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Controversial opinion: evaluation of *EGR1* and *LAMA2* loci for high myopia in Chinese populations*

Fang-yu LIN[1], Zhu HUANG[1], Ning LU[1], Wei CHEN[2], Hui FANG[1], Wei HAN[†‡1]

([1]*Department of Ophthalmology, the First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310003, China*)

([2]*Department of Immunology, School of Medicine, Zhejiang University, Hangzhou 310058, China*)

[†]E-mail: hanweidr@hotmail.com

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Abstract: Functional studies have suggested the important role of early growth response 1 (EGR1) and Laminin α2-chain (LAMA2) in human eye development. Genetic studies have reported a significant association of the single nucleotide polymorphism (SNP) in the *LAMA2* gene with myopia. This study aimed to evaluate the association of the tagging SNPs (tSNPs) in the *EGR1* and *LAMA2* genes with high myopia in two independent Han Chinese populations. Four tSNPs (rs11743810 in the *EGR1* gene; rs2571575, rs9321170, and rs1889891 in the *LAMA2* gene) were selected, according to the HapMap database (http://hapmap.ncbi.nlm.nih.gov), and were genotyped using the ligase detection reaction (LDR) approach for 167 Han Chinese nuclear families with extremely highly myopic offspring (<−10.0 diopters) and an independent group with 485 extremely highly myopic cases (<−10.0 diopters) and 499 controls. Direct sequencing was used to confirm the LDR results in twenty randomly selected subjects. Family-based association analysis was performed using the family-based association test (FBAT) software package (Version 1.5.5). Population-based association analysis was performed using the Chi-square test. The association analysis power was estimated using online software (http://design.cs.ucla.edu). The FBAT demonstrated that all four tSNPs tested did not show association with high myopia (P>0.05). Haplotype analysis of tSNPs in the *LAMA2* genes also did not show a significant association (P>0.05). Meanwhile, population-based association analysis also showed no significant association results with high myopia (P>0.05). On the basis of our family- and population-based analyses for the Han Chinese population, we did not find positive association signals of the four SNPs in the *LAMA2* and *EGR1* genes with high myopia.

Key words: Myopia, *EGR1*, *LAMA2*, Association study, Single nucleotide polymorphism
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1 Introduction

Myopia is a very common ocular disorder worldwide, resulting primarily from excessive ocular axial elongation. In some eastern countries, such as China, Japan, and Singapore, the prevalence of myopia has rapidly increased to more than 80% of high-school populations, among whom 10%–20% could be highly myopic (Pan *et al.*, 2012). Myopia, particularly high myopia, can bring about visual impairment due to the ocular morbidity of maculopathy, retinal detachment, cataract, and glaucoma, etc. The economic cost of refractive error management is substantial. Hence it has emerged as a major public health concern, especially in some Asian countries.

The etiology of myopia, except for syndromic myopia, is thought to be multifactorial with both genetic and environmental components involved. Although epidemiological studies have demonstrated that environmental factors, such as education, outdoor exposure, and near work, are important in the development of myopia, it is well established that genetics

‡ Corresponding author

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ORCID: Wei HAN, http://orcid.org/0000-0003-1696-0615

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also play a substantial role in the etiology of myopia, particularly high myopia (Wojciechowski, 2011).

In past decades, numerous studies have reported genes or loci which may predispose to myopia, including linkage or association analysis (Hawthorne and Young, 2013). Recently, advances in genome technology have facilitated loci identification in genes associated with a variety of ocular disorders including myopia using strategies of exome sequencing or genome-wide association studies (GWASs) (Aldahmesh *et al*., 2013; Guo *et al.*, 2015; Miyake *et al*., 2015). However, determining the genetic role of a specific gene/locus in the etiology of myopia is still difficult to achieve. At present, for research of mapping myopia genes/loci and the underlying mechanisms, replication of genetic association analysis is critically important.

In this study, we tested polymorphisms in two candidate genes, early growth response 1 (*EGR1*) and Laminin α2-chain (*LAMA2*), based on their biological functionality and previous data of GWAS analysis.

The *EGR1* gene encodes the protein which belongs to the EGR family of Cys2His2-type zinc finger proteins, a nuclear protein functioning as a transcriptional regulator. The EGR1 is essential for an individual's growth and development and has been found to be related to multiple disorders (Bhattacharyya *et al*., 2013). Studies have demonstrated its potential importance in myopia occurrence. The gene knockout mice model demonstrated that EGR1 played a role in experimental axial myopia (Schippert *et al*., 2007). Fischer *et al*. (1999) also found a significant association of the EGR1 expression level in the retina with eyeball growth in the animal model. Moreover, a recent myopia animal study indicated that EGR1 might play a role in signaling eye growth bidirectionally (Ashby *et al*., 2014). However, Li *et al.* (2008) screened mutations in the *EGR1* gene region in a small group of unrelated high myopia individuals (n=96), but found no causal variations in their work.

Another important protein, Laminin, is an extracellular protein and major component of the basement membrane. It is thought to mediate the attachment, migration, and organization of cells into tissues during embryonic development (Schéele *et al.*, 2007). The *LAMA2* gene encodes the Laminin α2 protein, which is the subunit of Laminin and plays an important role in the connection of sclera collagen fiber (Jonas and Xu, 2014). Extracellular matrix remolding of sclera is the major histological feature in eye growth or myopia (Harper and Summers, 2015). Therefore, LAMA2 is speculated to take part in the onset and progression of myopia. Recently, two multiple-center GWAS studies suggested the strong association of single nucleotide polymorphism (SNP) in the *LAMA2* locus with myopia in different ethnic populations (Cheng *et al*., 2013; Verhoeven *et al*., 2013). Another GWAS study in European populations also indicated the association of SNP in the *LAMA2* region (Kiefer *et al*., 2013). However, two recent association studies in Eastern Asian populations did not support the positive association of *LAMA* loci with myopia (Yoshikawa *et al*., 2014; Li *et al.*, 2015).

As discussed above, the two proteins, EGR1 and LAMA2, were both thought to be the potential key molecules involved in the cascade signaling the posterior sclera elongation and hence affecting eyeball growth. Many efforts have been made to elucidate their potential role in myopia onset. Therefore, we speculate that the variants in genes encoding EGR1 and LAMA2 may predispose the susceptibility to myopia, especially high myopia, for which the posterior pole expansion is the most significant lesion. However, the results regarding association analysis of the *EGR1* and *LAMA2* genes with myopia are still equivocal, despite the appealing role of the two proteins in ocular growth. The aforementioned literature reflected the complexity of myopia gene mapping and the importance of the replication of association analysis to achieve greater confidence in the association test results. The present study aimed to further evaluate the genetic association of potential SNPs in the two candidate genes with extremely high myopia (<10.0 diopters (D)) occurrence in the Han Chinese population.

To obtain a stronger genetic background for the subjects studied, our work used two types of dataset to test association of the tagging SNPs (tSNPs) in the *EGR1* and *LAMA2* genes: extremely high myopia nuclear family samples and independent case-control high myopia samples from the southern Han Chinese population. The tSNP markers used for association analysis were selected according to HapMap data (http://hapmap.ncbi.nlm.nih.gov) and also previous GWAS study data.

2 Subjects and methods

2.1 Subjects

Family-based analysis subjects were recruited by a method reported previously (Han *et al*., 2009). Meanwhile, the independent subjects including highly myopic cases and emmetropic controls for the case-control association study were also recruited from the Eye Department of the First Affiliated Hospital in Hangzhou, China, with written signed informed consent. All subjects were Han Chinese from southern China. The study was approved by the Human Subject Ethics Subcommittees of Zhejiang University and adhered to the tenets of the Declaration of Helsinki. Each nuclear family includes two parents and one affected myopic offspring. For all highly myopic siblings of the nuclear family study group and the highly myopic cases of the case-control study group, the entry criterion for high myopia was a spherical equivalence (SE) of −10.0 D or worse for both eyes, with astigmatism of less than 3 D. For all emmetropia controls of the case-control study group, the entry criterion was SE of ≥−0.75 D and ≤+1.0 D for both eyes, and astigmatism within 0.75 D.

2.2 Selection of genetic markers for association analysis

Genetic markers were selected based on the following strategies. For the genetic marker in the *EGR1* gene region used for analysis, one tSNP (rs11743810) was selected and tested in the genetic association study according to the tSNP database of HapMap. The *EGR1* is a small gene, and the one tSNP selected could be the sufficient linkage disequilibrium (LD) proxy covering the coding and flanking potential regulatory sequences (Fig. 1).

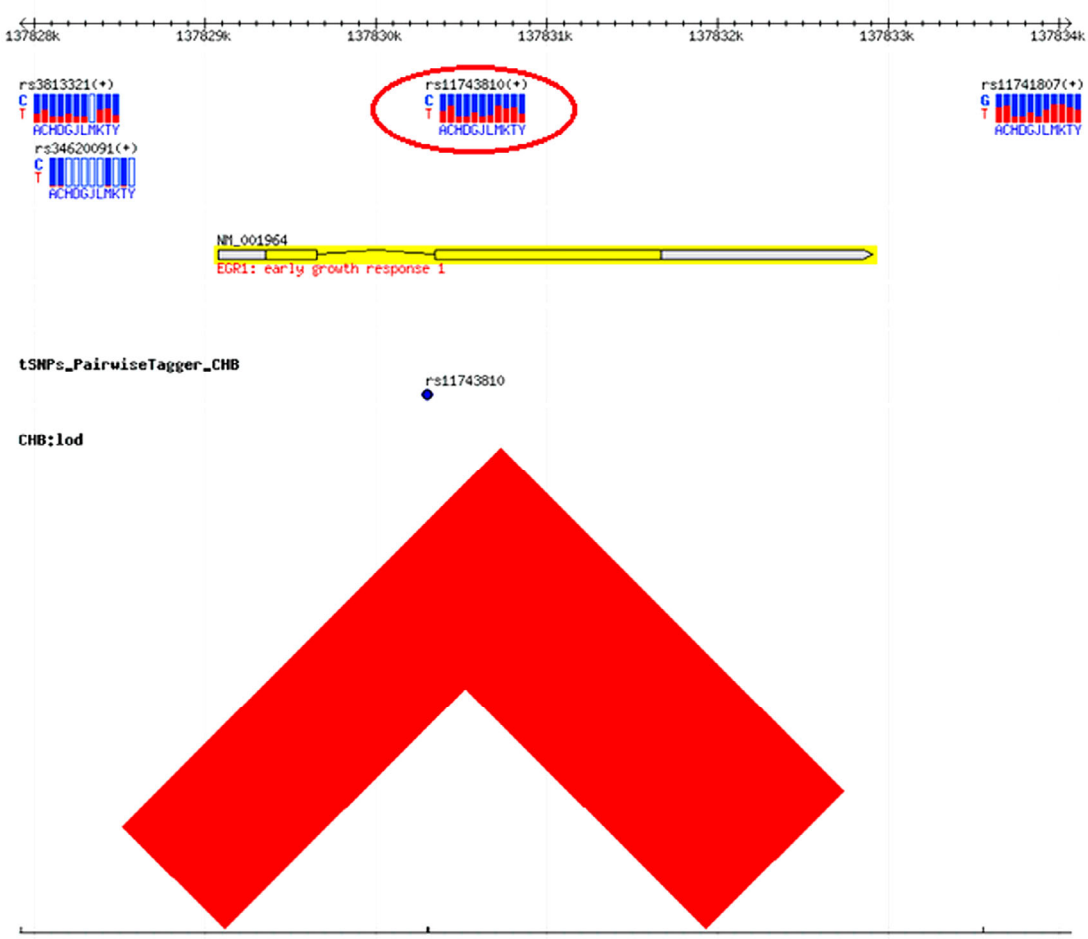

Fig. 1 Selection of tSNP rs11743810 in the *EGR1* gene based on the HapMap data
The tSNP selected for analysis is marked using a circle

For tSNPs in the *LAMA2* gene region, according to the previously reported data of GWAS analysis (Cheng *et al*., 2013; Kiefer *et al*., 2013; Verhoeven *et al*., 2013) and the tSNP suggested by the HapMap database, three tSNPs (rs2571575, rs9321170, and rs1889891) were selected, which were not rare polymorphisms in the Han Chinese population (minor allele frequency (MAF) >0.2) and were in the same LD plot as the SNPs showing significant association in the previous GWAS study (Fig. 2). The MAF cutoff value was set at 0.2 based on the updated high myopia prevalence data in Chinese as well as in Eastern Asian populations (Morgan *et al.*, 2012; Wu *et al*., 2015).

2.3 Genotyping of SNP markers for nuclear families and case-control subjects

DNA was extracted from blood samples with commercial kits (Han *et al*., 2009). The ligase detection reaction (LDR) approach was used to genotype the four tSNPs (rs11743810 in *EGR1*, and rs2571575, rs9321170, and rs1889891 in the *LAMA2* gene) for subjects of 167 Han Chinese nuclear families and an independent group with 485 highly myopic cases (<−10.0 D) and 499 controls. Direct sequencing was used to confirm the LDR results in twenty randomly selected subjects.

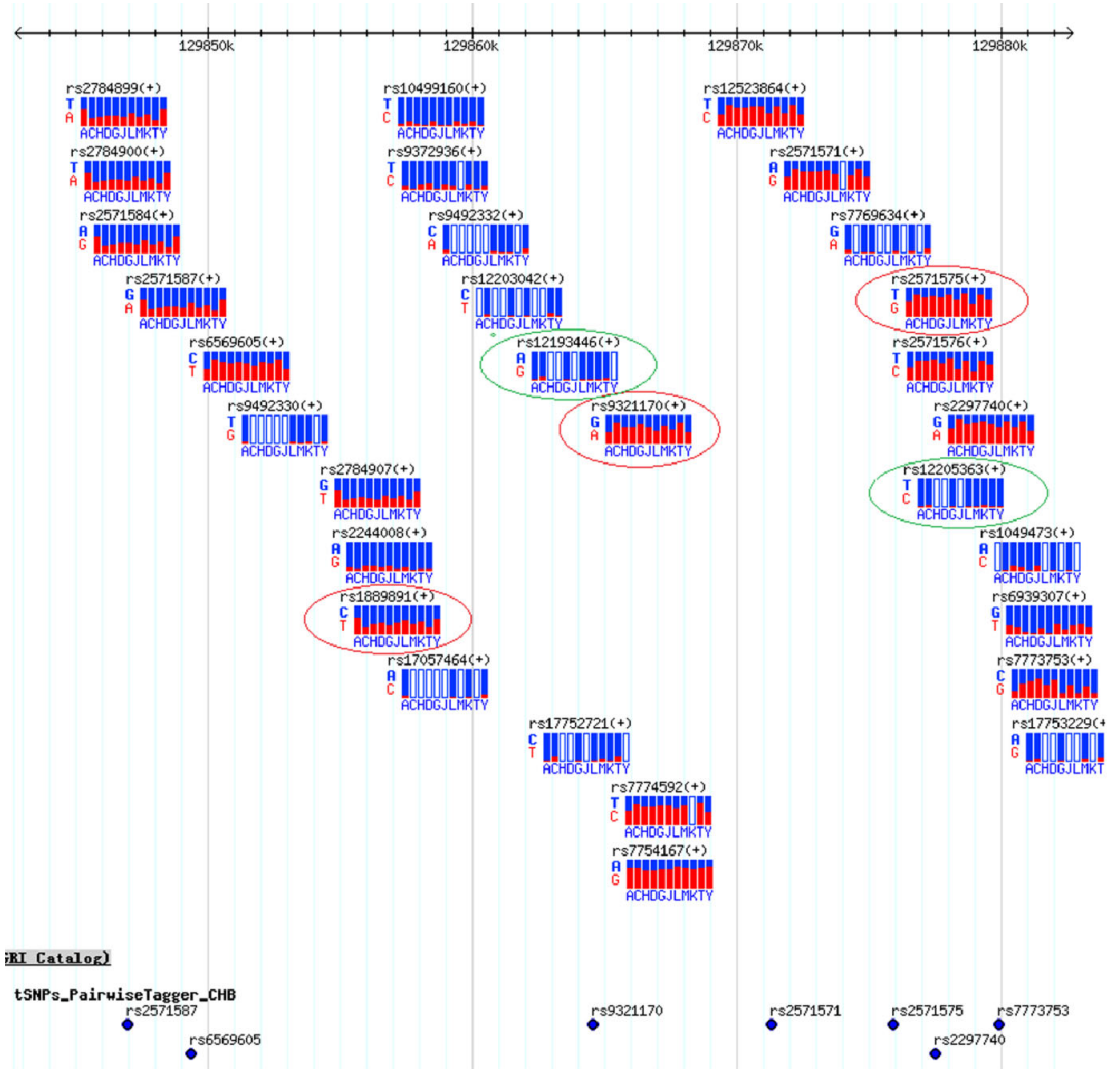

Fig. 2 Selection of tSNPs rs2571575, rs9321170, and rs1889891 in the *LAMA2* gene according to the HapMap data

The three tSNPs selected for analysis are marked using red circles, while the SNPs reported in previous GWAS data are circled in green (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

The 10 μl LDR mixture consists of 2 μl PCR product of target DNA fragments, 1 μl 10× Taq DNA ligase buffer, 0.125 μl Taq DNA ligase (40 U/μl) (New England Biolabs, Beverly, USA), 0.01 μl LDR probe (10 pmol), and distilled water. The LDR probes for each tSNP are listed in Table 1. The LDR mixture was then subjected to a PCR amplifier (Applied Biosystems, Foster City, USA) and altogether thirty cycles of the ligation reaction were completed with each cycle consisting of 30 s denaturing at 94 °C, followed by 3 min annealing at 56 °C. After the ligation reaction, 1 μl ligation product mixed with 10 μl loading buffer was denatured at 95 °C for 3 min and immediately bathed in an ice-water mixture. The mixture was subjected to an ABI 3730 XL sequencer (Applied Biosystems, Foster City, USA), and the SNP genotypes were then analyzed.

2.4 Statistical analysis of ocular data

Commercial software (SPSS Version 11.0; SPSS Inc., Chicago, IL, USA) was used to test the partial correlation between ocular components (axial length (AXL), anterior chamber depth (ACD), and corneal power (CP)) and mean spherical equivalent (MSE) of the affected siblings and independent high myopia cases.

2.5 Genetic association study

The family-based association test (FBAT) software package Version 1.5.5 (http://www.biostat.harvard.edu/~fbat/fbat.htm) was used for the association test for nuclear family data (Cordell *et al.*, 2004). The association of both single-marker SNPs and multiple-marker haplotypes for tSNPs in the *LAMA2* gene was tested. The linkage phase was resolved using an expectation-maximization algorithm. A matched case-control dataset was generated with each affected offspring matched to three possible pseudocontrols created from the untransmitted parental allele, if a positive association result was found. The association between the cases and control subjects was examined using the Chi-square test with Haploview software

Table 1 Clinical data of high myopia subjects including affected siblings from nuclear families group (n=167) and independent highly myopic cases from case-control group (n=485)

Parameter	Value*
All highly myopic subjects (n=167+485)	
Age at entry (year)	23.93±11.65
Onset of myopia (year)	7.05±3.25
Sex (male:female)	337:318
AXL (mm)	27.68±2.11 (r=−0.72, P<0.001)**
CP (D)	43.11±1.36 (r=−0.37, P=0.015)
ACD (mm)	3.67±0.33 (r=−0.29, P<0.001)
Affected highly myopic siblings (n=167)	
MSE (D)	−11.45±2.97
Families with no myopic parents	69
Families with 1 myopic parent	65
Families with 2 myopic parents	33
Families with 1 highly myopic parent	35
Families with 2 highly myopic parents	9
Rate of highly myopic fundus lesion (%)	45.3
Independent highly myopic cases (n=485)	
MSE (D)	−11.32±2.39
Cases with no myopic parents	236
Cases with 1 myopic parent	161
Cases with 2 myopic parents	39
Not be defined***	49
Rate of highly myopic fundus lesion (%)	63.1

* Values are expressed as mean±standard deviation (SD) or number. The values in brackets are the partial correlation test between mean spherical equivalent (MSE) and ocular indices of axial length (AXL), corneal power (CP), and anterior chamber depth (ACD). AXL, CP, and ACD were calculated in all high myopia subjects from both nuclear families and case-control groups. ** AXL shows the most significant correlation to MSE. *** These cases failed to provide their parental refractive error information. D: diopters

Version 3.3.2 (http://www.broadinstitute.org/haploview). Association test power was calculated using online software (http://design.cs.ucla.edu) based on the algorithm proposed by de Bakker *et al*. (2005).

3 Results

3.1 Clinical data analysis

For all highly myopic individuals including affected siblings and independent high myopia individuals, the average age at entry was 23.93 years and their average onset age of myopia was 7.05 years. AXL showed the strongest correlation with refractive error (P<0.001), suggesting the axial high myopia studied.

For the family-based association study, 167 nuclear families were recruited altogether. Each family had only one affected sibling (n=167). Of these nuclear families, 65 (38.9%) had one myopic (−0.75 D or less) parent and 33 (19.7%) had two myopic parents. The MSE of all affected siblings was (−11.45±2.97) D. For the high myopia-related eye fundus lesions, such as retina degeneration, hemorrhage, and hole, the incidence of morbidity was 45.3%.

For the case-control association study group, 485 high myopia subjects with MSE of (−11.32±2.39) D and 499 normal control subjects with MSE of (−0.25±0.75) D were recruited altogether. Among the 485 high myopia cases, 200 (41.23%) of them had one or two myopic parents. The incidence of the eye fundus morbidity was 63.1%. There was no significant difference in indices of sex ratio (male:female) or age between the healthy emmetropia controls and highly myopic cases (P>0.05). A strong significant difference of AXL was observed between highly myopic cases and emmetropic controls (P<0.0001). Detailed clinical data are listed in Table 1.

3.2 tSNP marker genotyping results

All four tSNPs were successfully genotyped using the LDR approach. The probes designed for tSNP genotyping by the LDR approach are listed in Table 2. The LDR genotyping results of twenty randomly selected subjects were all confirmed by direct sequencing (data not shown). Fig. 3 shows one example of the LDR results of four tSNPs for one genotyped high myopia subject. Our study also demonstrated the efficiency and validity of the LDR approach for SNP genotyping.

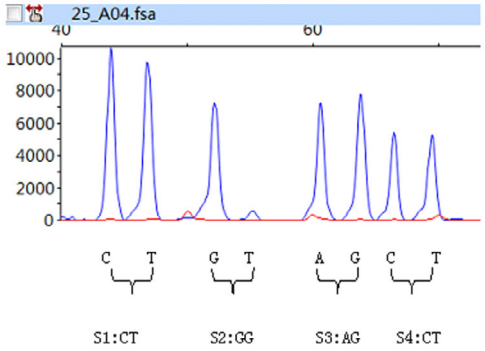

Fig. 3 LDR genotyping results of four tSNPs for one high myopia subject

S1: rs11743810; S2: rs2571575; S3: rs9321170; S4: rs1889891

3.3 Genetic association study

The four SNPs were all in Hardy-Weinberg equilibrium with P>0.10 (Tables 3 and 4). SNP rs11743810 in the *EGR1* gene and SNPs rs2571575,

Table 2 Probes used for genotyping tSNPs by LDR approach

SNP	Probe sequence
rs11743810	A171S1TC: GTCGTCCTCGTCCTCCAGTGATTGC
	A171S1TT: TTTTGTCGTCCTCGTCCTCCAGTGATTGT
	A171S1TR: -P-TTTCCAGTAACCAGGCCTCCCGCTT-FAM-
rs2571575	A350S2TG: TTTTCTAAACACCTCTTTCAGCCCTGCAG
	A350S2TT: TTTTTTTCTAAACACCTCTTTCAGCCCTGCAT
	A350S2TR: -P-TAAGACAACTTTGCACTATAGGCAGTTT-FAM-
rs9321170	A350S3TA: TTTTTTTTTAAGTGCAGATCTTAAAAACAGATA
	A350S3TG: TTTTTTTTTTTTAAGTGCAGATCTTAAAAACAGATG
	A350S3TR: -P-TGTTGGTGAGAGTGTGCAGGTTATTTTTTTT-FAM-
rs1889891	A350S4TC: TTTTTTTTTTTTGAGGGAAACAATGAGGATCTTCAAC
	A350S4TT: TTTTTTTTTTTTTTTGAGGGAAACAATGAGGATCTTCAAT
	A350S4TR: -P-TTAGACTGAGAAAAGCACACTAATGTTTTTTTTT-FAM-

rs9321170, and rs1889891 in the *LAM2* gene were all found not to be associated with high myopia using the FBAT (P>0.05; Table 3). For the case-control analysis, the four tSNPs tested in the *EGR1* and *LAMA2* genes also showed no significant association with high myopia (P>0.05; Table 4). Meanwhile, the haplotypes and subhaplotypes consisting of SNPs rs2571575, rs9321170, and rs1889891 in the *LAMA2* gene also did not show any significant association with high myopia for both family-based analysis and case-control analysis (P>0.05; detailed data not shown). The results of the association test power calculation are listed in Table 4. The sample size was 984, with 485 cases vs. 499 controls. The prevalence of high myopia was set at 0.15 (Morgan *et al*., 2012; Wu *et al*., 2015). The software for the association test power estimation gave the values of 0.872 31 for SNP rs1889891, 0.932 18 for SNP rs9321170, 0.788 46 for SNP rs2571575, and 0.997 83 for SNP rs11743810. The r^2 values of these SNPs with the tSNPs suggested by the HapMap were 0.727 27–1.000 00 (Table 4).

4 Discussion

4.1 Entry criteria and phenotyping

High myopia is usually defined as a refractive error of −6.00 D or worse (Curtin, 1985). Our study set an SE of −10.0 D or worse with early onset age (earlier than 12 years old) as the entry criterion for all highly myopic offspring and independent case subjects. This could enhance the genetic background for our high myopia subjects (Iribarren *et al*., 2005), as myopia is thought to be a multi-factorial phenotype determined by gene and environment factors and also their interaction. On the other hand, myopia is a refractive error phenotype which is determined by multiple ocular components including cornea curvature, lens power, and AXL, for which the underlying genetic background can be quite different (Guggenheim *et al*., 2013). These confounding factors may compromise the statistical efficiency of the association test and be responsible for the equivocal results observed in different myopia genetic studies (Farbrother *et al*., 2004; Hysi *et al*., 2014). It is well known that the elongation of AXL is the primary ocular component contributing to high myopia and has the strongest genetic component (Goss *et al*., 1997). The strongest correlation between AXL and SE for our subjects could also provide a strong and homogeneous genetic background for the cases in this study (Han *et al*., 2009). The incidence of high myopia-related eye fundus lesions was 45.3% and 63.1% for subjects of nuclear families and case-control groups, respectively (Table 1), indicating the pathological one for the myopia subjects in the present study. The discrepancy of fundus lesion morbidity between the two groups might be ascribed to the relatively younger age of the siblings in the nuclear family group, who still have not presented the fundus lesions. Our effort in recruiting extremely high myopia and stringent phenotyping is helpful for minimizing the heterogeneity of myopia trait and enhancing the efficiency of the subsequent association test.

Table 3 Family-based association test results of nuclear family subjects (n=167)

SNP	Franking sequence [SNP allele (major/minor)]	MAF	χ^2	P	HWE
rs11743810	GTCCTCCAGTGATTG**[C/T]**TTTCCAGTAACCAGG	0.414	1.494	0.2216	>0.10
rs2571575	TCTTTCAGCCCTGCA**[G/T]**TAAGACAACTTTGCA	0.240	0.174	0.6767	>0.10
rs9321170	ATCTTAAAAACAGAT**[A/G]**TGTTGGTGAGAGTGT	0.406	0.083	0.7738	>0.10
rs1889891	AATGAGGATCTTCAA**[C/T]**TTAGACTGAGAAAAG	0.386	0.136	0.7127	>0.10

MAF: minor allele frequency; HWE: Hardy-Weinberg equilibrium

Table 4 Association test results of case-control samples

SNP	Allele (major/minor)	Case (n=485)	Control (n=499)	P	HWE	Estimated power*	r^2 with tSNP**
rs11743810	C/T	270/215	551/447	0.5641	>0.10	0.99783	1.000 00
rs2571575	G/T	331/154	718/280	0.8375	>0.10	0.88846	0.739 20
rs9321170	A/G	267/218	560/438	0.9947	>0.10	0.93218	0.823 53
rs1889891	C/T	216/269	424/574	0.8322	>0.10	0.87231	0.727 27

HWE: Hardy-Weinberg equilibrium. * The association power was calculated with the sample size of 485 cases and 499 controls and assuming disease prevalence of 0.1. ** The r^2 denoted the correlation of the SNPs in the present study with the tSNPs suggested by the HapMap

4.2 Association analysis

The genetic association study is robust for mapping genes underlying complex diseases such as high myopia (Tang *et al*., 2008). Association analysis using family-based data can avoid confounding due to population stratification and heterogeneity in association studies using case-control data (Tang *et al*., 2008). However, the population-based association test is thought to be more powerful than family-based approaches, assuming no population stratification. Studies with replication data from independent populations are more powerful for drawing a more convincing result for association analysis than those from a single population. Replication and aggregate cohort studies are always desired to validate the candidate genes or loci (Hawthorne and Young, 2013). Therefore, we employed the study design with two independent groups of family-based and case-control samples.

4.2.1 *EGR1* gene results

However, we did not find significant association results for the SNP markers in the two candidate genes, in either nuclear family or case-control groups. We chose *EGR1* as a candidate gene for association analysis of myopia, based on its functionality evidence: (1) Animal model studies of induced myopia and gene knock-out have all demonstrated the important role of *EGR1* in myopia (Schippert *et al*., 2007); (2) EGR1 is a critical protein for eye growth and myopisation (Fischer *et al*., 1999; Ashby *et al*., 2014). The SNP rs11743810 selected here is itself the tSNP covering the *EGR1* gene region's LD plot with an r^2 of 1.000 00 and an estimated power for association test of 0.997 83 (Table 4), suggesting the high efficiency of the association analysis. Our negative results for the *EGR1* analysis of two independent sample groups are in line with the previous report (Li *et al*., 2008). It could be ascribed to the discrepancy of the underlying mechanism between animal and human eye growth. However, our data implied that the *EGR1* gene region may not contain the causal genetic locus predisposing to human myopia despite the key role of its encoding protein in eye growth or myopisation.

4.2.2 *LAMA2* gene results

Similarly, the selected SNPs in the *LAMA2* gene also did not show significant association with high myopia in both sample groups, either for single SNP markers or for relevant haplotypes. We selected to test the *LAMA2* gene based on two justifications: first, its biological function role in posterior sclera remolding, which is a crucial process for myopisation (Jonas and Xu, 2014; Harper and Summers, 2015); second, recent GWAS from multiple center data indicated a strong association of the SNP markers in the *LAMA2* gene region with myopia (Cheng *et al*., 2013; Kiefer *et al*., 2013; Verhoeven *et al*., 2013). Despite the robust data from previous works regarding functional and genomic research, we did not find the association of the adjacent selected SNPs in the *LAMA2* gene region with high myopia.

We selected the tSNPs of the *LAMA2* locus for testing according to the genetic information provided in the HapMap Phase database and previous GWAS studies. The three tSNPs of the *LAMA2* gene selected in this study are adjacent to and in the same LD plot as the SNPs which showed a significant association signal in previous reports. The tSNP rs2571575 is in the same haplotype block as SNP rs12205363, which showed a strong significant association level in a previous study by Verhoeven *et al*. (2013). Meanwhile, SNPs rs9321170 and rs1889891 are also in adjacent flanking sequence of SNP rs12193446, reported by Cheng *et al*. (2013) and Kiefer *et al*. (2013) (Fig. 2).

We did not select the same SNPs as the previous data, but chose the three tSNPs which are more common in the Han Chinese population with MAF= 0.2–0.4 and comparable to the genotyping data in the HapMap (Table 3). The SNPs reported in previous reports are very rare in the Chinese population, with MAF both being zero (Fig. 2), and the previous studies also reported that their MAFs were less than 0.01 in the Asian highly myopic population (Verhoeven *et al*., 2013). Myopia, even high myopia, is a common trait in the human population, particularly the Eastern Asian population, which is unlikely explained by very rare variants (Yang *et al*., 2010; Morgan *et al*., 2012). Therefore, we avoided using the rare SNPs rs12205363 and rs12193446, but instead chose to test the more common tSNPs in their flanking sequence for our analysis of the Han Chinese population. The selection strategy might allow the SNPs to carry more genetic information for the association test in the Han Chinese population and also to serve as the genetic proxy of the previously reported SNPs.

4.2.3 Efficiency of the association analysis

GWAS is currently thought to be the most robust approach for yielding valuable association test results. In the present work, we used both family-based and case-control approaches and only recruited extremely high-grade myopes with significant morbidity of fundus lesion and predominant refractive component of AXL (Table 1). These efforts could be helpful for enhancing the component and unanimity of the genetic background of the myopia subjects as well as the association test power. The estimated power values for the association test were 0.872 31–0.932 18 for the three SNPs in the *LAMA2* region (Table 4), suggesting high efficiency of the association analysis (Tang *et al*., 2008). Meanwhile, r^2 values with the tSNPs were 0.727 27–1.000 00 (Table 4), which denoted a strong LD between the selected SNPs and tSNPs covering the relevant region studied. Nevertheless, this work did not find positive association results regarding the *LAMA2* loci which were reported by the previous GWAS studies (Cheng *et al*., 2013; Kiefer *et al*., 2013; Verhoeven *et al*., 2013). Interestingly, our results were in agreement with two recent reports of Eastern Asian populations (Yoshikawa *et al*., 2014; Li *et al*., 2015). These two studies are also the single association investigation work testing specific potential loci. The inconsistency of association test results could be explained by the discrepancy of genetic backgrounds in different ethnic populations as well as the methodology of different studies employed. Except for the heterogeneity of myopia phenotype, for the genetic factor of myopia, high heterogeneity, penetration, and inherit mode may all give rise to the variation of the subjects used in different association studies (Guggenheim *et al*., 2013). As mentioned above, the pathological/extremely high myopia subjects used in this study might cause the underlying etiology to be different from that in the previous studies which also involved common myopia as their study cohort (Cheng *et al*., 2013; Kiefer *et al*., 2013; Verhoeven *et al*., 2013). This further reflects the complexity of myopia etiology and its genetic contribution.

4.3 Controversial opinion

The present study has involved two independent high-grade myopic subject groups, and we have replicated the results ourselves; however, the study has pointed to a negative association result. Our results have invited some controversy, as listed in the Appendix material. This is not surprising and must by no means lead to a final conclusion regarding the work on such a topic, as we all know that no research can ever be considered to be the final word. Replication and corroboration are always critical for association studies for myopia gene mapping, still a great challenge to date. Therefore, although this work has positive respects, as discussed above, of course limitations exist in our study. A larger sample size is always desirable when compared with a GWAS study using a large cohort. Selecting tSNPs in the particular region of interest based on previous genetic and prevalence data as well as the HapMap data also allowed us the chance to miss the genetic signal of "real" causal SNPs, because there was still the probability of SNP markers existing in the *LAMA2* gene region with a mild effect on myopia. These potential causal loci might be located in the other adjacent plot of the *LAMA2* gene region, as the *LAMA2* is a large gene comprising 66 exons and spanning a large sequence distance. To reconcile the contradictory results among the different studies (Cheng *et al*., 2013; Kiefer *et al*., 2013; Verhoeven *et al*., 2013; Yoshikawa *et al*., 2014; Li *et al*., 2015) including our work, firstly, further replication studies with larger sample sizes, more and denser SNP markers covering the whole *LAMA2* gene and in other independent populations are always desired to search potential loci and obtain more convincing results. Secondly, all the reports at this stage might actually reflect the complexity of myopia gene mapping, which can touch upon the issues of myopia phenotyping, genetic mode and component estimation, and genetic marker selection, etc. in different studies. This work provides association test results yielding from a single investigation by logical approaches, but should be contributory to the replication data for relevant myopia gene identification studies.

In conclusion, we tested the SNP polymorphisms of the *EGR1* and *LAMA2* gene regions in two independent sample groups with different approaches, but at present we have found no significant association for the SNPs selected in the regions of interest with extremely high-grade myopia, although the proteins encoded by the two genes are found to be very important for eye growth and myopisation.

Compliance with ethics guidelines

Fang-yu LIN, Zhu HUANG, Ning LU, Wei CHEN, Hui FANG, and Wei HAN declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study. Additional informed consent was obtained from all patients for whom identifying information is included in this article.

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Appendix

Our study has invited some controversy as follows:

> The authors have picked four tag SNPs from a gene that in the original studies (Verhoeven *et al*., 2013; Kiefer *et al*., 2013) were not associated with refraction or myopia, despite employing sample sizes more than 100 times larger than that in the current study; essentially they were doing the same thing, with lesser tools, but hoping for better results (extracted from reviewer's comments).

What this reviewer suggested is that perhaps it does not make much sense to select a cutoff of MAF=0.2 when the original studies found that the association was detected for alleles exclusively and significantly below this threshold (~0.1). While it is clear that the same polymorphisms associated in primarily European cohorts are too rare or even absent to show any association in Asians, the fact is that *LAMA2* polymorphisms are clearly on the lower end of the MAF spectrum. This study has not attempted to evaluate that end of the spectrum either through a more meaningful choice of tags, or indirectly through imputation and other associations. In addition, tagging was attempted using HapMap and not the data from the 1000 Genomes Project as a reference. This reviewer also is unconvinced by the heterogeneous and extremely post hoc criteria in the choice of candidates for genetic mapping.

中文概要

题　目：中国汉族人群病理性高度近视与*EGR1*和*LAMA2*基因多态位点的关联分析

目　的：检测分析 *EGR1* 和 *LAMA2* 基因序列中单核苷酸多态性位点（SNP）在中国汉族人病理性高度近视的遗传机制中的作用。

创新点：检测了 *EGR1* 基因外显子序列中的 SNP，验证了其与高度近视发病无关联；检测了 *LAMA2* 基因中热点区域中的三个 SNP，发现在我国汉族人群中，这几个 SNP 与高度近视发病的遗传机制无关，与先前国外报道结果不同。

方　法：收集 167 个高度近视核心家系以及 485 例散发高度近视患者和 499 例正视眼对照者。根据 HapMap 单倍型数据库以及先前的研究结果选择标签 SNP（图 1 和 2），测定所收集患者的相应基因型，采用 Haploview 和卡方分析软件作关联分析，并以关联分析效能软件计算本研究的计算效能（表 3 和 4）。

结　论：本研究在汉族人高度近视人群中未检测到阳性关联信号，需要进一步的研究深入验证。

关键词：高度近视；*EGR1* 基因；*LAMA2* 基因；关联分析；单核苷酸多态性