Genetic analysis of axial length genes in high grade myopia from Indian population

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**Article info**

**Abstract**

**Purpose**: To study the putative association of Membrane frizzled related protein (\textit{MFRP}) and Visual system homeobox protein (\textit{VSX2}) gene variants with axial length (AL) in myopia.

**Method**: A total of 189 samples with (\textit{N} = 98) and without (\textit{N} = 91) myopia were genotyped for the \textit{MFRP} and \textit{VSX2} variations in ABI Prism 3100 AVANT genetic analyzer. Genotype/haplotype analysis was performed using PLINK, Haploview and THESIAS softwares.

**Results**: Fifteen variations were observed in the \textit{MFRP} gene of which, rs36015759 (c.492C\textsuperscript{T}, T164T) in exon 5 was distributed at a high frequency in the controls and significantly associated with a low risk for myopia (\(P = 4.1 \times 10^{-7}\) OR \(b 1.0\)). An increased frequency for the coding haplotype block [CGT CGG] harboring rs36015759 was observed in controls (31\%) than cases (8\%) that also correlated with a decreased mean AL (\(-1.35085; P = 0.000444\)) by THESIAS analysis. The 'T' allele of rs36015759 was predicted to abolish the binding site for splicing enhancer (SRp40) by FASTSNP analysis.

**Conclusion**: Myopia is a complex disorder influenced by genetic and environmental factors. Our work shows evidence of association of a specific \textit{MFRP} haplotype which was more prevalent in controls with decreased AL. However, replication and functional studies are warranted to confirm these findings.

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Introduction

Myopia or nearsightedness is one of the most common human eye disorders with significant global public health concern. It is most prevalent in Taiwan, Japan, China, Korea (east Asia), and Singapore (south east Asia) affecting ~60–80% of young adults (Hsu et al., 2008; Kim et al., 2013a; Lam et al., 2012; Pan et al., 2013; Sawada et al., 2008; Yao et al., 2013). Long term myopia leads to irreversible eye problems which includes chorioretinal degeneration, retinal detachment, lattice degeneration (Asaminew et al., 2013; Koh et al., 2013), glaucoma (Detry-Morel, 2011) etc. and hence poses a serious socio-economic problem. The disease also exhibits an increased progression rate in females when compared to males (Donovan et al., 2012), and follows a complex inheritance pattern with a relative risk of 5 to 20 and 1.5 to 3 for high and low myopia respectively (Farbrother et al., 2004; Guggenheim et al., 2000) in the siblings of a myopic patient. In addition to genetic etiology, various environmental factors, that include near work, education levels (urban compared to rural location) and time spent outdoors have been shown to influence the development of myopic changes; however, the direct role of factors such as near work still remains controversial (Flitcroft, 2012; Jones et al., 2007; Rose et al., 2008).

Family based linkage studies have identified 23 loci till date (Wojciechowski, 2011) most of which have been replicated. Genome wide association/meta analyses (Kiefer et al., 2013; Verhoeven et al., 2013) have associated several loci that include genes involved in neurotransmission (GRIA4), ion transport (KCNQ5), retinoic acid metabolism (RDH5), extracellular matrix remodeling (LAMA2 and BMP2), eye development (SDX6 and PRSS56) and others. In addition to this single nucleotide polymorphisms (SNPs) in candidate genes like TGFβ (Ahmed et al., 2013), insulin like growth factor I (Yoshida et al., 2013), COL1A1 (Zhang et al., 2011), early growth response factor 1(Schippert et al., 2007), PAX6 (Miyake et al., 2012), matrix metalloproteinase genes (Wojciechowski et al., 2013), hepatocyte growth factor (Chen et al., 2012), MFRP (Metlapally et al., 2008), VSX2 (Aung et al., 2008) etc. have been studied for association with myopia in different populations. Studies pertaining to genetics of myopia from Indian subcontinent have been minimal which includes few candidate gene studies (TGFβ (Rasool et al., 2013), Fok1 (Annamaneni et al., 2011)), linkage studies (Ratnamala et al., 2011), etc.

In this scenario the newer trend of mapping quantitative traits (QT) rather than the disease itself proves to be a better approach for complex disorders such as myopia. The QT/endophenotypes of myopia include axial length (AL) (Cheng et al., 2013), refractive error (Klein et al., 2011), corneal curvature (CC) (Guggenheim et al., 2013), etc. and all these QTs have been studied for their heritability and their contribution to emmetropisation (Chen et al., 2011; Dirani et al., 2006). Among these, AL has been shown as an attractive endophenotype when compared to cornea and crystalline lens (Mutti et al., 2005). AL alone accounts for more than 40% of variation in refractive errors (Cheng et al., 2013; Ip et al., 2007; Pan et al., 2011) and exhibits high heritability factor than that for refraction (Kim et al., 2013b; Klein et al., 2009). The genes implicated with other endophenotypes like CC though important, are also associated with AL. Genetic studies have mapped various chromosomal loci for AL across different populations that include chromosomes 2p24 (Biino et al., 2005), 5q, 12q21 (MYP3), 4q12 (MYP9) (Zhu et al., 2008) and 1q41 (Fan et al., 2012).

So the present study was undertaken as a QTL approach to check for the association of two ocular development genes MFRP and VSX2 with AL in a disease scenario such as myopia where there is improper scaling. Sundin et al. (2008) proposed that MFRP had a role in regulation of ocular growth but not critical for retinal function and studies by Aung et al. (2008) and Metlapally et al. (2008) have shown their candidature as AL genes in POAG and myopic cases.

Materials and methods

Clinical examination

The study was approved by the institutional ethics board, adhered to the guidelines in the Declaration of Helsinki and was conducted at the Vision Research Foundation, Sankara Nethralaya, India. Cases were defined with a refractive error worse than −6.00D (N = 98) and controls with +0.50 to −0.50D (N = 91) in the least myopic eye. Subjects with other ocular diseases that predispose/associated with myopia were excluded from the study. Informed consent was obtained from the patients and controls for the research
use of peripheral blood samples after comprehensive ocular examination which included the standard retinoscopic technique to evaluate the refractive status and the corneal curvature values were obtained using Bausch and Lomb keratometer. Measurement of AL was performed with an optical biometer (IOLMaster; Carl Zeiss, Germany).

**Laboratory methods**

Venous blood was collected from all subjects after informed consent. Genomic DNA was extracted by Nucleospin Blood XL kit (Macherey-Nagel, Germany) as per the manufacturer’s instructions. The complete exonic and intronic regions of MFRP and VSX2 genes were PCR amplified with primers as described earlier (Ferda Percin et al., 2000; Wang et al., 2009) and genotyped by direct sequencing in ABI Prism 3100 AVANT genetic analyzer (Applied Biosystems, California).

**Data analysis**

**2.3.1. Genotype and haplotype analysis**

The genotypes were checked for Hardy–Weinberg equilibrium and statistical significance by PLINK (Purcell et al., 2007) and Fisher’s exact test (VassarStats, 1998–2013). Pairwise linkage disequilibrium (LD) was computed in Haplovie program, version 4.1 (Barrett et al., 2005). The parameters for LD computation include the correlation coefficient ($r^2$), haplotype estimation using accelerated EM algorithm similar to the partition/ligation method described in Qin et al., 2002. Logistic regression was performed for haplotype association using the sliding window method in PLINK; wherein for a given window size within a gene under consideration, the test was performed for all window sizes shifting one SNP at a time toward the 3′ end of the gene. This technique has been proven to be more powerful than single-marker analysis and especially for identifying SNPs lying in low LD regions (Guo et al., 2009).

Interaction of the haplotype with quantitative trait like AL was analyzed using the THESIAS software v3.1 (Tregouet and Garelle, 2007) that calculates the significance based on the maximum likelihood model and linked to the SEM algorithm which can perform the simultaneous estimation of haplotype frequencies and their associated effects on the phenotypes of interest.

**Bioinformatic analysis**

The pathogenic effect of the SNPs on the function of the protein was predicted using bioinformatic tools like SIFT, PolyPhen (Adzhubei et al., 2010; Ng and Henikoff, 2003), etc. The prediction of function of variations at transcriptional level, pre-mRNA splicing, protein structure, etc. were analyzed using the Function Analysis and Selection Tool for Single Nucleotide Polymorphisms (FASTSNPs) (Yuan et al., 2006). MUPro was used to test the protein stability changes due to single site mutations. Since the prediction was based on the sequence itself rather than the tertiary structure (Cheng et al., 2006), the proteins with out information on the tertiary structure (e.g.: MFRP) can also be analyzed by this software. Additionally the

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**Table 1**

Demographic data of the study cohort.

| Parameters                  | Controls | Cases |
|-----------------------------|----------|-------|
| Number                      | 91       | 98    |
| Sex (male/female)           | 40/53    | 57/45 |
| Age (mean ± SD, years)      | 26.5 ± 5 | 26.2 ± 6.2 |
| Axial length (mean ± SD, mm)| 22.95 ± 0.7 | 26.99 ± 1.86 |
| Anterior chamber depth (mean ± SD, mm) | 3.45 ± 0.21 | 3.56 ± 0.25 |
| Average K reading (mean ± SD, D) | 43.61 ± 1.47 | 44.54 ± 1.38 |
| Axial length/corneal radius (mean ± D) | 2.96 ± 0.06 | 3.56 ± 0.22 |
| Corneal radius (mean ± SD, mm) | 7.75 ± 0.26 | 7.58 ± 0.24 |

The demographic features and ocular dimensions of the 189 Indian myopic (98) and normal subjects (91) are shown. Only patients with non syndromic myopia were included in the study. SD: standard deviation.
Table 2
Genotype and allele frequency distribution of VSX2 and MFRP variants detected and investigated in myopia patients.

| Variation (rs ID) | Type                        | cDNA position | Amino acid change | Allelic distribution | Genotype distribution |
|------------------|-----------------------------|---------------|-------------------|----------------------|-----------------------|
|                   |                             |               |                   | Cases (N = 98) | Controls (N = 91) | Cases (N = 98) | Controls (N = 91) |
|                   |                             |               |                   | h  m  h  m       | h  m  h  m          | h  m  h  m       | h  m  h  m          |
|                   |                             |               |                   | P value |                   | P value |                   |
| VSX2              |                             |               |                   | 0.49    |                   | 0.49    |                   |
| rs62006815        | Synonymous                  | c.831G>A      | L277L              | 194     | 2 182 0            | 96      | 2 0 91            |
|                   |                             |               |                   | 0.49    |                   | 0.49    |                   |
| Novel*            | Non-synonymous              | c.866G>A      | G289D              | 194     | 2 182 0            | 96      | 2 0 91            |
|                   |                             |               |                   | 0.49    |                   | 0.49    |                   |
| rs75395981        | Non-synonymous              | c.871G>A      | D291N              | 194     | 2 180 2            | 96      | 2 0 89            |
|                   |                             |               |                   | 0.99    |                   | 0.99    |                   |
| rs137872696       | Non-synonymous              | c.1046C>T     | A349V              | 195     | 1 182 0            | 97      | 1 0 91            |
|                   |                             |               |                   | 1       |                   | 1       |                   |
| MFRP              |                             |               |                   |                     |                     |                     |                     |
| rs883247          | 5′UTR                       | c.−30G>A      | −                  | 47      | 149 59 123         | 7        | 33 58 11 37 43    |
|                   |                             |               |                   | 0.08    |                   | 0.21    |                   |
| rs79836575        | 5′UTR                       | c.−43G>A      | −                  | 191     | 5 175 7 0           | 94       | 3 1 84 7          |
|                   |                             |               |                   | 0.5     |                   | 0.2     |                   |
| Novel, rs143351376 | Intron1                     | c.55−15_17dup GTA | −                  | 189    | 7 175 7             | 94       | 1 3 84 7          |
|                   |                             |               |                   | 0.2     |                   | 0.2     |                   |
| Novel, rs199473708 | Exon 4, non-synonymous      | c.290C>T      | P97L               | 194     | 2 182 0 0.49       | 96       | 2 0 91 0          |
|                   |                             |               |                   | 0.49    |                   | 0.49    |                   |
| rs3814762         | Exon 4 non-synonymous       | c.406G>A      | V136M              | 158     | 38 155 27           | 65       | 28 64 27          |
|                   |                             |               |                   | 0.27    |                   | 0.1     |                   |
| rs36015759        | Exon 5 synonymous           | c.492C>T      | T164T              | 173     | 23 122 60 4.5×10^-7 | 76       | 21 1 37 48    |
|                   |                             |               |                   | 0.07    |                   | 6       | 4.10×10^-7       |
| rs2510143         | Exon 5 synonymous           | c.540T>C      | H180H              | 193     | 3 177 5             | 95       | 3 0 86 5          |
|                   |                             |               |                   | 0.32    |                   | 0.48    |                   |
| rs61736238        | Exon 6 non-synonymous       | c.770G>A      | R257H              | 195     | 1 182 0            | 97       | 1 0 91 0          |
|                   |                             |               |                   | 0.99    |                   | 0.99    |                   |
| rs10892350        | Intron7                     | c.898+86G>A   | −                  | 88      | 108 91 91 0.35     | 22       | 44 32 26 39 26    |
|                   |                             |               |                   | 0.6     |                   | 1       |                   |
| rs2509388         | Intron7                     | c.898+89C>G   | −                  | 88      | 108 92 90 0.3      | 22       | 44 32 26 40 25    |
|                   |                             |               |                   | 0.5     |                   | 0.5     |                   |
| rs35885438        | Exon 8 synonymous           | c.954G>A      | L318L              | 166     | 30 167 15 0.03     | 71       | 24 3 77 13 1      |
|                   |                             |               |                   | 0.11    |                   | 0.11    |                   |
| Novel, rs185451482 | Intron8                     | c.975+18T>C   | −                  | 195     | 1 179 3 0.3        | 97       | 1 0 88 3            |
|                   |                             |               |                   | 0.35    |                   | 0.35    |                   |
| Novel, rs199473709 | Intron9                     | c.1124+11C>G  | −                  | 191     | 5 177 5 1          | 94       | 3 1 86 5          |
|                   |                             |               |                   | 0.48    |                   | 0.48    |                   |
| Novel, rs199473711 | Intron 10                   | c.1255+33_34del TA | −                  | 195     | 1 182 0 1          | 97       | 1 0 91 0          |
|                   |                             |               |                   | 0.1     |                   | 0.1     |                   |
| rs11217241        | Intron 11 splice_site       | c.1387+3G>A   | −                  | 100     | 96 92 90           | 27       | 46 25 23 46 22    |
|                   |                             |               |                   | 0.89    |                   | 0.89    |                   |

Allele and genotype frequencies of the sequence variations within VSX2 and MFRP genes in the current study are shown. The distributions did not deviate from those predicted by the Hardy-Weinberg equilibrium. Proportions of groups were compared by the Fisher’s exact test. The criterion for statistical significance was p ≤ 0.05. DNA changes are documented based on cDNA sequences with +1 corresponding to the A of the ATG translation initiation codon in reference to NM_182894.1 (VSX2) and NM_031433.2 (MFRP) sequences. (h—ancestral allele, m—mutant allele).

*Identified in the current study.
ΔRSCU (Relative Synonymous Codon Usage) was also calculated using CAIcal software (Puigbo et al., 2008) to measure the effect of synonymous variation on the translation kinetics.

Results

Table 1 shows the demographic data of the study participants. The mean (SD) AL in myopes (26.99 ± 1.86 mm) was higher than the controls (22.95 ± 0.7 mm) with a $P = 1.45 \times e^{-08}$. Direct sequencing revealed 4 nucleotide variations in VSX2 gene that were equally distributed between cases and controls (Table 2). Five haplotype blocks were generated by Haplovie and haplotype analysis did not reveal any significant association with myopia.

Genotype, allele frequencies and haplotype analysis of MFRP gene variants in the study population

Fifteen variations (5 novel variations: c.55–15_17dup GTA, c.290C > T, c.975 + 18 T > C, c.1124 + 11C > G, c.1255 + 33_34del) were observed in the MFRP gene (Table 2). We observed significant difference in the genotype ($P = 4.10 \times e^{-07}$) and allele frequencies ($P = 4.5 \times e^{-07}$) of SNP rs36015759 (c.492C > T, T164T) in the controls when compared to myopic cases with OR < 1.0, CI: 95% (Table 2).

Haplovie v4.1 analysis showed 3 haplotype blocks that included all the MFRP variants (Fig. 1) that were corrected for multiple comparisons (15,000 permutations; Table 3). There wasn’t any significant difference in the distribution for blocks 1 and 2 between cases and controls. Two haplotypes (CGCAAG (cases: 55.7%, controls: 41.5%) and CTGCAAG (cases: 1.3%, controls: 13.2%) with wild type (C) and variant (T) allele for rs36015759) in block 3 showed significant association ($P_{perm} = 0.04$ and 0.0001 respectively; Table 3).

![Fig. 1. Linkage disequilibrium (LD) pattern for the 15 MFRP variants identified in Indian population under the current study. The plot was generated using Haploview 4.1 and pairwise r2 values are shown in diamonds that represent the pairwise LD between the 2 SNPs at the top left and right of the corresponding diamond. Colour Scheme: White, shades of pink for D<1; blue/bright red for D' = 1.](image-url)
haplotype was observed with the

Order of the SNPs for the respective blocks are: rs11217241, rs99473711, rs199473709 (Block 1); rs185451482, rs35885438, rs2509388, rs10892350 rs61736238 (Block 2); rs2510143, rs36015759, rs3814762, rs199473708, rs199473710, rs883247, rs79836575 (Block 3). \( P_{asymp} \) asymptotic \( P \) value, \( P_{emp} \) empirical \( P \) value for 15,000 permutations.

Table 3

| Block | Haplotype | Freq. | Case, control frequencies | Chi square | \( P_{asymp} \) | \( P_{emp} \) |
|-------|-----------|-------|---------------------------|------------|----------------|--------------|
| Block 1 | GAC       | 0.497 | 0.492, 0.502              | 0.04       | 0.8407         | 1            |
|        | AAC       | 0.474 | 0.478, 0.471              | 0.02       | 0.8885         | 1            |
|        | AAG       | 0.018 | 0.012, 0.024              | 0.766      | 0.3814         | 1            |
| Block 2 | TGGAG     | 0.491 | 0.516, 0.465              | 0.979      | 0.3225         | 0.9          |
|        | TCCGG     | 0.379 | 0.326, 0.436              | 4.868      | 0.0274         | 0.1          |
|        | TACGG     | 0.092 | 0.118, 0.064              | 3.301      | 0.0692         | 0.5          |
|        | TAGAG     | 0.024 | 0.030, 0.018              | 0.523      | 0.4697         | 1            |
|        | CGGAG     | 0.011 | 0.005, 0.016              | 1.168      | 0.2799         | 0.9          |
| Block 3 | CCGCAAG   | 0.488 | 0.557, 0.415              | 7.625      | 0.0058         | 0.04         |
|        | CCACAAG   | 0.144 | 0.180, 0.106              | 4.141      | 0.0419         | 0.3          |
|        | CTCGAG    | 0.128 | 0.099, 0.160              | 3.231      | 0.0723         | 0.5          |
|        | CCGCAG    | 0.106 | 0.108, 0.104              | 0.01       | 0.9202         | 1            |
|        | CTCCAG    | 0.07  | 0.013, 0.132              | 20.512     | 5.93 × 10^{-6} | 0.0001       |
|        | TCCCGA    | 0.018 | 0.015, 0.022              | 0.228      | 0.6333         | 1            |
|        | CTACAG    | 0.015 | 0.006, 0.024              | 2.01       | 0.1563         | 0.8          |

In addition, we also examined haplotypes using the sliding window strategy in PLINK and generated a total of 120 windows, of which 59 showed a significant \( P \) value (omnibus \( P_{emp} < 0.05 \)). The omnibus test result was significant for those SW consisting of rs36015759 (S6, Table 4) in the haplotype. The importance of rs36015759 (S6) was even more obvious when we probed more into the details of the most significant group of SW which was of size up to 6 SNPs per window. In window S1–S2–S3–S4–S5–S6, the most significant haplotype was observed with the first five SNPs in the wild type and the last SNP rs36015759 (S6) was either

Table 4

Summary of exhaustive haplotype analyses based on omnibus tests for sliding windows of all possible sizes across the 15 SNPs of the \( MFRP \) gene for the subjects under this study.\(^a\)

| Sliding window | SW with omnibus \( P_{emp} < 0.05 \) | Most significant results |
|----------------|-------------------------------------|-------------------------|
| SNPs no. | No. of SWs | No. of SWs | First SW | Last SW | SW | \( P_{asymp} \) | \( P_{emp} \) |
| 2        | 14       | 2          | S5–S6   | S6–S7   | S6–S7 | 1.95 × 10^{-6} | 6.67 × 10^{-5} |
| 3        | 13       | 5          | S4–S6   | S6–S8   | S6–S8 | 2.23 × 10^{-6} | 6.67 × 10^{-5} |
| 4        | 12       | 5          | S2–S6   | S6–S10  | S4–S8 | 5.37 × 10^{-6} | 6.67 × 10^{-5} |
| 5        | 11       | 6          | S1–S6   | S6–S11  | S1–S6 | 8.59 × 10^{-6} | 6.67 × 10^{-5} |
| 7        | 9        | 6          | S1–S7   | S6–S12  | S2–S8 | 7.04 × 10^{-6} | 6.67 × 10^{-5} |
| 8        | 8        | 6          | S1–S8   | S6–S13  | S6–S13 | 4.73 × 10^{-6} | 6.67 × 10^{-5} |
| 9        | 7        | 5          | S1–S9   | S6–S14  | S6–S14 | 0.00021     | 6.67 × 10^{-5} |
| 10       | 6        | 6          | S1–S10  | S6–S15  | S4–S14 | 0.00018     | 6.67 × 10^{-5} |
| 11       | 5        | 5          | S1–S11  | S5–S15  | S4–S14 | 0.00022     | 6.67 × 10^{-5} |
| 12       | 4        | 4          | S1–S12  | S4–S15  | S3–S14 | 0.00041     | 6.67 × 10^{-5} |
| 13       | 3        | 3          | S1–S13  | S3–S15  | S2–S14 | 0.00042     | 0.001333     |
| 14       | 2        | 2          | S1–S14  | S2–S15  | S1–S14 | 0.00061     | 6.67 × 10^{-5} |
| 15       | 1        | 1          | S1–S15  | S1–S15  | S1–S15 | 0.00745     | 0.00566      |

For easiness in representing the SNPs in haplotype they are named as S1, S2...S15. The SW is indicated as Sa–Sb, where 'a' is the first SNP and 'b' is the last SNP of the SW. For example, S2–S4 refer to the SW S2–S3–S4. Multiple comparisons were corrected by running 15,000 permutations. The minimum \( P \) value achievable with 15,000 permutations is 6.67 × 10^{-5}. For each fixed-size SW, the most significant results are shown in the 3 rightmost columns. Of the 120 sliding windows tested the S5–S6 ranks the first and S6–S8 ranks the second in providing evidence for association.

\(^a\) Abbreviation \( P_{asymp} \) asymptomatic \( P \) value; \( P_{emp} \) empirical \( P \) value; SW – Sliding window.
the wild allele ‘C’ or the variant ‘T’ \(P_{emp} = 6.67 \times e^{-0.05}\) and \(P_{asym} = 0.00042\) respectively. Interestingly the ‘T’ allele haplotype was more represented in controls (13%) whereas the haplotype with ‘C’ allele was seen more in cases (56%) (Table 4a).

Haplotype blocks that included only the 6 coding SNPs of the order rs199473708, rs3814762, rs36015759, rs2510143, rs61736238, rs35885438 (Table 5) were generated by THESIAS and analyzed for interaction with AL after adjusting for age and sex as covariates. The frequency distribution for the block CGT (rs36015759) CCG was high in controls (31%; \(P = 0.000001\), OR: 0.18240 95%CI: 0.09244–0.35988) and showed significant p value for decreased mean AL \(P = 0.000444\); difference in mean value = 0.10320; Table 5).

Bioinformatic analysis

PolyPhen/SIFT/SNAP analysis of the MFRP variants did not show any significant changes. FastSNP analysis revealed an alternative splicing regulatory region (either change in Transcription factor (TF) binding or Exonic splicing enhancer (ESE) site) for c.290C > T, c.975 + 18 T > C, rs11217241, rs35885438, rs61736238 and rs36015759. SNPs c.290C > T, rs3814762, rs61736238 showed a decrease in protein stability by MUpro analysis. Except for rs36015759 all other SNPs did not show any significant association with myopia. The significant SNP rs36015759 showed a loss of ESE binding site for SRp40 protein (Fig. 2).

Discussion

The coordinated growth changes of various components of the eye are required for achieving and maintaining emmetropia of which AL remains as one of the major determinants (Mutti et al., 2005). Emmetropisation is a process in the postnatal stage of development that regulates the eye growth, whereby the plane of the retina coincides with the focal point of the eye's optical system. An alteration in the visual stimuli initiates a signaling cascade originating in the sensory retina, traversing the retinal pigment epithelium and the vascular chorioid, and ultimately regulating eye growth through scleral remodeling (Flitcroft, 2013; Siegwart and Norton, 2011; Wallman and Winawer, 2004). Alterations in the sequence of these biochemical events might disrupt this finely tuned mechanism and lead to refractive errors. Hence, genes like, MFRP, VSX2 that plays important role in these complex signaling pathways could be studied for the genetic association with diseases manifesting altered AL (Fig. 3).

The present study is a comprehensive analysis of MFRP and VSX2 genotypes/haplotypes with AL, a biometric determinant in myopic cases and emmetropic controls. It is the first of its kind in Indian population. Fifteen variations were observed in the MFRP gene, of which, rs36015759 (T164T) in exon 5 showed significant difference in distribution among controls.

MFRP gene expression studies revealed that the protein is being produced in E11 stage which coincided with the development of the presumptive retina followed by significant increase at birth suggesting a critical role in the development of the anterior segment which eventually determines the eye size or AL (Mandal et al., 2006). Another rationale for its candidature is the presence of the frizzled domain at the ‘C’ termini suggesting that it could act as a regulator of the WNT signaling pathway during eye development.
Mutational studies in \textit{MFRP} have shown its association with hyperopia, nanophthalmos, and microphthalmos (Sundin et al., 2005; Ayala-Ramirez et al., 2006). Mutation in \textit{MFRP} has been correlated with shift towards both hyperopia and myopia. In a study by Sundin et al. (2008) heterozygotes for 1143insC frameshift mutation in \textit{MFRP} exhibited an increased AL. Recent studies have also shown that variations in genes like PRSS56 (small eye gene (Kiefer et al., 2013), hepatocyte growth factor (Chen et al., 2012), insulin like growth factor (Penha et al., 2011)) are strongly associated with both myopia and hyperopia conditions (increased or decreased AL).

In the current study we tested for the possible interaction of the \textit{MFRP} variants with biometric indices like AL by haplotype analysis using THESIAS software. Haplotype CGCTGG, bearing the \textit{T} allele of rs36015759 was represented at a higher frequency in controls (31%) when compared to cases (8%) and also associated with decreased mean AL ($P = 0.000444$, difference in mean value $= -1.35085$). Since the SNP was represented in a low LD region, we adopted a sliding window (SW) strategy in PLINK analysis which allows an effective evaluation of haplotypic effects for such SNPs (Jiang et al., 2011). 120 SWs were tested for the 15 \textit{MFRP} variants identified in the current study. SWs with SNPs of the order, rs883247–rs79836575–rs199473710–rs199473708–rs3814762–rs36015759, ranked first followed by rs36015759–rs2510143–rs61736238 providing evidence for the significant association of rs36015759 (\textit{T} allele), in non myopic controls. This region has been suggested as a mutation hotspot and deletion of C allele at c.492 position, terminating the protein prematurely after 25 residues, was observed in an Indian family (Kannabiran et al., 2012).

The synonymous SNP rs36015759 results in loss of binding site for SRp40 protein to the exonic splicing enhancer region for the \textit{T} allele (FASTSNP analysis; Fig. 2). Such concept of improper splicing has been studied earlier in neurofibromatos gene (\textit{NF1}) and functionally proven to result in an incomplete protein by skipping of the exon. Similarly a synonymous variation of T255T in \textit{ASB10} gene affected the exon splicing enhancer site, altered the mRNA expression in lymphoblasts and was also associated with primary open angle glaucoma (Fingert et al., 2012). The haplotype (CTGCCG) bearing the \textit{T} allele of rs36015759 could have a similar effect on the expression/function of the gene by loss of splice site enhancer region. Recent review on significance of synonymous variations, suggests a putative effect of such SNPs via improper splicing accuracy, translation fidelity, mRNA structure or protein folding (Sauna and Kimchi-Sarfaty, 2011) and has given a formulae for measuring the effect of synonymous variations on the translation kinetics, $\Delta$RSCU (Relative synonymous codon usage). In our study $\Delta$RSCU is 0.28 between wild type (TAC = 1.14) and variant (TAT = 0.86) codon of rs36015759 calculated using CAICal software (Puigbo et al., 2008). A positive $\Delta$RSCU implies increased translation kinetics, thus providing a putative functional relevance for this SNP.

### Table 5

Frequency distribution of \textit{MFRP} haplotypes and their interaction with AL as generated by THESIAS software v3.1.

| Haplotype (P97L,V136M, T164T, H180H, R257H, L318L) | Frequency in controls (N = 91) | Frequency in cases (N = 98) | $P$ value | OR (95% CI) | $P$ value | Difference in mean value |
|-------------------------------------------------|-------------------------------|-----------------------------|-----------|-------------|-----------|-------------------------|
| CGCCGG                                          | 0.437594                     | 0.550016                    | Intercept |             |           |                         |
| CGCGGA                                          | 0.068211                     | 0.110641                    | 0.427961  | 1.43337     | (0.58850–3.49120)      | 0.978239 0.01693  (–1.19965–1.23351) |
| CGCTGG                                          | 0.022222                     | 0.020833                    | –         |             |           |                         |
| CGTCGG                                          | 0.315006                     | 0.088661                    | 0.000001  | 0.18240     | (0.09244–0.35988)      | 0.000444 1.35085  (–2.10460–0.59711) |
| CGTCGA                                          | 0.006967                     | 0.016307                    | –         |             |           |                         |
| CACCGG                                          | 0.130483                     | 0.169417                    | 0.692164  | 1.16495     | (0.54707–2.48068)      | 0.813546 0.10320  (–0.75438–0.96078) |
| CACCCA                                          | 0.008156                     | 0.018885                    | –         |             |           |                         |
| CACCAG                                          | 0.00520                      | 0.009615                    | –         |             |           |                         |
| CATCGG                                          | 0.011361                     | 0.010417                    | –         |             |           |                         |
| TGCCGG                                          | 0.00520                      | 0.009615                    | –         |             |           |                         |

Haplotypes are indicated in ACGT format. The data are boldfaced if their corresponding haplotypes show significant $P$ value (<0.05). All data were adjusted for gender and age. AL — axial length.

(Katoh, 2001).
In conclusion the results of the current study contribute to the data on genetics of myopia in Indian population. The haplotype (CTGC GG) bearing the T allele of rs36015759 in MFRP, shows significant association with decreased AL even after multiple correction and no significant variations in VSX2 were

Fig. 2. The ESE binding sites for wild type (upper panel) and variant (c.492C>T; lower panel) of MFRP cDNA (NM_031433.2) are shown as red, blue, green and yellow boxes. The binding site for SRp40 protein is lost for the variant (2nd green bar, lower panel).

**Conclusion**

In conclusion the results of the current study contribute to the data on genetics of myopia in Indian population. The haplotype (CTGC GG) bearing the T allele of rs36015759 in MFRP, shows significant association with decreased AL even after multiple correction and no significant variations in VSX2 were
observed. However, the study bears the limitation of smaller sample size and warranties further exploration with hyperopes as well.

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