Int J Biochem Mol Biol 2021;12(1):35-48
www.ijbmb.org /ISSN:2152-4114/IJBMB0124527

Original Article

MYP2 locus genes: Sequence variations, genetic association studies and haplotypic association in patients with High Myopia

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Received October 20, 2020; Accepted February 12, 2021; Epub February 15, 2021; Published February 28, 2021

Abstract: High Myopia (HM) is a common complex-trait eye disorder. There is essential evidence that genetic factors play a significant role in the development of nonsyndromic high myopia. Identification of susceptibility genes of high myopia will shed light on the pathophysiological mechanism underlying their genesis. This was a case control study examining the prospect of association of *DLGAP1, EMILIN2 & MYOM1* genes on *MYP2* locus in purely ethnic (Kashmiri) population representing a homogeneous cohort. Genomic DNA was extracted using phenol chloroform and salting out method. Extracted DNA was genotyped for polymorphic variations in *MYOM1, EMILIN2 and DLGAP1* genes involving Sanger di-deoxy method. Allele frequencies were tested for Hardy-Weinberg disequilibrium in 224 cases and compared with 220 emmetropic controls. In *DLGAP1,* documented single nucleotide polymorphism (SNP); *Pro517Pro* was observed. A previously reported *Asn451Asn* SNP was observed in *EMILIN2*. *MYOM1* showed five polymorphic variations; two in coding region *(Gly333Gly & Gly341Ala)* and three intronic (c.1022+23, G>A; c.3418+44 G>T & c.3418+65; C>G). All of the elucidated SNPs were having statistical significant role in increasing or decreasing the risk of disease. Although not statistically significant, a novel *Glu507Lys* SNP was observed in *DLGAP1* (P>0.05). In silico predictions showed *MYOM1 Gly341Ala* to be benign & tolerated substitution while as *DLGAP1 Glu507Lys* to be possibly damaging substitution. The studied SNPs followed Over-Dominant, Recessive and Co-Dominant mode of inheritance with specific haplotypes associated with the disease. Our study reveals the involvement of *MYP2* locus candidate gene polymorphism in the pathogenesis of HM.

Keywords: High myopia, *MYP2* Locus, *DLGAP1*, *EMILIN2*, ethnic, *MYOM1*, novel, polymorphism

Introduction

Myopia is the multi-factorial ocular disorder with highest prevalence globally branded by spherical error of refraction (RE) and retinal defocus causing reduced visual insight. Approximately 60-80% of adult population in Taiwan, China, Korea, Japan and Singapore is hit by the disorder [1-6]. Pathological or high Myopia (RE>6D) is different from simply being "short sighted" [7]. HM is an advanced variant causing retinal detachment, lattice degeneration and chorio-retinal degeneration which are irreversible eye damages that cannot be corrected with glasses or contact lenses [7, 8]. It can also lead to glaucoma [9] and incur a huge financial burden, even on developed countries [10]. The development and progression of HM is directly or indirectly influenced by various environmental factors including *"near vision stimulus"* in students and in some professions; educational status and time spent outdoors [11, 12].

Genetic mapping involving successful efforts to determine disease genes rely on the use of functionally implicated candidate genes or probes, and the detection of significant cytogenetic findings of deletions, insertions, or translocations that consistently pair with the dis-

ease's phenotype [13]. Genetic and environmental factors play a very important role in shaping refractive progress of an individual which is backed up by the fact that Myopia has a higher prevalence in developed Asian countries compared to Western world [14, 15].

The influence of the genetic factors in the development of HM has also been demonstrated in Population-based association studies (or case-controlled studies) which have linked various genomic polymorphisms to HM phenotypes and pathogenesis. Recent multigenerational linkage studies have reported at least 23 Myopia susceptibility loci (MYP; MYP2 to MYP5) [16-19]. A genome-wide linkage analysis exposed a significant linkage of 18 centimorgan (cM) in *MYP2* for non-syndromic autosomal dominant HM on 18 p11.31, which has been refined to 7.6 cM interval between markers D18S59 and D18S1138 by Haplotype analysis [16] and further tapered to an interval of 0.8 cM between markers D18S63 and D18S52 [17].

MYP2 locus harbor the genes involved in sclera formation and regulation, which are candidate genes for HM [20, 21]. Numerous candidate genes for HM have been recognized within *MYP2* which are involved in growth, maintenance and remodeling of sclera [20-22] which comprise Myomesin 1 *(MYOM1)*, Elastin Microfibril Interfacer 2 *(EMILIN2)*, Large Drosophila Homolog Associated Protein 1 *(DLGAP1)*, Transforming Growth B-Induced Factor *(TGIF1)*, Lipin 2 *(LPIN2)*, Myosin Regulatory Light Chain 2 *(MRLC2)*, Myosin Regulatory Light Chain 3 *(MRCL3)*, Clusterin-like 1 *(CLUL1)*, and Zinc Finger Protein 161 Homolog *(ZFP161)* [20, 21]. Definite role of *MYP2* locus SNPs in the etiopathogenesis of HM has been established in different populations [23-25]. Yet, several studies have not shown any association between *MYP2* locus SNPs and HM [20, 21, 26]. So, our study aimed to clarify this relationship with a case-control design keeping in view the ethnic purity of our population which may lead to effective therapies for severe forms of this potentially blinding eye disease.

Materials and methods

Study design

This is a Case-Control study conducted by the Department of Biotechnology, University of Kashmir and Department of Ophthalmology, Government Medical College (GMC) Srinagar and associated SMHS Hospital, Kashmir, India over a period of two years (2017-2019). The study has been approved by Ethical Committee of GMC Srinagar under ref no. 11A/ETH/GMC/ICM dated 22-02-2017 strictly adhering to the guidelines of the Declaration of Helsinki. Informed consent was obtained from the study subjects after explaining the nature and possible consequences of the study.

Study subjects

The study included cases with high Myopia (n= 224) attending the Department of Ophthalmology, Govt. Medical College Srinagar and associated SMHS Hospital. Individuals with known ocular disease such as retinopathy, cataract or genetic disease associated with Myopia, such as Stickler or Marfan syndrome and any sort of genetic disorder were excluded from the study. Ophthalmic evaluation of each patient was done which included measuring visual acquity, keratometry, retinoscopy, slit lamp examination of the anterior segment, fundus examination and measurement of axial length. Controls (n=220) were randomly selected from a pool of healthy volunteers who visited the hospital for health check-up during the same period and enrolled in the study.

Sample collection and DNA extraction

05 ml of blood was collected from each patient and healthy control in EDTA vials; refrigerated at -80°C till further processing. Deoxyribonucleic acid (DNA) extraction of samples was carried out by standard procedures like phenol chloroform extraction and salting out. Extracted DNA was dissolved in tris-EDTA buffer for further use. The quality of DNA was checked on 2% agarose gel electrophoresis whereas purity and concentration was measured by using the NanoDrop 2000c Spectrophotometer (ThermoScientific, USA).

Polymerase chain reaction (PCR)

PCR was carried out in a total volume of 50 µl, using 50-100 ng genomic DNA, 2-6 pmole of each primer, 1× PCR buffer (Sigma Aldrich, USA) and 0.5 units of Taq DNA polymerase (Sigma Aldrich, USA). The PCR cycling conditions were as follows: one cycle of denaturation at 95°C for 5 min, 30 cycles of denaturation at

Table 1. Primers used for amplification of *DLGAP1, EMILIN2 & MYOM1* gene and their annealing temperature and product size

Gene	Exon	Primer sequence	T_a (°C)	Product size (bp)
MYOM1	4	F; 5' CATGAAGTTGTTTACACTTCAACTTAC 3' R; 5' CTCAGTGTGATCACACAGCAT TGG 3'	63	260
	19	F; 5' TGCTTCTACACCTGCTTCTA CAG 3' R; 5' TTATATTCAGATAGCACACATTGA 3'	56	259
	29	F; 5' CCATTTCCTTTCAACCAGAAAGGG 3' R; 5' CATACATCTGCATG CCCTCCTGG 3'	52	218
EMILIN2	4	F; 5' TTGGTCAACAGATCAAGACATTGGACC 3' R; 5' GAACGCTCCCCAGACGGTCTTCCAGAG 3'	66.7	300
DLGAP1	2	F; 5' GTCCACGGCATCCAAGCAGACCAC 3' R; 5' TGTTTTCCTCAGGGACAGGCG 3'F	67.8	223
	4	F; 5' CTGGAGTCGCAGGCCGTGGAAGCG 3' R; 5' ACATGGGTGGTATCTTGTTCCTGG 3'	67.8	300

95°C for 45 s, annealing at t°C for 45 s, and extension at 72°C for 45 s, and one final 6 min extension cycle at 72°C, for amplifying different genes. All PCR products were verified on 2% agarose gel. The primer sequences, annealing temperatures and their corresponding amplicon size is shown in **Table 1**.

DNA sequencing

PCR products were purified by sodium iodide method. All the Purified PCR products were sequenced, using the automated DNA sequencer ABI prism 310 (Applied Bio systems, USA) involving Sanger di deoxy method. DNA sequences of the amplicons were obtained in FASTA and PDF formats. The FASTA files were analyzed using *ClustalX version 2 software* (European Bioinformatics Institute, Cambridgeshire, UK) for sequence alignment and by *ChromasPro version 1.49 beta2 software* (Technelysiumpty Ltd, Australia) for the detailed inspection of the chromatograms individually.

Computational prediction tools

3D structure of protein in pdb format was predicted by an automated server (I-TASSER; zhang.bioinformatics.ku.edu/I-TASSER) [27]. *Swiss PDB Viewer* computed free energy of predicted 3D structures [28]. We also used *Sorting Intolerant From Tolerant (SIFT) version 2*, a program which predicts the tolerant and deleterious substitutions within a given sequence. Possible impact of an amino acid substitution on the structure and function of a human protein was predicted using *PolyPhen-2 (Polymorphism Phenotyping version 2)* [29].

Genetic association study and haplotyping

Adjusted odds ratios (ORs) were assessed using co-dominant, dominant, recessive and over-dominant inheritance models. The inheritance model with the lowest AIC (Akaike information criterion) is considered appropriate for the individual SNP data. Haplotype analysis for haplotypes with frequencies >1% was conducted using *HAPSTAT 3.0 software* and the risks were compared to the reference haplotype (Most common haplotype in control group). Haplotype frequencies were estimated from the genotyping data after stratification by gender and age.

Statistical analysis

For each polymorphism, the allelic and genotypic frequencies of cases and controls were compared by χ^2-test with two degrees of freedom. The association of *MYOM1*, *EMILIN2* & *DLGAP1* genotypes with risk of disease was calculated by employing the logistic regression analysis. The relative risk was estimated by odds ratios (OR) and 95% confidence intervals (95% CI), $P \leq 0.05$ was considered as significant. Statistical analysis was done using SPSS 23.0 statistical package (SPSS Inc., Chicago IL, USA).

Results

Patient characteristics

Socio-demographic and clinicopathological parameters of cases and controls are revealed in **Table 2**. All cases and controls were matched as per their age, gender and smoking status.

Table 2. Demographic and clinicopathological characteristics of Cases and controls enrolled for the study

Characteristics	Cases N = 224 (%)	Controls N = 220 (%)	*P* Value
Age			
≤30 years	137 (61.0)	130 (59.0)	
>30 years	87 (39.0)	90 (41.0)	0.3
Gender			
Male	130 (58.0)	140 (63.6)	
Female	94 (42.0)	80 (36.4)	0.1
Smoking Status			
Non-smoker	40 (17.8)	45 (20.4)	
Passive smoker	140 (62.5)	135 (61.5)	0.5
Active smoker	44 (19.7)	40 (18.1)	
Occupation			
Students	80 (35.7)	50 (22.7)	
Near workers	100 (44.6)	80 (36.3)	≤0.05
Others	44 (19.7)	90 (41.0)	
Family history			
No	80 (35.7)	-	-
Yes	144 (64.3)	-	
Degree of Myopia			
<-6 D	124 (55.3)	-	-
≥-6 D	100 (44.7)	-	

D; Diopters.

The calculated mean age of the high Myopia patients and control groups were 35.6±6.1 and 34.04±5.3 respectively. 61% (137 of 224) of patients were ≤30 of age and compared to controls where 59% (130 of 220) were ≤30 years old. Maximum number of cases and controls were passive smokers (62.5% vs 61.5%). 64.3% (144 of 224) of patients were having family history of high Myopia (**Table 2**).

Sequence analysis of MYP2 loci genes

This study detects sequence variations in *MYOM1, EMILIN2* & *DLGAP1* in a pure ethnic Kashmiri population. Mutational screening discovered a total of 8 polymorphic variations (05 in exons and 03 in introns) as shown in **Table 3**. *MYOM1* showed five polymorphic variations; two in coding region and three intronic, *EMILIN2* showed one polymorphic variation while as *DLGAP1* showed two polymorphic variants (**Table 3**). *MYOM1 Gly333Gly, MYOM1 c.1022+23, EMILIN2 Asn451Asn SNPs* are significantly associated with the decreased risk while as *MYOM1 Gly341Ala, MYOM1 c.3418+44, MYOM1 c.3418+65,* and *DLGAP1 Pro517Pro* SNPs are associated with the increased risk of high myopia in study subjects (P≤0.05).

Genotype and allele frequencies of *MYP2* locus gene polymorphisms in cases and controls is shown in **Table 4**. In case of *MYOM1 Gly333Gly (G>A; rs2230162)* SNP, the frequency of variant genotype (GA+AA) in cases was 25% (56 of 224) as compared to controls (92 of 220; 42%) (P≤0.05). The partial electrophoretogram depicting change from G to A is shown in **Figure 1A**. Although the results suggest the possible association of homozygous variant state (AA) with disease phenotype but the combined effect of variant genotype (GA+AA) plays a protective role in predisposing an individual to risk of high Myopia. In case of *MYOM1 Gly341Ala (G>C; rs8099021)* SNP, homozygous wild genotype (GG) was absent in both cases and controls. The partial electrophoretogram depicting change from G to C is shown in **Figure 1B**. When variant genotype (GC+CC) was compared between cases and controls significance was not noted (P>0.05) however on comparing individual alleles (G vs C) statistical significance was noted (P≤0.05) with variant allele (C) conferring 2.3 times more risk of contracting high myopia. In case of *MYOM1 c.1022+23 (G>A; rs17177479)* SNP, the frequency of variant genotype (GA+AA) is almost double in controls when compared with cases (24% vs 13%) (P≤0.05). **Figure 1C** depicts the sequence variation from G to A. Although homozygous variant allele (AA) is present in 13% (28 of 224) of cases but the overall protective role of SNP is mediated by higher frequency of heterozygous genotype (GA) in controls (24%; 52 of 220). Among all studied SNPs, *MYOM1 c.3418+44 (G>T; rs55779127)* and *MYOM1 c.3418+65 (C>G; rs8096379)* SNPs in intronic region pose highest degree of risk to high Myopia as there is total absence of variant genotype (GT & TT; CC & GG) in controls and the effect is mediated by heterozygous genotypes (GT; 87%) being present only in cases (P≤0.05). **Figure 1D**, **1E** shows the nucleotide change from G to T and C to T respectively. In case of, *EMILIN2 Asn451Asn (T>C; rs3810067),* heterozygotes (TC) are absent in both cases and controls. The nucleotide change from T to C is shown in **Figure 2**. The frequency of variant genotype (TC+CC) is higher in controls proving this SNP to

Table 3. Variations detected in *DLGAP1, EMILIN2 & MYOM1* genes of HM patients

Gene	Wild nucleotide	SNP	rs number	Codon change (Amino acid change)	Codon Position
MYOM1					
NM_003803	G	G/A	rs2230162	GGG to GGA (Gly>Gly)	Gly333Gly
NP_003794	G	G/C	rs8099021	GGA to GCA (Gly>Ala)	Gly341Ala
MYOM1					
NM_003803.3	G	G /A	rs17177479	-	Intronic c.1022+23 G>A
	G	G/T	rs55779127	-	Intronic c.3418+44 G>T
	C	C/G	rs8096379	-	Intronic c..3418+65 C>G
EMILIN2					
NM_032048	T	T/C	rs3810067	AAT to AAC (Asn>Asn)	Asn451Asn
NP_114437					
DLGAP1					
NM_004746	G	G/A	Novel	GAG to AAG (Glu>Lys)	Glu507Lys
NP_004737	G	G/A	rs3745051	CCG to CCA (Pro>Pro)	Pro517Pro

Glu; Glutamic acid, Lys; Lysine, Pro; Proline, Asn; Asparagine, Gly; Glysine, Ala; Alanine.

Table 4. Genotype and allele frequencies of *MYP2* locus gene polymorphisms in 224 cases and 220 controls

Gene/variation	Genotype	Cases N = 224 (%)	Controls N = 220 (%)	OR (95% CI)	*P* value
MYOM1 Gly333Gly (G>A; rs2230162)	GG	168 (75.0)	128 (58.0)	Ref. (1.00)	
	GA	28 (12.5)	92 (42.0)	0.2 (0.14-0.37)	<0.0001
	AA	28 (12.5)	00 (00.0)	22.14 (2.9-38.6)	<0.0001
	(GA+AA)	56 (25.0)	92 (42.0)	0.4 (0.3-0.7)	<0.0001
	G	364 (81.0)	348 (79.0)	Ref. (1.00)	
	A	84 (19.0)	92 (21.0)	0.9 (0.6-1.2)	0.2
MYOM1 Gly341Ala (G>C; rs8099021)	GG	00 (00.0)	00 (00.0)	Ref. (1.00)	
	GC	32 (14.0)	68 (31.0)	0.46 (0.02-7.6)	0.3
	CC	192 (86.0)	152 (69.0)	1.2 (0.07-20.3)	0.4
	(GC+CC)	224 (100.0)	220 (100.0)	1.01 (0.06-16.3)	0.5
	G	32 (7.0)	68 (16.0)	Ref. (1.00)	
	C	416 (93.0)	372 (84.0)	2.3 (1.5-3.7)	<0.0001
MYOM1 c.1022+23 (G>A; rs17177479)	GG	196 (87.0)	168 (76.0)	Ref. (1.00)	
	GA	00 (00.0)	52 (24.0)	6.7 (1.8-45.1)	<0.0001
	AA	28 (13.0)	00 (00.0)	24.8 (3.3-184.2)	<0.0001
	(GA+AA)	28 (13.0)	52 (24.0)	0.46 (0.27-0.76)	<0.0001
	G	392 (87.0)	398 (88.0)	Ref. (1.00)	
	A	56 (13.0)	52 (12.0)	1.09 (0.7-1.6)	0.3
MYOM1 c.3418+44 (G>T; rs55779127)	GG	30 (13.0)	220 (100.0)	Ref. (1.00)	
	GT	194 (87.0)	00 (00.0)	1390 (188-10280)	<0.0001
	TT	00 (00.0)	00 (00.0)	7.1 (0.4-11.6)	0.12
	(GT+TT)	194 (87.0)	00 (00.0)	1390 (188-10280)	<0.0001
	G	254 (57.0)	440 (100.0)	Ref. (1.00)	
	T	194 (43.0)	00 (00.0)	33.7 (4.6-42.4)	<0.0001
MYOM1 c.3418+65 (C>G; rs8096379)	CC	30 (13.0)	220 (100.0)	Ref. (1.00)	
	CG	194 (87.0)	00 (00.0)	1390 (188-10280)	<0.0001
	GG	00 (0.0)	00 (00.0)	7.1 (0.4-11.6)	0.12
	(CG+GG)	194 (87.0)	00 (00.0)	1390 (188-10280)	<0.0001

	C	254 (57.0)	440 (100.0)	Ref. (1.00)	
	G	194 (43.0)	00 (00.0)	33.7 (4.6-42.4)	<0.0001
EMILIN2 Asn451Asn (T>C; rs3810067)	TT	186 (83.0)	160 (72.0)	Ref. (1.00)	
	TC	38 (17.0)	60 (28.0)	0.54 (0.34-0.86)	0.004
	CC	00 (0.0)	00 (00.0)	0.86 (0.05-13.8)	0.4
	(TC+CC)	38 (17.0)	60 (28.0)	0.54 (0.34-0.86)	0.004
	T	410 (91.0)	380 (86.0)	Ref. (1.00)	
	C	38 (9.0)	28 (14.0)	1.25 (0.75-2.09)	0.2
DLGAP1 Glu507Lys (G>A; Novel)	GG	00 (00.0)	00 (00.0)	Ref. (1.00)	
	GA	224 (100.0)	220 (100.0)	1.02 (0.06-16.4)	0.5
	AA	00 (00.0)	00 (00.0)	1.0 (0.01-50.3)	0.5
	(GA+AA)	224 (100.0)	220 (100.0)	1.02 (0.06-16.4)	0.5
	G	224 (50.0)	220 (50.0)	Ref. (1.00)	
	A	224 (50.0)	220 (50.0)	1.0 (0.7-1.3)	0.5
DLGAP1 Pro517Pro (G>A; rs3745051)	GG	144 (64.0)	220 (100.0)	Ref. (1.00)	
	GA	80 (36.0)	00 (00.0)	124.3 (17.1-903.1)	<0.0001
	AA	00 (00.0)	00 (00.0)	1.5 (0.09-24.5)	0.4
	(GA+AA)	80 (36.0)	00 (00.0)	124.3 (17.1-903.1)	<0.0001
	G	368 (82.0)	440 (100.0)	Ref. (1.00)	
	A	80 (18.0)	00 (00.0)	96.8 (13.4-698.9)	<0.0001

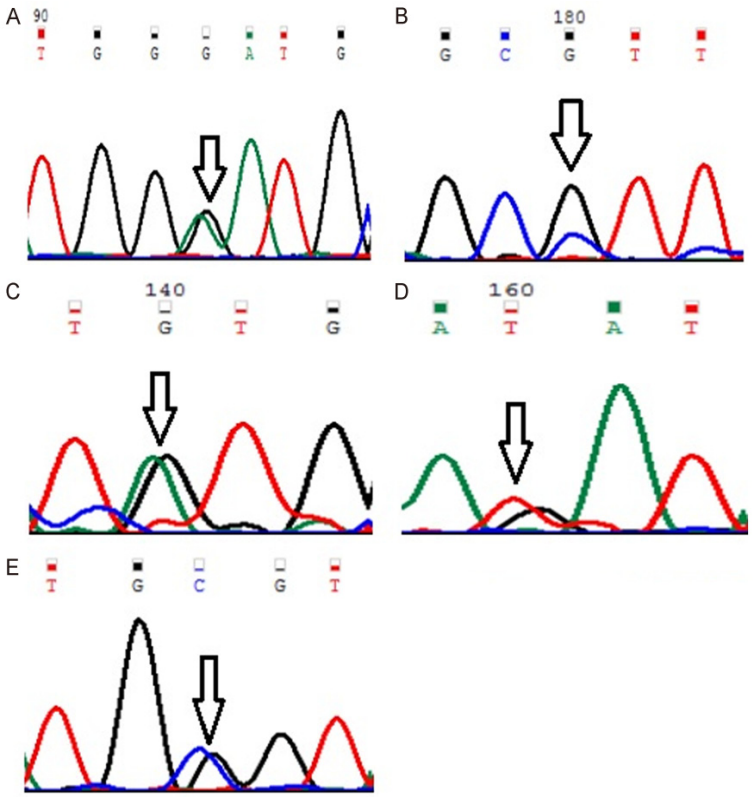

Figure 1. Representative partial chromatograms of affected samples showing sequence variations in *MYOM1* (indicated by arrows). (A) Gly333Gly (G>A; rs2230162) (B) Gly341Ala (G>C; rs8099021) (C) c.1022+23 (G>A; rs17177479) (D) c.3418+44 (G>T; rs55779127) and (E) c.3418+65 (C>G; rs8096379).

be protective against disease risk and the effect is mediated by homozygotes (CC) only (P≤0.05). In case of *DLGAP1 Pro517Pro (G>A; rs3745051)* SNP, homozygous variant genotype (AA) was absent in both cases and controls and presence of heterozygous genotype (GA; 36%) in cases increases risk to high Myopia to a very high degree (P≤0.05). The nucleotide change from G to A is shown in **Figure 3**. Although a novel non-synonymous SNP, *DLGAP1 Glu507Lys (G>A),* was also found in our study but there was only presence of heterozygotes in cases as well as controls rendering this SNP statistically insignificant (*P*≥ 0.05; **Table 4**).

Stratification analysis of MYP2 loci gene variations and risk of High Myopia

To further assess the effect of *MYOM1*, *EMILIN2* & *DLGAP1* genotypes on disease risk with

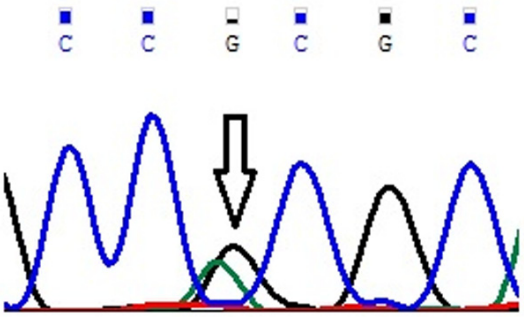

Figure 2. Representative partial chromatogram of affected samples showing sequence variations in *EMILIN2 Asn451Asn (T>C; rs3810067)* (indicated by arrows).

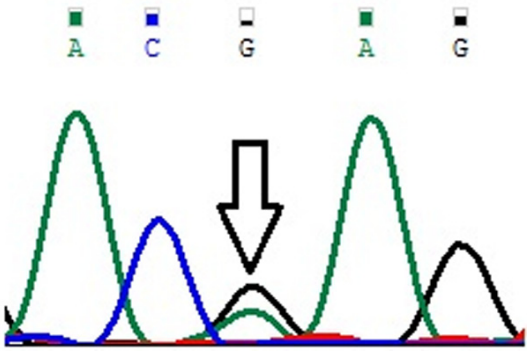

Figure 3. Representative partial chromatograms of affected samples showing sequence variations in *DLGAP1 Pro517Pro (G>A; rs3745051)* (indicated by arrows).

respect to various demographic and clinicopathological parameters of cases and controls, stratification analysis was carried out as shown in **Tables 5**-**7** respectively. *MYOM1 Gly333Gly* SNP showed a statistically significant association with age, gender, smoking status, occupation, family history and degree of myopia respectively with the frequency of variant genotype (GA+AA) higher in males, passive/active smokers, patients with no family history and degree of Myopia ≥-6 D (*P≤0.05*, **Table 5**). No significant association of any parameter with *MYOM1 Gly341Ala* SNP was found (*P*≥0.05; **Table 5**). *MYOM1 c.1022+23* SNP showed statistical significance with occupation and family history with frequency of variant genotype higher in near workers and study subjects with no family history (*P*≤0.05, **Table 6**). Age, occupation, family history and degree of myopia were significantly associated with *MYOM1 c.3418+44* SNP and the frequency of variant allele (GA+AA) was higher in study subjects with ≤30 years of age, family history of disease, ≥-6 D myopia and near workers (*P*≤0.05, **Table 6**). Statistical significance of *MYOM1 c.3418+65* SNP was noted with age, occupation and family history of disease with frequency of variant allele (CG+GG) higher in study subjects with ≤30 years of age, family history of disease and in near workers (*P*≤0.05, **Table 6**). In case of *EMILIN2 Asn451Asn* SNP statistical significance was observed with smoking status, occupation, family history and degree of Myopia with the frequency of variant allele higher in passive smokers, near workers and in subjects with no family history and degree of Myopia ≥-6 D (*P*≤0.05, **Table 7**). *DLGAP1 Pro517Pro* SNP was significantly correlated with age, smoking status, family history and degree of Myopia with the frequency of variant allele (GA+AA) higher in study subjects with ≤30 years of age, family history of disease, <-6 D Myopia and non-smokers (*P*≤0.05, **Table 7**).

In silico prediction analysis

MYP2 locus genes were modeled by *I-TASSER* to obtain the PDB structures and predicted analysis (energy calculations) was done using *PDB Viewer*. In case of MYOM1, wild protein showed higher energy (-9702.442 kJ/mol) compared to mutant (-11496.317 kJ/mol) for codon *Gly341Ala* variation. *PolyPhen* Analysis for mutation *MYOM1 Gly341Ala* is predicted to be benign according to both *HumVar* and *HumDiv* datasets. Additionally *SIFT* analysis predicted the amino-acid substitution as tolerated with the score of 0.94. *Provean score* of 0.648 also predicts the mutation to be neutral.

Although statistically insignificant, the assessment of the *I-TASSER* protein structure showed higher energy for mutant DLGAP1 protein (20206.113 kJ/mol) compared to wild DLGAP1 protein (23265.684 kJ/mol). *PolyPhen* Analysis for mutation *DLGAP1 Glu507Lys* is predicted to be possibly damaging according to *HumDiv* dataset and benign according to *HumVar* dataset. *SIFT* analysis also predicts the amino acid substitution as damaging with the score of 0.01 and likewise, *Provean score* of 2.712 also predicts the mutation to be deleterious.

Genetic association studies and haplotyping

Various genetic association models have been applied on MYOM1, EMILIN2 and DLGAP1 gene polymorphisms; details of which are contained

Table 5. Association of *MYOM1* gene alterations with demographic and clinicopathological variables in 444 subjects (224 cases and 220 controls)

Parameters	*MYOM1 Gly333Gly (G>A; rs2230162)*		OR (95% CI)	*P* value	*MYOM1 Gly341Ala (G>C; rs8099021)*		OR (95% CI)	*P* value
	GG	GA+AA			GG	GC+CC		
Age								
≤30 years	189	78	Ref. (1.00)		00	267	Ref. (1.00)	0.3
>30 years	107	71	0.6 (0.4-0.9)	0.01	00	177	1.5 (0.09-24.2)	
Gender								
Male	163	107	Ref. (1.00)		00	270	Ref. (1.00)	0.4
Female	133	41	2.1 (1.3-3.2)	0.0002	00	174	1.5 (0.09-24.9)	
Smoking Status								
Non-Smoker	70	15	Ref. (1.00)		00	85	Ref. (1.00)	0.2
Passive Smoker	195	80	0.52 (0.28-0.96)	0.01	00	275	0.3 (0.01-5.03)	0.4
Active Smoker	31	53	0.12 (0.06-0.25)	<0.0001	00	84	1.01 (0.06-16.4)	
Occupation								
Student	83	47	Ref. (1.00)		00	130	Ref. (1.00)	
Near Workers	105	75	0.80 (0.49-1.26)	0.1	00	180	0.7 (0.04-11.6)	0.4
Others	108	26	2.3 (1.34-4.12)	0.001	00	134	0.97 (0.06-15.6)	0.5
Family History								
No	48	32	Ref. (1.00)		00	80	Ref. (1.00)	0.3
Yes	120	24	3.3 (1.7-6.2)	<0.0001	00	144	0.5 (0.03-8.9)	
Degree of Myopia								
<-6 D	100	24	Ref. (1.00)		00	124	Ref. (1.00)	0.4
≥-6 D	68	32	0.51 (0.27-0.94)	0.01	00	100	1.2 (0.07-20.03)	

D; Diopters.

in **Table 8**. MYOM1 Gly333Gly, MYOM1 c.1022+23 and MYOM1 c.3418+44 SNPs follow Overdominant mode of inheritance. MYOM1 Gly341Ala and MYOM1 c.3418+65 SNPs follow Recessive mode while as EMILIN2 Asn451Asn SNP follows Co-dominant mode of inheritance.

The haplotype frequency estimation and its association with disease phenotype was done after adjustment by gender and age and the most common haplotype was taken as the reference group. **Table 9** demonstrates MYOM1, EMILIN2 and DLGAP1 haplotype frequencies and its association with the disease.

Discussion

MYP2 is a candidate locus of the non-syndromic autosomal dominant HM, first identified by Young *et al.* who performed a genome-wide linkage investigation for *MYP2*; localized to chromosome 18p11.31 [30]. Candidate genes that map to *MYP2* locus show expression in eye tissues and are vital for fundamental organization and preservation of connective tissue function [3]. The genes sheltering in this locus are also expressed in retina and impact the growth of sclera [31]. Experimental work indicates that neural control machinery is partly restricted to the retina itself, but how retinal signals directly regulate the growth of the outer coats of the eye is presently unknown [21].

Although some case control studies have been conducted relating *MYP2* locus SNPs and HM, but tiny evidence has been collected from these studies owing mainly to insufficient sample size, lack of replica studies and heterogeneous nature of the populations studied. Previously *MYP2* locus candidate genes like *MYOM1, EMILIN2, TGIF, DLGAP1, CLUL1, LPIN2, MRCL3, MRLC2 and ZFP161* have been screened for polymorphic variations [21, 26, 32]. Population from Kashmir represents a homogeneous cohort of common ethnicity and provided an opportunity to revalidate the significance of *MYP2* locus candidate gene variations (if any) for defining their relevance in the pathogenesis of the disease. Numerous studies indicate the association of *MYP2* locus SNPs with HM which is in line with the results

Table 6. Association of intronic *MYOM1* gene alterations with demographic and clinicopathological variables in 444 subjects (224 cases and 220 controls)

Parameters	*MYOM1 c.1022+23 (G>A; rs17177479)*		OR (95% CI)	*P* value	*MYOM1 c.3418+44 (G>T; rs55779127)*		OR (95%CI)	*P* value	*MYOM1 c.3418+65 (C>G; rs8096379)*		OR (95%CI)	*P* value
	GG	GA+AA			GG	GT+TT			CC	CG+GG		
Age												
≤30 years	213	54	Ref. (1.00)		140	127	Ref. (1.00)		140	127	Ref. (1.00)	
>30 years	151	26	1.4 (0.88-2.4)	0.06	110	67	1.5 (1.01-2.1)	0.02	110	67	1.5 (1.01-2.1)	0.02
Gender												
Male	220	50	Ref. (1.00)		152	118	Ref. (1.00)		150	120	Ref. (1.00)	
Female	144	30	1.09 (0.66-1.7)	0.3	98	76	1.0 (0.68-1.4)	0.5	100	74	1.08 (0.7-1.5)	0.3
Smoking Status												
Non-Smoker	70	15	Ref. (1.00)		65	20	Ref. (1.00)		60	25	Ref. (1.00)	
Passive Smoker	230	45	1.09 (0.57-2.0)	0.3	140	135	0.3 (0.2-0.5)	0.001	145	130	0.4 (0.2-0.78)	0.001
Active Smoker	64	20	0.6 (0.3-1.4)	0.16	45	39	0.3 (0.2-0.7)	<0.001	45	39	0.5 (0.2-0.9)	0.01
Occupation												
Student	115	15	Ref. (1.00)		55	75	Ref. (1.00)		55	75	Ref. (1.00)	
Near Workers	135	45	0.4 (0.2-0.7)	0.001	85	95	1.2 (0.7-1.9)	0.2	85	95	1.2 (0.7-1.9)	0.1
Others	114	20	0.7 (0.3-1.5)	0.2	110	24	6.2 (3.5-10.7)	<0.001	110	24	6.2 (3.5-10.9)	<0.001
Family History												
No	60	20	Ref. (1.00)		20	60	Ref. (1.00)		20	60	Ref. (1.00)	
Yes	136	08	5.6 (2.3-13.5)	<0.001	10	134	0.2 (0.09-0.5)	<0.001	10	134	0.2 (0.09-0.5)	<0.001
Degree of Myopia												
<-6 D	106	18	Ref. (1.00)		22	102	Ref. (1.00)		20	104	Ref. (1.00)	
≥-6 D	90	10	1.5 (0.6-3.4)	0.15	08	92	0.4 (0.17-0.9)	0.01	10	90	0.5 (0.2-1.2)	0.09

D; Diopters.

Table 7. Association of *EMILIN2* and *DLGAP1* gene alterations with demographic and clinicopathological variables in 444 subjects (224 cases and 220 controls)

Parameters	*EMILIN2 Asn451Asn (T>C; rs3810067)*		OR (95% CI)	*P* value	*DLGAP1 Pro517Pro (G>A; rs3745051)*		OR (95%CI)	*P* value
	TT	TC+CC			GG	GA+AA		
Age								
≤30 years	204	63	Ref. (1.00)		207	60	Ref. (1.00)	
>30 years	142	35	1.2 (0.78-1.9)	0.1	157	20	2.2 (1.3-3.9)	<0.001
Gender								
Male	215	55	Ref. (1.00)		220	50	Ref. (1.00)	
Female	131	43	0.7 (0.5-1.2)	0.1	144	30	0.86 (0.5-1.4)	0.2
Smoking Status								
Non-Smoker	60	25	Ref. (1.00)		65	20	Ref. (1.00)	
Passive Smoker	215	60	1.5 (0.8-2.5)	0.07	235	40	1.8 (0.9-3.3)	0.03
Active Smoker	70	13	2.2 (1.0-4.8)	0.01	64	20	0.9 (0.4-2.0)	0.4
Occupation								
Student	95	35	Ref. (1.00)		115	15	Ref. (1.00)	
Near Workers	135	45	1.1 (0.6-1.8)	0.3	140	40	0.4 (0.2-0.8)	0.007
Others	116	18	2.3 (1.2-4.4)	0.003	109	25	0.5 (0.2-1.1)	0.06
Family History								
No	52	28	Ref. (1.00)		60	20	Ref. (1.00)	
Yes	134	10	7.2 (3.2-15.9)	<0.001	84	60	0.4 (0.2-0.8)	0.006
Degree of Myopia								
<-6 D	112	12	Ref. (1.00)		69	55	Ref. (1.00)	
≥-6 D	74	26	0.3 (0.1-0.6)	<0.001	75	25	2.3 (1.3-4.2)	0.001

D; Diopters.

Table 8. Appropriate genetic association models for SNPs in *MYOM1*, *EMILIN2* and *DLGAP1* genes with response to HM phenotype (n = 444, adjusted by gender and age)

Gene/variation	Model	Genotype	Case; n (%) n=224	Control; n (%) n=220	*P* Value	AIC
MYOM1 Gly333Gly (G>A; rs2230162)	Overdominant	G/G+A/A	196 (87.0)	128 (58.0)		
		G/A	28 (13.0)	92 (42.0)	<0.0001	1319.44
MYOM1 Gly341Ala (G>C; rs8099021)	Recessive	G/G+G/C	32 (14.0)	68 (31.0)		
		C/C	192 (86.0)	152 (69.0)	<0.0001	1081.20
MYOM1 c.1022+23 (G>A; rs17177479)	Overdominant	G/G+A/A	224 (100.0)	168 (76.0)	<0.0001	1196.02
		G/A	00 (0.0)	52 (24.0)		
MYOM1 c.3418+44 (G>T; rs55779127)	Overdominant	G/G+T/T	30 (13.0)	220 (100.0)	<0.0001	144488.4
		G/T	194 (87.0)	00 (0.0)		
MYOM1 c.3418+65 (C>G; rs8096379)	Recessive	C/C+C/G	224 (100.0)	220 (100.0)		
		G/G	00 (0.0)	00 (0.0)	<0.0001	44797.3
EMILIN2 Asn451Asn (T>C; rs3810067)	Codominant	T/T	186 (83.0)	160 (73.0)		
		T/C	38 (17.0)	60 (27.0)	<0.0001	1087.4
		C/C	00 (0.0)	00 (0.0)		
DLGAP1 Pro517Pro (G>A; rs3745051)	Recessive	G/G+G/A	224 (100.0)	220 (100.0)		
		A/A	00 (0.0)	00 (0.0)	<0.001	514.14

reported from our study [26, 32]. In our study no association of *DLGAP1 SNP* with the risk of HM was found which is in coherence with previous studies [21].

Table 9. *MYOM1*, *EMILIN2*, *DLGAP1* Haplotype frequencies estimation and haplotype association with HM (adjusted by gender and age)

Haplotype	Controls n=220	Cases n=224	Combined
MYOM1			
00000	0.6209	0.4118	0.5083
00010	0.0000	0.0548	0.0273
00011	0.0045	0.3093	0.1649
00100	0.0109	0.0000	0.0058
00101	0.0000	0.0179	0.0082
01000	0.1545	0.0000	0.0874
01011	0.0000	0.0187	0.0000
10000	0.1018	0.0233	0.0640
10001	0.0000	0.0057	0.0025
10011	0.0000	0.0076	0.0034
10100	0.1073	0.0611	0.0841
10101	0.0000	0.0223	0.0121
10111	0.0000	0.0148	0.0070
11000	0.0000	0.0101	0.0000
11001	0.0000	0.0090	0.0045
11011	0.0000	0.0247	0.0162
11100	0.0000	0.0059	0.0025
11111	0.0000	0.0030	0.0020
EMILIN2			
0	0.1308	0.0957	0.1126
1	0.8692	0.9043	0.8874
DLGAP1			
00	0.5000	0.4128	0.4572
01	0.0000	0.0872	0.0428
10	0.5000	0.4128	0.4572
11	0.0000	0.0872	0.0428

MYOM1 is a structural constituent of cytoskeleton thought to integrate the thin and thick filaments and confer elasticity to the M-band of sarcomere in striated muscle [33]. In this study, two exonic *(Gly333Gl; Gly341Ala)* and 3 intronic *(c.1022+23; c.3418+44; c.3418+65)* polymorphic variations of *MYOM1* gene were observed to be significantly associated with HM ($P \leq 0.05$, **Table 4**). *MYOM1* intronic SNPs are reported to fall outside splice sites and outside promoter regions hence do not affect splicing [34].

EMILIN2 confers elasticity to the extracellular matrix [35]. Broadly expressed in connective tissues with cell adhesion promoting functions, it is abundant in blood vessels, skin, heart, lung, kidney, and cornea suggesting its central role in the process of elastogenesis in association with other extracellular matrix constituents [36]. EMILIN2 plays an important role in scleral wall elasticity seen in HM with elongated axial lengths [23]. Previously, *EMILIN2 Asn451Asn* SNP has not been associated with risk of HM [21] which is in contradiction with our study wherein we have found a significant association of disease with *EMILIN2 Asn451Asn* SNP.

DLGAP1 is a member of the PSD95 domain containing family of molecules that are collectively known as “chapsyns” for their function as channel associated proteins. It is known to be highly enriched in synaptosomal preparations of the brain, and is present in the post synaptic density [37]. Scavello *et al.* [21] have shown the expression of this gene in the retina of eye and have further proposed its role in regulating eye growth. The novel *Glu507Lys* SNP in *DLGAP1* observed in our study group is apparently population specific and does not segregate with the disease phenotype (P=0.5) while as an additional documented *Pro517Pro* SNP appeared to associate significantly with the risk of HM (P<0.0001). Computational analysis and SIFT suggest *DLGAP1 Glu507Lys* polymorphism as damaging. *DLGAP1 Glu507Lys* SNP observed in our study must have a potentially significant implication among species because of the fact that sequences of different species like *Homo sapiens, Oryctolaguscuniculus & Rattusnorvegicus* were found to be completely conserved with respect to observed variation when aligned, whereas in *Mus musculus* G is replaced by A (G>A) which is a conserved variation in terms of protein coding, as GAA and GAG both code for same amino acid proving that the region has even been preferably conserved during evolution in terms of amino acid sequence [37]. None of the polymorphisms in *DLGAP1* gene have been segregated with risk of HM as per previous studies [21].

When stratified with respect to demographic and clinicopathological characteristics of HM patients, majority of SNPs were significantly associated with occupation or family history ($P \leq 0.05$). Relevance of genetic factors in HM has been substantiated by various twin and familial studies indicating correlations between refractive error in parents and siblings [38, 39]. A detailed assessment of confounding effects and interactions between hereditary and envi-

ronmental influences in HM by various researchers has shown that near work describes very little of the variance in refractive error compared to parental myopia [40]. In addition, near work exerted no confounding influence on the association between parent and child myopia, indicating that children do not become myopic by adopting parental reading habits. More importantly, there was no significant interaction between parental myopia and near work [45]. Reading has been weakly and equally associated with HM regardless of the number of myopic parents by various studies [41, 42] which suggest that children inherit HM as a trait from parents. Multiple familial aggregation studies report a positive correlation between parental myopia susceptibility and myopia in their children, indicating heritable myopia [41-43]. Previous studies reported that children with a family history of myopia on an average had less hyperopia, deeper anterior chambers, and longer vitreous chambers even before becoming myopic [34, 39]. In our study, most of *MYP2* loci SNPs were significantly associated with smoking ($P \leq 0.05$). Cigarette smoke has around 60 cancer-causing compounds, including nitrosamines, aromatic amines and polycyclic aromatic hydrocarbons (PAH) which can form DNA adducts by metabolic activation leading to the mutations in genes and development of various diseases including HM [44]. As per earlier studies, genetic factors interact with cigarette smoke, signifying that diverse risk approximations relate to diverse genetic tendencies [45].

In this study, the screened *MYP2* loci SNPs follow *Overdominant, Recessive and Co-dominant* mode of inheritance. An Over-dominant model assumes that heterozygote has the strongest impact on disease predisposition; Recessive model presumes the highest impact of homozygous mutant genotype while as Co-dominant model hypothesize that homozygous wild, heterozygous, homozygous mutant genotypes are associated with highest, intermediate and lowest risk respectively [46, 47].

Conclusion

Our study supports the idea that the *MYP2* locus candidate gene polymorphism contributes to the pathogenesis of HM. Since these SNPs appear to change the energy state of protein indicated by *in silico* analysis, a biological corroboration would be needed to elucidate the actual effect of these changes on the function of these proteins. The identification of *MYP2* locus genes will not only provide insight into the molecular basis of this significant eye disease, but will also identify pathways that are involved in eye growth and development. In addition, this information may implicate other genes as possible myopia disease gene candidates. This effort may lead to effective therapies for the severe forms of this potentially blinding eye disease.

Acknowledgements

We are grateful to the patients who have willingly participated in the study. This work was financially supported by Department of Science and Technology, New Delhi, by grants to SR under Young Women Scientist scheme (Project No: -SR/WOS-A/LS-232/2007).

The study was performed with informed consent and following all the guidelines for experimental investigations required by the Institutional Review Board or Ethics Committee of which all authors are affiliated.

Disclosure of conflict of interest

None.

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