Association of Polymorphisms in *ZFHX1B* and *PAX6* With Anisometropia in Chinese Children: The Hong Kong Children Eye Genetics Study

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Purpose. To identify gene variants associated with anisometropia development in children.

Methods. This is a population-based, cross-sectional, and longitudinal genetic association study involving 1057 children aged 6 to 10 years with both baseline and 3-year follow-up data. Six single nucleotide polymorphisms (SNPs), *ZC3H11B* rs4373767, *ZFHX1B* rs13382811, *KCNQ5* rs7744813, *SNTB1* rs7839488, *PAX6* rs644242, and *GJD2* rs524952 were analyzed in all children. Anisometropia was defined by an interocular difference in SE of ≥1 diopter (D) (Aniso-SE) and an interocular difference in axial length (AL) of ≥0.3 mm (Aniso-AL), respectively. Genetic associations of individual SNPs and joint SNP effects were analyzed.

Results. *ZFHX1B* rs13382811 was associated nominally with Aniso-AL (odds ratio [OR], 1.66; $P = 0.003$) at baseline. At 3 years, rs13382811 was significantly associated with Aniso-AL (OR, 1.49; $P = 0.001$) and became nominally associated with Aniso-SE (OR, 1.40; $P = 0.01$). In addition, *PAX6* rs644242 was significantly associated with Aniso-AL at 3 years (OR, 1.45; $P = 0.002$). At the 3-year follow-up, *PAX6* rs644242 was associated significantly with Aniso-AL development (OR, 1.61; $P = 0.0003$) and nominally with Aniso-SE development ($P = 0.03$) in children who were not anisometropic at baseline, whereas *ZFHX1B* rs13382811 was associated nominally with Aniso-AL development ($P = 0.02$). An additive SNP analysis indicated children carrying the risk allele T of *ZFHX1B* rs13382811 and allele A of *PAX6* rs644242 might have a 4.33- and 6.90-fold of increased risk of Aniso-SE and Aniso-AL development by 3 years, respectively.

Conclusions. This study identified two susceptible gene variants, *ZFHX1B* rs13382811 and *PAX6* rs644242, for anisometropia development in Hong Kong Chinese children, implicating their role in imbalanced refractive change and axial elongation between both eyes.

Keywords: anisometropia, refractive error, *ZFHX1B*, *PAX6*, genetic association

Anisometropia is referred to as a difference in the refractive powers between the two eyes of an individual. Severe anisometropia is a cause of strabismus and amblyopia in children.[1] Anisometropia is correlated with the severity of myopia and hyperopia, indicating the risk factors that influence the development of refractive errors can impose risk to anisometropia.[2] However, unlike common refractive disorders, anisometropia is usually not apparent and underdiagnosed in young children if the better eye provides enough visual acuity, resulting in a miss of the best treatment time.[3,4] Therefore, identifying the risk factors for anisometropia development is important.

Genetic predisposition to anisometropia had been suggested before.[5] A higher rate of cylindrical anisometropia was found in East Asian children (5.2%) compared with European Caucasians (1.9%).[4] Also, anisometropia was more commonly found in Hispanic children (7.8%) than in African American children (4.8%) at the age of 6 to 72 months.[6] In family studies, the co-occurrence of severe anisometropia was found in twins and siblings.[7,8] These findings suggested the involvement of ethnic backgrounds, lifestyles, and genetic factors in anisometropia development. Genetic case reports had revealed the implication of *SLIT2* and *UNC5D* gene variants in anisometropia.[9,10] So far, however, there has

been no report about the genetic factors of anisometropia development in longitudinal population-based cohorts.

Genes associated with anisometropia could be involved in the imbalanced development and/or progression of refractive errors between the eyes of an individual. Therefore, genes associated with different severities of myopia and/or myopia progression can be excellent candidate genes for anisometropia. Genome-wide association studies have identified single nucleotide polymorphisms (SNPs) in multiple genes and loci for myopia and refractive errors.[11–14] However, considering that SNPs associated with myopia progression are not available in adult cohorts and that the genetic effects of different age spans are heterogenous, which could be due to the fact that myopia in adults is an outcome influenced by long-time lifestyles, decreasing or masking the effects of genes.[15] Therefore, SNPs associated with different severities of myopia and/or myopia progression in children were selected for this study, rather than those only associated with myopia in adults. The SNPs *PAX6* rs644242, *SNTB1* rs7839488, *GJD2* rs524952, *ZFHX1B* rs13382811, *ZC3H11B* rs4373767, and *KCNQ5* rs7744813 met such criteria.[16–18] Among them, *ZC3H11B* rs4373767, *KCNQ5* rs7744813, and *GJD2* rs524952 were associated with different severities, endophenotypes, and fast myopia progression in Chinese children.[16,18] Besides, *KCNQ5* rs7744813 and *GJD2* rs524952 were associated with early and later onset myopia in a large cohort of European children. In replication cohorts, *KCNQ5* rs7744813 was associated with refractive error in European children and *GJD2* rs524952 in Asian children,[19] indicating the effects of myopia-associated SNPs might differ with age and ethnicities; there is a need for further exploration of the potential roles of these SNPs in children refractive error and related traits. In this study, we investigated the association of the six gene variants with anisometropia development in a population-based Chinese children cohort with 3 years of follow-up.

Methods

Subjects

This study involved 1057 unrelated Hong Kong schoolchildren aged from 6 to 10 years at enrolment. They were recruited from the Hong Kong Children Eye Study and have completed 3 years of follow-up as described.[16] This study was approved by the Ethics Committee of the Chinese University of Hong Kong, and all procedures conducted were adhered to the tenets of the Declaration of Helsinki.

All participants were given a detailed ocular examination and were confirmed to have no major eye diseases, such as amblyopia or strabismus, except for refractive errors. Ocular biometric investigations were conducted after complete cycloplegia following our protocol.[16,18] The spherical and cylindrical refractive errors were measured by an autorefractor (Nidek ARK-510A, Gamagori, Japan). The SE was calculated as a summation of the spherical power and half of the cylinder power. The axial length (AL) was measured using the Zeiss IOL Master 500 (Carl Zeiss Meditec, Dublin, CA). Refractive errors of the children were corrected by spectacles. No additional regimen, such as atropine eye drops or orthokeratology lens, was prescribed.

Candidate SNP Selection and Genotyping

Six candidate SNPs were selected based on their associations of myopia progression in Chinese children and/or with different myopia severities in Chinese adults and children: *GJD2* rs524952, *KCNQ5* rs7744813, *ZFHX1B* rs13382811, *SNTB1* rs7839488, *ZC3H11B* rs4373767, and *PAX6* rs644242.[16–18]

Genomic DNA was extracted from buccal swabs obtained from the children by using the QIAamp DNA kit (Qiagen, Hilden, Germany). All the eight SNPs were genotyped by using TaqMan assays (Applied Biosystems, Foster City, CA) on a Roche LightCycler 480 Real-Time PCR System (Roche, Basel, Switzerland).

Data Analysis

In this study, refractive power was presented as SE. Anisometropia was defined by SE and AL. The difference between the two eyes was presented as the absolute value of the right eye parameter minus the left eye parameter. An interocular difference of SE (IOD of SE) $\geq$1 diopter (D) was defined as anisometropia by SE (Aniso-SE), otherwise as isometropia by SE (Iso-SE). Because a 1-mm increase in AL is correlated with an myopia shift of approximately -3 D,[20] an interocular difference (IOD) of AL (IOD of AL) $\geq$0.3 mm was defined as anisometropia by AL (Aniso-AL), otherwise as isometropia by AL (Iso-AL).[21] To investigate the genetic association of newly developed anisometropia, children with Iso-SE and Iso-AL at baseline were selected for the longitudinal study. Those who became Aniso-SE and Aniso-AL at the 3-year follow-up were considered as having newly developed Aniso-SE and Aniso-AL, and the change in IOD of SE and AL per year was defined as the IOD rate of SE and AL, respectively.

All selected SNPs were tested for Hardy–Weinberg equilibrium by PLINK (V.1.9; https://www.cog-genomics.org/plink2/). Categorical variables and genotype distribution were analyzed by the χ^2 test. The odds ratio (OR) and 95% confidence intervals (CI) of associations between individual SNP and the phenotype, that is, anisometropia and newly developed anisometropia, were estimated by logistic regression (Supplementary Methods). The IOD of ocular quantitative traits, including the IOD of SE and AL at baseline and at 3-year follow-up, were analyzed using linear regression (Supplementary Methods). Genetic models of the six candidate SNPs analyzed by the SNPStats software (Catalan Institute of Oncology, Barcelona, Spain; http://bioinfo.iconcologia.net/SNPstats). Nonparametric Kruskal–Wallis or Mann–Whitney tests were used for genotype–phenotype correlation analyses. Firth regression was used to analyze rare genotypic variants. In this study, the primary outcomes were the allelic associations of individual SNP with anisometropia in the form of Aniso-SE and Aniso-AL at baseline and 3-year follow-up. A nominal significance was defined when the P was <0.05, and a study-wide significant association was defined if the P value was <0.0021 (0.05/6/4, where "6" was the number of SNPs and "4" the number of outcome variables involved in association analyses for the primary outcomes) based on Bonferroni correction for multiple testing. This threshold was also adopted for secondary outcomes, including the association of SNPs with anisometropia development over 3 years and genotypic association. To evaluate the joint effects of the candidate SNPs on anisometropia development, we tested all possi-

ble two-SNP combinations in the additive analysis, using logistic regression. We conducted multiple-testing correction by adjusting the number of pairwise combinations of SNPs ($n = 15$), comparison times among joint wide type and other types within each pairwise SNP ($n = 8$) and forms of anisometropia (Aniso-SE and -AL) ($n = 2$). Thus, the study-wise significance threshold is a P value of $<2.1 \times 10^{-4}$ for the joint SNP analysis. Age, sex, and the mean of baseline SE or AL of both eyes were adjusted wherever appropriate. The software SPSS (version 26.0; IBM Corp.) and R (version 4.2.0; The R Foundation, Vienna, Austria) were used for data analyses.

Results

Demographics of Study Subjects

At baseline, the mean age of the children was 7.51 ± 0.88 years; 50.2% were boys. The median of IOD of SE was 0.25 D (interquartile range, 0.125–0.375 D) and an AL of 0.10 mm (interquartile range, 0.05–0.18 mm). The prevalence of Aniso-SE and Aniso-AL were 6.43% and 9.47% at baseline, increased to 17.03% and 24.80%, respectively, by the 3-year follow-up. As only a small part of anisometropic children at baseline became isometropia after 3 years (17 of 68 in Aniso-SE and 21 of 97 in Aniso-AL), further genetic association was not applied. Of note, although all 1057 children had SE data, 33 of them had missing data for the AL (Table 1).

Association of SNPs With Anisometropia at Baseline and the 3-year Follow-up

All six candidate SNPs were genotyped with call rates of >99% and conformed with Hardy–Weinberg equilibrium in the whole group ($P > 0.05$). At baseline, no SNP was associated with Aniso-SE, although the risk allele T of *ZFHX1B* rs13382811 showed a nominal association with Aniso-AL (OR, 1.66; 95% CI, 1.18–2.34; $P = 0.003$; Table 2). At the 3-year follow-up, the T allele of *ZFHX1B* rs13382811 had a nominal association with Aniso-SE (OR, 1.40; 95% CI, 1.07–1.83; $P = 0.01$), but a significant association with Aniso-AL (OR, 1.49; 95% CI, 1.17–1.89; $P = 0.001$) (Table 3). At 3 years, the risk allele A of *PAX6* rs644242 became significantly associated with Aniso-AL (OR, 1.45; 95% CI, 1.14–1.83; $P = 0.002$) (Table 3).

In linear regression, *ZFHX1B* rs13382811 showed nominal associations with IOD of SE and AL ($\beta = 0.05$/D; $P = 0.04$ and $\beta = 0.02$/mm; $P = 0.02$, respectively) at baseline, and the associations maintained at the 3-year follow-up (IOD of SE: $\beta = 0.09$/D; $P = 0.003$; IOD of AL: $\beta = 0.03$/mm; $P = 0.002$) (Table 4). In addition, *PAX6* rs644242 had a nominal association with IOD of AL at the 3-year follow up ($\beta = 0.03$/mm; $P = 0.008$) (Table 4). Of note, we also used the logarithmic formula $Y = \ln(X + 1)$ to transform the IOD of AL and SE (Supplementary Table S1), and the results remained consistent, suggesting the robustness of our findings.

Association of SNPs With Anisometropia Development Over 3 Years

To investigate the association of SNPs with anisometropia development from baseline to the 3-year follow-up, longitudinal SE and AL data were analyzed from those who were Iso-SE and Iso-AL at baseline. The T allele of *ZFHX1B* rs13382811 conferred a 1.38-fold of risk toward Aniso-SE and Aniso-AL development, respectively, but the P values did not achieve study-wide significance ($P = 0.04$ and 0.02, respectively) (Table 5). In contrast, the A allele of *PAX6* rs644242 conferred a 1.61-fold of significantly increased risk toward Aniso-AL development ($P = 0.0003$) (Table 5). *PAX6* rs644242 also showed a nominal association with Aniso-SE development (OR, 1.39; 95% CI, 1.03–1.87; $P = 0.03$) (Table 5).

Genotypic Effects of Individual and Additive Effects of SNPs on Anisometropia

Genotypic association of individual SNP with the longitudinal changes of anisometropia were analyzed in three genetic models: namely, codominant, dominant, and recessive. The risk allele T of *ZFHX1B* rs13382811 in the recessive model showed the most significant association with Aniso-SE and Aniso-AL development, but could not withstand multiple

Table 1. Demographics of Study Subjects at Baseline and the 3-Year Follow-Up

	$n = 1057$		$n = 1057$				$n = 1024$			
	All Subjects		Iso-SE		Aniso-SE		Iso-AL		Aniso-AL	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
No. at baseline (n, %)	1057	–	989	93.57	68	6.43	927	90.53	97	9.47
Sex (male, %) at baseline	531	50.2	496	50.15	35	51.47	456	49.19	58	59.79
Age at baseline (years)	7.51	0.88	7.49	0.87	7.86	0.87	7.49	0.88	7.78	0.88
Average SE at baseline (D)	0.28	1.43	0.31	1.38	−0.06	1.99	0.29	1.37	0.20	1.89
Average AL at baseline (mm)	23.06	0.89	23.05	0.89	23.09	0.90	23.05	0.88	23.22	0.93
IOD of SE at baseline (D)*	0.25	0.125–0.375	0.25	0.125–0.375	1.50	1.125–1.875	0.13	0.125–0.375	1.00	0.625–1.5
IOD of AL at baseline (mm)*	0.10	0.05–0.18	0.09	0.04–0.16	0.52	0.37–0.695	0.09	0.04–0.15	0.47	0.36–0.61
No. at 3 years (n, %)	1057	93.57	877	82.97	180	17.03	770	75.20	254	24.80
Sex (male, %) at 3 years	531	50.2	439	50.06	92	51.11	385	50.00	129	50.79
Age at 3 years (years)	10.67	0.92	10.68	0.92	10.63	0.91	10.65	0.92	10.75	0.93
Average SE at 3 years (D)	−0.97	1.99	−0.9	1.92	−1.33	2.3	−0.87	1.97	−1.32	2.06
Average AL at 3 years (mm)	23.87	1.08	23.85	1.07	23.99	1.11	23.84	1.07	24.01	1.07
IOD of SE at 3 years (D)*	0.38	0.125–0.75	0.25	0.125–0.5	1.38	1.125–1.75	0.25	0.125–0.5	1.06	0.75–1.5
IOD of AL at 3 years (mm)*	0.16	0.07–0.29	0.13	0.06–0.22	0.50	0.39–0.66	0.11	0.05–0.19	0.45	0.36–0.62

* Presented as median and IQR.

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Table 2. Association of Candidate SNPs With Anisometropia at Baseline

			MAF (n = 1057)						MAF (n = 1024)					
SNP	Gene	MA	Iso-SE (n = 989)	Aniso-SE (n = 68)	*OR (95% CI)	*P Value	†OR (95% CI)	†P Value	Iso-AL (n = 927)	Aniso-AL (n = 97)	OR (95% CI)	*P Value	‡OR (95% CI)	‡P Value
rs4373767	*ZC3H11B*	C	0.29	0.35	1.31 (0.91–1.88)	0.15	1.39 (0.96–2.03)	0.08	0.29	0.31	1.10 (0.80–1.52)	0.56	1.12 (0.81–1.54)	0.49
rs13382811	*ZFHX1B*	T	0.21	0.26	1.41 (0.94–2.11)	0.10	1.50 (0.99–2.25)	0.05	0.21	0.29	1.64 (1.17–2.30)	0.004	1.66 (1.18–2.34)	0.003
rs7744813	*KCNQ5*	C	0.20	0.14	0.67 (0.41–1.1)	0.11	0.69 (0.42–1.14)	0.14	0.20	0.20	1.04 (0.72–1.50)	0.84	1.05 (0.73–1.52)	0.79
rs7839488	*SNTB1*	A	0.24	0.25	1.06 (0.71–1.59)	0.77	1.04 (0.69–1.57)	0.86	0.24	0.25	1.07 (0.76–1.51)	0.71	1.08 (0.76–1.52)	0.67
rs644242	*PAX6*	A	0.21	0.15	0.64 (0.40–1.04)	0.07	0.57 (0.34–0.95)	0.03	0.21	0.21	0.96 (0.67–1.37)	0.81	0.95 (0.66–1.37)	0.80
rs524952	*GJD2*	T	0.44	0.43	0.96 (0.68–1.37)	0.84	1.02 (0.70–1.47)	0.92	0.44	0.43	0.95 (0.70–1.28)	0.72	0.94 (0.69–1.26)	0.67

MA, minor allele; MAF, minor allele frequency.
* P values were adjusted for age and sex.
† P values were adjusted for age, sex, and SE.
‡ P values were adjusted for age, sex, and AL.

Table 3. Association of Candidate SNPs With Anisometropia at the 3-Year Follow-Up

			MAF (Total = 1057)						MAF (Total = 1024)					
SNP	Gene	MA	Iso-SE (n = 877)	Aniso-SE (n = 180)	*OR (95% CI)	*P Value	†OR (95% CI)	†P Value	Iso-AL (n = 770)	Aniso-AL (n = 254)	*OR (95% CI)	*P Value	‡OR (95% CI)	‡P Value
rs4373767	*ZC3H11B*	C	0.29	0.30	1.04 (0.81–1.33)	0.76	1.06 (0.82–1.35)	0.66	0.29	0.31	1.12 (0.9–1.39)	0.33	1.13 (0.91–1.40)	0.28
rs13382811	*ZFHX1B*	T	0.20	0.26	1.39 (1.06–1.81)	0.02	1.40 (1.07–1.83)	0.01	0.20	0.27	1.48 (1.16–1.88)	**0.001**	1.49 (1.17–1.89)	**0.001**
rs7744813	*KCNQ5*	C	0.20	0.18	0.86 (0.64–1.16)	0.32	0.86 (0.64–1.16)	0.33	0.20	0.18	0.88 (0.68–1.13)	0.32	0.90 (0.69–1.16)	0.40
rs7839488	*SNTB1*	A	0.24	0.24	1.00 (0.77–1.30)	0.99	0.99 (0.76–1.29)	0.95	0.23	0.26	1.16 (0.92–1.46)	0.21	1.17 (0.93–1.47)	0.19
rs644242	*PAX6*	A	0.20	0.23	1.18 (0.90–1.54)	0.23	1.16 (0.89–1.52)	0.28	0.20	0.26	1.45 (1.15–1.82)	**0.002**	1.45 (1.14–1.83)	**0.002**
rs524952	*GJD2*	T	0.44	0.43	0.94 (0.74–1.18)	0.58	0.96 (0.76–1.21)	0.72	0.44	0.43	0.97 (0.79–1.18)	0.74	0.96 (0.79–1.18)	0.71

MA, minor allele; MAF, minor allele frequency.
* P values were adjusted for age and sex.
† P values were adjusted for age, sex, and magnitude of SE.
‡ P values were adjusted for age, sex, and magnitude of AL.

Table 4. Association of Candidate SNPs With IOD of SE and AL

			Baseline								3-Year Follow-Up							
			IOD of SE				IOD of AL				IOD of SE				IOD of AL			
SNP	Gene	MA	*β	*P Value	†β	†P Value	*β	*P Value	‡β	‡P Value	*β	*P Value	†β	†P Value	*β	*P Value	‡β	‡P Value
rs4373767	*ZC3H11B*	C	0.02	0.33	0.03	0.20	0.009	0.28	0.01	0.23	0.03	0.36	0.03	0.30	0.01	0.48	0.01	0.49
rs13382811	*ZFHX1B*	T	0.05	0.05	0.05	0.04	0.02	0.02	0.02	0.02	0.09	0.004	0.09	0.003	0.03	0.004	0.03	**0.002**
rs7744813	*KCNQ5*	C	−0.03	0.23	−0.03	0.24	−0.003	0.79	−0.002	0.87	−0.02	0.62	−0.02	0.64	−0.02	0.08	−0.02	0.11
rs7839488	*SNTB1*	A	0.01	0.65	0.01	0.80	−0.001	0.89	−0.0003	0.97	−0.02	0.49	−0.02	0.43	0.01	0.61	0.005	0.65
rs644242	*PAX6*	A	−0.01	0.76	−0.02	0.50	0.01	0.54	0.01	0.53	0.01	0.85	0.001	0.97	0.03	0.007	0.03	0.008
rs524952	*GJD2*	T	−0.02	0.24	−0.02	0.44	−0.01	0.43	−0.01	0.36	0.01	0.62	0.02	0.49	−0.01	0.34	−0.01	0.43

MA, minor allele.
* *P* values were adjusted for age and sex.
† *P* values were adjusted for age, sex, and SE.
‡ *P* values were adjusted for age, sex, and AL.

Table 5. Association of Candidate SNPs With Anisometropia Development

			IOD of SE						IOD of AL					
			MAF (n = 989)						MAF (n = 927)					
SNP	Gene	MA	Iso (n = 860)	Aniso (n = 129)	*OR (95% CI)	*P Value	†OR (95% CI)	†P Value	Iso (n = 749)	Aniso (n = 178)	*OR (95% CI)	*P Value	‡OR (95% CI)	‡P Value
rs4373767	*ZC3H11B*	C	0.29	0.28	0.95 (0.71–1.27)	0.74	0.97 (0.73–1.30)	0.85	0.29	0.31	1.10 (0.85–1.41)	0.46	1.11 (0.86–1.43)	0.41
rs13382811	*ZFHX1B*	T	0.20	0.26	1.35 (0.99–1.84)	0.06	1.38 (1.01–1.88)	0.04	0.20	0.25	1.36 (1.03–1.79)	0.03	1.38 (1.04–1.82)	0.02
rs7744813	*KCNQ5*	C	0.20	0.19	0.92 (0.66–1.29)	0.63	0.96 (0.69–1.35)	0.82	0.20	0.18	0.88 (0.65–1.18)	0.39	0.90 (0.67–1.21)	0.49
rs7839488	*SNTB1*	A	0.24	0.23	0.93 (0.68–1.27)	0.65	0.95 (0.70–1.31)	0.77	0.23	0.28	1.26 (0.97–1.65)	0.08	1.27 (0.98–1.66)	0.07
rs644242	*PAX6*	A	0.21	0.27	1.39 (1.04–1.87)	0.03	1.39 (1.03–1.87)	0.03	0.20	0.28	1.61 (1.24–2.09)	$\mathbf{4 \times 10^{-4}}$	1.61 (1.24–2.10)	$\mathbf{3 \times 10^{-4}}$
rs524952	*GJD2*	T	0.44	0.41	0.86 (0.66–1.13)	0.27	0.83 (0.64–1.09)	0.19	0.44	0.43	0.96 (0.76–1.21)	0.73	0.96 (0.76–1.21)	0.71

MA, minor allele; MAF, minor allele frequency.
* *P* values were adjusted for age and sex.
† *P* values were adjusted for age, sex, and SE.
‡ *P* values were adjusted for age, sex, and AL.

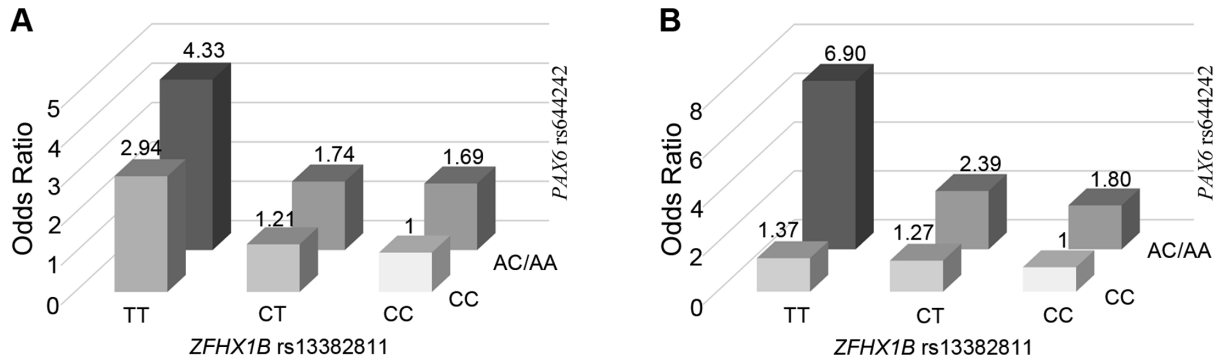

Figure. Additive analysis of *ZFHX1B* rs13382811 and *PAX6* rs644242 with anisometropia development. (**A**) Association of *ZFHX1B* rs13382811 and *PAX6* rs644242 with Aniso-SE development. (**B**) Association of *ZFHX1B* rs13382811 and *PAX6* rs644242 with Aniso-AL development. Joint CC of *ZFHX1B* rs13382811 and CC of *PAX6* rs644242 (CC–CC) were set as reference. The OR values in bold were statistically significant.

testing (Supplementary Tables S2 and S3). *PAX6* rs644242 was significantly associated with Aniso-AL development in the codominant models (AC vs. CC; OR, 1.95; 95% CI, 1.38–2.75; AA vs. CC, OR, 1.85; 95% CI, 0.93–3.69; $P = 0.0005$) and in the dominant models (AC/AA vs. CC, OR, 1.93; 95% CI, 1.39–2.69; $P = 0.0001$) (Supplementary Table S3).

Based on the genotypic associations, we analyzed the SNP × SNP interactions to explore the potential joint effects of the six SNPs. The results showed that all SNPs were independent from each other (Supplementary Table S4). We then performed joint-SNP analysis for all possible combinations of these six SNPs to investigate the effects of paired SNPs on Aniso-SE and -AL development (Supplementary Tables S6–S20). For joint analysis between *ZFHX1B* rs13382811 and *PAX6* rs644242, the wild-type genotypes of *PAX6* rs644242 and *ZFHX1B* rs13382811, that is, CC-CC, were used as a reference. Considering the small cell counts of the genotypes of *ZFHX1B* rs13382811 TT and *PAX6* rs644242 AA, that is, TT-AA ($n = 3$), the Firth regression was applied when it was compared with CC-CC. The TT-AA genotype conferred a 12.43-fold risk toward Aniso-AL development ($P = 0.01$) (Supplementary Table S5), and a mild but insignificant risk toward Aniso-SE development (OR, 1.63; 95% CI, –1.51 to 4.77; $P = 0.76$) (Supplementary Table S5). Because *PAX6* rs644242 showed the strongest association with both Aniso-SE and Aniso-AL in the dominant model, the AA genotype was pooled with the AC genotype for further analysis. The joint AC/AA-TT genotype of *PAX6* rs644242 and *ZFHX1B* rs13382811 was associated nominally with Aniso-SE (OR, 4.33; 95% CI, 1.39–13.48; $P = 0.01$) (Fig. A and Supplementary Table S6) and Aniso-AL (OR, 6.90; 95% CI, 2.23–21.36; $P = 0.001$) (Fig. B and Supplementary Table S6). The joint AC/AA-CT genotype was also nominally associated with Aniso-AL development (OR, 2.39; $P = 0.001$) (Fig. B and Supplementary Table S6). In addition, nominal associations were found for the joint genotype of *ZFHX1B* rs13382811-*KCNQ5* rs7744813 and Aniso-SE (TT-AA vs. CC-AA, OR, 3.29; $P = 0.007$) (Supplementary Table S8), and that of *ZFHX1B* rs13382811-*SNTB1* rs7839488 and Aniso-SE (TT-GG vs. CC-GG, OR, 3.01; $P = 0.02$) (Supplementary Table S9) and Aniso-AL (TT-AG vs. CC-GG, OR, 6.54; $P = 0.002$) (Supplementary Table S9). Nominal and significant associations were also found for the joint genotype of *ZC3H11B* rs4373767-*PAX6* rs644242 (TT-AC/AA vs. TT-CC, OR, 2.57; $P = 0.0001$; CT-AC/AA vs. TT-CC, OR, 2.05; $P = 0.007$; CC-AC/AA vs. TT-CC, OR, 2.64) ($P = 0.03$; Supplementary Table S11), *KCNQ5* rs7744813-*PAX6* rs644242 (AA-AC/AA vs. AA-CC, OR, 1.93; $P = 0.001$; CC-AC/AA vs. AA-CC, OR, 3.99; $P = 0.008$) (Supplementary Table S12), *SNTB1* rs7839488-*PAX6* rs644242 (AG-AC/AA vs. GG-CC, OR, 2.47; $P = 0.0001$; AA-AC/AA vs. GG-CC, OR, 2.58; $P = 0.03$) (Supplementary Table S13), and *GJD2* rs524952-*PAX6* rs644242 (AA-AC/AA vs. AA-CC, OR, 2.65; P =0 .002; TA-AC/AA vs. AA-CC, OR, 2.42; $P = 0.002$; TT-AC/AA vs. AA-CC, OR,2 .22; $P = 0.03$) (Supplementary Table S14) and Aniso-AL.

Genotypic Associations of SNPs With IOD Rate in a Longitudinal Study

Children carrying the *PAX6* rs644242 AC/AA genotypes showed a faster IOD rate of SE and AL than those carrying the wild-type genotype (0.133D/y vs. 0.114D/y; $P = 0.003$; and 0.046mm/y vs. 0.038mm/y; $P = 0.036$, respectively) (Supplementary Fig. 1A, B and Supplementary Table S21), although the associations were of nominal significance.

Discussion

In this population-based, cross-sectional, and longitudinal genetic association study, we have identified two polymorphisms, *ZFHX1B* rs13382811 and *PAX6* rs644242, for anisometropia in children. Children carrying the risk allele T of *ZFHX1B* rs13382811 and allele A of *PAX6* rs644242 had a higher risk of anisometropia development. The consistent results of IOD rates of SE and AL suggested that these two variants may exert their influence on Aniso-SE by regulating an imbalanced axial elongation between both eyes.

Refractive anisometropia was found to increase with age and occur mainly through the changes of the posterior segment rather than the cornea in children,[22] suggesting that imbalanced axial elongation should play a role in anisometropia development. Our study showed that *ZFHX1B* rs13382811 was associated with Aniso-AL both at baseline (OR, 1.66) and the 3-year follow-up (OR, 1.49), with the β value for the IOD of AL increasing from 0.02 to 0.03. Moreover, *ZFHX1B* rs13382811 became nominally associated with Aniso-SE at the 3-year follow-up (OR, 1.40; $P = 0.01$), with the β for the IOD of SE increasing from 0.05 to 0.09. Thus, the association of *ZFHX1B* rs13382811 with

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anisometropia in children may also increase with age, and the Aniso-SE could result from the development of interocular AL difference. *ZFHX1B* was associated with high and extreme myopia in Chinese, Japanese, and Europeans.[23–25] In a Hong Kong children cohort, *ZFHX1B* rs13382811 was associated with longitudinal SE progression.[16] In the present study, the longitudinal data indicated marginal associations of the T allele of *ZFHX1B* rs13382811 with Aniso-SE ($P = 0.04$) and Aniso-AL ($P = 0.02$). It also showed an association with the development of Aniso-SE (OR, 2.55; $P = 0.018$) and a trend toward Aniso-AL development (OR, 1.90; $P = 0.10$) in a recessive model. These accordant findings suggested *ZFHX1B* rs13382811 as a genetic risk factor for both myopia and anisometropia.

ZFHX1B encodes the Zinc finger E-box-binding homebox2, a transcriptional corepressor in the TGF-β signaling pathway, which is involved in regulating sclera metabolism.[26] TGF-β1 was associated positively with AL in myopic patients and with increased lens size in highly myopic eyes owing to the up-regulation of β/γ-crystallin expression.[27] However, the exact mechanism of *ZFHX1B* and the TGF-β signaling pathway in anisometropia development remains unknown. Epigenetic events might be involved, and the expressions of the same genes in different sites of an individual can be influenced by age and environmental factors, leading to imbalanced phenotypes.[28] The ZFHX1B protein level can also be affected by DNA methylation levels and, thus, influences the activities of the TGF-β signal pathway that lead to asymmetric development of ocular structures.

PAX6 is crucial for ocular development and involved in several ocular diseases. So far, the association of *PAX6* polymorphisms with myopia and refractive error remains controversial. No association between *PAX6* and myopia was found in White populations,[29] whereas two SNPs in *PAX6*—rs3026390 and rs3026393—were found to be significantly associated with high myopia in Chinese.[30,31] *PAX6* polymorphisms were also associated with extreme myopia in Chinese adults.[32] Of note, however, no association was found between *PAX6* and high/extreme myopia in Chinese children.[18] In the present study, *PAX6* rs644242 was associated with anisometropia in children, especially with the development of Aniso-AL (OR,1.61; $P = 0.0003$), with the risk allele A contributing to faster IOD rate of SE and AL than those carrying the wild-type genotype (0.133 D/y vs. 0.114 D/y; $P = 0.003$; and 0.046 mm/y vs. 0.038 mm/y; $P = 0.036$, respectively), implying greater imbalanced refractive change between the both eyes of an individual. Of note, however, only the association of *PAX6* with Aniso-AL can withstand the correction for multiple testing. This finding might be due to the limited sample size. Another possible explanation is that the genetic effects on SE, a composed parameter, might be diluted by other ocular parameters, such as the corneal curvature, anterior chamber depth, and lens thickness. In contrast, AL is an independent parameter and a major component of ocular dimensions, and the elongation of eyeball is the primary mechanism of children myopia. Therefore, AL can be more directly and strongly affected by *PAX6* in the development and/or progression of refractive error.

Asymmetric ocular phenotypes have been identified in ocular malformations caused by mutations in genes such as *SOX2* and *PRR12*.[33,34] A unilateral Peters anomaly was associated with a *PAX6* mutation.[35] The underlying causes may be protein truncation- and nonsense mediated decay-related haploinsufficiency,[34,35] but these explanations are inadequate to account for the asymmetric phenotypes of two eyes sharing the same variants. Several genome-wide epigenetic studies identified heterogeneous roles of gene methylation levels in myopia children and animal models. Decreased levels of DNA methylation in specific CpG sites had been proposed as a risk factor for early onset myopia in young children.[36] *PAX6* was found to be regulated by its promoter methylation and its over-expression upregulated *MMP2* and *MMP9*.[37] Because MMPs are enzymes that degrade the extracellular matrix components, this mechanism could be related to scleral remodeling. Similarly, higher levels of scleral DNA methylation at the *COL1A1* promoter were detected in a monocular form deprivation mouse myopic model.[38] The subsequent inhibition of COL1A1 protein production might be the cause of monocular myopia. Therefore, it is likely that different degrees of *PAX6* promoter methylation subjected to environmental factors may lead to different profiles of scleral metabolism and axial elongation between the two eyes of an individual. Further studies on *PAX6* methylation and the imbalanced refraction changes are warranted.

This population-based study in children is the first to reveal the involvement of *ZFHX1B* and *PAX6* in anisometropia development. The consistent results on Aniso-SE and Aniso-AL in both cross-sectional and longitudinal settings suggested that the findings should be robust. However, there are several limitations. First, although having a total sample size of 1057, the number of anisometropic children subjects is small, that is, 68 Aniso-SE and 97 Aniso-AL, and 180 Aniso-SE and 254 Aniso-AL at the first and second visits, respectively. Therefore, the statistical power might be insufficient for certain analyses, leading to some nominal associations. Second, we only had two refractive measurements—at baseline and the 3-year follow-up—so the IOD rate was averaged by the change of SE and AL over 3 years and may not reflect the exact situation in individual years accurately. Third, only nominally significant P values were obtained from *ZFHX1B* rs13382811 in longitudinal analysis. Nevertheless, because both categorical analyses of two types of anisometropia and QTL analyses of IOD SE and AL changes provided consistent findings, and in the joint SNP analysis, the pairing of *ZFHX1B* rs13382811 and *PAX6* rs644242, respectively, with other SNPs showed stronger associations with Aniso-SE or Aniso-AL development than those combinations without *ZFHX1B* rs13382811 or *PAX6* rs644242 (Supplementary Tables S6–S20), these two SNPs should, therefore, be major susceptibility variants for anisometropia development, although the effects of some joint genotypes could not withstand the multiple-testing correction. Future studies in larger, multicenter samples with more longitudinal follow-up visits are warranted. Last, only a few candidate SNPs were included in this study, providing limited genomic coverage. Future genome-wide studies, such as genome-wide association studies, would be helpful in identifying more gene variants for anisometropia.

In summary, we have revealed the associations of two polymorphisms, *PAX6* rs644242 and *ZFHX1B* rs13382811, with anisometropia in a population-based children cohort with longitudinal follow-up. The two SNPs also showed a joint effect on Aniso-SE and Aniso-AL development. Along with their associations with IOD rate of SE and AL, our findings consistently suggested the involvement of *PAX6* and *ZFHX1B* in the asymmetric refractive error shift and axial elongation between the both eyes in children.

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Contributors: XJZ, JCY, and LJC conceived the idea and designed the study. XJZ, JCY, LJC, KWK, FYT, WWKY, and ALY recruited the study subjects and performed clinical examinations. JCY and LJC contributed to data acquisition of the study; YYW and YZZ contributed data analysis of the study; YYW, ZJC, FFL, and POST performed the experiments; YYW and XJZ draft the manuscript; WWY, ALY, CCT, CPP, JCY, and LJC provided critical review on the manuscript. All authors approved the submitted version.

Ethics Approval: The study was approved by the Ethics Committee of the Chinese University of Hong Kong (Research Ethics Committee Reference Number: 2015.033). All procedures were conducted according to the tenets of the Declaration of Helsinki. All children and their parents signed an informed consent on participation.

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