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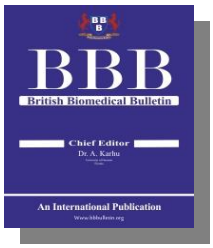

Original

Lack of Association between Fibromodulin rs7543418 Gene and High Myopia

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A R T I C L E I N F O

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A B S T R A C T

Background: The development of high myopia is associated with altered extracellular matrix (ECM) biochemistry. The ECM Fibromodulin (FMOD) is expressed in ocular tissue and regulates assembly of collagen network. The FMOD null mice show certain features of high myopia. The purpose of this investigation was to test the variation between high myopia and FMOD rs7543418 in the human cohorts.
Methods: This case control study compared genotype distribution of 157 subjects including myopic cases (n=105) with spherical equivalent of -6.0 diopters (D) or more negative refractive error and control cases (n=52) with a spherical equivalent within ± 1.0 D. Genotyping of single nucleotide polymorphisms (SNPs) was conducted by polymerase chain reaction (PCR) and temporal temperature gradient gel electrophoresis (TTGE) followed by DNA sequencing.
Results: DNA sequencing of fibromodulin amplicons yielded the expected sequence with identification of a single silent polymorphism G→A variation at c.237G>A without any statistical difference.
Conclusions: *Fmod* null mice showed certain features of high myopia, however our human cohort did not show affected status association with fibromodulin. This study has shown the sequence variation in FMOD is not related to high myopia, allowing its exclusion as a candidate gene for studying high myopia in Kashmiri ethnic populace.

Acronyms:

FMOD	:	Fibromodulin
ECM	:	Extracellular Matrix
PCR	:	Polymerase Chain Reaction
TTGE	:	Temporal Temperature Gradient Gel Glectrophoresis
D	:	Diopter

Introduction

Myopia (nearsightedness) is the most pervasive eye disorder in the current world, with the prevalence of 82% in Asian countries and approximately 16% to 27% in western countries[1-3]. High myopia is characterized as -6.00 or more diopters and is associated with the incremented risk of several ocular diseases, such as glaucoma, retinal detachment, visual impairment and sightlessness[4,5]. The studies have proposed that myopia is a complicated disease with different reasons, including the interaction of various genes with environmental stimuli. Hence, it is obligatory to consider both genes and environment to comprehend myopia[6,7]. One methodology to comprehension the molecular players underlying myopia is to identify the myopia susceptibility genes[8].

In human myopia essentially develops from extortionate enlargement of the eye, overwhelmingly in axial dimension[9]. On the other hand, the animal models have exhibited that dynamic remodeling of sclera assumes a crucial role in the pathogenesis of ocular ailment[10,11]. Scleral remodeling involves regulation of extracellular matrix resulting from diminished engenderment of collagen and proteoglycans and from increased collagen degradation with concomitant increased activities of matrix metalloproteinase 2 (MMP2) and lessened action of tissue inhibitors of MMP[12-16].

Fibromodulin is a member of glycoproteins/proteoglycans, comprising of central area of leucine-rich repeat (LRR) motif, flanked with 4 keratan sulfate chains. Fibromodulin is expressed in ocular tissue and take part in assembly of extracellular matrix by virtue of its capability to associate with type I and type II collagen fibrils and to restrain fibrillogenesis in vitro. A recent mouse knockout study, implicated the proteoglycans fibromodulin (FMOD) as functional candidate genes for high myopia. The study focused on the morphological and structural changes in collagen fibrils of sclera in *Lum*$^{-/-}$*Fmod*$^{-/-}$ double mutant mice. The results showed that the double null in Lum and Fmod manifest certain features of high myopia which incorporate retinal detachment, diminishing of sclera, incremented ocular axial length and variation in diameter of collagen fibrils[17].

So as to examine whether the FMOD rs7543418 polymorphism is corresponded with high myopia in Kashmiri ethnic populace, the polymorphism of high myopic patients (myopia in excess of - 6.0 D) and volunteers in the control group were tested utilizing polymerase chain reaction and Temporal Temperature Gradient Gel Electrophoresis to reveal whether the frequency of *FMOD* differs between control subjects and patients with high myopia.

Material and methods

Subjects

A total of 157 subjects (105 subjects with high myopia -6D or higher and 52 healthy control subjects) are recruited from local hospital (Ophthalmology unit) as well from our ophthalmologist's clinic. Informed consent was obtained from the study subjects after an explanation of the nature and possible consequences of the study. Criteria for selection included a history of onset of myopia in all affected subjects. Individuals were excluded if there was known ocular disease such as retinopathy, cataract or if they had a known genetic disease associated with myopia, such as stickler or Marfan syndrome. An ophthalmic examination of the participating subjects was performed by our ophthalmologist. Ophthalmic evaluation included measuring visual acuity, keratometry, retinoscopy, slit lamp examination of the anterior segment, fundus examination and measurement of axial length. Auto-refraction was taken and

A- scan was done on both eyes. Subjects were encouraged to narrate all the details relevant to this study. This included age of subject, history of onset of myopia, any associated ocular complications and information regarding close work. The study was approved by Research Ethics Committee. Informed consent in accordance with the Declaration of Helsinki was obtained from all participants or their parents and controls.

Polymerase chain reaction

Genomic DNA was extracted from venous blood samples using standard protocols. Primer pairs for individual exons were designed using the primer3 program. Amplifications were performed in 50 µl reaction volume containing 50-100 ng of genomic DNA, 400 nM of each primer, 250 µM of dNTPs, 2.5mM MgCl2, and 0.2 U Taq DNA polymerase in the standard PCR buffer provided by the manufacturer (Sigma Aldrich). Following primer sequences were used for amplification: Forward Primer: 5′-GCTGG CTTGC TCTGT TCTCT-3′ and Reverse Primer: 5′- CGCCGCCGC CGCCCG GCCAAGGTCTCACCATTGAT -3′ and GC-Primer 5'-CGCCCGCC GCGCGCCGCGCCCGTCCCGCCGCCGC CCGCCGCCGCCGCCCG-3'. PCR amplification consisted of a denaturation step at 96°C for 5 min followed by 20 cycles, each consisting of 96°C for 45s followed by 55 °C for 45 s and 72°C for 1 min. Expected PCR product of 1048 bp was generated successfully. The actual size of fragment is 15bp longer because of the linker sequence of Reverse Primer. A GC-clamped PCR product was generated by second round of PCR amplification. One microliter of PCR product was transferred to a second round of amplification with Forward Primer and GC-Primer using conditions as described above for 35 cycles. PCR products were analyzed on 1.5% agarose gel and purified using purification kit (Sigma Aldrich) or NaI.

Temporal Temperature Gradient Gel Electrophoresis (TTGE)

TTGE was performed on the D Code TM mutation detection system and temperature range for TTGE for each PCR fragment was determined empirically with the aid of computer simulation using WinMeltTM (Bio-Rad Laboratories). PCR products were analyzed on 8% polyacrylamide (acrylamide:bis = 37.5:1) gels prepared in 50x Tris acetate-EDTA buffer (2mol/L Tris Base, 1 mol/L glacial acetic acid and 50mM EDTA, pH 8.0) containing 6 mol/L urea. Approximately 15µl purified PCR product and 5 µL of 2x gel loading dye (700 mL/L glycerol, 1 g/L bromphenol blue, 1 mL/L xylene cyanol) were applied to each lane of the gel. The electrophoresis was performed at 150V for 4h at an initial temperature of 60°C and a ramp rate of 2°C/h. The gels were stained in a silver stain.

Sequencing

Samples that showed presence of heteroduplex bands were sent for sequencing to confirm the presence of sequence variations.

Sequence analysis

Sequence results obtained in fasta and pdf formats were analysed using ClustalX version 2 software (Thompson JD *et al*, 1997 & Larkin MA *et al*, 2007) and by Chromas Pro version 1.49 beta 2 software for the detailed inspection of individual chromatograms.

Statistical analysis

Genotypes were obtained by direct counting with subsequent calculation of allele frequencies. Statistical analysis was undertaken using the $\chi 2$ test and significance

value (p). A p value of <0.05 was considered significant. Adherence to the Hardy-Weinberg equilibrium constant was tested using the $\chi 2$ test with one degree of freedom. Odds ratio and confidence interval was also calculated.

Results

Screening for mutations

The rs7543418 fragment of FMOD was analyzed by WinMelt software, a computer algorithm that uses the nucleotide sequence of a DNA fragment to prognosticate melting behavior. The melting map, is a plot of tm (the temperature at which each base pair in a molecule has a 50% chance of being helical or melted) versus the nucleotide sequence of the molecule shows this DNA fragment melts in two distinct melting domains, with a tm of 90°C (designated domain 1) and 76°C (designated domain 2) (Fig. 2A). On the basis of melting predictions, rs7543418 fragment carrying mutations in domain 2 should be resolved by TTGE (Fig. 2A); experiments carried out on a denaturing gradient gel have confirmed these predictions (Fig. 2B).

The amplification of this fragment was carried by a subsequent two rounds of PCR. The analysis of the generated DNA fragments on a 1.5% agarose gel demonstrates that the amplification yields a single DNA fragment of 1083bp, indicating that the 50-bp GC-clamp is attached to this fragment (Fig. 1).

Identified mutations

TTGE analysis of the FMOD gene detected heterozygous mutations, example of the pattern produced by various mutations in the heterozygous states are presented in Fig. 2. The fragments that showed multiple bands in TTGE were subjected to DNA sequencing for characterization of mutations.

In this case-control study, conducted for the first time in this population, we analyzed 105 myopic subjects in cognation to 52 control subjects in order to examine the role of the *SNP* in the *FMOD* gene in Myopic risk in the Kashmiri populace. Only 1 variants G/A (rs7543418) (Fig.3) was observed which corresponded with previously reported SNPs in public databases (Table 1). The genotypic dispersion and allelic dissemination of FMOD did-not contrast significantly between high myopia subjects and normal controls. We found the recurrence of the two different genotypes of *FMOD gene in* our ethnic Kashmir population, i.e., AA and AG to be 17.30 % and 82.70 % among the general control population and 20% and 80% among the myopic cases, respectively (Table 1).

Genotype analysis of individual variants revealed the presence only of heterozygous G>A transition at c.237G>A in exon 2 (Fig. 4). This transition is a silent mutation occurring at glumatic residue at amino acid 79 and would not affect the encoded protein (Fig 4).

Discussion

The *FMOD* gene is a 59kd protein located at 1q32.1 and is composed of 3 exons. Nonetheless, the interpreted region of the gene is composed just out of two exons. An exon in the 5'-non translated region is separated from the next exon by a 1 kb intron. This exon encodes the major part of the deciphered area and is trailed by an approx. 5 kbp intron. The last 50 nucleotides of the translated region as well as the 3'-nontranslated region are spotted on the last exon. The two interpreted exons in the human FM gene encode a protein of 376 amino acid residues[18]. Fibromodulin is a member of the leucine-rich repeat glycoprotein family and was initially described as a corneal proteoglycan

responsible for the control of collagen fibrillogenesis and interaction[19, 20]. This role implicates FMOD in determining the biomechanical properties of the sclera. Results in other studies have proposed that scleral diminishing in the exceedingly nearsighted eye is connected to dissociation of the collagen fiber bundle, and transmutes in the biochemical structure of the sclera have been accounted for in patients with high myopia[21]. The studies from animal models implicate that high myopia is additionally caused by exorbitant axial elongation associated with altered proteoglycan synthesis.

Gene-knockout studies in mice have demonstrated that the fibromodulin genes may be candidate genes for high myopia, on account of incremented axial length in double-null mice[21]. However, Paluru PC et.al omitted the possibility of the association between high myopia and the *FMOD* gene[22]. In our study, using human cohorts, sequence analysis of *FMOD* revealed a SNP which could not be associated with myopia. The results propose that FMOD is unrealistic to be the candidate gene for high nearsightedness in these patients.

In knockout animals the interpretation of the impacts of gene inactivation relies on phenotype examination of wild type contrasted with mutant animal. There is a plausibility that the section of the chromosome that carries the knockout gene might likewise carry nearby influenced genes that may impact the phenotype. In this manner the contrasts observed between the mutant and control mice may be due to genetic differences directly cognate to the linked background gene, and not necessarily due to the null mutation as observed by Chakravarti S *et al*.[17]. This may be a conceivable clarification for the human accomplice not to show influenced status association with this gene[23,24].

Conclusion

This is a novel study carried out for the first time in Kashmir Valley, for screening mutation in fibromodulin gene. The sequence analysis of *FMOD* revealed a novel SNP which could not be associated with myopia and excluding the FMOD as a candidate gene for studying high myopia in these patients. The clinical significance of our findings ought to be further evaluated in larger cohorts with longer catch up to evaluate the effect of these findings.

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Conflict of interest

The authors declare that they have no competing interests.

Author's Contributions

Aeijaz Ul Noor: Preparation of manuscript, formulated and performed all the lab work.
Shabhat Rasool: Helped in sample processing
Sabia Rashid: Provided High Myopia samples
Khurshid I Andrabi: Designed the work, edited the manuscript, co-ordinated the group and overall invigilator of the study.

All authors have read and approved the manuscript.

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Table 1: Polymorphism detected in exon-2 of FMOD gene in ethnic Kashmiri Population

	Genotype	Cases **(n= 105)**	Control **(n= 52)**	χ2	P-Value	OR 95 % CI
SNP rs991967	A (Normal)	126 (60)	61 (58.65)			
	G (Mutant)	84 (40)	43 (41.34)			
	AA(Refrent)	21 (20%)	9 (17.30)		1.00	0.8372
	AG	84 (80)	43 (82.70)	0.84	0.68	(0.3532 - 1.9845)

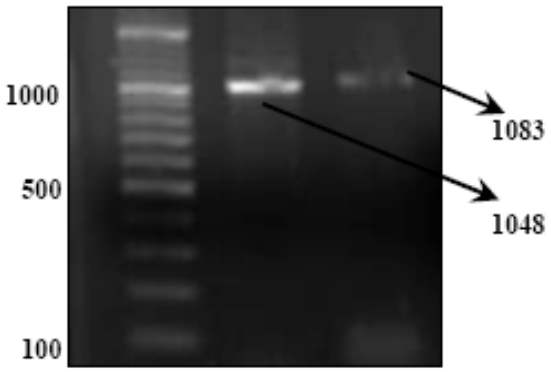

Figure 1: Ethidium bromide staining of amplified DNA fragments size separated on a 1% (wt/vol) agarose gel. The PCR products of a 1033-bp FMOD EXON2 fragment is amplified by primers A and B. The actual size of fragment is 15bp longer because of the linker sequence of primer B. GC-clamped PCR products of the *FMOD EXON2* fragments generated by two consecutive amplifications. The size marker is a 100-bp DNA ladder (FERMENTAS).

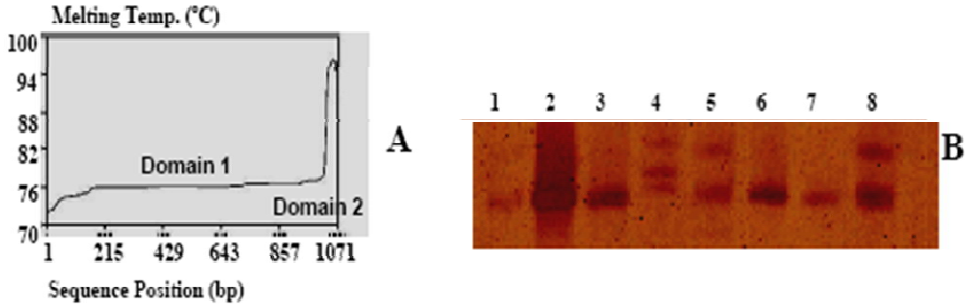

Figure 2: TTGE analysis of FMOD Exon-2 fragment with attachment of G/C nucleotides: Theoretical melt maps and experimental TTGE analysis for *FMOD Exon-2.* (**A**) Melt map of a 1083bp fragment with GC-clamp shows two melting domains. (B) TTGE analysis of the 1083bp fragment showing the presence of heteroduplex. Lane 1, 2, 3, 6 and 7 represents control samples, whereas Lane 4, 5 and 8 represents heteroduplex. The mutation is detected.

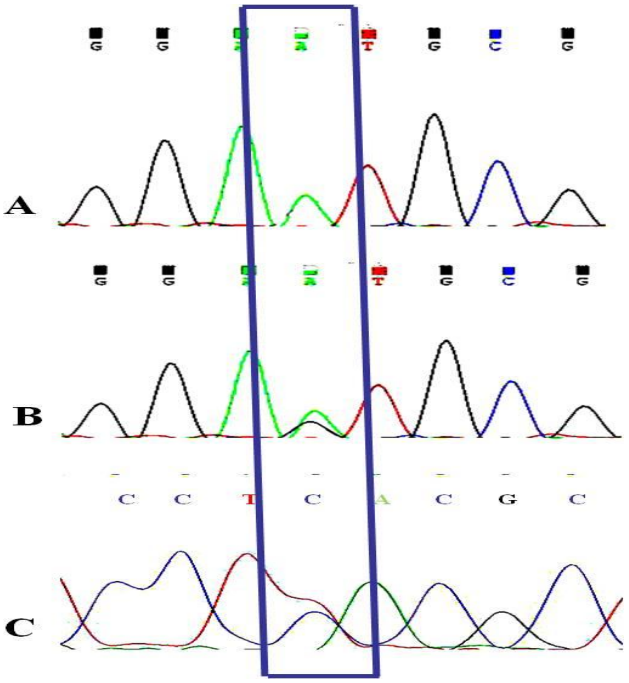

Figure 3: FMOD exon-2 sequence in an unaffected and affected individual: (A) Sequence chromatograms of an unaffected individual showing the wild type allele A. (B) Sequence chromatogram of an affected individual showing the heterozygous variation A to G. (C) Sequence chromatogram of an affected individual using reverse primer. The position of the wild type nucleotide A and the variation A/G in an unaffected and affected individual is indicated in box.

FMOD (Wt)

Nt	217					237						255
	CCC_CGC_GAC_TGC_CCC_CAG_GAG_TGC_GAC_TGC_CCA_CCC_AAC											
	P	R	D	C	P	Q	*E*	C	D	C	P	P	N
AA	73						79						84

FMOD (Mut)

Nt	217					237						255
	CCC_CGC_GAC_TGC_CCC_CAG_GAA_TGC_GAC_TGC_CCA_CCC_AAC											
	P	R	D	C	P	Q	*E*	C	D	C	P	P	N
AA	73						79						84

Figure 4: A partial fragment of the corresponding genomic sequence of the *FMOD* gene of exon 2 is given. The mutated sequence is also shown. The wild-type base and amino acid are marked in green, and the mutated forms in red.