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Genome wide association studies reveal genetic variants in *CTNND2* for high myopia in Singapore Chinese

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Abstract

Objective—To determine susceptibility genes for high myopia in Singaporean Chinese.

Design—A meta-analysis of two genome wide association (GWA) datasets in Chinese and a follow-up replication cohort in Japanese.

Participants and Controls—Two independent datasets of Singaporean Chinese individuals aged 10–12 years (SCORM -- Singapore Cohort Study of the Risk factors for Myopia: cases=65, controls=238) and aged > 21 years (SP2 -- Singapore Prospective Study Program: cases=222, controls=435) for GWA studies, and a Japanese dataset aged >20 years (cases=959, controls=2128) for replication.

Methods—Genomic DNA samples from SCORM and SP2 were genotyped using various Illumina Beadarray platforms (> HumanHap 500). Single-locus association tests were conducted for each dataset with meta-analysis using pooled z-scores. The top-ranked genetic markers were examined for replication in Japanese dataset. Fisher's P was calculated for the combined analysis of all three cohorts.

Main outcome measures—High myopia, defined by spherical equivalent (SE) ≤ −6.00 diopters (D); controls defined by SE between −0.50D and +1.00D.

Results—Two SNPs (rs12716080 and rs6885224) in the gene *CTNND2* on chromosome 5p15 ranked top in the meta-analysis of our Chinese datasets (meta- $P = 1.14\times10^{-5}$ and meta- $P = 1.51\times10^{-5}$, respectively) with strong supporting evidence in each individual dataset analysis (Max $P = 1.85.x10^{-4}$ in SCORM: Max $P = 8.8\times10^{-3}$ in SP2). Evidence of replication was observed in Japanese dataset for rs6885224 ($P = 0.035$, *meta-P* of three datasets: 7.84×10^{-6}).

Conclusion—This study identified strong association of *CTNND2* for high myopia in Asian datasets. The *CTNND2* gene maps to a known high myopia linkage region on chromosome 5p15.

Keywords

myopia; genome wide association; *CTNND2*; single nucleotide polymorphism; genetics

Myopia is a common eye disorder and a major public health concern in urban East Asian populations, affecting nearly 40% of Chinese persons aged 40 to 79 years[1–3]. High myopia, defined by spherical equivalent (SE) ≤ −5.00 diopter (D) or SE ≤ −6.00 D for at least one eye, is associated with significant ocular morbidity, including retinal detachment and myopic macular degeneration[4;5].

The genetic etiologic basis of myopia and high myopia is supported by data from familial aggregation, segregation, and twin studies[5–12]. The relative risk of myopia in siblings of a person with myopia (λ_s) has been estimated to be strongest in high myopia (SE ≤ −6.00 D; $\lambda_s = 5 – 20$), and moderate for lower degrees of myopia (SE: −1.00 to −3.00D; λ_s=1.5–3)[5;12]. To date, more than 15 chromosomal regions (or genetic loci, designated as MYP loci) have been mapped for myopia-related phenotypes by genome wide linkage scans, and many candidate genes have been reported by association and sequencing studies[13]. However, no gene implicated in myopia has been consistently replicated.

Genome wide association (GWA) studies have become an important and unbiased approach to aid in the search for causal sequence variants by screening upwards of a million single nucleotide polymorphisms (SNPs) spaced across the genome. This is exemplified by the

recent GWA studies of seven complex trait disorders by the Wellcome Trust Case-Control Consortium14. Recently, Nakanishi et al15 reported the first GWA study for pathological myopia (axial length > 26 mm of both eyes; equivalent to refractive error<−6.00 D16), and detected a novel susceptibility locus at chromosome 11q24.1. At the time of writing this paper, no GWA studies have been reported for myopia in a Chinese population.

The aim of the present study is to identify genetic variants that may account for individual susceptibility to high myopia using a GWA approach in two well-characterized population studies of Chinese in Singapore: the Singapore Cohort Study of the Risk factors for Myopia (SCORM) and the Singapore Prospective Study Program (SP2) study. The Chinese in Singapore are primarily immigrants from southern provinces of China, and a recent study on the genomic variations of Singapore Chinese demonstrates a homogenous group with minimal substructure17. Utilizing GWA approach in this homogenous population provides a viable means to discover susceptibility genes for high myopia in Chinese persons. We therefore performed a meta analysis using the SCORM and SP2 genotyped datasets to identify top-ranked 'susceptibility' markers for high myopia. The Nakanishi et al15 Japanese dataset (Japan) was used as a replication cohort to confirm these top-ranked markers.

Patients and Methods

Study populations

SCORM and SP2 are the primary datasets in this study with genome-wide high-density SNP data. SCORM is one of few cohorts with precise longitudinal ocular phenotypic data from predominantly Chinese Singaporean children18. SP2 is a population-based study of primarily Chinese adults aged 21 years and above with refractive error data19–22. Our study adhered to the Declaration of Helsinki. SCORM was approved by the Institutional Review Boards of the National University of Singapore and the Singapore Eye Research Institute, while SP2 was approved by the Singapore Eye Research Institute and Singapore General Hospital. Written informed consent was obtained from all parents (SCORM) and participants (SP2). The Japan adult dataset is enriched for high myopia and control samples, and was used as the replication dataset[15].

SCORM—A total of 1979 children in grades 1, 2, and 3 from three schools were recruited from 1999 to 200118;23. The children were examined on the school premises every year by a team of eye care professionals. Three drops of 1% cyclopentolate were administered 5 minutes apart. At least 30 minutes after the third drop, the refractive error was measured using a stand-alone autorefractor (Canon RK-F1, Japan). Contact ultrasound biometry measures were performed using one of two biometry machines (Echoscan model US-800; Nidek Co, Ltd, Tokyo, Japan). To reduce genetic heterogeneity derived from different racial groups in SCORM, the GWA study was conducted in a subset of children, specifically 1116 Chinese, comprising 56% of the whole cohort,. The high myopia phenotype used in this study was based on the refractive error obtained on the 4th annual examination of the study (children at age 10 to 12 years).

SP2—Samples of SP2 were from a revisit protocol of two prior population-based surveys, the 1992 National Health Survey and the 1998 National Health Survey19–22. Both studies recruited a random sample of individuals from the Singapore population. Disproportionate sampling was stratified by ethnicity to increase the number of minority ethnic groups (Malaysians and Asian Indians). A total of 8266 subjects were invited to participate in the follow-up survey. 6301 (76.1% response rate) subjects completed the questionnaire and of these, 4056 also attended the health examination and donated blood specimens (64.4% of

those who completed the questionnaire). The protocol for obtaining refractive error measurements was identical to that of SCORM. Refractive error was measured using the stand-alone autorefractor (Canon RK-F1, Japan). The GWA genotyping for SP2 involved only individuals of Chinese descent (n=2867).

Japan—The Japan dataset consists of 959 high myopia cases and 2128 population controls. Details of the Japan data have been reported15. Briefly, the high myopia cases were primarily selected based on axial length > 26 mm of both eyes. It is known that excessive increase in axial length of the eye ball is the key contributor to myopic refractive. For instance, an axial length > 26 mm is equivalent to SE < −6.00D in general[16], which corresponds to the criteria for high myopia used in SCORM and SP2. Cases were recruited at the Center for Macular Disease of Kyoto University Hospital, the High Myopia Clinic of Tokyo Medical and Dental University, and Fukushima Medical University Hospital. All subjects underwent comprehensive ophthalmologic examinations, including dilated pupillary indirect ophthalmoscopy of the fundus, slit-lamp biomicroscopy of the anterior chamber, automatedrefraction evaluation, and measurement of the axial length by applanation A-scan ultrasonography (UD-6000, Tomery, Nagoya, Japan) or partial coherence interferometry (IOLMaster, Carl Zeiss Meditec, Dublin, CA). Controls were obtained from the JSNP database[24;25] and recruited at the Aichi Cancer Center Research Institute. The Institutional Review Board and the Ethics Committee of each institution approved the study protocols.

Phenotype studied

The threshold state of high myopia was determined by SE [refractive sphere + cylinder/2 (in plus cylinder)]. For both SCORM and SP2, high myopia cases were defined as SE ≤ −6.00 D in at least one eye, and controls were defined as SE between −0.50D and +1.00D in both eyes (emmetropia).

Genotyping

SCORM & SP2—Genome-wide SNP genotyping was conducted for both SCORM and SP2 using Illumina Beadarrays (http://www.illumina.com/, December 2007). For SCORM, a total of 1116 DNA samples (1037 from buccal swab and 79 from saliva) were genotyped using Illumina HumanHap 550 or 550 Duo Beadarrays®. For SP2, a total of 2867 blood-derived samples were genotyped using Illumina HumanHap 550v3, 610Quad, and 1Mduov3 platforms. That is, 392 samples were genotyped by 550v3, 1459 samples by 610Quad, 817 samples by 1Mduov3, 191 samples by both 550v3 and 1Mduov3, and 8 samples by 610 Quad and 1Mduov3. Genotyping consistency across platforms was addressed using 199 samples placed on the two different Beadarrays.

Japan—The SNPs to be validated were genotyped in the Japanese samples. Genotyping was performed with the Taqman SNP assay using the ABI PRISM 7700 system (Applied Biosystems, Foster City, CA).

Quality control (QC) criteria

The Illumina BeadStudio program (Illumina Inc., San Diego CA) was used for genotyping calls of each marker. To ensure high quality genotype data, we instituted a series of marker filtering criteria. Markers were excluded if they significantly deviated from Hardy-Weinberg equilibrium (HWE) in the control dataset ($P<10^{-5}$), had a minor allele frequency (MAF) < 1%, or had missing genotype calls >10% across samples. Samples were excluded from further analysis if the overall genotype call rate was <98%, and more than 6 standard deviation in the population structure analysis using EIGENSTRAT[26] programs.

Association analysis

Since the genotype data were not generated from a single Illumina Beadarray platform (two for SCORM and three for SP2), we analyzed all common markers in the dataset(s) tested. The software PLINK[27] served as the primary analytical tool. Single-locus association tests were performed under a logistic regression framework for all SNPs in SCORM and SP2. Genotypes of each marker were coded as 0, 1, and 2 for the number of minor alleles carried, and a trend test for association was conducted within a logistic regression framework. Age and gender covariates were included in the logistic regression model.

The z-test[28] was used for meta-analysis of both the SCORM and SP2 cohorts. Markers were considered as genome wide significant if $P < 5\times10^{-8}$ (individual p-value or meta p-value), which is the mostly commonly accepted significance threshold for GWA studies[29;30]. In addition to this stringent criteria, a relaxed significance threshold was applied for choosing markers for the replication analysis in the Japan dataset, That is, markers need to satisfy both (1) a meta p-value of $P < 5\times10^{-5}$, and a combination of study-specific p-values of $P < 10^{-3}$ and $P < 0.01$ in either dataset. For the Japan dataset analysis, the trend test for association was performed for high myopia[31]. $P < 0.05$ was considered significant for replication in the Japan dataset. To assess the effect size of the target marker, the per-allele odds ratio (OR) and its accompanying 95% confidence interval (CI) was calculated from the trend test for the replicated SNP(s) for all datasets.

Results

GWAS analysis datasets

The basic demographic data for both SCORM and SP2 datasets is summarized in Table 1, and the description of SNPs selection process for meta-analysis of both cohorts is shown in Figure 1.

The post-QC SCORM GWA dataset was comprised of 929 subjects (481 males and 448 females) with refractive error data, of which 65 subjects have high myopia and 238 subjects are emmetropic controls. Due to two types of Illumina chips used in SCORM, only 541,849 autosomal SNPs were investigated. We excluded 69,801 markers based on the marker exclusion criteria described in the Methods section. After implementation of the exclusions and filtering, 472,048 SNPs remained in the SCORM dataset for the analysis.

The post-QC SP2 GWA dataset comprised of 2008 subjects (921 male, 1087 female), for which 222 have high myopia and 455 are emmetropic controls (Table 1). Three Illumina chips were used in the SP2 GWA study, and 489,028 common autosomal SNPs were evaluated. A total of 26,737 SNPs were excluded due to the violation of genotype missingness > 10%, gross departure from HWE, and monomorphism, which left 462,291 SNPs for GWA analysis.

For the meta-analysis of the SCORM and SP2 GWA results, we analyzed 459,687 markers genotyped in common for both datasets.

Meta-analyses and replication studies

The full list of markers with p-value less than 0.001 from the GWA analyses of SCORM, SP2, and meta-analysis of SCORM and SP2, respectively, is listed in Tables 2, 3 and 4 (available at http://aaojournal.org). Figure 2 provides an overview of the meta-analysis of SCORM and SP2 for high myopia. Although no markers met the genome wide significance level ($P<5\times10^{-8}$), three markers, rs10508626 (chromosome 10 (chr10), *meta-P*=9.24×10^{-6}), rs6885224 (chr5, *meta-P*=2.25×10^{-5}), and rs12716080 (chr5, *meta-P*=2.61×10^{-5}), met our

marker selection criteria (see methods) to be brought forward for replication in the Japan dataset. Among the three markers, rs12716080 and rs6885224 (both from chromosome 5) showed evidence of association in the Japan dataset (Table 5). That is, we observed nominal significant for rs6885224 (P=0.035) in Japan, thus supporting association evidence of this marker in SCORM and SP2 meta-analysis. The second SNP (rs12716080) showed weaker evidence of association at P=0.11, in the same direction as that of rs6885224 (Table 5). When these two markers were examined in detail, we found that both displayed statistically significant association even when analysed within the specific SCORM and SP2 cohorts: rs12716080 (SCORM: $P=7.06\times10^{-5}$; *OR = 2.41, 95% CI: 1.56 – 3.72*, SP2: $P = 8.8\times10^{-3}$; *OR = 1.48, 95% CI:1.1 – 1.97*), and rs6885224 (SCORM: $P=1.85\times10^{-4}$, *OR=2.25, 95% CI: 1.47 – 3.43*, SP2: $P=7.6\times10^{-3}$, *OR=1.5, 95% CI:1.11 – 2.01*). Figure 3 depicts the p-values from the meta-analysis of SCORM and SP2, and p-values of individual dataset for all markers within *CTNND2* region. The linkage disequilibrium (LD) plot clearly shows that both rs6885224 and rs12716080 are in the same LD block.

Discussion

This study utilized two GWA datasets of Singaporean Chinese with 287 high myopia cases and 693 controls out of 2937 GWA samples, and a follow-up replication study in 3087 (959 high myopia cases and 2128 controls) Japanese. As we combined refractive error data from children and adults, high myopia is likely the more robust phenotype, as children with high myopia are very likely to remain highly myopic for life and phenotype reversal is extremely rare.

We found significant association of the *CTNND2* gene on chromosome 5p15 to high myopia. The minor allele of rs6885224 was consistently associated with increased susceptibility to high myopia in SCORM (OR = 2.25, 95% CI:1.47–3.43) and SP2 (OR = 1.5, 95% CI:1.11–2.01), with evidence of replication in Japan dataset (OR = 1.14, 95%CI: 1.02–1.27), thus suggesting that this gene is a potential candidate for high myopia across pediatric and adult age groups in two East Asian populations. A second *CTNND2* SNP rs12716080 also had evidence of association to high myopia for the SCORM and SP2 cohorts (Figure 2), with weaker evidence in the Japan cohort (Table 5). Both SNPs are in LD with r^2 of 0.89 in Chinese and 0.937 in Japanese. In the HapMap and Human Genome Diversity projects, Chinese and Japanese have high similarity in population structure[32;33]. Intuitively, *CTNND2* may also be a genetic determinant for childhood high myopia, leading to high-grade myopia in adulthood. This finding may be useful for developing interventions in high risk children who carry the *CTNND2* risk allele.

The *CTNND2* gene spans 933 Kb with 22 coding exons and resides within a 17.45 cM region on chromosome 5p15 previously found to be linked to high myopia in a family segregation study of three Hong Kong Chinese pedigrees (LOD = 4.68)[34]. More recently, evidence of linkage replication for that region was determined in a small Asian subset of families with high-grade myopia (N = 10 families) (LOD=1.34)4 (Figure 2). Of note, in non-human models, *CTNND2* has been documented to play a crucial role in retinal morphogenesis, adhesion, and retinal cell architectural integrity via regulation of adhesion molecules[35;36]. Interestingly, *CTNND2* was one of five biologically plausible genes for high myopia examined by Lam et al.[34], who conducted direct sequencing of their coding regions to determine possible segregation with high myopia. In their study, five SNPs in the vicinity of *CTNND2* were genotyped in a case-control association analysis using 94 cases with high myopia (SE at least −6.00 D) and 94 non-myopic controls. Evidence of segregation or association was not determined for *CTNND2* in the Lam et al. study. It should be noted that both rs6885224 and rs12716080 were not assessed by Lam et al., and that the underpowered small sample size may have influenced the ability to detect evidence of association.

The strengths of our GWA studies are the reporting of association of unique genetic variants in two datasets of Singapore Chinese with very high rates of myopia, followed by the availability of the Japanese dataset for direct replication. The epidemic of myopia, especially in Chinese populations in Asia, may be due to either environmental factor (e.g., competitive educational systems with intensive near-work activity at an early age, limited outdoor activity)[37;38], genetic susceptibility, or both. We are, therefore, mindful that myopia is a complex disease with (up till now) no major, striking susceptibility locus. Rather, the disease susceptibility may be accounted for by multiple loci each exerting very small effect sizes. Therefore, it was not surprising that genome wide significance for single-marker analysis was not observed in our SCORM and SP2 datasets due to the small sample sizes, which have insufficient power to detect markers with small genetic effects (e.g., OR < 1.2). In order to reach the formal genome-wide threshold ($P < 5\times10^{-8}$), we estimate that we would need a sample size of > 9000, which we are currently working actively to achieve.

Despite missing this formal threshold, our observations with the *CTNND2* SNPs are significantly consistent across all 3 cohorts (2 Chinese and 1 Japanese), and this argues against it being a false positive finding. Overall, our study suggests that although genetic factors could be influential (e.g., *CTNND2*), they are in all likely to be very modest [1;2;23].

In summary, we report novel association to high myopia of a common polymorphism of the gene *CTNND2* in Chinese and Japanese cohorts. This new locus may inform functional variants for myopia, and provide insights into the eventual development of early intervention strategies to retard the progression of myopia in high risk populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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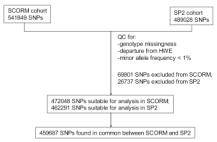

Figure 1.

The flowchart of the number of markers genotyped, excluded, and analyzed for Singapore Cohort Study of the Risk factors for Myopia (SCORM) and the Singapore Prospective Study Program (SP2) datasets. SNPs = single nucleotide polymorphisms; QC = quality control; HWE = Hardy-Weinberg equilibrium.

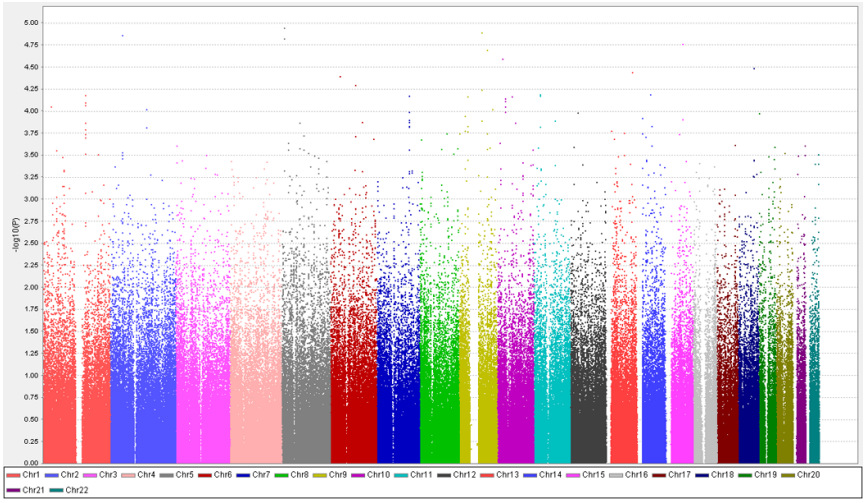

Figure 2.
Manhattan plots of meta-analyses of Singapore Cohort Study of the Risk factors for Myopia (SCORM) and Singapore Prospective Study Program (SP2) datasets for high myopia. Chr = chromosome.

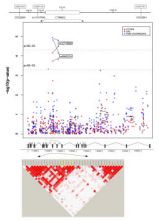

Figure 3.
Location, Association Results, and linkage disequilibrium (LD) Pattern of *CTNND2*. The relative location of *CTNND2* to the target markers for the chromosome 5 linkage region reported in Lam et al. (2005) (D5S2004 and D5S2081) and Li et al. (2009) (rs13157690). Two single nucleotide polymorphisms (SNPs) (rs12716080 and rs6885224) shown promising replication evidence are located in the same LD block. LOD=logarithm (base 10) of odds.

Table 1

Summary statistics for spherical equivalent and sample size for each phenotypic category from the Singapore Cohort Study of Risk Factors for Myopia and Singapore Prospective Study Program datasets.

Cohorts	**Singapore Cohort Study of Risk Factors for Myopia (SCORM)**		**Singapore Prospective Study Program (SP2)**	
Male/Female	481/448		921/1087	
Mean Age* **(SD§)**	10.83 (0.83)		47.9 (11.18)	
Phenotypes	**No. Samples (Percentage)**	**Both Eye Avg SE Mean (SD§)**	**No. Samples (Percentage)**	**Both Eye Avg SE Mean (SD§)**
SE*,§	929	−2.02 (2.26)	1931	−1.67 (2.89)
High myopia**	65 (6.9%)	−6.88 (1.06)	222 (11.5%)	−7.51 (2.02)
Emmetropia (Controls)**	238 (25.6%)	0.42 (0.53)	455 (23.6%)	0.19 (0.35)

* For the SCORM dataset, age and spherical equivalent were based on the 4th annual examination of the study.

§ SE = Spherical equivalent; SD = Standard deviation

** High myopia: SE ≤ −6.00D for at least one eye; Control: −0.50D< SE< +1.00D for both eyes; D=Diopter

Table 5

Summary of top single nucleotide polymorphisms from the Singapore Cohort Study of the Risk factors for Myopia* and Singapore Prospective Study Program* datasets, and the replication results in Japan dataset for high myopia.

Chr*	SNP*	BP*	Gene	Minor Allele		Meta-SCORM* and SP2*	SCORM*	SP2*	Japan	Meta-SP2* SCORM* and Japan
5	rs6885224	11222945	CTNND2	C	**Sample**					
					sizes	1070	303	767	3083	4153
					MAF*,§	0.28 (0.21)	0.38 (0.20)	0.25 (0.21)	0.26 (0.23)	
					p-values					
						1.51E-05	1.85E-04	7.60E-03	0.035	7.84E −06**
					OR*	1.71	2.25	1.50	1.14	1.24
					[95% CI*]	[1.34,2.18]	[1.47,3.43]	[1.11,2.01]	[1.02,1.27]	[1.11,1.39]
5	rs12716080	11219948	CTNND2	G	**Sample**					
					sizes	1070	303	767	3085	4155
					MAF*,§	0.31 (0.23)	0.42 (0.22)	0.28 (0.23)	0.29 (0.27)	
					p-values					
						1.14E-05	7.06E-05	8.80E-03	0.11	1.05E-05*
					OR*	1.72	2.41	1.48	1.10	1.20
					95% CI*]					
						[1.35,2.19]	[1.56,3.72]	[1.10,1.97]	[0.98,1.22]	[1.08,1.34]

*SCORM = Singapore Cohort Study of the Risk factors for Myopia; SP2 = Singapore Prospective Study Program; Chr = chromosome; SNP = single nucleotide polymorphisms; BP = basepair position; MAF = minor allele frequencies; OR = per-allele odds ratio for high myopia; 95% CI = 95% confidence interval of OR.

§MAF is listed for high myopia cases and controls, respectively, where the one for controls is in parenthesis.

**Fisher p values[39] by adding Japan dataset.