract surgery, ocular surgery other than cataract surgery and ocular laser treatment, including photocoagulation, was obtained using a questionnaire. For the replication cohorts, detailed information is given in the Supplementary Methods.

Genome-wide SNP genotyping. Genome-wide SNP genotyping was performed on samples from 3,710 participants who joined the Nagahama cohort from 2008 to 2009. A series of BeadChip DNA arrays, namely HumanHap610 Quad (1,828 samples), HumanOmni2.5-4 (1,616 samples), HumanOmni2.5-8 (378 samples), HumanOmni2.5s (192 samples) and HumanExome (192 samples; Illumina, San Diego, CA, USA) were used for the analysis. Some of the samples were repeatedly genotyped using different arrays, and 296 samples that had been genotyped using HumanHap610 Quad, HumanOmni2.5-8, HumanOmni2.5s and HumanExome were used as a reference panel in the genotype imputation. SNPs with a call rate <99%, MAF <1% and significant deviation from Hardy-Weinberg Equilibrium (*P* value for deviation < 1.0×10^{-7}) were excluded from further statistical analysis.

Samples with a call rate <95% (*n* = 162) were excluded from the analysis. Among the remaining 3,548 subjects, 295 estimated to have a first- or second-degree kinship within this population (π -hat > 0.35, PLINK ver. 1.07 (<http://pngu.mgh.harvard.edu/~purcell/plink/>)) and seven ancestry outliers identified by principal component analysis with the HapMap Phase 2 release 28 JPT data set as a reference (EIGENSTRAT ver. 2.0 (<http://www.hsph.harvard.edu/alkes-price/software/>)) were also excluded from the analysis.

Genotype imputation was performed using MACH ver. 1.0.16 software (<http://www.sph.umich.edu/csg/abecasis/MACH/tour/imputation.html>) and 1,792,015 SNPs from the 296-sample reference panel. Imputed SNPs for which the MAF was <0.01% or R^2 <0.5 were excluded from the following association analysis. Finally, 1,773,334 SNPs from 3,248 individuals were fixed.

Replication genotyping. We conducted a two-stage replication study. The first stage was the replication within the Nagahama Study participants and the second stage was the trans-ethnic replication.

For the first stage, we genotyped the subset of remaining samples from the Nagahama Study (*n* = 3,460) whose phenotypes were all available using TaqMan allelic discrimination probes (Applied Biosystems). For the second stage, genotypes of rs200329677 and/or rs10453441 were determined by one of the following methods: using the HumanOmni1M chip (Illumina), using TaqMan allelic discrimination probes (Applied Biosystems) or imputation from the existing data. The particular method used is described in Supplementary Table 2. We first used direct genotyping data. However, if only imputed data were available, we included them only when the imputation quality (R^2) was >0.5.

Statistical analysis. Genome-wide QTL analysis was conducted for the three myopia-related traits: axial length, spherical equivalent and corneal curvature. We used the mean values of both eyes for each trait. If one eye was not applicable, measurement of the other eye was used in the analysis. For every post-QC SNP, we evaluated the association between the genotypes and the trait using a multivariable linear regression, assuming an additive model. This regression framework allowed us to adjust for covariates such as age, sex and height. Experimental-wide significance was set at 2.82×10^{-8} , corresponding to *P* = 0.05 divided by the 1,773,334 SNPs included in the QTL analysis. We also carried SNPs with *P* values < 1.00×10^{-6} forward to the replication stage if their MAFs were at least 5%. Meta-analysis was conducted using inverse variance weights for 8,981 Asians, 2,835 Caucasians and all subjects. This process was performed using the METAL software (<http://www.sph.umich.edu/csg/abecasis/Metal/>). Estimates of effect size with standard error were compared using the *z*-test. Manhattan plots and forest plots (package 'metafor') were generated using R version 2.15 (<http://www.r-project.org>).

Association with extreme myopia. To investigate the association between *WNT7B* rs10453441 and extreme myopia, we performed two association tests. In these analyses, we defined extremely myopic patients as patients whose average axial lengths in both eyes were >28.00 mm, according to a previous report². Samples whose average axial lengths ranged from 22.00 to 24.00 mm were used as control samples.

We used two case-control data sets of the Japanese population. The first one was the Kyoto/Nagahama data set consisting of 938 highly myopic cases recruited from the Kyoto High Myopia cohort^{9,29,30} and 1,902 controls extracted from the Nagahama Study samples genotyped by the TaqMan method. The Kyoto High Myopia samples were also genotyped using the TaqMan method. The second cohort consisted of Yokohama samples, recruited from Yokohama City University, which contained 109 extreme myopia patients and 395 controls. They were both genotyped using Illumina OmniExpress at Yokohama City University. The demographics of the Kyoto High Myopia cohort and the Yokohama samples are shown in Supplementary Table 3. For further replication, three Chinese cohorts, that is, SCES, Hong Kong and Sichuan, were used for the same analysis. Chinese samples were also genotyped using the TaqMan method.

The analyses were performed using logistic regression. Meta-analyses were conducted using inverse-variance weights for two Japanese cohorts, three Chinese cohorts and all five cohorts.

Experimental myopia mouse model. To induce form-deprivation myopia in mice^{16,69,70}, we occluded the right eyes of male C57BL/6 mice (Japan SLC Inc., Shizuoka, Japan) with diffuser goggles from postnatal day 21. The mice were housed individually in standard mouse cages for 5 days at 25 °C on a 12 h:12 h light:dark cycle, with standard rodent chow pellets and water freely available. The

animal study was approved by the Kyoto University Animal Experimentation Committee. All the procedures performed in this study complied with the Association of Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmology and Vision Research.

Immunohistochemical staining. After a mouse was killed, eyes were enucleated and fixed with 4% paraformaldehyde in 0.1 M PB for 20 min. The expression of WNT7B in the retina was examined in vertical sections. For this, the eyes were cryoprotected in a sucrose gradient (10 and 30% w/v sucrose in 0.1 M PB), and 14- μ m cryostat sections were cut. The sections were blocked for 1 h in a solution containing 10% normal goat serum (Millipore), 0.3% Triton X-100 (Bio-Rad, Hercules, CA, USA) and 0.1 M PB. The primary antibodies were diluted in the blocking solution and incubated overnight at 4 °C, followed by incubation for 1 h in the secondary antibody solution. The primary antibodies used were 1:200 dilution of rabbit anti-Wnt7b (NOVUS, NBP1-59564) and 1:100 dilution of mouse anti-Brn-3a (CHEMICON, MAB1585). For secondary antibodies, 1:1,000 dilutions of Alexa Fluor 594 goat anti-rabbit IgG (H + L) and Alexa Fluor 488 goat anti-mouse IgG (H + L) antibodies (catalogue numbers A-11012 and A-11001, respectively, Invitrogen, Eugene, OR, USA) were used. All the steps were carried out at 4 °C. All the images were obtained using a KEYENCE BZ-9000 microscope.

Quantitative real-time polymerase chain reaction. To evaluate changes in the expression level of WNT7B at the early stage of myopia, we used experimental mice treated for 5 days. Gene expression in the corneas and retinas of treated and untreated eyes were compared using a Wilcoxon test.

Total RNA was isolated from mouse corneas or neural retinas using QIAzol Reagent (Qiagen, Tokyo, Japan) for the experimental eyes ($n = 5$) and untreated eyes ($n = 5$). RNA was purified using the RNeasy Micro kit (Qiagen).

Purified RNA was reverse transcribed into cDNA by using a ReverTra Ace quantitative real-time polymerase chain reaction (qPCR) RT Kit (Toyobo, Hiroshima, Japan). We performed qPCR using ThunderBird SYBR qPCR mix (Toyobo) to validate the gene expression. The sequences of the qPCR primers are shown in Supplementary Table 4. The reactions were run using an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) for 40 cycles under the following conditions: 95 °C for 15 s and 60 °C for 60 s. To normalize for input load of cDNA between samples, β -actin was used as an endogenous internal control. Threshold cycle (CT) was determined automatically, and relative change in mRNA expression was calculated using $\Delta\Delta$ CT values. All cDNA samples were tested in triplicate and the average values were used in statistical analysis.

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Author contributions

M.M., K.Y., Y.T., P.N.B. and N.Y. designed the study. M.M., K.Y., K.S., S.M., H.N., I.N., Y.A.-K., N.G., A.T., S.K., M.M. and K.O.-M. gathered the clinical data. Y.T., C.-C.K., P.C., F.Q., A.M., O.P., C.H., A.F.W., H.C., A.J.R., M.S., M.T., D.A.M., A.W.H., G.C., Y.S., L.H., Z.Y., K.H.L., P.Y.P.K., M.K.H.Y., S.P.Y., N.M., S.M.G., V.V., T.A., S.-M.S., E.-S.T., T.Y.W. and C.-Y.C. generated the genetic data. M.M., P.C., F.Q., A.M. and R.Y. analysed the data. M.M., K.Y., Y.T., P.N.B. and R.Y. interpreted the data. M.M., K.Y., S.P.Y., V.V. and P.N.B. drafted the paper. All the authors contributed towards revision of the paper.

Additional information

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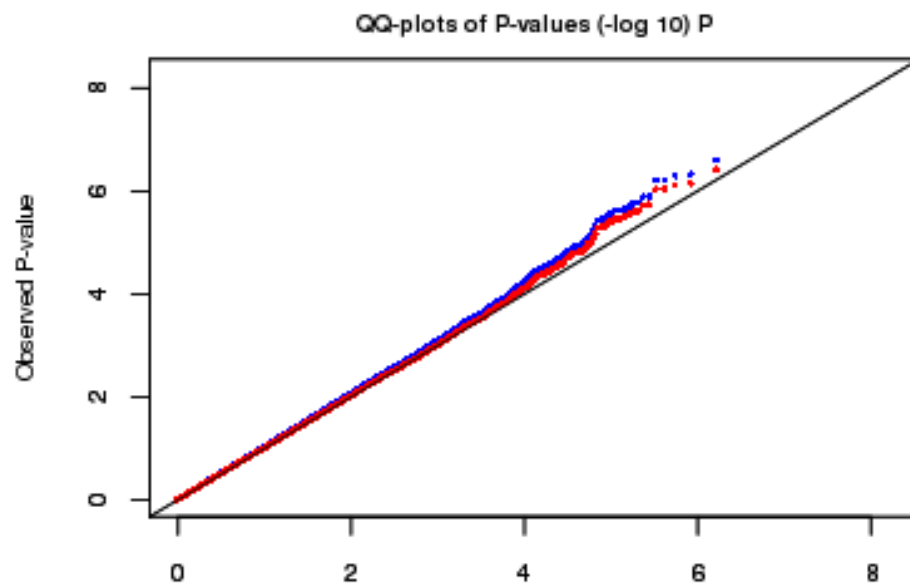
Takeo Nakayama²⁵, Akihiro Sekine²⁶ and Shinji Kosugi²⁷

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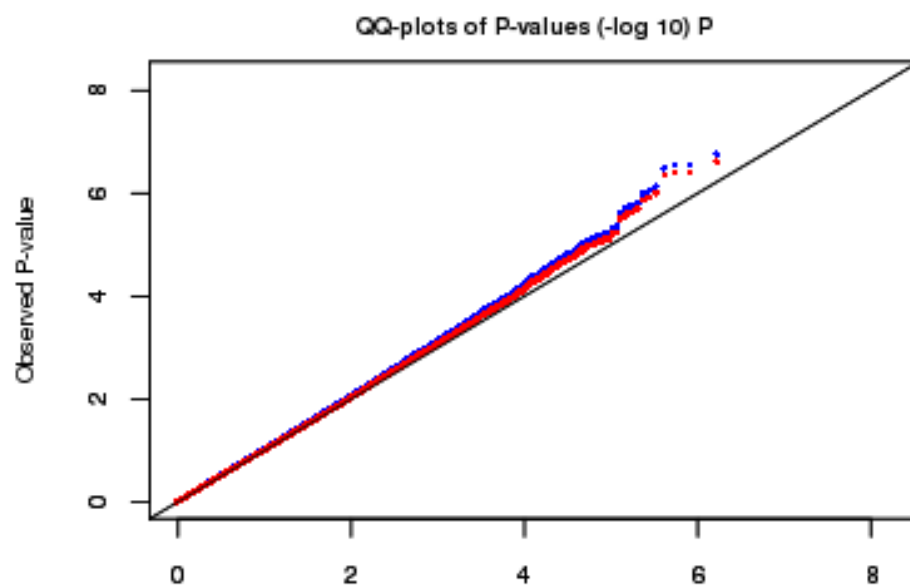
Supplementary Figures

Supplementary Figure 1

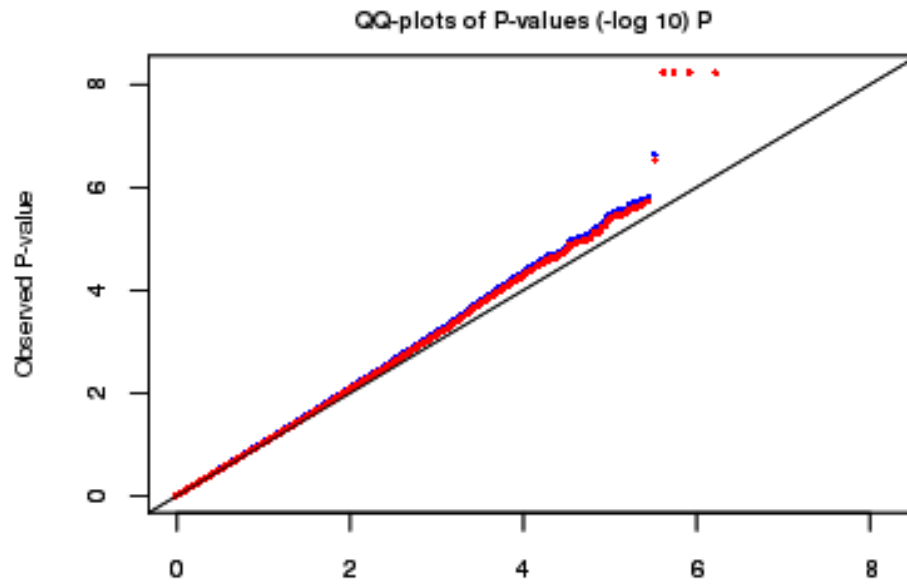
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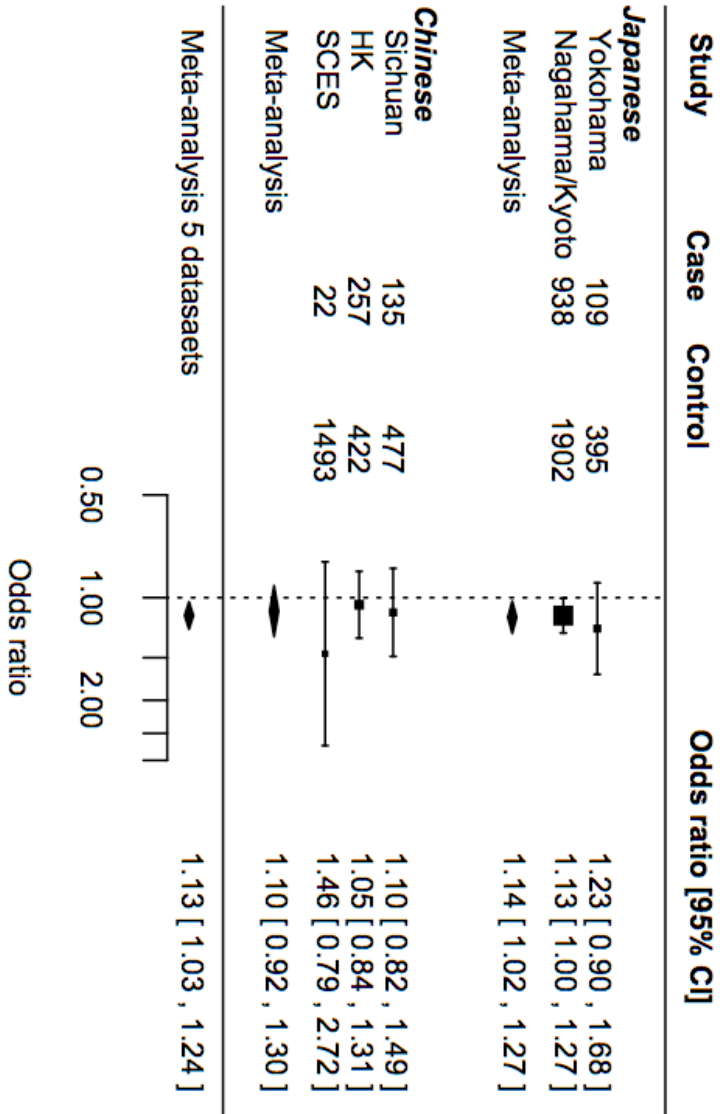
C



Supplementary Figure 1. Quantile-quantile (QQ) plot for association between all SNPs analyzed and 3 traits within the Nagahama study. Each blue dot represents an observed statistic (defined as $\log_{10} P$) versus the corresponding expected statistic before genomic control, while each red dot represents the observed statistic versus the corresponding expected statistic after genomic control. The black line corresponds to the null distribution. **A.** QQ plot for the QTL analysis for axial length. **B.** QQ plot for the QTL analysis for spherical equivalent. **C.** QQ plot for the QTL analysis for corneal curvature.

Supplementary Figure 2

WNT7B rs10453441 and extreme myopia



Supplementary Figure 2. Forest plot of the association between WNT7B rs10453441 and extreme myopia.

Supplementary Tables

Supplementary Table 1. Description of the cohorts.

	Cohort ^a	n	Ethnicity	Age (years)	Sex (male:female)	Axial length ^b	Spherical equivalent ^b	Corneal curvature ^b
Discovery	Nagahama Study	3248	Japanese	52.2 ± 14.1	1092:2154	24.10 ± 1.38	-1.69 ± 2.78	7.68 ± 0.26
Replication	Nagahama Study	3460	Japanese	54.7 ± 12.5	1103:2247	23.90 ± 1.28	-1.25 ± 2.66	7.66 ± 0.26
	SCES	1923	Chinese	57.8 ± 9.0	987:936	23.98 ± 1.37	-0.80 ± 2.66	7.66 ± 0.26
	SP2	851	Chinese	46.7 ± 10.2	543:308	-	-1.80 ± 2.84	7.75 ± 0.28
	ORCADES	735	Caucasian	54.3 ± 14.7	254:481	23.54 ± 1.03	0.09 ± 2.09	-
	GEM	635	Caucasian	48.9 ± 15.0	413:222	24.45 ± 1.63	-0.55 ± 2.39	8.00 ± 1.08
	CROATIA-Korcula	969	Caucasian	56.3 ± 14.2	346:623	23.20 ± 1.12	-0.24 ± 1.82	7.77 ± 0.31
	CROATIA-Split	472	Caucasian	51.5 ± 14.0	169:303	23.42 ± 1.00	-1.07 ± 1.70	7.72 ± 0.28
	RAINE	108	Caucasian	20.0 ± 0.4	64:44	23.65 ± 0.93	-0.19 ± 1.86	7.75 ± 0.27

a. Abbreviations are described in the Supplemental notes. b. Data from samples included in the analysis.

Supplementary Table 2. Summary of the genotyping method of each cohort in the replication stage.

Cohort name	Genotyping method
Nagahama Study (replication)	Taqman assay
SCES	Taqman assay
SP2	Illumina 1M-Duov3
ORCADES	Illumina HumanOmniExpress
GEM	Taqman assay
CROATIA-Korcula	Imputed data
CROATIA-Split	Illumina HumanOmniExpressExome
RAINE	Illumina HumanOmniExpress

Supplementary Table 3. Demographics of the highly myopic cases and controls.

Items	Kyoto High Myopia	Yokohama Study		Hong Kong		Sichuan	
		case	control	case	control	case	control
Patients, n	1340	471	519	257	422	135	477
Age in years, mean ± SD	57.1 ± 14.9	40.4 ± 12.5	59.7 ± 11.3	30.7 ± 8.6	30.1 ± 9.3	38.9 ± 19.4	64.6 ± 4.9
Sex, n (%)							
Male	188 (39.9%)	188 (39.9%)	220 (42.4%)	99 (38.5%)	133 (31.5%)	63 (46.7)	173 (36.3)
Female	283 (60.1%)	283 (60.1%)	298 (57.4%)	158 (61.5%)	289 (68.5%)	72 (53.3)	304 (63.7)
Axial length ^a , mm							
	27.25	27.25	23.20	28.65	23.36	28.81	23.20
Right eyes	(26.60, 28.01)	(26.60, 28.01)	(22.63, 23.74)	(28.28, 29.24)	(23.01, 23.69)	(28.24, 30.21)	(22.59, 23.62)
	27.21	27.21	23.13	28.62	23.28	28.77	23.15
Left eyes	(26.46, 27.98)	(26.46, 27.98)	(22.63, 23.71)	(28.22, 29.16)	(22.93, 23.64)	(28.25, 29.86)	(22.60, 23.64)
Refraction of the phakic eyes ^a , D							
	-10.25	-10.25	0.50	-11.13	0.25	-13.0	-0.50
Right eyes	(-11.50, -9.50)	(-11.50, -9.50)	(0.00, 1.00)	(-13.25, -9.68)	(-0.13, 0.50)	(-18.0, -10.0)	(-0.75, -0.50)
	-10.00	-10.00	0.50	-11.00	0.25	-13.0	-0.50
Left eyes	(-11.25, -9.25)	(-11.25, -9.25)	(0.00, 1.00)	(-12.88, -9.86)	(-0.13, 0.50)	(-17.0, -10.0)	(-0.75, -0.50)
Genotyping	Taqman	illumina OmniExpress		Taqman		Taqman	

a. Data are displayed as the median (first quartile, third quartile)

SD: standard deviation, D: diopter

Supplementary Table 4. qPCR primers

Gene	Direction	Sequence
Wnt7b	Forward	TGTCAGGCTCCTGTACCACC
	Reverse	AGTTGGGCGACTTCTCGATG
β -Actin	Forward	CATCCGTAAAGACCTCTATGCCAAC
	Reverse	ATGGAGCCACCGTCCACA

Supplementary Note 1

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Supplementary Methods

Subjects and genotyping

SP2 (Singapore Prospective Study Program)

SP2 samples were from a revisit of two previously conducted population-based surveys carried out in Singapore between 1992 and 1998, including the National Health Survey 1992 and the National Health Survey 1998¹. These studies comprised random samplings of individuals stratified by ethnicity from the entire Singapore population. A total of 8,266 subjects were invited to participate in this follow-up survey, and 6,301 (76.1% response rate) subjects completed the questionnaire, of which 4,056 (64.4% of those who completed the questionnaire) also attended the health examination and donated blood specimens. Autorefractometry and keratometry were measured using an autorefractor (Canon RK-5 Auto Ref-Keratometer, Canon Inc. Ltd., Japan). The study followed the principles of the Declaration of Helsinki, and the protocol was approved by the SingHealth Centralized Institutional Review Board. Informed consent was obtained from all participants.

The present genotyping for SP2 involved individuals of Chinese descent only ($n = 2,867$)². Of the 2,867 blood-derived DNA samples, 1,016 samples were genotyped on the Illumina 1M-Duov3. We excluded samples based on the following conditions: sample call rates of less than 95%, excessive heterozygosity, cryptic relatedness by IBS, population structure ascertainment, and gender discrepancies. During the SNP quality control procedure, we excluded SNPs with low genotyping call rates ($> 5\%$ missingness), monomorphic genotypes, MAFs less than 1%, or significant deviation from Hardy-Weinberg equilibrium (HWE; $P < 10^{-6}$ by chi-square test). A total of 851 samples with phenotype information were available after genotype quality controls.

SCES (Singapore Chinese Eye Study)

SCES was a population-based cross-sectional study of eye diseases in Chinese adults 40 years of age or older residing in the southwestern part of Singapore. The methodology of the SCES has been described in detail in previous reports.³ In brief, between 2009 and 2011, 3,353 (72.8%) of 4,605 eligible individuals underwent a comprehensive ophthalmologic examination. Autorefractometry, keratometry, and ocular biometry were measured using an autorefractor (Canon RK-5 Auto Ref-Keratometer, Canon Inc. Ltd.) and the IOL Master (Carl Zeiss; Meditec AG Jena, Germany), respectively. Venous blood samples were collected for DNA archival. Extracted DNA samples were separated into aliquots and stored at -80°C for further genetic analysis. Individuals were excluded from the study if they had cataracts or previous refractive surgery in both eyes or missing refraction or

ocular biometry data. The study followed the principles of the Declaration of Helsinki, and ethical approval was obtained from the SingHealth Centralized Institutional Review Board. Informed consent was obtained from all participants.

CROATIA-Split Study

The CROATIA-Split study, Croatia, was a population-based, cross-sectional study in the Dalmatian City of Split that included 1,012 examinees ages 18 to 95 years. The study received approval from relevant ethics committees in Scotland and Croatia and followed the tenets of the Declaration of Helsinki. Keratometry and noncycloplegic autorefraction were measured on each eye using a NIDEK Ark30 hand-held autorefractometer/keratometer. Axial length was measured with the Echoscans US-1800 instrument from Nidek. Measurements on eyes with a history of trauma, intra-ocular surgery, LASIK operations, and keratoconus were excluded, and analyses were conducted using the average of both eye measurements or on one eye measurement only when the measurement for the fellow eye was missing. Only the half of the cohort that had been genotyped with the Illumina HumanOmniExpressExome was analyzed here. Association analysis was performed using the GenABEL package using an additive SNP allelic effect model and correcting for individual relatedness using the `polygenic_hglm` function.

CROATIA-Korcula Study

The CROATIA-Korcula study, Croatia, was a population-based, cross-sectional study that included a total of 969 adult examinees, ages 18 to 98 years (mean age = 56.3 years), and most participants (N = 930) underwent a complete eye examination. The study received approval from relevant ethics committees in Scotland and Croatia and followed the tenets of the Declaration of Helsinki. Non-cycloplegic autorefraction was measured for each eye using a Nidek Ark30 hand-held autorefractometer. Measurements were performed on eyes with a history of trauma, intra-ocular surgery, or LASIK operations were excluded, and the analyses were conducted using the average spherical equivalent of both eye measurements or one eye measurement only when the measurement for the fellow eye was missing. Keratometry and non-cycloplegic autorefraction were measured on each eye using a Nidek Ark30 hand-held autorefractometer/keratometer. Axial length was measured with the Echoscans US-1800 instrument from Nidek. Measurements on eyes with a history of trauma, intra-ocular surgery, LASIK operations, or keratoconus were excluded, and the analyses were performed using the average of both eye measurements or one eye measurement only when the measurement for the fellow eye was missing. Data were genotyped using an Illumina HumanCNV370-Duo and imputed to the 1000Gv3-Phase1 integrated (March 2012 release) references using `shapeit2` and `impute2` software with all ancestry references. Association analysis was performed in the GenABEL package using SNP doses from the 1000Gv3 imputation (after

converting imputations to mach format) as a predictor variable and correcting for individual relatedness using the polygenic_hglm function.

Orkney Complex Disease Study (ORCADES)

The Orkney Complex Disease Study (ORCADES) was a population-based, cross-sectional study in the Scottish archipelago of Orkney, including 1,285 individuals with eye measurements. The study received approval from relevant ethics committees in Scotland and followed the tenets of the Declaration of Helsinki. Autorefractive measurements were obtained using a Kowa KW 2000 autorefractometer, and axial length was obtained using IOLmaster. Measures on eyes with a history of trauma, intra-ocular surgery, or LASIK operations were excluded, and the analyses were performed using the average of both eye measurements or one eye measurement only when the measurement for the fellow eye was missing. Over half of the participants with eye measurements had been genotyped with the Illumina HumanOmniExpress array, and the others had been genotyped with a mixture of Illumina Human Hap3000 v2 and Illumina HumanCNV370-Quad. Association analysis was performed in the GenABEL package using either directly genotyped rs10453441 (ORCADES-OMNI) as described for the CROATIAS-Split data or imputed doses from 1000Gv3 imputation in the whole dataset (ORCADES_1000G) as described for CROATIA-Vis and CROATIA-Korcula.

GEM

The methodologies used for recruitment of subjects and obtaining informed consent were published previously^{4,5}. Individuals were included in the study if they did not have any other known ocular or systemic disease, did not have anisometropia of greater than 2D difference between both eyes, and were of European ancestry. Myopia was defined as less than or equal to -0.5 D in the right eye. Controls were defined as greater than -0.5 D in the right eye. DNA from all consenting individuals was collected from venous blood samples. Written informed consent was obtained from all individuals prior to any clinical examination, and ethics approval was provided by the Human Research and Ethics Committee of the Royal Victorian Eye and Ear Hospital, Melbourne. This study adhered to the tenets of the Declaration of Helsinki. Genotyping was undertaken by Kyoto University. Samples were genotyped as stated in the manuscript.

RAINE

The Western Australian Pregnancy Cohort (Raine) Study originated as a randomized-controlled trial of 2,900 women recruited from the state's largest maternity hospital. DNA was collected from their offspring and genotyped using Human660W-Quad BeadChip (N = 1,592) and HumanOmniExpress BeadChip (N = 310). After our standard quality control and exclusion of individuals from non-Caucasian descent, genomic imputation was performed on 1,208 of the individuals genotyped with the Human660W-Quad BeadChip. A total of 1,344 of the participants completed a comprehensive eye assessment at the age of 20 years. Axial length

and corneal curvature were measured with IOLMaster V.5 (Carl Zeiss Meditec AG). For axial length, five consecutive measurements were taken until the following criteria were satisfied: measurements within ± 0.02 mm of each other; good waveform, i.e., no double peaks; and acceptable signal-to-noise ratio > 2.0 . Any measurement outside the mentioned criteria was deleted and repeated. During keratometry, three measurements within 0.3 D within each meridian with careful alignment and focus were recorded. Finally, a total of 1,105 individuals with genotypes (i.e., 108 genotyped and 997 imputed) and phenotypes were included in the study.

Kyoto High Myopia cohort

A total of 1,340 unrelated Japanese patients with high myopia (axial length ≥ 26 mm in both eyes) were recruited from the Kyoto University Hospital, Tokyo Medical and Dental University Hospital, Fukushima Medical University Hospital, Kobe City Medical Center General Hospital, and Ozaki Eye Hospital. All the patients underwent a comprehensive ophthalmic examination, including dilated indirect and contact lens slit-lamp biomicroscopy, automatic objective refraction, and measurements of axial length by applanation A-scan ultrasonography or partial coherence interferometry (IOLMaster, Carl Zeiss Meditec, Dublin, CA, USA).

Yokohama Study

A total of 471 unrelated Japanese patients with high myopia (refractive error ≤ -9.00 D in at least one eye) were recruited from the Yokohama City University and Okada Eye Clinic. All the patients were diagnosed by comprehensive ophthalmologic tests, including spherical power and axial length using autorefractors (ARK-730A [NIDEK, Gamagori, Japan], ARK-700A [NIDEK], or KP-8100P [TOPCON, Tokyo, Japan]) and an ultrasound pachymeter (AL-2000 [Tomey Corporation, Nagoya, Japan]). The study details were explained to all the patients before obtaining their consent for genetic analysis. DNA was collected from peripheral blood cells and genotyped with the Illumina HumanOmniExpress array using the standard protocol recommended by Illumina.

Myopia Genomics Study of Hong Kong

For the Myopia Genomics Study of Hong Kong, unrelated Chinese subjects ages 18 to 45 years were recruited via the Optometry Clinic at The Hong Kong Polytechnic University, as has been described previously⁶⁻⁸. Briefly, ophthalmic examination was carried out with refraction measured by cycloplegic autorefraction in addition to the measurement of lens thickness, axial length and corneal curvature. Any subjects showing signs of eye diseases or other inherited diseases associated with myopia were excluded from the study. In the original study,

a case was defined by a refractive error of spherical equivalent of -8.0 D or worse for both eyes, while a control was defined by a refractive error of spherical equivalent within ± 1.0 D. All samples were genotyped for the SNPs concerned, but samples from subjects fulfilling the specific criteria of this international collaborative study were selected for association analysis by PLINK. Cases were defined as having a spherical equivalent of -10 D or less or an axial length of 28 mm or more, and controls were defined as having a spherical equivalent within ± 1.0 D or with an axial length ranging from 22 to 24 mm (inclusive). The SNP rs10453441 was genotyped by unlabeled probe melting analysis, as described previously⁸⁻¹⁰.

Sichuan

The details of this cohort are described elsewhere^{11,12}. Briefly, patients in this cohort consisted of the Shanghai Han Chinese dataset of 419 unrelated individuals with high myopia and 669 unrelated normal controls. These participants came from the Shanghai and Anhui region and were recruited at the Xinhua Hospital Ophthalmic Clinic, Shanghai Jiao Tong University and at the clinics of the hospital affiliated with Anhui Medical University in China. The diagnosis of high myopia in this study required the spherical equivalent to be less than or equal to -6.0 DS in at least one eye and the axial length of the eye globe to be greater than or equal to 26.0 mm.

Individuals were excluded from the study if they had undergone ocular procedures that might alter refraction or if they had other symptoms besides high myopia (e.g., in addition to eye problems, individuals with Stickler syndrome suffer from distinctive facial abnormalities, hearing loss, and joint phenotypes). Individuals with retinopathy of prematurity (ROP) were also excluded from the study if an individual was prematurely born according to the criteria of the International Classification of Retinopathy of Prematurity (ICROP). For the controls, the criteria were a spherical equivalent from -0.5 to $+1.0$ DS and no evidence of disease in either eye. The institutional review board of Xinhua Hospital, Shanghai Jiao Tong University and the institutional review board of Anhui Medical University, Anhui, China both approved this project. Informed consent was obtained from all participants of this cohort.

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Genome-wide association study identifies *ZFHX1B* as a susceptibility locus for severe myopia

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Severe myopia (defined as spherical equivalent < -6.0 D) is a predominant problem in Asian countries, resulting in substantial morbidity. We performed a meta-analysis of four genome-wide association studies (GWAS), all of East Asian descent totaling 1603 cases and 3427 controls. Two single nucleotide polymorphisms (SNPs) (rs13382811 from *ZFHX1B* [encoding for ZEB2] and rs6469937 from *SNTB1*) showed highly suggestive evidence of association with disease ($P < 1 \times 10^{-7}$) and were brought forward for replication analysis in a further 1241 severe myopia cases and 3559 controls from a further three independent sample collections. Significant evidence of replication was observed, and both SNP markers surpassed the formal threshold for genome-wide significance upon meta-analysis of both discovery and replication stages ($P = 5.79 \times 10^{-10}$, per-allele odds ratio (OR) = 1.26 for rs13382811 and $P = 2.01 \times 10^{-9}$, per-allele OR = 0.79 for rs6469937). The observation at *SNTB1* is confirmatory of a very recent GWAS on severe myopia. Both genes were expressed in the human retina, sclera, as well as the retinal pigmented epithelium. In an experimental mouse model for myopia, we observed significant alterations to gene and protein expression in the retina and sclera of the unilateral induced myopic eyes for *Zfhx1b* and *Sntb1*. These new data advance our understanding of the molecular pathogenesis of severe myopia.

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INTRODUCTION

Myopia is a world-wide eye disability, suspected to be due to both genetic and environmental influences. The heritable elements responsible for predisposition to myopia are difficult to identify and dissect due to substantial confounding by environmental factors. This is particularly complicated in studies of common myopia, typically defined as spherical equivalent (SE) of < -0.5 D, in which the robust identification of genetic determinants requires the deployment of very large sample sizes (1–3).

Natural variation in ocular biometric quantitative traits has been shown to be significantly influenced by a large number of genetic sequence variants, each conferring modest effect sizes (4–7). Thus far, three genome-wide association studies (GWAS) on SE quantitative trait have been performed on ever larger sample sizes, revealing very strong evidence of association between multiple single nucleotide polymorphism (SNP) markers and inter-individual SE variation (8–11). Notably, the effect sizes for all of these identified loci are modest, suggesting a multi-factorial etiology and possible presence of unmeasured environmental confounding, in keeping with other common diseases (12). To a more limited extent, GWAS on severe myopia (defined as SE < -6.0 D in either eye) has also been performed (13–16), and robustly associating genetic loci surpassing the formal threshold for genome-wide significance ($P < 5 \times 10^{-8}$) are now beginning to emerge (15,16).

Severe myopia is more common in East Asians than whites, affecting up to 10% of the population aged 40 years and older, and can be associated with vision loss due to myopic macular degeneration, glaucoma and retinal detachment. To date, successful GWAS of severe myopia have been conducted in individuals of Chinese descent from China (15,16), the most recent of which involved 665 cases and 960 controls in the discovery stage (15). To identify further genetic determinants for severe myopia, we conducted a meta-analysis of four genome-wide association studies across four independent Asian collections enrolled from mainland China, Hong Kong, Japan and Singapore (Table 1). The collections from China and Hong Kong have been described in prior publications. Altogether, these total 1603 severe myopia cases and 3427 emmetropic controls. Replication experiments were conducted in a further 1241 severe myopia cases and 3559 controls from Hong Kong, Japan and Singapore.

RESULTS

After stringent quality control filters on samples and SNPs (see Materials and Methods), a total of 494 215 SNPs (see Supplementary Material for summary statistics of the complete GWAS dataset) were observed to be successfully genotyped in two or more sample collections, and 250 531 SNPs were found common across all four study collections. Statistical associations between each SNP genotype and severe myopia were measured using logistic regression, modeling for a trend per-copy effect of the minor allele. A quantile–quantile (QQ) plot of the P -values obtained from the four-collection meta-analysis showed low evidence of genomic inflation of the association test statistics ($\lambda_{gc} = 1.057$), giving minimal evidence of cryptic population stratification or differential genotyping success rates between cases and controls which could confound the association results. Observed against this background was several point P -values showing extreme deviation

at the tail end of the QQ distribution. These data points could represent true positive associations with severe myopia, and reflected two distinct genetic loci ($P < 1 \times 10^{-7}$) (Supplementary Material, Figs S1 and S2). The first locus is rs13382811 mapping within *ZFHX1B* (also known as *ZEB2*; per-allele odds ratio (OR) = 1.33, $P = 7.44 \times 10^{-9}$) (Supplementary Material, Fig. S3). The second marker is rs6469937 mapping within *SNTB1* (per-allele OR = 0.75, $P = 6.08 \times 10^{-8}$) (Supplementary Material, Fig. S4), which is located within the same linkage disequilibrium region as the three SNP markers recently described as strongly associated with severe myopia (15).

A power calculation based on a per-allele OR of between 1.20 and 1.30 (due to the winner's curse phenomenon), minor allele frequency between 0.20 and 0.30, and a replication sample size of 1232 severe myopia cases and 3559 emmetropic controls showed that only SNPs surpassing $P = 1 \times 10^{-7}$ in the GWAS discovery stage would be sufficiently powered to achieve genome-wide significance upon meta-analysis should the effect be a true positive (Supplementary Material, Table S1). Based on these estimations, we proceeded to conduct replication experiments of both SNPs in a further 1232 severe myopia cases and 3559 emmetropic controls enrolled across three countries (Table 1). Both SNP markers showed significant evidence of replication (Fig. 1A and B, Supplementary Material, Table S2a and b), with minimal to no heterogeneity across the three study collections ($0\% \leq I^2 \text{ index} < 25\%$). Meta-analyzing data from all seven collections, we note genome-wide significant association with severe myopia ($P = 5.79 \times 10^{-10}$, per-allele OR = 1.26 for rs13382811 and $P = 2.01 \times 10^{-9}$, per-allele OR = 0.79 for rs6469937) for both SNP markers, again with no evidence of inter-collection heterogeneity.

Differential gene expressions for *ZEB2* and *SNTB1* from the tissues of myopic (with SE < -5.0 D) and fellow non-occluded eyes of the experimental mice were compared with age-matched control tissues (Fig. 2). The mRNA levels of *ZEB2* and *SNTB1* were significantly downregulated in myopic retina/RPE compared with naive control retina/RPE and this was upregulated in the myopic sclera. Fold change for *ZEB2* in retina/RPE/sclera $-3.1/-7.8/2$; $P = 0.00004$; $P = 0.0002$ and $P = 0.0003$, respectively. Fold change for *SNTB1* in retina/RPE/sclera $-5.6/-22/17.4$; $P = 0.0001$; $P = 0.0008$ and $P = 0.00006$, respectively.

We performed additional analysis assessing for association within the current severe myopia dataset for previously reported severe myopia loci (Supplementary Material, Table S3), as well as previously reported loci influencing SE (Supplementary Material, Table S4) identified via the GWAS approach. All SNP markers showing evidence of association surpassing $P < 1 \times 10^{-4}$ in the GWAS meta-analysis discovery stage are shown in Supplementary Material, Table S5.

DISCUSSION

Severe myopia is much more extreme phenotype compared with common myopia (defined as SE < -0.5 D) and the study of individuals at the more severe end of the quantitative phenotype spectrum has documented usefulness (17,18). Furthermore, severe myopia can cause significant visual impairment via myopic macular degeneration, retinal detachment and glaucoma. Severe myopia is more common in East Asians, affecting 5–10% of persons 40 years and older. In an attempt to minimize

Table 1. Sample collections used in both stages of the study.

Collection	Cases	Age (mean; [range])	Controls	Age (mean; [range])	Genotyping platform
GWAS stage					
China (Sichuan)	419	34.3 [12–76]	669	32.7 [22–55]	Illumina 610K
Hong Kong	232	50.6 [12–87]	244	69.0 [39–94]	Illumina 370K
Japan	500	57.8 [14–91]	1194	50.0 [20–79]	Illumina 550K
Singapore	452	40.9 [10–75]	1320	43.5 [10–85]	Illumina 610K
Total GWAS	1603		3427		
Replication stage					
Hong Kong	106	56.2 [21–85]	178	75.0 [55–99]	
Japan	728	56.6 [11–86]	3248	52.2 [30–75]	
Singapore	407	19.5 [16–37]	133	18.7 [16–25]	
Total replication	1241		3559		
Total samples	2844		6986		

spurious associations due to population stratification or environmental effects (as >70% Asians are myopic to some degree), we utilized emmetropic adults ($-0.5 \text{ D} < \text{SE} < +1.0 \text{ D}$) as far as possible, adjusted for genetic stratification, and included multiple populations in the study design. Also, we only considered markers showing low inter-cohort heterogeneity, in order to avoid genotyping artifacts driven by specific sample collections. This current study in 2844 severe myopia cases and 6986 controls identified *ZFHX1B* as a new susceptibility locus for severe myopia, and confirmed a recent observation at *SNTB1* where the minor allele of several SNP markers were significantly associated with decreased susceptibility towards severe myopia.

Many different experimental animal models (chick, rabbit, tree shrew, macaque and tree shrew) (19,20) had been used for the studies of emmetropization and myopia generation. These animal models were used to characterize the optical parameters of and study the mechanisms of induced myopia (21). Studies of the chick eye have formed the basis for several hypotheses of myopic development, but the chick does not possess a fovea or retinal blood supply. It is thus unclear whether these differences alter the pathways of emmetropization. Even closely related primate species can exhibit different responses to form deprivation conditions, suggesting differing mechanisms of eye growth control. Monocular occlusion of the rhesus macaque, for instance, results in myopia when the ciliary muscle is paralyzed or the optic nerve cut, but does not in the stump tailed macaque, suggesting a role of excessive accommodation in the development of myopia in the stump tail but not the rhesus.

Given such variability in the models, a persisting element of continued myopia research must be an evaluation of the relevance of any given model to the human condition. In this regard, the study of changing patterns of gene expression within and among species during emmetropization and myopic progression may offer a productive avenue for future research. Experimental myopia has been induced in the mouse by us and others (22–24). The mouse myopia model was developed and assayed because of the availability of the whole-genome sequence, comprehensive protein database and more importantly, the availability of molecular tools like whole-genome gene chip. With our mouse models and noninvasive methods for measuring and monitoring axial length, we were able to monitor the progress of myopia in the same animal without the need to sacrifice it.

ZFHX1B encodes for Zinc finger E-box-binding homeobox 2, also known as SMAD-interacting protein-1 (SMADIP1). It

functions as a transcriptional co-repressor involved in the transforming growth factor- β (TGF- β) signaling pathway, and has also been implicated in Mowat–Wilson syndrome (25,26). Members of this TGF- β signaling pathway, such as *BMP2* and *BMP3*, have only recently been implicated in a large multi-ethnic GWAS ($N > 45\,000$) as quantitative trait loci for SE (27). Notably, multiple genes encoding for zinc finger proteins (including *ZIC2* (27), *ZC3H1B* (28) and *ZNF644* (29)) have been shown to associate with refractive error in recent GWAS and exome sequencing studies. Mowat–Wilson syndrome is a genetic condition that could affect multiple distinct parts of the body. Common manifestations of this disorder frequently include characteristic facial features, intellectual disability, delayed development, Hirschsprung disease and other associated birth defects. This syndrome is often associated with an unusually small head (microcephaly), structural brain abnormalities and intellectual disability ranging from moderate to severe. Although ocular disorders are not particularly pronounced in patients with Mowat–Wilson syndrome apart from wide-set eyes, DNA sequencing experiments performed on a patient presenting with Down syndrome, Hirschsprung disease, high myopia and ocular coloboma revealed a non-synonymous amino acid substitution (953Arg→Gly) which was not present in 200 matched normal chromosomes (30). This significant association observed between natural genetic variation within *ZFHX1B* and severe myopia is thus in keeping with current knowledge on the biological pathways for refractive errors. The second locus, *SNTB1*, has only been recently described as a susceptibility locus for severe myopia [15]. It encodes for Beta-1 syntrophin. The Syntrophin family of proteins associate with ion channel and signaling proteins of the dystrophin-associated protein complex. Syntrophins have a diverse role of acting as molecular adaptors for many cellular signaling pathways (31,32). Beta-1 syntrophin, one of the homologous isoforms, has been implicated in the regulation of ABCA1 protein levels in human fibroblast cell lines (33).

We were able to show nominal evidence of association ($0.001 < P < 0.05$) at some of the previously reported GWAS loci for high myopia, namely *CTNND2*, *MIPEP-C1QTNF9B* on Chromosome 13q12 and *VIPR2* (Supplementary Material, Table S1). For the previously reported SE loci, we could observe this level of association at directly genotyped SNPs found at Chromosome 15q14 and 15q25, which were the first SE loci identified via GWAS. The allele associated with

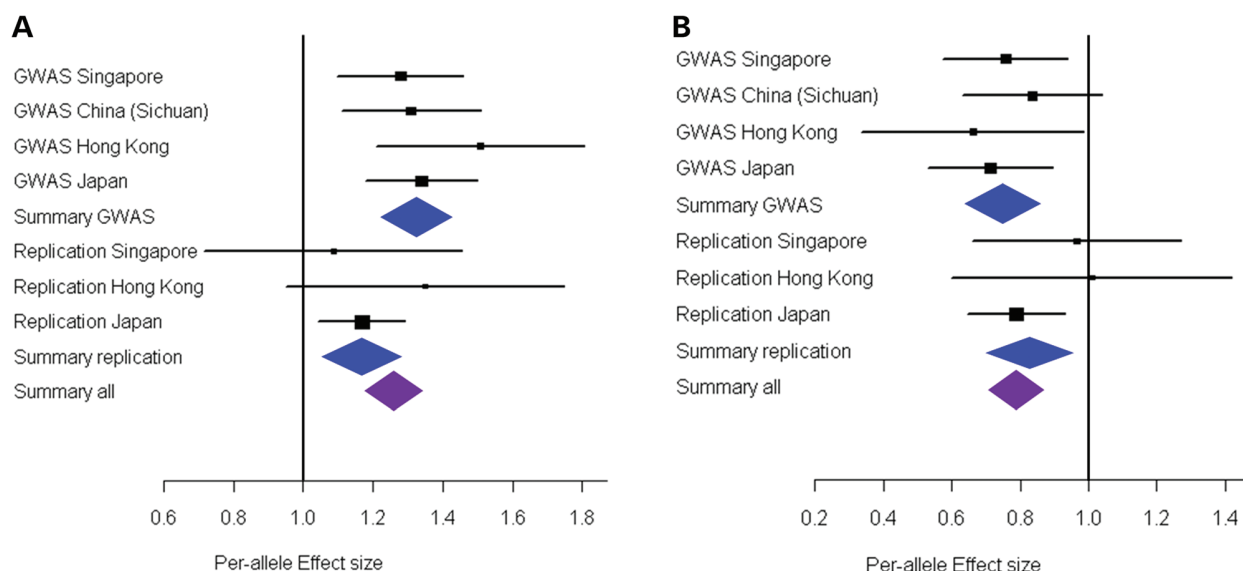


Figure 1. (A) Forest plot of the meta-analysis for all sample collections at *ZFHX1B* rs13382811. (B) Forest plot of the meta-analysis for all sample collections at *SNTB1* rs6469937.

increased negativity of SE in quantitative trait analysis (8,9) was also found to be associated with the increased risk of severe myopia in this current study.

In summary, our GWAS on seven independent sample collections of East Asians (five Chinese and two Japanese) identify *ZFHX1B* as a new susceptibility locus for severe myopia. It offers new information into the pathogenesis of severe myopia in Chinese and Japanese, and further implicates the TGF- β biological pathway as an important determinant of severe extreme of refractive error disorders.

MATERIALS AND METHODS

Samples for GWAS stage

Details of the GWAS from Sichuan (419 severe myopia cases and 669 emmetropic controls) and Hong Kong (232 severe myopia cases and 244 emmetropic controls) have been described elsewhere (15,16). The 452 severe myopia cases from Singapore are of Chinese descent, and were enrolled from the SCORM, SP2 and SCES collection. The emmetropic controls were enrolled from the same studies. For Japan, a total of 500 severe myopia cases with axial length ≥ 28 mm in both eyes and 1194 controls were enrolled for this study.

Sample for replication stage

We enrolled a further 1232 severe myopia cases and 3559 controls from Hong Kong, Japan and Singapore.

Hong Kong

A total of 338 unrelated individuals with severe myopia (refractive error in at least one eye ≤ -6 D and axial length in at least one eye ≥ 26 mm) and 422 unrelated emmetropic control individuals were recruited from the CUHK Eye Centre (Chinese University of Hong Kong), Hong Kong Eye Hospital and the eye

clinics of the Prince of Wales Hospital, Hong Kong. As 232 severe myopia cases and 244 emmetropic controls have undergone GWAS genotyping and were included in the discovery stage, there remained 106 severe myopia cases and 178 emmetropic controls for the replication stage.

Japan

The 728 individuals with severe myopia (axial length ≥ 26 mm in both eyes) were recruited from Kyoto University Hospital, and Tokyo Medical and Dental University Hospital. For the controls, we used 3248 unrelated individuals recruited from the Nagahama Prospective Genome Cohort for the Comprehensive Human Bioscience (The Nagahama Study).

Singapore

A total of 407 individuals with severe myopia and 133 emmetropic controls were volunteers recruited by DSO National Laboratories from military personnel. The severe myopia has spherically equivalent refractive error of at least -6 D in either eye. All subjects were of self-declared Chinese ancestry (all four grandparents), with an age range of between 16 and 25 years.

Genotyping and quality checks

Genome-wide genotyping for the Sichuan (China) and Hong Kong sample collections were performed as previously described (15). The Japanese severe myopia cases were genotyped using the Illumina 550K and 660 W Bead chips, whilst the Japanese controls were genotyped using the Illumina 610K Bead chip. The Singaporean severe myopia collections were genotyped using the Illumina 610K platform according to manufacturer's instructions. Post-genotyping quality checks were conducted on a per-SNP and per-sample basis. SNP markers showing any one of these characteristics were removed from further analysis:

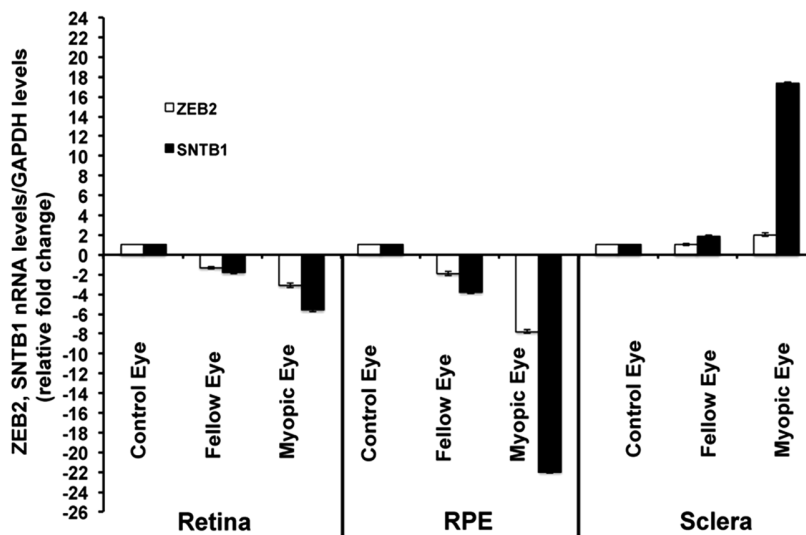


Figure 2. Transcription quantification of *ZEB2* and *SNTB1* in mouse retina, RPE and sclera in induced myopic eyes, fellow eyes and independent control eyes. (transcription quantification of *ZEB2* and *SNTB1* in mouse retina, RPE and sclera in induced myopic eyes, fellow eyes and independent control eyes. Myopia was induced using -15 D negative lenses in the right eye of mice for 6 weeks. Uncovered left eyes were served as fellow eyes and age-matched naive mice eyes were controls. Quantification of mRNA expression in mice retina, RPE and sclera is via qRT-PCR. The bar represents the fold changes of mRNA for *ZEB2* and *SNTB1* genes after normalization using *GAPDH* as reference. The mRNA levels of *ZEB2* and *SNTB1* in myopic and fellow retina, RPE and sclera are compared with independent controls).

- (i) call rate $< 95\%$,
- (ii) minor allele frequency $< 1\%$ and
- (iii) significant deviation from Hardy–Weinberg equilibrium ($P < 10^{-6}$).

Samples showing any of the following characteristics were removed from further analysis:

- (i) call rate $< 95\%$,
- (ii) extremes of heterozygosity,
- (iii) significant ancestral outlier on principal component analysis (PCA) and
- (iv) first degree relatives in which case the sample with the lower call rate within the pair would be excluded.

Replication genotyping was performed using the Taqman allelic discrimination assay (Applied Biosystems) according to manufacturer's instructions in all three sample collections.

Statistical analysis

Statistical analyses were conducted using PLINK version 1.07 (34) and the R statistical software package (<http://www.r-project.org/>). SNP association analysis with severe myopia was performed using logistic regression testing for a trend per-copy of the minor allele, within a multiplicative model for the OR. Additional adjustments compensating for the significant axes of genetic stratification were also performed, when data are available (e.g. in the GWAS stage). PCA and quantile–quantile distributions were plotted using the R statistical software package. PCA assessing for genetic stratification was performed using EIGENSTRAT, and has been described previously for the Chinese (Sichuan) and Hong Kong collections (15,16). The plots contrasting principal component scores between severe myopia

cases and controls for each collection are presented in Supplementary Material, Figures S5–S8. Good genetic matching between cases and controls was observed along the top five axes of variation of genetic ancestry.

Ethical approval for animal study

Animal study approval was obtained from the Sing Health IACUC (AAALAC accredited). All procedures performed in this study complied with the Association of Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmology and Vision Research.

Differential gene expression in a mouse model of myopia

Experimental myopia was induced in B6 wild-type mice ($n = 36$) by applying a -15.00 D spectacle lens on the right eye (experimental eye) for 6 weeks since post-natal Day 10. The left eyes were uncovered and served as contra-lateral fellow eyes. Age-matched naive mice eyes were used as independent control eyes ($n = 36$). Eye biometry, refraction and data analysis were followed as described previously (22,35).

We used quantitative real-time PCR (qRT-PCR) to validate the gene expression. Tissue collection, RNA extraction, qRT-PCR methods and analysis were followed as described previously (28). qRT-PCR primers were designed using Probe Finder 2.45 (Roche Applied Science, Indianapolis, IN, USA) and this was performed using a Lightcycler 480 Probe Master (Roche Applied Science). The primer sequences for *ZEB2* and *SNTB1* were forward: 5'-ccagaggaaacaaggatttcag-3' and reverse: 5'-aggcctgacatgtagctctgtg-3' (NM_015753.3) and forward: 5'-tttggaggcaaagaaggaga-3' and reverse: 5'-aggagtggatgatgaaacga-3' (NM_016667.3), respectively.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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Association of *paired box 6* with high myopia in Japanese

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Purpose: The objective of this study was to investigate whether genetic variations in the *paired box 6* (*PAX6*) gene are associated with high myopia in Japanese subjects.

Methods: A total of 1,307 unrelated Japanese patients with high myopia (axial length ≥ 26 mm in both eyes) and two independent control groups were evaluated (333 cataract patients without high myopia and 923 age-matched healthy Japanese individuals). We genotyped three tag single-nucleotide polymorphisms (SNPs) in *PAX6*: rs2071754, rs644242, and rs3026354. These SNPs provided 100% coverage of all phase II HapMap SNPs within the *PAX6* region (minor allele frequency ≥ 0.10 ; r^2 threshold: 0.90). Chi-square tests for trend and multivariable logistic regression were conducted.

Results: Genotype distributions in the three SNPs were in accordance with the Hardy-Weinberg equilibrium. After adjusting for age and sex, evaluation of cataract control showed a marginal association with high myopia in rs644242 (odds ratio [95% confidence interval]=0.69 [0.49–0.96], $p=0.026$), and a significant association was observed in healthy Japanese controls (0.79 [0.66–0.96], $p=0.015$). We pooled two control cohorts to evaluate the association. This analysis revealed a strong association between rs644242 and high myopia (0.78 [0.65–0.92], $p=0.0045$). The rs644242 A allele was a protective allele for development of high myopia. Subanalysis also revealed that rs644242 was significantly associated with extreme high myopia (0.78 [0.64–0.95], $p=0.0165$). The other two SNPs did not show a significant association with this condition.

Conclusions: The current study showed a significant association of *PAX6* with high and extreme myopia in Japanese participants. The A allele of rs644242 is a protective allele.

Myopia is the most common visual disorder in the world and presents major public health concerns, especially in East Asian populations. Eyes with long axial lengths (≥ 26 mm) or a high degree of myopic refractive error (≤ -6 diopter [D]) were diagnosed with high myopia [1]. High myopia is associated with various ocular complications [2], and pathological myopia is one of the leading causes of legal blindness in developed countries [3–5]. Therefore, clarifying the pathological pathway that leads to high myopia and developing methods for preventing or delaying its onset are important.

Myopia is a complex disease caused by environmental and genetic factors. Although linkage analysis studies have revealed more than 20 myopia-susceptibility loci and various candidate genes have been evaluated, most of these genes were not consistently responsible for high myopia. Recently, several groups performed genome-wide association studies (GWAS); we determined a susceptible locus at 11q14.1 [6] and 5p15 [7], while studies of Caucasians revealed

myopia-susceptibility loci on chromosome 15 [8,9]. We demonstrated the association of these susceptibility loci on chromosome 15 with high myopia in Japanese [10], and a Chinese study successfully replicated the association between high myopia and the *catenin $\delta 2$* (*CTNND2*) gene polymorphism in the susceptibility loci 5p15 we determined [11]. However, although the C allele of *CTNND2* single nucleotide polymorphism (SNP) rs6885224 was a risk allele for high myopia in our study, the replication study showed this allele was protective against high myopia. Since the expression of the *catenin $\delta 2$* protein is regulated by transcription factor Pax6 [12] and *PAX6* is another myopia-susceptibility gene, *PAX6* and *CTNND2* might cooperatively affect myopia development. Although several studies have examined the association between *PAX6* and myopia, whether *PAX6* is a susceptibility gene for myopia remains controversial [13–20]. To determine whether *PAX6* is associated with high myopia, we conducted a large-cohort case-control study of Japanese participants.

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METHODS

All procedures adhered to the tenets of the Declaration of Helsinki. The Institutional Review Board and the Ethics Committee of each participating institute approved the protocols. All the patients were fully informed of the purpose and procedures of the study, and written consent was obtained from each patient.

Patients and control subjects: In total, 1,307 unrelated Japanese patients with high myopia from the Kyoto University Hospital, Tokyo Medical and Dental University Hospital, Fukushima Medical University Hospital, Kobe City Medical Center General Hospital, and Ozaki Eye Hospital were included in the study. Comprehensive ophthalmic examinations were conducted on all the patients, which included dilated indirect and contact lens slit-lamp biomicroscopies, automatic objective refractions, and measurements of axial length using applanation A-scan ultrasonography or partial coherence interferometry (IOLMaster; Carl Zeiss Meditec, Dublin, CA). An axial length of at least 26 mm in both eyes confirmed the patient had high myopia.

Two control cohorts were recruited for this study. The first cohort was categorized as the selected control group, and comprised 333 cataract patients with axial lengths of less than 25.0 mm in both eyes (control 1). These patients were recruited from the Department of Ophthalmology at Kyoto University Hospital, the Ozaki Eye Hospital, the Japanese Red Cross Otsu Hospital, and the Nagahama City Hospital. In this group, the mean age $\pm$ standard deviation (SD) was 75.2 ± 7.9 years; 37.2% were men, and 59.5% were women. Axial length was measured with applanation A-scan ultrasonography or partial coherence interferometry before cataract surgery, and post-surgery, a dilated fundus examination was performed. If the fundus examination revealed that myopic changes had occurred, such as lacquer cracks/peripapillary atrophy, staphyloma, or choroidal neovascularization, the subject was eliminated from the group.

The second cohort was recruited as a general-population control. In total, 923 healthy unrelated Japanese individuals were recruited from the Aichi Cancer Center Research Institute (control 2). Only individuals at least 35 years of age were selected to participate in this group, meaning that the controls were age-matched with the high-myopia cohort. The mean age $\pm$ SD of this cohort was 56.9 ± 11.4 years ($p=0.855$ compared with the high-myopia cohort); 39.3% were men, and 60.7% were women.

Genotyping and statistical analyses: Genomic DNAs were prepared from peripheral blood using a DNA extraction kit (QuickGene-610L; Fujifilm, Minato, Tokyo, Japan) according

to the manufacturer's protocol, and the A260/A280 optical density was measured. Extracted DNA was stored at -80°C until used. Three tag SNPs ([rs2071754](#), [rs644242](#), and [rs3026354](#)) were selected using Tagger software, and provided 100% coverage for all common phase II HapMap SNPs (minor allele frequency: $>10\%$; Build: 36.1) within a 22.4-kb region that covered the *PAX6* gene on chromosome 11 (r^2 threshold: 0.90). The samples from patients with high myopia and the cataract controls were genotyped using a commercially available assay (TaqMan SNP assay with the ABI PRISM 7700 system; Applied Biosystems, Foster City, CA). The individuals recruited from the Aichi Cancer Center Research Institute were genotyped using Illumina HumanHap 610 Chips (Illumina Inc., San Diego, CA). The genotype for [rs3026354](#) was obtained from imputed data using [MACH software](#) because it was not included in the Illumina BeadChip. Phase II HapMap (Build: 36.1) was referred to for reference sequences.

Deviations from the Hardy-Weinberg equilibrium (HWE) in genotype distributions were assessed for each group using the HWE exact test. The chi-square test for trend or its exact counterpart was used to compare the genotype distributions of the two groups. Multiple regression and logistic regression analysis were performed to adjust for age and sex. These statistical analyses were conducted using Software R (R Foundation for Statistical Computing, Vienna, Austria). A p value of less than or equal to 0.05 was considered statistically significant. Bonferroni correction was used for multiple comparisons.

RESULTS

The demographics of the study population are shown in Table 1. The mean axial length of the 2,614 eyes with high myopia was 29.17 ± 1.84 mm. Of the eyes in this group, 1,878 (71.8%) were phakic, with a mean refraction of -12.71 ± 4.57 D. In the control 1 group, the mean axial length of the 666 eyes was 22.87 ± 0.80 mm, and the mean refraction of the phakic eyes in this group was -0.355 ± 2.96 D.

The genotype counts, associations, and odds ratios (ORs) for the three SNPs in the high-myopia and control groups are shown in Table 2. The genotype distributions of the three SNPs were in HWE ($p>0.05$). After corrections for age and sex differences had been made, based on a logistic regression model, [rs644242](#) showed a marginal association ($p=0.026$) with high myopia when evaluated with control 1 ($n=333$), and a significant association ($p=0.015$) when evaluated with control 2 ($n=923$); further analysis demonstrated that this association was still significant after Bonferroni correction. For the high-myopia group, the odds ratios were 0.69 (95%

TABLE 1. CHARACTERISTICS OF THE STUDY POPULATION.

Population characteristics	High myopia*	Case		Control	
		Control 1†	P value	Control 2	P value
Patients (n)	1307	333		923	
Age (mean±SD; years)	57.1±15.0	75.2±7.9	<0.0001‡	56.9±11.4	0.8549‡
Sex (n)					
Male	427 (32.7%)	124 (37.2%)	0.05626§	363 (39.3%)	0.0015§
Female	879 (67.3%)	198 (59.5%)		560 (60.7%)	
Axial length (mm±SD)					
Right eyes	29.23±1.85	22.84±0.81	NA		
Left eyes	29.10±1.82	22.88±0.78	NA		
Refraction of the phakic eyes (D)					
Right eyes	-12.86±4.44	-0.411±3.15	NA		
Left eyes	-12.57±4.71	-0.296±2.77	NA		

* Axial length >26.00 mm in both eyes. † Individuals who underwent cataract surgery and who had an axial length of <25.00 mm in both eyes. ‡ Unpaired t test. Compared with the high-myopia group. § Chi-square test. Compared with the high-myopia group. SD: standard deviation, D: diopter, NA: Not applicable.

confidence interval [CI]: 0.49–0.96) for the [rs644242](#) A allele when evaluated with control 1, and 0.79 (95% CI: 0.66–0.96) when evaluated with control 2. Chi-square tests for the trend also showed that [rs644242](#) was significantly associated with high myopia when this group was evaluated with control 2 ($p=0.015$). The two other SNPs did not have any significant associations with the condition.

Since the allele frequency and the genotype frequency of the three SNPs were not significantly different ($p>0.20$) between control 1 and control 2, we pooled the controls for further analysis (Table 3). The genotype distributions in the pooled control were still within HWE. This analysis revealed that the [rs644242](#) polymorphism was strongly associated with high myopia. The p value of a chi-square test for the trend was 0.011, and was 0.0045 after adjusting for age and sex with a logistic regression model. Since previous studies have reported on SNP associations with extreme myopia, the genotype distributions of the three SNPs between the extreme myopia cases were compared (axial length ≥ 28 mm in both eyes) as a pooled control. After age and sex adjustment and Bonferroni correction, this analysis also showed a significant association between [rs644242](#) and extreme myopia ($p=0.0165$). The OR of this analysis was similar to the OR for the high-myopia analysis (0.78 [95% CI: 0.64–0.95]). To investigate whether there are more appropriate genetic association models, we applied other possible ones: dominant, recessive, and codominant. However, we did not find a more significant association than the additive model.

Comparisons between the results of the current study and those of previous studies are summarized in Table 4. The current study is the first study to prove significant associations between a *PAX6* SNP and high myopia and extreme myopia.

DISCUSSION

In the present study, using a relatively large cohort of 2,563 individuals, we showed that *PAX6* is associated with high and extreme myopia in Japanese. The minor A allele of [rs644242](#) was a protective allele for high and extreme myopia.

The association of *PAX6* with common myopia was first evaluated in a Caucasian cohort. Although genome-wide linkage scans in a twins study suggested the *PAX6* region was strongly linked to common myopia, further case–control studies using tag SNPs rejected the hypothesis of an association between *PAX6* and common myopia [13–15]. Regarding high myopia, although Han et al., in a Chinese nuclear family study, reported that two SNPs in *PAX6* were associated with the condition [17], the subsequent case–control study did not replicate these associations, while haplotype analyses using 16 SNPs revealed the association [19]. Two Chinese reports also denied an association of *PAX6* with high myopia, while the subgroup analysis showed *PAX6* was associated with extreme myopia [16,20]. However, only 67 and 55 cases were used in these subgroup analyses, respectively, and therefore, caution should be applied when interpreting the findings, as pointed out by Zayats et al. [21].

TABLE 2. GENOTYPE COUNTS, ASSOCIATIONS, AND ODDS RATIOS IN PARTICIPANTS WITH HIGH MYOPIA AND CONTROL PARTICIPANTS.

Single nucleotide polymorphisms	Genotype	High myopia			Control 1			Control 2		
		n	n	Adjusted p value†	Nominal p value*	Adjusted p value†	Adjusted OR (95% CI)	n	Nominal p value*	Adjusted p value†
rs2071754 (C/T)	CC	326	90	0.26	0.61	0.26	1.12 (0.92-1.38)	232	0.485	0.497
	CT	632	156					466		
	TT	344	87					225		
rs644242 (C/A)	CC	1052	258	0.026	0.12	0.026	0.69 (0.49-0.96)	710	0.0153	0.0152
	CA	237	68					195		
	AA	14	7					18		
rs3026354 (A/G)	AA	544	142	0.78	0.33	0.78	1.03 (0.83-1.29)	376	0.611	0.638
	AG	590	155					421		
	GG	171	34					126		

* Differences in the observed genotypic distribution were examined by a chi-square test for trend. † Age and sex adjustment were performed based on a logistic regression model. CI: Confidence interval, OR: Odds ratio.

TABLE 3. GENOTYPE COUNTS, ASSOCIATIONS, AND ODDS RATIOS IN PATIENTS WITH HIGH MYOPIA, EXTREME MYOPIA AND POOLED CONTROL PARTICIPANTS.												
SNP	Genotype	Pooled control			High myopia			Extreme myopia			Adjusted odds ratio (95% confidence interval)	Adjusted p value‡
		n	Genotype frequency p value*	Allele frequency p value*	HWE P value	n	Nominal p value†	Adjusted p value‡	Adjusted odds ratio (95% confidence interval)	n	Nominal p value†	Adjusted p value‡
rs2071754 (C/T)	CC	322	0.53	0.99	0.74	326	0.44	0.343	1.06 (0.94–1.18)	196	0.327	0.317
	CT	622				632				397		
	TT	312				344				215		
rs644242 (C/A)	CC	968	0.95	0.95	0.15	1052	0.0105	0.00445	0.78 (0.65–0.92)	651	0.0294	0.0165
	CA	263				237				149		
	AA	25				14				8		
rs3026354 (A/G)	AA	518	0.28	0.22	1	544	0.99	0.834	0.99 (0.88–1.11)	346	0.656	0.585
	AG	576				590				359		
	GG	160				171				104		

* The difference in genotype and allele frequency between control 1 and control 2 were analyzed using a chi-square test. † Differences in the observed genotypic distribution were examined by chi-square test for trend. ‡ Age and sex adjustment were performed based on a logistic regression model. SNP: Single-nucleotide polymorphism, HWE: Hardy–Weinberg equilibrium

TABLE 4. SUMMARY OF PREVIOUS REPORTS THAT EVALUATED AN ASSOCIATION BETWEEN *PAX6* AND HIGH MYOPIA.

Author (year)	Definition of cases			Reported single-nucleotide polymorphisms*				Remarks
	Criteria of high myopia	Affected eye	Cases	Controls	rs667773‡ rs644242 rs662702	rs3026390 rs3026393 rs2071754	rs3026354 rs628224	
Tsai et al., 2008	SE<6 D	Both	255	87	n.s.	-	-	Significant association of AC- and AG-repeat lengths in the P1 promoter
Ng et al., 2009	SE≤6 D	-	379	349	n.s.	-	-	Significant association in haplo-type analysis
Han et al., 2009	SE<6 D	Both	FBAT† with 164 nuclear family	n.s.	p=0.0011	n.s.		
Liang et al., 2011	SE≤6 D	At least one eye	1083	1096	n.s.	n.s.	n.s.	
Jiang et al., 2011	SE≤8 D	Both	300	300	n.s.	n.s.	n.s.	
	SE≤8 D	Both	299	299	-	n.s.	-	
Current Study	AI≥26 mm	Both	1307	1256	p=0.0045	n.s.	n.s.	
Tsai et al., 2008	SE<10 D	Both	67	87	p<0.001	-	-	
Liang et al., 2011	SEM≤11 D	At least one eye	55	619	p=0.0074	n.s.	n.s.	
Current Study	AI≥28 mm	Both	810	1256	p=0.0165	n.s.	n.s.	

* Single-nucleotide polymorphism pairs with r2 ≥ 0.90 in HapMap Phase II are in the same column. † Family-based association test ‡ Although this single-nucleotide polymorphism is not included in HapMap SNP, it is reported to be in strong linkage disequilibrium (r2=0.92) with rs644242. D: diopter, n.s.: Not significant, SE: Spherical equivalent, AI: Axial length.

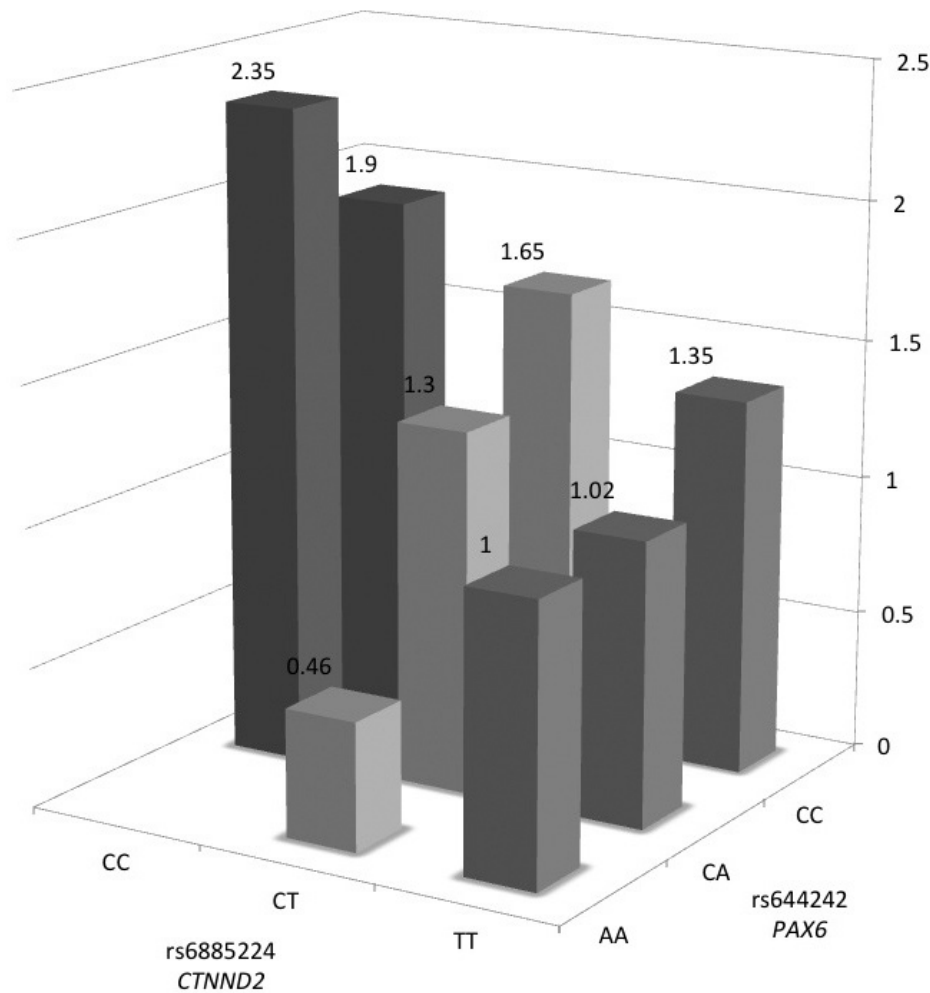


Figure 1. Collaborative effect of *CTNND2* rs6885224 and *PAX6* rs644242 on high myopia. The odds ratio of each genotype-pairs was calculated adjusting for age and sex. Patients with both the rs644242 AA genotype (non-risk homo) and the rs6885224 TT genotype (non-risk homo) are set as the reference (odds ratio=1.0). The number of subjects with rs6885224 CC and rs644242 CC were 55 in the case group and 42 in the control group, 286 in the case group and 252 in the control group with rs6885224 CT and rs644242 CC, 392 in the case group and 416 in the control group with rs6885224 TT and rs644242 CC, 15 in the case group and nine in the control group with rs6885224 CC and rs644242 CA, 70 in the case group and 78 in the control group with rs6885224 CT and rs644242 CA, 76 in the case group and 108 in the control group with rs6885224 TT and rs644242 CA, three in the case group and nine in the control group with rs6885224 CT and rs644242 AA, and six in the case group and nine in the control group with rs6885224 TT and rs644242 AA.

Table 4 summarizes the SNPs that have been evaluated previously to discover if *PAX6* is associated with high/extreme myopia. Rs667773 and rs662702 are reportedly in strong linkage disequilibrium with rs644242 [20], which showed significant association with high/extreme myopia in the present study. The association of these SNPs with extreme myopia was reported by Tsai et al. and Liang et al. [16,20], as well as in the current study, and the direction was the same in these three studies.

There are three possible reasons previous studies did not identify the association of rs644242 (or SNPs in strong linkage disequilibrium with rs644242) with high myopia. First, the parameter used to define high myopia was axial length, while all of the previous studies used standard error of the mean (SEM). Currently, *PAX6* is considered the “master gene” in eye development, owing to the gene’s pivotal role during the induction of lens and retina differentiation [22].

At an early stage of eye development, *PAX6* expression alone forms the eyeball and, with *SOX2*, affects the crystalline lens [23]. Hence, using SEM to define high myopia, which is affected by lens and eye shape, does not convey the direct effects of *PAX6*. However, high myopia defined by axial length, which is determined by changes to the shape of the eye only, demonstrates the direct effects of *PAX6*. This is why previous studies showed a significant association only in extreme myopia; almost all cases of extreme myopia present an abnormal eye shape. The second reason for the discrepancy between the studies is the number of cases. All the previous studies (except the study by Liang et al. [20]) had fewer than 600 participants [16,17,19], which is less than half the number of cases we included in our study. The variance in the inclusion criteria for patients with high myopia is the last possible reason previous studies failed to identify the association. Although Liang et al. included more than 1,000 cases, the researchers defined high myopia as SEM no greater

than -6 D in at least one eye [20]. Considering the effect of the *PAX6* gene, the current inclusion criterion, which is to enroll patients who have two highly myopic eyes, is more suitable for selecting genetic-dependent high myopia. Indeed, the inclusion criteria of other studies are the same as in the current study [16,17,19].

Recently, our GWAS showed that *CTNND2* is a susceptibility gene for high myopia [7]. *CTNND2* encodes catenin $\delta 2$, also known as δ -catenin. Catenin $\delta 2/\delta$ -catenin belongs to the catenin $\delta 1/p120$ -catenin protein family, which regulates cell adhesion and intracellular signaling pathways [24-26]. P120-catenin and β -catenin bind to the cytoplasmic tail of cadherin, which stabilizes the adherence junctions composed of cadherin, p120-catenin, β -catenin, α -catenin, and the actin cytoskeleton. δ -catenin competes with p120-catenin for interaction with cadherin and destabilizes the adherens junction [26,27]. In addition, δ -catenin can also affect the gene expression of other molecules associated with the wntless (Wnt)/ β -catenin signaling pathway [28]. Since *CTNND2* expression is regulated by Pax6 [12], and that the distribution of Pax6 and δ -catenin/catenin $\delta 2$ is remarkably similar [29,30], the collaboration of *PAX6* and *CTNND2* might be associated with myopia. In genetic studies on age-related macular degeneration (AMD), its association with the *CFH* gene led to the discovery that other molecules in the complement pathway were also associated with the condition, such as *C2/CFB*, *C3*, and *CFI* [31-34]. Similar to these collaborative associations of several complement factors to AMD, molecules associated with the adherence junction and Wnt/ β -catenin signaling might contribute to the development of myopia. When we calculate the odds ratio of each genotype-pairs of *PAX6* and *CTNND2* using samples shared between the present study and our previous study [7], the C allele of *CTNND2* rs6885224 seems to be a risk allele for high myopia in populations with the CC/CA genotype in *PAX6* rs644242, while the T allele of *CTNND2* rs6885224 seems to be a risk allele in populations with the AA genotype in *PAX6* rs644242 (Figure 1). However, since the number of patients with the *PAX6* AA genotype are small, replication studies are needed.

In conclusion, we proved the significant association of rs644242 in *PAX6* with high and extreme myopia. The A allele for rs644242 is protective for high and extreme myopia, and the collaboration of *PAX6* and *CTNND2* might be associated with the development of this condition. The adherens junction and Wnt/ β -catenin signaling are possible attractive targets for further study of myopia development.

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