Genotypic variants of MYP2 locus: Analysis for association with high myopia

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DECLARATION

I solemnly declare that the research work entitled, *"Genotypic variants of MYP2 Locus: Analysis for association with High Myopia"*, presented in the thesis is an original piece of work submitted for the award of Ph.D degree in Biochemistry. This work has not been submitted in part or in full for any other degree or diploma.

Shabhat Rasool

CERTIFICATE OF ORIGINAL AUTHORSHIP

The work contained in this thesis entitled, *"Genotypic variants of MYP2 Locus: Analysis for association with High Myopia"*, is the bonafide research work of Ms. Shabhat Rasool, and is worthy of consideration for the award of Doctor of Philosophy in Biochemistry.

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Shabhat Rasool

CONTENTS

LIST OF ABBREVIATIONS

AD	Autosomal dominant
AEL	Axial eye length
bp	Base pair
CIA	Chloroform Isoamyl Alcohol
CSGE	Conformation sensitive gel electrophoresis
D	Dioptre
DLGAP1	Large Drosophila homolog associated protein 1
dNTP	Deoxyribose nucleotide triphosphate
ECM	Extracellular matrix
EMILIN2	Elastin microfibril interfacer 2
HGF	Hepatocyte growth factor
IOL	Intraocular lenses
Kb	Kilobase
LOD	Log of odds
MMP	Matrix metalloproteinases
MYOM1	Myomesin1
MYP2	nonsyndromic autosomal dominant high myopia Locus
M	Molar
mg	Milligram
ml	Millilitre
mM	Millimolar
ng	Nanogram
^{o}C	Degree centrigrade

pm	Picomolar
rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulfate
SER	Spherical Equivalent Refraction
SNP	Single Nucleotide Polymorphism
TAE	Tris acetate ethylene diamine tetra acetae
Taq	Thermus aquatiqus
TE	Tris-ethylene diamine tetra acetate
TGIF1	Transforming Growth Factor β-induced factor1
TGF β1	Transforming Growth Factor Beta-1
UV	Ultra Violet
V	Volt
µg	Microgram
µl	Microlitre
µM	Micromolar

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Abstract

Abstract

Identification of genes involved in the progression of myopia is largely hampered by challenges inherent in mapping genes due to high prevalence, genetic heterogeneity, and wide clinical spectrum of the condition. Genetic mapping studies have identified at least 24 chromosomal loci suspected of harboring genes for myopia progression, MYP1–MYP24 of which MYP2 is considered to be a strong candidate gene locus. Environmental and genetic factors together are attributed to explain the spectrum of geographical and population dependent variations in the incidence of high myopia. Incidently researchers have come up with controversial results with regard to the association of MYP2 locus despite a varied spectrum of polymorphic changes reported in the genes harboured by the locus. The controversy is largely attributed to population heterogeneity. The purity of genetic traits associated with Kashmiri population is likely to minimize the influence of mixed risk/resistance alleles to reliably establish their potential association.

One of the three SNPs observed in codon 10 of TGFβ1 showed a significant difference between patients and control subjects (rs1982073: p genotype=0.003, p allele=0.001). There were no statistically significant differences between patients and control subjects for the other two SNPs, rs1800471 at codon 25 and a novel variant at codon 52.

In TGIF1 three adjacent novel intronic variations (T>C/A; p=0.04: T>G; p=0.02: G>C; p=0.01) and one novel missense sequence variation G26A (p = <0.001) were observed that show possible association with high myopia. G26A also segregates with gender and degree of myopia (p = 0.05).

DLGAP1 gene revealed a total of two polymorphic variations among which G507A (P=1) was novel and one reported polymorphic variation G517A with a significant (P=<0.001) occurrence in affected population. G517A show association with gender and degree of myopia (p=<0.0001).

A previously reported variant T451C observed in EMILIN2 gene did not appear to associate with disease phenotype.

MYOM1 showed five polymorphic variations; two in coding region (G333A; P=<0.0001: G341C; P=0.005) and three intronic (G>A; P=< 0.0001: G>T & C>G; P=

< 0.001) that potentially segregate with the disease phenotype. G333A shows a statistically significant association with gender (p = 0.01) and degree of myopia (p = 0.01) while G341C does not associate with any of the clinical parameters. Among intronic variations, G>T (rs55779127) and C>G (rs8096379) showed significant association with degree of myopia (p=<0.0001 & p=<0.001). The assessment of the I-TASSER predicted protein structure showed change in energy for almost all mutants compared to wild type proteins. The results are indicative that the energy changes due to these polymorphic variations may have significant functional consequences.

Introduction

Myopia, the most prevalent multifactorial ocular disorder, is characterized by refractive error and retinal defocus resulting in decreased visual acuity. High or pathological myopia (RE > 6D) is associated with blinding conditions like glaucoma, macular degeneration, retinal detachment, and choroidal neovascularization, which when left untreated may eventually cause permanent vision loss (Young, 2009). High myopia is the fourth common cause of irreversible blindness (Sandhya, *et al.,* 2011), that occurs due to excessive axial growth of the eye for which active remodeling of the ocular sclera has been shown to play a crucial role (McBrien & Gentle, 2003; Rada, *et al*., 2006). In simple terminology, myopia is a refractive error of the eye that causes focused image to fall anterior to the retinal photoreceptor layer of the eye (Scavello, *et al*., 2005). Ocular refractive components precisely undergo coordinated physical alterations during ocular growth, to attain and maintain normal emmetropic visual acuity, so that image focuses directly on retinal plane (Wildsoet, 1997). Any discordance between axial length and other optical refractive components, such as corneal and lenticular curvatures would result in ametropia and blurred visual acuity (Zhou, *et al*., 2006). Myopia can be detected by visual acuity testing, retinoscopy, autorefraction, or photorefraction during vision screening or clinical examination (Goss, *et al*., 1997). People with myopia can be classified in two groups, those with low to modest degrees of myopia (referred to as “simple” or “school” myopia, 0 to - 6 dioptres) and those with high or pathological myopia (greater than - 6 dioptres). Simple myopia can be corrected with spectacles or contact lenses, whereas high myopia, also referred to as pathological myopia, represents a significant public health burden due to associated ocular complications, which may result in substantial vision loss and even blindness (Su, *et al*., 2010; Saw, *et al*., 2005). The complications associated with high myopia render it to be one of the leading causes of blindness in the world. Myopia-related blindness, in contrast to

other causes, often afflict people earlier in life when they may still be active professionally adding to the agony even more (Jacobi, *et al*., 2005).

It is considered to be a complex, multifactorial condition in which several nongenetic/environmental components like near work, excess illumination, nutritional deficiencies, mechanical stress and mental stress, along with the genetic components influence normal emmetropisation mechanisms of the eye contributing to ocular refraction in myopia (Feldkamper & Schaeffel, 2003). Genetic studies have identified 24 gene loci for myopia till date providing an array of potential candidate genes, but have failed to identify any single causative mutation (Ng *et al*., 2009).

Myopia is the most prevalent ocular disorder globally being on rise and reaching epidemic proportions. In Asia, the prevalence is 1% to 5%, even ranging to 9.1% in some regions (Wong, *et al*., 2000). Considerable increase in myopia prevalence has been observed in East Asian countries like Japan, Singapore, Taiwan and China (Saw, 2003; Xu, *et al*., 2005). The prevalence varies moderately in Western countries, ranging from 16% in Australia and 18% in Netherlands, to an average value of 25% in USA among adults (Kempen, *et al*., 2004). Asian population seems to be more affected than Western populations, over 38% of urban adults from Singapore China (Wong, *et al*., 2000) and up to 80% of teenagers (16–18 years old) in urban Taiwan (Lin, *et al*., 2004) are myopic. The prevalence is 4.5% in populations of Western European origin (Kempen, *et al*., 2004) as compared to 8%–9% (Wong, *et al*., 2000; Iwase, *et al*., 2006) in Eastern Asian adults over the age of 40 years.

The prevalence in India is found to be 19% with 4% in Kashmir (Ahmed, *et al*., 2008). Ahmed I *et al*, 2008, reported effect of age, gender and socioeconomic conditions on myopia prevalence and showed an increase in its prevalence with increased age (3.76% in the age group of 6-10, 4.9% and 6.16% in age groups 11-15 and 16-22). Additionally Girls on average were 1.52 times more likely to have myopia than boys. The prevalence of myopia among girls was 5.54% compared with 3.6% in boys. Socioeconomic conditions also had an impact on the prevalence of myopia with only 3.23% students from medium and high socioeconomic strata having cb cr567tcb cr567t myopia, it was about three times more in students from low socioeconomic strata (8.60 %) (Ahmed, *et al*., 2008).

The treatment options investigated include various types of spectacles and contact lenses, refractive surgery, pharmaceutical agents like atropine and pirenzepine. Experimental evidences show that most of the therapies have small benefits that either last for a relatively short period of time or have significant side effects (Gwiazda, 2009). World Health Organization set goal to eliminate preventable blindness associated with high myopia by year 2020 (Dandona & Dandona, 2001). High prevalence of myopia and its prominence as a public health problem emphasize the importance of understanding the mechanisms of eye growth and finding effective treatments to slow down its progression (Gwiazda, 2009). Laser refractive surgery as a myopia-related cost was estimated to be 4.6 billion dollars for United States in 1990 (Javitt & Chiang, 1994). Stambolian *et al*., estimated this to be doubled by year 2005 (Stambolian, *et al*., 2005; Paget, *et al*., 2008).

Studies of high myopia in animal models have demonstrated that increasing eye size facilitated by remodeling of sclera as one of the most important etiologies in the progression of myopia associated pathologies (Lin, *et al*., 2009). The wide spectrum of myopia-associated disorders strongly argues for an etiologically heterogeneous nature of myopic refractive errors, where multiple factors with genetic and epigenetic effects contribute at different stages during development (Feldkaemper & Schaeffel, 2003). There is a long-standing dispute on the relative role of genetic versus environmental factors in the development of myopia (Saw, *et al*., 2000). Strategies to limit the problem of multiple gene and gene-environment interaction confounding the results in the genetic mapping of myopia are therefore necessary (Ibay, *et al*., 2004). The concept that environmental factors influence ocular development has been well established in epidemiological and experimental animal studies (Saw, *et al*., 2002; Schaeffel, *et al*., 1988). The frequent manifestation of myopia during school and college years, as well as in some occupations requiring intense and prolonged near work, has suggested the critical role of near vision stimulus in the development of myopia. Although the precise nature of this stimulus remains elusive, one current theory is that a lag in accommodation shifts the image focus during near vision behind the retina (Schor, 1998).

Despite the recognized importance of visual experience in the development of myopia there is abundant evidence for genetic factors determining refractive development

(Francois, 1961; Zadnik, *et al*., 1994). First, high myopia prevalence in developed Asian countries compared with the Western world suggests a genetic susceptibility to myopia development. Further, myopic parents are more likely to give rise to offspring with myopia than non-myopic parents (Goldschmidt, 1981). This finding has been confirmed by recent large-scale epidemiological studies, according to which heritable factors account for 80% of juvenile myopia development (Mutti, *et al*., 2002). Strong evidence for the role of inheritance is also provided by twin studies (Teikari, *et al*., 1991; Hammond, *et al*., 2001), where in identical twins display a higher similarity in their refractive status than fraternal twins (Jacobi, *et al*., 2005).

Identification of genes involved in the development and progression of complex disease like myopia has been hampered by challenges inherent in mapping genes due to high prevalence, genetic heterogeneity, and wide clinical spectrum of the condition (Chen, *et al*., 2007). Genetic mapping studies have identified at least 24 chromosomal loci suspected of harboring genes for myopia progression (Ng, *et al*., 2009). Among them, 11 have been implicated in high myopia viz., MYP1– MYP5, MYP11, MYP12, MYP13, MYP15, MYP16, MYP18 (Nallasamy, *et al*., 2007; Zhang, *et al*., 2005; Zhang, *et al*., 2006; Wojciechowski, *et al* 2006; Naiglin, *et al*., 2002; Paluru, *et al*., 2003; Paluru, *et al*., 2005; Young, *et al*., 1998a; Young, *et al*., 1998b; Young, *et al*., 2001; Nishizaki, *et al*., 2009; Lam, *et al*., 2008) and seven in myopia viz., MYP6–MYP10, MYP14, MYP17 (Hammond, *et al*., 2004: Stambolian, *et al*., 2004; Ciner, *et al*., 2008). Five of these loci viz., MYP2, MYP3, MYP6, MYP10, MYP13 have been confirmed through replication analysis in independent family studies (Zhang, *et al*., 2007; Lam, *et al*., 2003; Stambolian, *et al*., 2006; Klein, *et al*., 2007; Nurnberg, *et al*., 2008).

MYP2 is a candidate locus of the nonsyndromic autosomal dominant high myopia first identified by Young, Ronan, Drahozal *et al.* (1998) who performed a genome-wide linkage analysis for myopia susceptibility loci in 8 multigenerational families with an autosomal dominant mode of myopia (more than −6.00 diopters), and showed a significant linkage to 18p. Haplotype analysis further refined this myopia locus to a 7.6 centi-Morgan (cM) interval between markers D18S59 and D18S1138 on 18p11.31. Afterwards Young *et al.* (2001) narrowed the candidate region to the interval of 0.8 cM between markers D18S63 and D18S52. This locus on chromosome

18p11.31 is believed to harbour genes involved in sclera formation or regulation thereby making it most preferential locus with potential to harbor the candidate genes for the disease (Young, 2004: Yamane, *et al*., 2007). The genes localized to MYP2 locus may be expressed in retina and influence growth of sclera (Wallman, 1993). Genes that map to MYP2 critical region include clusterin-like 1 (CLUL1), elastin microfibril interfacer 2 (EMILIN2), lipin 2 (LPIN2), myomesin 1 (MYOM1), myosin regulatory light chain 3 (MRCL3), myosin regulatory light chain 2 (MRLC2), transforming growth β-induced factor (TGIF), large Drosophila homolog associated protein 1 (DLGAP1), and zinc finger protein 161 homolog (ZFP161) (Scavello, *et al*., 2005).

The relationship between MYP2 locus genes and scleral remodeling during the development of myopia has come to be established of late (Honda, *et al*., 1996; Kusakari, *et al*., 2001), due primarily to the evidence that genes existing in the locus are expressed in retina and influence the growth of sclera (Wallman, 1993). The reason for prioritizing these genes is that they are important for constituent organization and maintenance of connective tissue function. This hypothesis emanates mainly from animal studies of experimental myopia as the induction of myopia in juvenile animals by form deprivation demonstrates a visual feedback mechanism in eye growth control. Experiments indicate this neural control mechanism to be partly localized to retina, but how retinal signals directly control the growth of the outer coats of the eye is presently unknown. Mutational screening of MYP2 locus genes like MYOM1, EMILIN2, TGIF, DLGAP1, CLUL1, LPIN2, MRCL3, MRLC2, ZFP161 detected polymorphic variations in all these genes but none of the mutations segregated with the affected status (Scavello, *et al*., 2005). Additionally, recent studies investigated the association of single-nucleotide polymorphisms (SNPs) of the TGFβ1 gene and high myopia but produced conflicting results (Hayashi, *et al.*, 2007). Numerous studies indicate the association of series of SNPs in these genes with high myopia in populations like Chinese living in Hong Kong and Italian Sardinian cohorts (Heath, *et al*., 2001), whereas certain other studies have found no such association, (Young, 2009). Earlier mutational screening study of MYP2 locus genes reports negative association of genes like EMILIN2, TGIF, CLUL1 and MLCB with high myopia (Young, 2004; Scavello, *et al*., 2005). However the locus has shown

significant association with high myopia in two Chinese families (Lam, *et al*., 2002) and Consistent association of this locus with high myopia is also reported in an Italian population (Heath, *et al*., 2001; Lam, *et al*., 2003). This inconsistency in association has largely been attributed to population heterogeneity, wherein purer ethnic cohorts tend to associate with the disease phenotype more frequently than the others.

The purity of genetic traits associated with a population like Kashmiri could serve as an ideal study group to establish any possible association of such SNPs with the disease. This population would minimize the influence of mixed risk/resistance alleles influencing the outcome of the study. Further the identification of the MYP2 genotypes will not only provide insight into the molecular basis of high myopia, but will also help identify pathways that are involved in eye growth and development. In addition, this information may implicate other genes as possible myopia disease gene candidates (Scavello, *et al*., 2005). Information derived from this effort will be useful for submissions to the ever growing SNP database and other researchers screening for myopia candidate genes in this interval may wish to avoid repeat screening of those genes that have been excluded. Additionally identification of the implicated genes for myopia susceptibility will provide a fundamental molecular understanding of how myopia occurs, that may possibly lead to directed physiologic (e.g., pharmacologic or gene therapy) interventions.

Review of Literature

2.1. Myopia: Insights and Challenges

Shabhat Rasool[1], Rubiya Dar[1], Rehana Ismayeel[1], Mehvish Showkat[1], Sabia Rashid[2], Mahboob-ul-Hussain[1], Khurshid I. Andrabi[1]

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2.2. Abstract

Myopia development is a consequence of mismatch between the power of optical components and the axial length of the eye. Lower grades of myopia (< -6 diopters) are not associated with blinding conditions but higher or pathological grades often associates with blinding conditions like macular degeneration, retinal detachment and glaucoma. Ethnic diversity plays a great role in the development of myopia and comparative prevalence rates of high myopia from diverse parts of the world show considerable variability. Identification of candidate genes for high myopia is hampered by challenges inherent in mapping genes due to high prevalence, genetic heterogeneity, and wide clinical spectrum of the condition. Genetic mapping studies have identified at least 24 chromosomal loci suspected of harboring genes for myopia progression. MYP2 is a candidate locus for nonsyndromic autosomal dominant high myopia. Environmental and genetic factors are attributed to explain the spectrum of geographical and population dependent variations in the incidence of high myopia. Researchers over the world have come up with controversial results regarding the association of MYP2 locus genes, MYOM1, EMILIN2, TGIF, DLGAP1, CLUL1, LPIN2, MRCL3, MRLC2, ZFP161 with high myopia. These genes show variation both in the profile and frequency of mutations reported for high myopia subjects, the world over. The treatment options investigated include various types of spectacles and contact lenses, refractive surgery, pharmaceutical agents like atropine and pirenzepine. Experimental evidences show that most of the therapies have small treatment benefits that last for a relatively short period of time or have significant side effects. High prevalence of myopia and its prominence as a public health problem emphasize the importance of understanding the mechanisms of eye growth and finding effective treatments to slow down its progression.

2.3. Myopia Overview

Myopia is most prevalent multifactorial ocular disorder, characterized by refractive error (RE) and retinal defocus resulting in decreased visual acuity. It defines a state of refraction where only nearby objects can be focused to produce a clear retinal image. In other words, myopia occurs when overall optical power of the eye exceeds that required for the axial length of the eye as a result light rays entering the eye are over-convergent and the retinal image is focused in front of retina (Hung, *et al*., 2010).The prevalence has been increasing mostly in East Asian countries like Japan, Singapore, Taiwan and China (Saw, 2003; Xu, *et al*., 2005). High myopia (RE >6D) is associated with vision threatening pathologies like glaucoma, macular degeneration, retinal detachment, and choroidal neovascularization, which may eventually lead to permanent vision loss if left untreated, making it fourth most common cause of irreversible blindness (Young, 2009). The prevalence of high myopia in Asia is 1% to 5%, even ranging to 9.1% in some regions (Wong, *et al*., 2000). Several environmental factors such as near work, excessive illumination, nutritional deficiencies, mechanical stress and mental stress, along with genetic factors influence normal emmetropisation mechanisms of the eye making it a complex, multifactorial disorder (Feldkamper & Schaeffel, 2003). Till date 24 gene loci have been identified for myopia providing an array of potential candidate genes, but unfortunately failed to identify single causative mutation (Ng, *et al.,* 2009). Excessive axial growth of the eye and active remodeling of ocular sclera has been shown to play a crucial role in myopia progression (McBrien & Gentle, 2003; Rada, *et al.,* 2006).

The World Health Organization has grouped myopia and uncorrected refractive error with cataract, macular degeneration, infectious disease, and vitamin A deficiency among the leading causes of blindness and vision impairment in the world (Fredrick, 2002; Pararajasegaram, 1999). In terms of physical optics, myopia is mismatch of the optical power of the eye and its axial length so that parallel rays from distant objects are brought into focus in front of the retina.

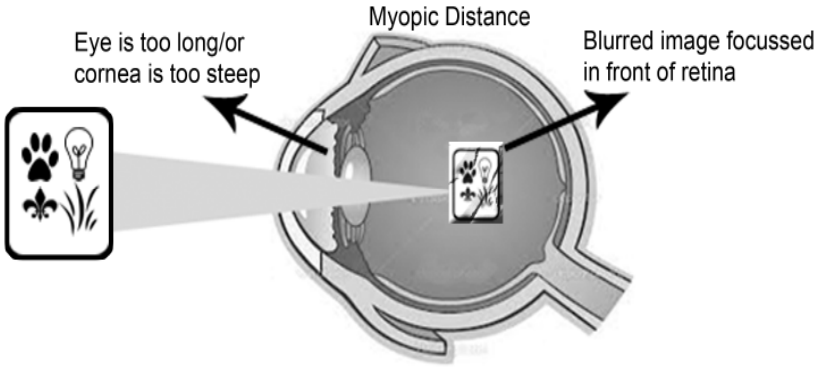

Figure 2.1. Depicts the refractive status of a nearsighted/myopic eye

Refractive errors are expressed as the power of corrective lens expressed in diopters (dpt.) that is necessary to bring image back onto the retina. In case of myopia negative lens reduces total optical power of the eye, higher the negative value, greater is the degree of myopia. The refractive status of human eye is determined by refractive powers of cornea and lens and axial length of the globe. Humans and most animals are born with moderate hyperopic errors caused by short axial length of the eye. Axial elongation is regulated by the process of emmetropization during eye growth that matches the refractive components to axial length to produce normal vision (Troilo & Wallman, 1991). Low and moderate refractive errors frequently result due to mismatch of refractive components of the eye, while the magnitude of these components fall generally into the range of normal distribution (Sorsby, *et al*., 1962a). Whereas in high myopia usually the axial length is out of normal limits. Besides this morphological distinction different types of myopia have been distinguished clinically (Jacobi, *et al*., 2005). Juvenile-onset myopia mostly develops between the ages of 10 and 16 years, whereas pathologic myopia begins to develop in the perinatal period and is associated with rapid refractive error myopic shifts before 10 to 12 years of age (Curtin, 1985; Curtin, 1970; Grosvenor, 1987; Mantyjarvi, 1985).

2.4. Classification of Myopia

2.4.1. Simple Myopia

Axial length and optical power are inversely correlated in emmetropic eyes, **(**Stenstrom, 1948; Sorsby, *et al*., 1957; Alphen, 1961; Araki, 1962; Francois & Goes, 1977; Larsen, 1979). Eyes with simple myopia (refractive error of less than 6

diopters) are either too long for optical power or optically more powerful for axial length.

2.4.2. Anisometropic myopia

Anisometropic myopia or anisomyopia is a condition in which degree of myopia is unequal in two eyes.

2.4.3. Simple myopic anisometropia

The condition in which one eye is emmetropic and the other is myopic is known as Simple myopic anisometropia. It is not significant clinically until the difference between the two eyes reaches about 1 D (Goss, *et al*., 1997).

2.4.4. Nocturnal Myopia

Nocturnal or night myopia is primarily due to increased accommodative response associated with low levels of light as it occurs only in dim illumination. In this condition a person has difficulty to see in low illumination areas having normal daytime vision. Here far point of an individual's focus varies with the level of light. This type of myopia is caused due to dilation of pupil to accommodate more light (Leibowitz & Owens, 1975; Owens & Leibowitz, 1976; Epstein, 1983; Hope & Rubin, 1984).

2.4.5. Pseudo myopia

Overstimulation of the eye's accommodative mechanism or ciliary spasm causes increase in refractive power resulting in a condition called pseudo myopia; it is so named because the patient only appears to have myopia due to an inappropriate accommodative response (Goss & Eskridge, 1987; Alexander, 1940; Stenson & Raskind, 1970).

2.4.6. Induced myopia

Exposure to pharmaceuticals, increase in glucose levels, nuclearsclerosis in addition to other anomalous conditions result in Induced myopia. The encircling bands used in the repair of retinal detachments may induce myopia by increasing the axial length of the eye (Vukojevic, *et al.*, 2005).

2.4.7. Degenerative myopia

Degenerative myopia also known as pathological or progressive myopia is associated with high refractive error in addition to subnormal visual acuity after correction (Cline, *et al*., 1997). It worsens over time and has been reported as one of the main causes of visual impairment. It is very common in Chinese, Japanese, Arab, and Jewish people (Li, *et al*., 2002; Verma & Singh, *et al*., 2005).

2.5. Etiology of Myopia

There is compelling evidence that both environmental and genetic factors are involved in the etiology of myopia (Bear, 1991). The role of environment in myopia progression, represented by near visual activity remains debatable (Mutti, *et al*., 1996), however recent analysis of the contribution of near work and parental history of myopia shows that parental history makes the greater contribution (Mutti, *et al*., 2002). Near work explains little variance in the refractive error, in the range of 2% to 12% (Angle & Wissmann, 1978; Angle & Wissmann, 1980; Richler & Bear, 1980; Zadnik, *et al*., 1994).

Table 2.1. Showing Possible Etiologies of Myopia by Classification.

Type of Myopia	Etiologies
Simple Myopia	1. Inheritance 2. More near work activities 3. Unknown
Nocturnal Myopia	1. Dark focus of accommodation
Pseudomyopia	1. Accommodative disorder 2. High exophoria 3. Cholinergic agonist agents
Degenerative Myopia	1. Inheritance 2. Retinopathy of prematurity 3. Interruption of light passing through ocular media
Induced Myopia	1. Age-related nuclear cataracts 2. Exposure to sulfonamides and other pharmaceutical agents 3. Variability in blood sugar level

Source: Goss DA, *et al*., 1997

2.6. Symptoms and associated Complications

Blurred distance vision is the most common symptom associated with myopia. Distance blur is constant in simple and pathologic myopia, while in nocturnal myopia,

it occurs only in dim light conditions. In pseudo myopia, the blurred distance vision may be constant or intermittent with occurrence of greater distance blur after near work while in induced myopia it varies from transient to constant, depending upon the causative condition (Hirsch, 1945; Crawford, *et al*., 1945; Peters, 1961). Patients with nocturnal myopia mostly suffer difficulty in driving and blurred distance vision at night. The common sign of pseudo myopia is more minus power on manifest refraction than on cycloplegic refraction. This additional minus power is hard to eliminate with standard refraction procedures. Pathological myopia is congenital or of early onset and corrected visual acuity may be reduced due to pathological changes in the posterior segment (Curtin, 1985; Karlin & Curtin, 1976; Curtin, 1977; Levy, *et al*., 1977; Curtin, 1982; Shapiro & Chandra,1985; Hoffman & Heath, 1987; Goldschmidt, *et al*., 1990; Celorio & Pruett, 1991).

Patients with degenerative myopia are more likely to have retinal detachment than patients with hyperopia, and the risk for retinal detachment increases with increase in degree of myopia (Perkins, 1979). High myopia affected individuals mostly have different forms of glaucoma and loss of vision can occur at lower intraocular pressures when the patient is myopic (Perkins, 1960a; Perkins & Jay, 1960b; Daubs & Crick, 1981; Perkins & Phelps, 1982). All these associated pathologies together make high myopia one of the leading causes of blindness in the United States, United Kingdom, and Canada (MacDonald, 1965; Sorsby, 1972; Hatfield, 1975; Curtin, 1985).

2.7. Refractive parameters

To understand myopia it is necessary to have a basic knowledge of the eye's focusing system: cornea, lens, and retina. Cornea is a tough, transparent, dome-shaped tissue that covers the front of the eye and lies in front of iris. Lens is transparent, double-convex structure located just behind the iris. Retina is a thin membrane lining the rear of the eyeball. Light-sensitive retinal cells are destined to convert incoming light rays into electrical signals which are sent through the optic nerve to brain, to interpret the images (Fallon, 2007). In normal vision, parallel rays of light entering the eye are bent by cornea and lens focusing precisely on the retina, providing a crisp, clear image. Whereas in myopic eyes, the focusing power of the cornea and lens is too strong with

respect to length of eyeball and light rays are bent too much so that they converge in front of retina. This inaccuracy is called refractive error. Several studies (Curtin, 1985; Alphen, 1961; Curtin & Karlin, 1971; Jansson, 1963) have shown that the refractive status of an eye is determined primarily by axial eye length (AEL). The average refractive error at birth is approximately 1 to 2 diopters (D) of hyperopia, and AEL approximately measures 17 mm. By adulthood, the AEL grows to about 24 mm resulting in little change in refractive error, because the radius of curvature of the cornea increases and the refractive power of the lens decreases (Curtin, 1985; Sorsby, *et al*., 1962).

2.8. Ocular morbidity

High myopia is associated with progressive and excessive elongation of the globe, accompanied by degenerative changes in the choroid, sclera, Bruch's membrane, retinal pigment epithelium, and neural retina (Young, 2004). Myopia occurs frequently in association with infant prematurity (Palmer, *et al*., 1994) and has been linked to juvenile chronic arthritis (JCA) (Fledelius, *et al*., 2001). A more serious ocular involvement is feared in either of the conditions, i.e. retinopathy of prematurity following preterm birth and anterior uveitis in JCA. In a number of inherited X-linked retinal disorders like retinitis pigmentosa linked to the RP2 and RP3 locus (corresponding to Xp11.23 and Xp21.1, respectively) (Flaxel, *et al*., 1999; Yokoyama, *et al*., 2001) and X-linked congenital stationary night blindness (CSNB1, CSNB 2) (Pusch, *et al*., 2000; Strom, *et al*., 1998), moderate to high degrees of myopia are frequently observed both in carrier females and affected males.

2.9. Prevalence and Economic Impact

Myopia the most common eye disorder and significant ocular health burden is associated with increased risk of vision loss around the world (Fredrick, 2002). The prevalence varies by country and by ethnicity, reaching as high as 70-90% in Asian populations (Curtin, 1985; Leibowtiz, *et al*., 1980). Nearly epidemic levels (up to 80%) have been reported in Hong Kong (Lam & Goh, 1991; Yap, *et al*., 1993; Edwards, 1999; Lam & Edwards, 1999), Singapore (Tan, *et al*., 2000; Wong, *et al*., 2000; Hui-Min, *et al*., 2001), Taiwan (Lin, *et al*., 1998; Lin, *et al*., 1999) and Japan (Matsumura & Hirai, 1999). The prevalence varies between 30- 40% in Europe and

America and 10-20% in Africa (Katz, *et al*., 1997). Myopia affects 25% population in United States (Burton, 1990). Economically myopia is a burden to society due to expenses for regular eye examinations, cost of spectacles and contact lenses and refractive surgery charges. The prevalence of myopia has been estimated at roughly 25% of adults in the United States, with associated costs of examination and treatment in excess of $4.6 billion (Javitt & Chiang, 1994; Sperduto, *et al*., 1983; Jones, *et al*., 2007).

2.10. Environmental vs Genetic factors

Epidemiological and experimental animal studies add support to the concept that environmental factors influence ocular development. Myopia development during school and college years and in some occupations requiring prolonged near work, suggests the critical role played by near vision stimulus in the development of myopia (Stambolian, *et al*., 2006). Although precise nature of this stimulus remains elusive one current theory suggests that lag in accommodation shifts the image focus during near vision behind the retina (Schor, 1998), which is consistent with the observation that myopia can readily be induced in animals experimentally by hyperopic defocus, i.e. by fitting concave lenses. The chicken/egg dilemma in myopia pathogenesis is highlighted by the concurring theory that assumes the opposite, i.e. that excessive instead of insufficient accommodation results in axial elongation by exerting mechanical pressure on the eye wall (Morgan, 2003; Jacobi, *et al*., 2005). Evidences support the notion that genetic factors play an important role in the development of high myopia (Feldkamper & Schaeffel, 2003; Tang, *et al*., 2008; Young, *et al*., 2007; Schaeffel, *et al*., 2003; Wang, *et al*., 2008). Myopic parents more often give rise to myopic children than nonmyopic parents. Also, multiple studies with twins confirmed the higher similarity in identical twins compared to fraternal twins in terms of myopia development. Myopia susceptibility genes should be screened to identify possible allelic association of these genes with the expression of the disease. Genetic polymorphic studies provide the most information with respect to elucidating the mechanism for myopia progression (Stambolian, *et al*., 2006; Han, *et al*., 2006; Lin, *et al*., 2006).

2.11. Animal Models

Animal studies in juvenile and newborn monkeys, chick models, and tree shrew have revealed emmetropization mechanism that normally maintains a match of AEL to optical power so that photoreceptors are in focus for distant objects. All studies add support to the observation that eye growth is affected by the quality of visual experience in early period of life. In animal models of myopia, active remodeling of sclera plays a crucial role in axial elongation (McBrien & Gentle, 2003; Rada, *et al.*, 2006). Scleral remodeling involves reduced production of extracellular matrix which results from reduced production of collagen and proteoglycans and from increased collagen degradation along with concomitant increased activity of matrix metalloproteinase 2 (MMP2) and a reduction in the activity of tissue inhibitors of MMP. Transforming growth factor β (TGFβ) together with its receptor expressed in eye tissues (Saika, 2006) also regulates the proliferation of fibroblasts and production of collagen, MMP2, and tissue inhibitors of MMP (Overall, *et al.*, 1989). Experimentally induced myopia is achieved by many ways, such as lens-induced optical defocus, form deprivation and restricted visual environment conditions (Sherman, *et al*., 1977; Troilo & Wallman, 1991; Wallman & Mc Fadden, 1995a; Wallman, *et al*., 1995b; Wildsoet, 1997; Norton, 1999; Raviola & Wiesel, 1985; McBrien & Norton, 1992). Lens-induced optical defocus is based on shifting the eyes focal plane posteriorly (with minus lenses) or anteriorly (with plus lenses) (Figure 2.3). Negative lenses cause axial elongation of the eye, which continues until retinal location shifts by the amount that almost matches the shift of the focal plane (Irving, *et al*., 1991), however positive lenses act inversely to decrease the axial length elongation rate in tree shrews (Siegwart & Norton, 1993) and chicks (Irving, *et al*., 1995). The concept of recovery from induced myopia emerged after it was reported that induced chick axial elongation due to form deprivation showed recovery when patterned light was restored in young animal groups (Wallman & Adams, 1987; Young, *et al*., 2007).

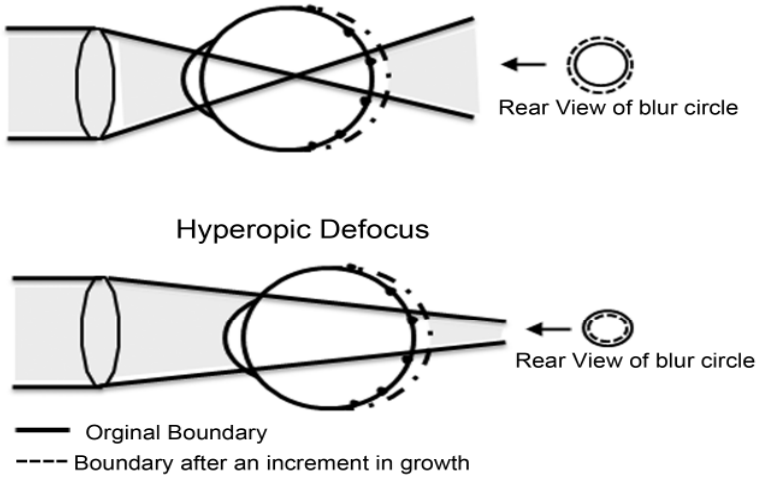

Figure 2.2. Change in blur circle size following an increment in axial growth. The solid eye ball boundry indicates the original boundry of the sclera, the dashed boundry indicates the boundry with an increment in ocular growth. Dots on boundries indicate the positions of nodes in the retinal region of interest. For myopic defocus (focal plane located in front of retina), incremental changes in growth result in increased blur circle size at nodes in the retinal region. For hyperopic defocus (focal plane located behind retina), incremental changes in growth result in increased blur circle size. Hyperopic defocus will cause myopia due to excessive axial growth, while myopic defocus will cause relative hyperopia due to reduced growth.

2.12. Molecular Genetics

Genetic mapping for a complex common disorder like myopia has been progressive, an X-linked recessive form of myopia has been mapped and designated the first myopia locus, MYP1 (Schwartz, *et al*., 1990). Young *et al* studied several medium to multigenerational families with autosomal dominant high myopia and reported significant linkage to chromosome 18p11.31, MYP2 locus (Young, *et al*., 1998a) and 12q23.1-24, MYP3 locus (Young, *et al*., 1998b) and long arm of chromosome 17 (Paluru, *et al*., 2003). Niaglin reported a novel locus for autosomal dominant high myopia on 7q36 (Naiglin, *et al*., 2002). Fourth autosomal dominant locus on chromosome 17q21-q22 (MYP5) was determined in a large multigenerational English-Canadian family (Paluru, *et al*., 2003). Paluru, *et al*., 2005, identified autosomal dominant high myopia locus on chromosome 2q37 in a large, multigenerational white US family. Loci on chromosome Xq23-q25 and 4q have also recently been identified by Zhang, *et al.* (Zhang, *et al*., 2006; Zhang, *et al*., 2005) in ethnic Chinese families. Eight additional regions (14q, 4q22-q28, 8q22.2, 10q22,

11q23, 13q22, 14q32, and 17qter) showed nominal linkage evidence. Hammond, *et al*., 2004 evaluated 221 dizygotic twin pairs with moderate myopia and found significant linkage to 4 loci, with a Maximum LOD score of 6.1 on chromosome11p13 and a recent study group (Wojciechowski, *et al*., 2006) found significant evidence for linkage of refractive error to a novel quantitative trait locus on chromosome 1p36 in an Ashkenazi Jewish population (Young, *et al*., 2007).

2.13. Candidate Genes

In addition to enhancing our understanding of the underlying biology of myopia, a better understanding of genetic factors in myopia might lead to improvements in prediction of myopia onset, treatment and prevention. Identification of the genetic factors involved in complex traits is complicated by the involvement of a number of genes, genetic epistasis, and population heterogeneity. Despite these issues, several research groups have made strides in the last eight years towards identification of genetic regions of interest with respect to myopia. The studies have been of families with histories of pathological myopia (Mutti, *et al*., 2007). These regions include 18p11.31 in eight American (Young, *et al*., 1998a) and 15 Chinese families (Lam, *et al*., 2003a) , 17q21-22 in an English/Canadian family (Paluru, *et al*., 2003) , 12q21-23 in a German/Italian family (Young, *et al*., 1998b), 7q36 in 21 French and two Algerian families (Naiglin, *et al*., 2002; Mutti, *et al*., 2007) and 2q37.1 in an American family of Northern European extraction (Paluru, *et al*., 2005). Linkage analysis studies have also implicated association of various chromosomal loci with high myopia including MYP2 locus on chromosome 18p11.31. This locus is believed to harbour genes involved in sclera formation and regulation (Young, 2004) thereby making it most preferential locus with potential to harbor the candidate gene for the disease. MYP2 locus has been screened and multiple candidate genes for high myopia identified within this critical region and within the other mapped loci by Young *et al*. Some genes have been excluded based on screening results. There are 9 known and 6 hypothetical genes considered to be candidates based on mapped position within MYP2 interval. All sequences within this region have been labeled as “finished”, and there are no known gaps within the interval. The genes (Figure) that map to the MYP2 critical region include clusterin-like 1(CLUL1), elastin microfibril interfacer 2

(EMILIN2), myomesin 1 (MYOM1), lipin 2 (LPIN2), myosin regulatory light chain 3 (MRCL3), myosin regulatory light chain 2 (MRLC2), large Drosophila homolog associated protein 1 (DLGAP1), transforming growth β-induced factor (TGIFβ) and zinc finger protein 161 homolog (ZFP161) (Scavello, *et al*., 2005).

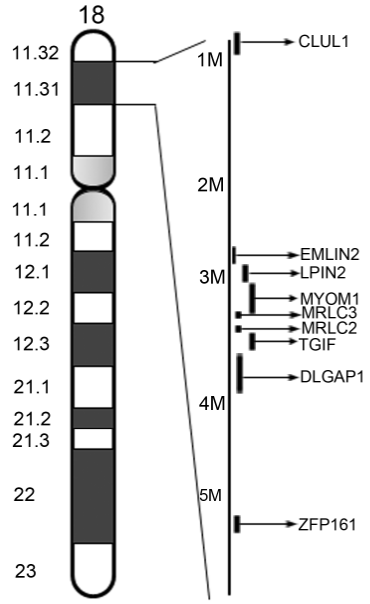

Figure 2.3. Ideogram of chromosome 18 highlighting known candidate genes within the MYP2 interval.

These genes are important for constituent organization and maintenance of connective tissue function and are additionally expressed in retina and influence the growth of sclera (Wallman, 1993). This retinal hypothesis emanates mainly from animal studies of experimental myopia. The induction of myopia in juvenile animals by deprivation of form vision demonstrates a visual feedback mechanism in eye growth control. Experimental work indicates that this neural control mechanism is at least partly localized to the retina, but how retinal signals directly control the growth of the outer coats of the eye is presently unknown. Transcription factors and regulatory genes expressed in retina like CLUL1, MRLC2, MRCL3, TGIF, ZFP161 and DLGAP1 may play a role in regulating eye growth (Scavello, *et al*., 2005).

EMILIN proteins are a group of extracellular matrix multimeric glycoproteins (Colombatti, *et al*., 2000) including EMILIN1, EMILIN2 and Multimerin1, Multimerin2. They share four protein domains: C-terminal C1q domain, collagenous domain, coiled-coil domain and N-terminal cysteine-rich domain (EMI domain). The

domain organization suggests shared in addition to some specific functions for each of these EMILIN proteins. The proline-rich domain of EMILIN2 provides structural flexibility and unique protein-protein interaction sites. EMILIN2 most closely resembles EMILIN1 (Doliana, *et al*., 2001), sharing 70% and 75% identity at N- and C-terminal domains, respectively. C1q is the target recognition domain of the classical pathway of complement activation and a connecting link between innate and acquired immune systems (Hayward, *et al*., 1995). The C1q domains bind to form homo and hetero-multimers. EMILIN2 forms heterotrimers with EMILIN1 and partially co-localizes with EMILIN1 in cell culture (Sa & Hoover-Plow, 2011).

EMILIN2 encodes for an elastic fiber interacting protein that confers elasticity to the extracellular matrix (Doliana, *et al*., 2001). It spans 68 kb and has 9 exons encoding a 4009 bp transcript. It has a unique multimodular organization with C1q-like globular domain at the C terminus, a short collagen-like region, a long segment of about 650 residues with a high potential for forming coiled-coil α-helices, and a cysteine rich domain at N-terminus. It is deposited extracellularly as a fine network and broadly expressed in connective tissues having cell adhesion promoting functions and is particularly abundant in blood vessels, skin, heart, lung, kidney, and cornea (Bressan, *et al*., 1983; Colombatti, *et al*., 1988). The expression profile, pro-adhesive functions, and domain characteristics are suggestive of its fundamental role in the process of elastogenesis in association with other extracellular matrix constituents (Doliana, *et al*., 2001). This may be an important association in scleral wall elasticity seen in high myopia. Mutation screening of EMILIN2 resulted in 8 polymorphisms, 4 silent, 1 missense, and 3 were in the untransilated region (UTR). None of these polymorphisms segregated with the affected status (Scavello, *et al*., 2005).

MYOM1 alternatively known as skelemin is a 36 exon gene spanning 128 kbps. Protein being structural constituent of cytoskeleton is thought to integrate the thin and thick filaments while conferring elasticity to M-band of sarcomere in striated muscle (Wang, 1985; Maruyama, 1986; Trinick, 1991). It is a member of immunoglobin super family (Price & Gomer, 1993) binding extracellular matrix proteins (Diamond, *et al*., 1991). MYOM1 may also play an important role in the assembly and stabilization of myofibrils (Speel, *et al*., 1998). Mutation screening of this gene (Scavello, *et al*., 2005) resulted in 39 polymorphismss, out of which 5 were silent, 4

missense, 29 Intronic, and 1 in UTR. Eight of the polymorphic variations were novel but none of these segregated with the disease status. Myomesin 1, like other myofibrillar proteins contains structural modules with strong homology to either fibronectin type III (motif I) or immunoglobulin C2 (motif II) domains. Myomesin 1 and myomesin 2 each have a unique N-terminal region followed by 12 modules of motif I or motif II, in the arrangement II-II-I-I-I-I-I-II-II-II-II-II. Further the two proteins share 50% sequence identity in this repeat-containing region. The head structure formed by these 2 proteins on one end of the titin string extends into the center of the M band (Entrez Gene).

TGIF is a 46 kb DNA binding homeo-domain protein containing 10 exons and belongs to three amino acid loop extension homeobox family (Wotton, *et al*., 1999a; Wotton, *et al*., 1999b). It is a transcription repressor with multiple actions including a role in retinoid-responsive transcription (Bertolino, *et al*., 1995). TGIF mutations are associated with holoprosencephaly, a congenital craniofacial and brain anomaly disorder (Overhauser, *et al*., 1995; Muenke & Beachy, 2000; Gripp, *et al*., 2000; Chen, *et al*., 2002; Scavello, *et al*., 2005). Genetic evidence supporting a role for TGIF in myopia pathogenesis came from analysis of a Chinese cohort where six single nucleotide polymorphisms (SNPs) were significantly associated with high myopia (Lam, *et al*., 2003b). However, a significant association with this gene could not be replicated in a second Chinese case control study of high myopia population (Li, *et al*., 2003). A Japanese case control study of high myopia individuals also analyzed this gene by using 13 SNPs across the TGIF gene and failed to identify significant association (Hasumi, *et al*., 2006).Till date, it was only in Caucasians that coding regions, and intron/exon boundaries of TGIF were sequenced in 10 cases (< -6.00 D) from European high-myopia families and 10 unrelated emmetropic control individuals (0.00 D). Surprisingly no significant sequence variants were detected in the high myopia subjects compared to controls (Scavello, *et al*., 2005). Currently published studies of TGIF gene have concentrated on the myopia phenotype (refraction) as the trait of interest (Pertile, *et al*., 2008).

DLGAP1 (DISCS large associated protein 1: also known as DAP1 or GKAP) is a member of the PSD95 domain containing family of molecules that are collectively known as “chapsyns” for their function as channel associated proteins. Chapsyns are

known to have one to three conserved domains: a binding domain found in the amino (NH2) or the carboxyl (COOH) regions, a sulfhydryl (SH3) group, and a guanylate kinase domain in the carboxyl region (Kim, *et al*., 1997). It is known to be highly enriched in synaptosomal preparations of the brain in addition to its presence in the post synaptic density (NCBI). Mutation screening of this gene resulted in 3 polymorphisms, 2 silent, and 1 missense. One polymorphic variation was novel and none of these segregated with the affected status (Scavello, *et al*., 2005).

2.14. Ethnicity

Ethnicity makes a great contribution to the development and progression of myopia. Indeed, it is the ethnic diversity that appears to distinguish different populations with regard to prevalence. Myopia is the most prevalent ocular disorder globally that is on rise reaching epidemic proportions. Singapore has the dubious reputation of having the highest prevalence in the world. Whereas the prevalence of myopia in the United States is estimated to be 25%, and the prevalence in India to be 19%, in the Asian cities of Singapore, Hong Kong and Taiwan, prevalence rates of myopia are considerably higher (Sperduto, *et al*., 1983; Dandona, *et al*., 1999). A study of 4,000 Taiwanese schoolchildren revealed myopia prevalences to be 40% at age 12 years, and 70% at age 15 years (Lin, *et al*., 1988). All these studies provide evidences for the ethnic background of the disease (Tan, 2004).

2.15. Therapeutic interventions

The prevalence of myopia has been estimated at 25% with increasing trends toward surgical correction the most widespread surgical procedure for the correction of myopia has been laser in situ keratomileusis (Hamilton, *et al*., 2004, Duffey, *et al*., 2004). It provides a safe and effective procedure for the surgical correction of myopia, but it also carries a number of potential limitations in the treatment of eyes with high myopia (-8.0 diopters), including corneal ectasia, severe night glare and worsened best corrected visual acuity (BCVA) (El Danasoury, 1998; Oshika, *et al*., 1999; Seiler, *et al*., 1998; Stulting, *et al*., 1999). These concerns have prompted the expansion of refractive surgery options to include other procedures, like photorefractive keratectomy, clear lens extraction and phakic intraocular lens (IOL) implantation. Photorefractive keratectomy initially showed great promise, but recent

studies citing poor long-term stability of BCVA and visual disturbances from excess corneal ablation and smaller optical zones have blunted enthusiasm for photorefractive keratectomy, which uses older and conventional excimer lasers (Hersh, *et al*., 1998; Pop & Payette, 2004; Tahzib, *et al*., 2007). Newer excimer lasers and wave front guided ablations have shown promise and there is renewed interest in performing surface ablation for high myopia (Bilgihan, *et al*., 2004, Kim, *et al*., 2004; O'Brart, *et al*., 2006). Clear lens extraction has also been employed for many years for the correction of high myopia but has some significant complications limiting its widespread adoption, the primary concern being an increased risk of retinal detachment (Fernandez-Vega, *et al*., 2003; Kubalogclu, *et al*., 2004; O'Brien, *et al*., 2002). Several categories of phakic IOLs, including posterior chamber lenses, angle-supported anterior chamber lenses and iris-fixated lenses are available. The most promising type of phakic IOL is the iris fixated lens (Silva, *et al*., 2008).

Material and Methods

3.0. Chemicals & Reagents

Chemicals and reagents used were of standard analytical molecular biological grade unless otherwise specified (Supplrmentary data: S1.1-S1.10)

3.1. Methodology

3.1.1. Sample collection

A total of 423 venous blood samples (247 with high myopia of > -6D and 176 healthy controls) were recruited for the study (Sample details, Annexure I-VII). During the survey for high myopia, subjects were recruited from S.H.M.S. (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, with degree of myopia more than -6D in one or both eyes. 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. Non-Kashmiri and non-Muslim subjects were also excluded. The control subjects had no or very little degree of myopia in one or both the eyes.

An ophthalmologic examination of the participating subjects was performed by our ophthalmologist. The ophthalmologic evaluation included measuring visual acuity, keratometry, and retinoscopy, a 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 the subject, history of onset of myopia, any associated ocular complications and information regarding close work.

Venous blood samples were collected in 0.5M EDTA for DNA extraction and high molecular weight DNA was extracted by proteinase K method (Blin, *et al*., 1976) and Salting out method (Nasiri, *et al*., 2005)

3.1.2. DNA extraction from blood

I. DNA extraction using proteinase K

5 ml EDTA treated blood was taken in a 50 ml sterile falcon tube. To it 15 ml of lysis solution was added, followed by the incubation at -20^{o}C for 30 minutes. After incubation, it was centrifuged at 3000 rpm for 10 minutes at 4^{o}C. The supernatant obtained at this step was discarded and the pellet was washed three times by adding 5 ml of erythrocyte lysing buffer and steps 4 and 5 were repeated. Now 10 ml of SE buffer (75mM NaCl; 20mM Na_2EDTA; pH 8.0) and proteinase K (100µg/ml) were added to the pellet and mixed, followed by the addition of 2 ml of 10% SDS.

The tube was left for overnight incubation at 37^{o}C in a water bath. After overnight incubation, Equal volume of TE equilibrated phenol was added to the tube and mixed gently on overhead shaker for 20 minutes followed by centrifugation at 4000 rpm for 10 minutes at 4^{o}C. The Supernatant obtained at this stage was taken in fresh sterile falcon tubes with the help of micropipette fitted with wide bored tip and equal volume of phenol and CIA (1:1) was added (CIA=Chloroform-isoamylalcohol; 24:1).This mixture was shaken on overhead shaker for 20 minutes and steps 11 and 12 were repeated. Now equal volume of CIA was added to the supernatant and the mixture was shaken on overhead shaker for 20 minutes and steps 11 and 12 repeated again.

Now 1/10 volume of chilled 3M sodium acetate solution and 2.5 volumes of chilled absolute ethanol or equal volume of isopropanol was added to the supernatant and mixed gently. White thread like precipitate of genomic DNA appearing at this step was transferred to 1.5 ml microfuge tube and centrifuged at 6000 rpm for 5 minutes. The pellet obtained was washed twice with 500µl of 70% ethanol and recentrifuged. Further, DNA pellet in the microfuge tube was allowed to dry at room temperature and finally the air-dried pellet was dissolved in 500 µl of autoclaved ddH_2O and incubated at 65^{o}C for 10 minutes. The DNA solution thus obtained was stored at -20 for further use.

II. DNA extraction by modified salting out method

5 ml EDTA treated venous blood was taken in 50ml sterile falcon tubes and mixed with 8 ml of lysis buffer and the tubes were incubated on ice for 10 minutes, followed by centrifugation for 5 minutes at 2500 g (4 ^{o}C).The Supernatant was discarded and to the pellet, 300µl of 10mM tris-HCl , pH 8 was added. Pellet was released from the bottom of the tube by vortexing, followed by Centrifugation for 15 minutes at 7000 g. The supernatant was again discarded. Now to the pellet 330µl of 10mM tris-HCl, pH 8 and 330µl of laundry powder solution (conc. 30mg/ml) and a glass bead was added in each tube. Vortexing was done for 1 minute followed by addition of 250µl of 5 or 6 M NaCl and vortexed for another 20 seconds.

Then it was centrifuged for 5 minutes at 15000g. The supernatant was transferred carefully to fresh 1.5µl microfuge tubes with the help of micropipettes and equal volume of 96% ethanol was added to the supernatant for precipitation. Then DNA precipitate was retrieved carefully and washed 2-3 times with 500µl of 70% ethanol. Then the precipitated DNA pellet was allowed to air dry at room temperature. Then air dried pellet was dissolved in mq water or Tris – HCl, pH=8. It was incubated for 5 minutes at 70^{o}C, followed by storage at -20^{o}C.

3.1.3. Agarose gel electrophoresis

Agarose gel eletrophoresis (Aaiji *et al*., 1972) was carried out to establish the quality of the genomic DNA using 0.8% agarose and for comfirming the specificity of the amplicon on 1.5% agarose.

Agarose gel was prepared by dissolving 0.4g agarose in 50 ml 1x TAE and allowed to cool to 50-60°C before adding ethidium bromide to a final concentration of 0.5 µg/ml. The gel solution was mixed by gentle swirling. The solution was poured into an electrophoresis plastic tray (with sealed edges) with comb inserted and gel was allowed to cool for approximately 20 min. Then 200ng of DNA sample was mixed with 1µl of loading dye and loaded in to the slots of submerged gel using a micropipette, using 1x TAE as running buffer. A DNA marker was loaded in the last lane. Electrophoresis was carried out at 100 volts for 40 minutes. DNA band pattern was visualized by placing the gel under UV light in a transilluminator and

photographed (Supplementary data Fig. S2). The samples showing bright, intact bands with no fragmentation or shearing and without any apparent contamination or streaking were chosen for further analysis.

3.1.4. Determining the concentration and purity of isolated genomic DNA

The concentration of genomic DNA was determined by measuring the absorbance at wavelength 260nm against a blank using double beam spectrophotometer. DNA samples were diluted (1:100) with distilled water before recording the absorbance. The absorbance of 1 OD at 260 nm is approximately equivalent to 50µg/ml of ds DNA. The formula for the calculation of DNA concentration is depicted below.

$$\text{DNA conc. } (\mu g/ml) = OD_{260} \times \text{dilution factor} \times 50\ \mu g/ml.$$

Purity of extracted genomic DNA was established by OD_{260}/OD_{280} .The samples having this ratio between 1.7-1.9 are considered pure and free from contamination.

3.2. PCR Amplification

PCR amplifications were carried out in a reaction volume of 50µl containing 50-100ng of genomic DNA, 200 µM of each dNTP, 0.6 pm of each primer, and 1.0 unit of Taq polymerase in Taq Buffer containing Tris-HCl, KCl, $(NH_4)_2SO_4$, 1.5mM $MgCl_2$; pH 8.7). The primer details and PCR protocol and programme are given in table 3.1, 3.2, and 3.3 respectively.

Table 3.1. Primer characteristics

Gene	Exon	Primer Sequence	Annealing	Product size(bp)
TGFβ1	1	F; 5'GCCTCCCCACCACACCAG 3' R; 5'ATCCTGTCCAAGCTGCGGC 3'	60	237
TGIF	2	F; 5'CCCAAATTGTCTATCGGTG 3' R; 5' GACTAGGTTCAAGCCAATG 3'	54	247
	6	F; 5' GGGAATAAGTGAGGGGCTCT 3' R; 5' CCTGAACCAGTCGCAAAGTT 3'	60	472
DLGAP I	4	F; 5'CTGGAGTCGCAGGCCGTGGAAGCG 3' R; 5'ACATGGGTGGTATCTTGTTCCTGG 3'	67.8	300
	2	F; 5 'GTCCACGGCATCCAAGCAGACCAC 3' R; 5' TGTTTTCCTCAGGGACAGGCG 3'	67.8	223
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' TTGGTCAACAGATCAAGACATTGGACC3' R; 5' GAACGCTCCCCAGACGGTCTTCCAGAG 3'	66.7	300

Table 3.2. Giving volume and final concentration of different reagents used in amplification process

Reagent	Final concentration	Volume required
Taq Buffer (10X)	1 X	5.0 µl
dNTP mix (2mM)	0.2 Mm	5.0 µl
Forward Primer 10 pmoles/µl	0.4 pmoles/µl	2 µl
Reverse Primer 10 pmoles/µl	0.4 pmoles/µl	2 µl
Taq DNA Polymerase 5U/µl	0.01 units/ µl	0.1 µl
Genomic DNA	50-100ng	2.0 µl
MilliQ water		33.9 µl
Total Volume		**50.0 µl**

Table 3.3. PCR Cycling Parameters X= Different for different primer sets, respectively.

STEP	TEMPERATURE (°C)	TIME	
Initial Denaturation	94	10 min	
Denaturation	94	45sec	
Annealing	X	45 sec	**35 Cycles**
Extension	72	45 sec	
Final extension	72	5 min	

To check the concentration and the quality of the PCR products, a 5µl aliquot was analysed on (1.5%) agarose gel (Supplementary data Fig. S3).

3.2.1. Purification of PCR products

Prior to sequencing, PCR products were purified. For the purification, PCR products were mixed with loading dye and loaded on 2% agarose gel and run at 100 volts for almost 60 minutes. The bands were visualized under UV transilluminator. Sharp and specific bands (compared with marker run on same gel) were excised from the gel with a sharp sterilized surgical blade and purified either using the kit or with glass beads.

3.2.2. Purification using glass beads (SiO_2)

Each excised gel piece was weighed and 3 volumes of sodium iodide were added. The tubes were incubated in a water bath at 45-55°C for 5 minutes or till the gel melts completely. The tubes were gently shaken during this process and 10-15 µl of glass milk was added to each tube and incubated on ice for 15 min, vortexing slightly 2-3 times during incubation. Then the tubes were centrifuged in a

microfuge at maximum speed for 45 sec and the supernatant was discarded. Now to the glass milk pellet, 500µl of Wash buffer was added to resuspend the pellet completely by vortexing, followed by centrifugation at 14,000 rpm. This washing step was repeated twice. Third time washing was done without resuspending the pellet. After washing, the pellet was allowed to air dry. The air dried pellet was reconstituted in appropriate volume of MilliQ water to adjust the concentration range between 25-50ng/µl. Purified PCR products were stored at -80 ^{o}C till sequencing.

3.3. CSGE (Conformation Specific Gel Electrophoresis)

Amplicons were subjected to heteroduplex assay for which heteroduplex formation was accomplished by denaturing the PCR products (100-200ng) at 95^{o}C followed by random annealing at 68^{o}C. The heteroduplexes were analysed on a 10%/12% acrylamide gel using standard conformation sensitive gel electrophoresis (CSGE) protocol (Ganguly, *et al*., 1993). The gel was silver stained according to standard procedure.

There are at least two principles on which the CSGE method works. First, single-base mismatches can produce conformational changes in the double-stranded DNA, leading to the differential migration of heteroduplex and homoduplex. Second, mildly denaturing solvents in an appropriate buffer can intensify the conformational changes produced by single-base mismatches, resulting in the increased differential migration of heteroduplexes and homoduplexes. The CSGE method involves heteroduplex analysis of PCR products in a novel, mildly denaturing polyacrylamide gel matrix using a different cross-linker, bis-acrolyl-piperazine, instead of the conventional bis-acrylamide. Essentially, the protocol involves amplification of the entire coding region in small fragments and analyzing them by CSGE. The presence of additional slow or differentially migrating bands in comparison to normal sample indicates the presence of heteroduplex bands, which are suggestive of presence of mutation (Supplementary data Fig. S4). The samples that show heteroduplex bands have to be sequenced to locate and identify the nature of mutations (Lakhotia and Somasundaram, 2003).

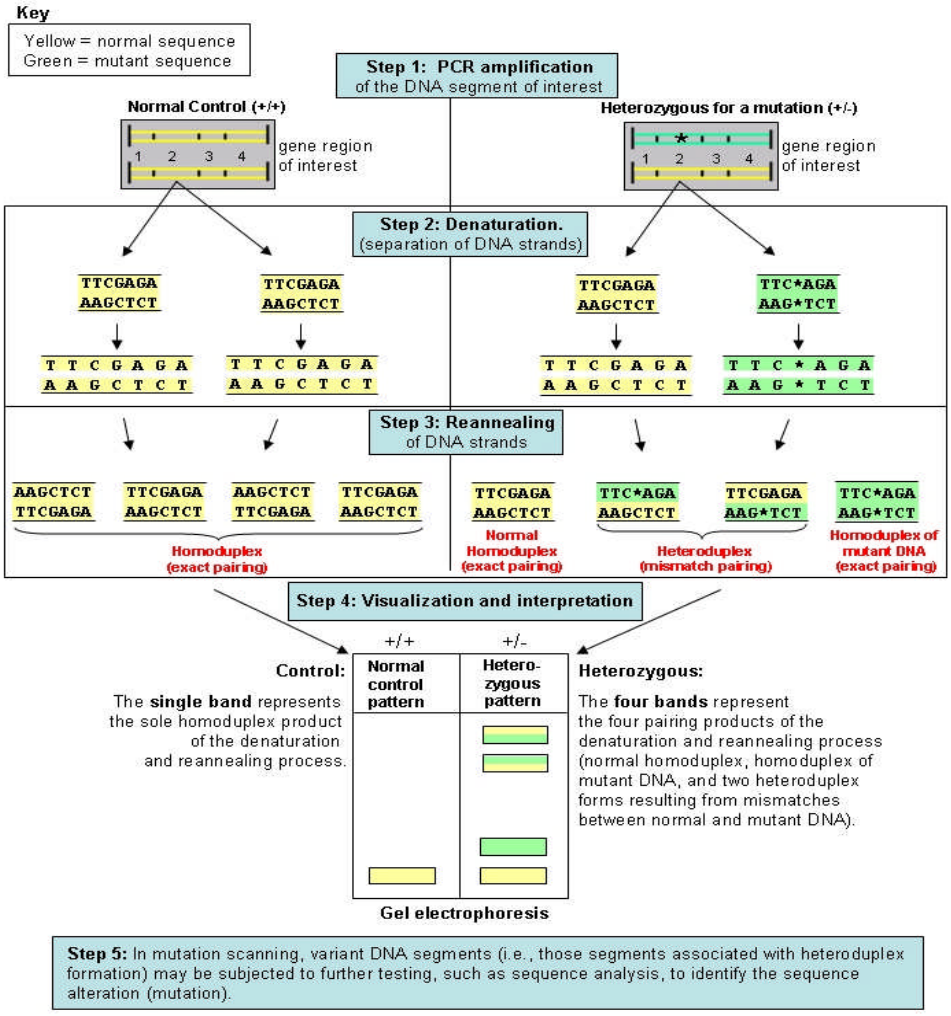

Figure 3.1. Detection of mutations by using conformation-sensitive gel electroporesis (CSGE). One band appears in the normal sample, while additional bands appear in the case of the patient. The additional slow or differentially migrating bands represent heterodulplx DNA species. Source website: nstitutoroche.es.

3.3.1. Protocol for performing CSGE: Preparation of 12% Acrylamide Gel

A 0.75-mm thick gel with 15-well comb prepared with 12% polyacrylamide (99:1 ratio of acrylamide to 1,4-bis(acryloyl) piperazine), 10% ethylene glycol, 15% formamide, 0.1% ammonium persulfate, and 0.07% N,N,N',N'-tetramethylethylenediamine in 0.5x TTE buffer (44 mM Tris/14.5 mM Taurine/0.1 mM EDTA buffer, pH 9.0) was prepared, using the protocol given in (Table 3.4)

Table 3.4. Volume & final concentration of different reagents used in the preparation of 12% acrylamide gel

Component	Volume (ml)
Acrylamide Mix	9
Ethylene Glycol	3
Formamide	4.5
20X TTE (pH 9.0)	0.75
Double Distt. Water	12.429
10% APS	0.3
TEMED	0.021
Total Volume	**30 ml**

3.3.2. Running the gel

The optimal polymerization time was about 1 hr. PCR products containing heteroduplexes were mixed with 3 µl of 10x stock loading buffer (10x stock solution of 30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF). The gel was pre-electrophoresed for 45 min. Samples were separated by electrophoresis at room temperature on a maxiformat apparatus (Biorad; 20 x 16 mm, with 1mm thickness) using 0.5x TTE as the electrode buffer. The samples were electrophoresed at 150 volts overnight (16-18 hours), after electrophoresis, the gel was silver stained and photographed (Supplementary data Fig. S4).

3.3.3. Silver Staining of the gel

The silver staining technique (Bassam & Gresshoff, 2007) is an efficient method for visualizing DNA fragments and other organic molecules. This method measures DNA in picogram concentrations reliably, further integrity of the protocol is proved by its simple implementation and fast approach. The staining of the polyacrylamide gel was done on a clean plastic tray. Sufficient volume of fixing solution was poured in to the tray to cover the gel completely. For fixation, tray containing the gel was kept on rocking platform for about 10 minutes. Following the fixation, the solution was decanted carefully without touching the surface of gel. Washing was done with double distilled water for 2 minutes, keeping the gel tray on rocker. The wash step was repeated two times for a total of three washes. After the completion of washing steps, formaldehyde solution was

added to completely cover the gel and placed on rocking platform for 10 minutes after which the solution was removed completely. Now silver nitrate solution was added to the gel in the staining tray, and kept on rocking platform for ~20 minutes. Staining was followed by decanting carefully without touching the gel surface. The gel was briefly rinsed to remove residual silver solution from its surface for 10 seconds. Chilled developer solution (4°C) was added to the gel in the staining tray, and the tray was agitated throughout image development which takes from 3-10 minutes. Then the developer solution was decanted carefully without touching the gel surface. To stop the reaction, the developer stop solution was added to the gel in the staining tray, gel being kept in stop solution for 5-10 minutes. Finally, the stop solution was decanted carefully without touching the gel surface and the gel was again rinsed with double distilled water. The gel was dried and covered with cellophane wrap for preservation.

3.4. DNA Sequencing

The concentration of purified PCR products was again approximated by intensity comparison on an agarose gel, with that of the corresponding molecular weight marker band. Product concentration was adjusted between 20 to 50ng by adding appropriate volume of MilliQ water.

3.4.1 Sequencing of purified PCR products

Sequencing was done commercially using the services of Scigenom Cochin, Kerala. DNA sequences of the amplicons were obtained in fasta and pdf formats. For analysing of the sequencing data in fasta format, software programs ClustalX version 2 (for sequence alignment; Supplementary data Fig. S5A, S5D, S5H, S5K, S5N, S5P, S5S, S5V) (Thompson, *et al.*, 1997; Larkin, *et al.*, 2007) and Chromas Pro Version 1.49 beta 2 (for detailed inspection of the individual chromatograms, Supplementary data Fig. S5B, S5C, S5E-S5G, S5I, S5J, S5L, S5M, S5O, S5Q, S5R, S5T, S5U, S5W- S5Y), were used. The pdf file of each DNA sequence was used for visual inspection of the sequencing chromatogram using Acrobat Reader 8.0.

3.5. Statistical Analysis

Genotypes were obtained by direct counting with subsequent calculation of allele frequencies. Statistical analysis was undertaken using the χ^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 χ^2 test with one degree of freedom. Odds ratio and confidence interval was also calculated.

3.6. Sequence submission for 3D modeling

The amino acid sequence of the protein in fasta format obtained from (NCBI) (www.ncbi.nlm.nih.gov) was submitted to an automated server (I-TASSER) (zhang.bioinformatics.ku.edu/I-TASSER) for 3D structure prediction (Zhang, 2007b; 2008). The server furnishes the predicted 3 D structure in a pdb format.

I TASSER server furnished five PDB files in each case, wild type and mutant, representing the probable tertiary structures of the protein, with C-Scores**,** respectively. C-score is a confidence score for estimating the quality of predicted models by I-TASSER. C-score is typically in the range of [-5, 2], where a C-score of higher value signifies a model with a high confidence and vice-versa.

3.6.1. Viewing the PDB files and free energy calculations

Swiss PDB Viewer was used for viewing pdb files and computing the free energy (Supplementary data Fig. S6.1-S6.3 & table S6.1-S6.3) of the predicted 3D structures (Camacho, *et al*., 2000; Camacho, *et al*., 2003; Comeau, *et al*., 2004).

RESULTS

Section 1

4.1. TGFβ1 Codon 10 Polymorphism Contributes to High Myopia in an Ethnic Kashmiri Population from India

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4.2. Abstract

This study looks at novel variants of the TGFβ1 gene and their potential association with high myopia in an ethnic population from Kashmir, India. Allele frequencies of 247 Kashmiri subjects (from India) with high myopia and 176 ethnically matched healthy controls were tested for Hardy–Weinberg disequilibrium. The genotype and allele frequencies were evaluated using chi-square or Fisher's exact tests. One of the three SNPs in codon 10 showed a significant difference between patients and control subjects (rs1982073: p genotype=0.003, p allele=0.001). There were no statistically significant differences between patients and control subjects for the other two SNPs, rs1800471 at codon 25 and a novel variant at codon 52. SNP rs1982073, substituting proline with leucine, appeared to associate with high myopia significantly ($p < 0.05$). Insilico predictions show that substitutions are likely to have an impact on the structure and functional properties of the protein, making it imperative to understand their functional consequences in relation to high myopia.

Key words: Myopia; Ethnic; Polymorphism; CSGE; TGFβ1; Novel

4.3. Introduction

Development of myopia is a consequence of an incongruity between the power of optical components and the axial length of the eye (Wensor, *et al.*, 1999). While lower grades of myopia (< -6 diopters) are not associated with blinding conditions, higher or pathological grades with a refractive error of >-6 diopters often associate with blinding conditions like macular degeneration, retinal detachment and glaucoma (Lin, *et al.*, 2006). Ethnic diversity plays a great role in the progression of myopia reaching as high as 70-90% in some parts of Asia, 30-40% in Americans and Europeans and

upto 20% in Africans (Chow, *et al.*, 1990; Lin, *et al.*, 2006). Comparative prevalence rates of high myopia from different parts of the world show considerable variability, but still confirm that it affects a significant proportion of the population in different countries (Wang, *et al.*, 1994; Curtin, 1970; Tokoro & Sato, 1982; Lin, *et al.*, 1988; Wilson & Woo, 1989; Fledelius, 1988; Paluru, *et al.*, 2005). The prevalence in India is found to be 19%, with 4% in Kashmir (Ahmed, *et al.*, 2008). Ahmed, *et al.*, 2008 also, reported the effects of age, gender, and socioeconomic conditions on the prevalence of myopia and showed an increase in myopia prevalence with increased age (3.76% in the age group of 6-10, 4.9% and 6.16% in age groups 11-15 and 16-22). Additionally, girls on average were 1.52 times more likely to have myopia than boys (5.54% of girls and 3.6% of boys). Socioeconomic conditions had an impact on the prevalence of myopia. While only 3.23% students from medium and high socioeconomic strata had myopia, it was about three times more in students from low socioeconomic strata (8.60 %).

Despite many decades of research there is little knowledge about the precise molecular defects and abnormal biochemical pathways that result in myopia. It is a highly prevalent and complex phenotype involving both genetic and environmental factors (Ibay, *et al.*, 2004).

Recent studies have mapped 14 genomic loci associated with myopia (MYP1on Xq28, MYP2 on chromosome18p, MYP3 on chromosome 12q, MYP4 on chromosome 7q, MYP5 on chromosome 17q, MYP6 on chromosome 22q12, MYP7 on chromosome 11p13, MYP8 on chromosome 3q26, MYP9 on chromosome 4q12, MYP10 on chromosome 8p23, MYP11 on chromosome 4q22–q27, MYP12 on chromosome 2q37.1, MYP13 on Xq23–q25, and MYP14 on chromosome 1p36) (Paluru, *et al.*, 2005; Hammond, *et al.*, 2004; Naiglin, *et al.*, 2002; Paluru, *et al.*, 2003; Schwartz, *et al.*, 1990; Stambolian, *et al.*, 2004; Wojciechowski, *et al.*, 2006; Young, *et al.*, 1998a; Zhang, *et al.*, 2006; Inamori, *et al.*, 2007). A high heritability of myopia does not mean that environmental factors have no effect on the development of myopia. Close visual work in childhood has been hypothesized as an environmental risk factor for myopia progression (Saw, *et al.*, 2001). Initially one of the studies indicated a strong association between myopia and nightlight exposure

(Quinn, *et al.*, 1999) but recent research has shown contradictory results (Saw, *et al.*, 2001; Zadnik, *et al.*, 2000; Gwiazda, *et al.*, 2000; Guggenheim, *et al.*, 2003).

Myopia develops mainly because of excessive elongation in axial dimension rather than changes in corneal or lens power in human beings (Zadnik, 1997). In animal models of myopia, active remodeling of sclera plays a crucial role in axial elongation (McBrien & Gentle, 2003; Rada, *et al.*, 2006). Scleral remodeling involves reduced production of extracellular matrix which results from reduced production of collagen and proteoglycans and from increased collagen degradation along with concomitant increased activity of matrix metalloproteinase 2 (MMP2) and a reduction in the activity of tissue inhibitors of MMP. Transforming growth factor β (TGFβ) together with its receptor expressed in eye tissues (Saika, 2006) also regulates the proliferation of fibroblasts and production of collagen, MMP2, and tissue inhibitors of MMP (Overall, *et al.*, 1989). TGFβ is an obvious player in the regulation of scleral remodeling and accordingly has been implicated in the development of myopia (Zha, *et al.*, 2009). TGFβ exists in three isomeric forms (TGFβ1, TGFβ2, and TGFβ3), and during myopia development the expression of TGFβ1 was found to be reduced in an isoform and time-specific manner in the sclera (Kusakari, *et al.*, 2001) and retina/choroid (Song, *et al.*, 2000) of chickens whereas the TGFβ2 level increased in both the retina/choroid and sclera of the chickens (Kusakari, *et al.*, 2001; Song, *et al.*, 2000). Cultured human retinal pigment epithelial cells have been shown to express atleast TGFβ1 and TGFβ2 isoforms (Lam, *et al.*, 2003; Andrew, *et al.*, 2004; Tanihara, *et al.*, 1993; Seko, *et al.*, 1995). TGFβ1 belongs to a family of polypeptides that display a broad range of multifunctional activities like transcriptional activation and increase in synthesis and secretion of matrix proteins (Guggenheim & McBrien, 1996). It is encoded on chromosome l9ql3.1-ql3.3 and contains seven exons (Patel, *et al.*, 2005). Recent studies investigated the association of single-nucleotide polymorphisms (SNPs) of the TGFβ1 gene and high myopia but produced conflicting results (Hayashi, *et al.*, 2007). Our study serves to clarify this relationship with a case-control design and ethnic purity of our population.

4.4. Materials and Methods

4.4.1. Subjects

Preliminary conclusions by evaluation of 48 samples during pre doctoral work *(M.Phil, Thesis Shabhat Rasool),* were validated using a larger sample size of 423 samples (247 with high myopia of < -6D and 176 healthy control subjects, Annexure I) recruited from the local hospital (Ophthalmology unit) as well as from our ophthalmologist's clinic. Although we were interested in doing a familial study, it was very difficult to find ample numbers of families with high myopia. We therefore designed a case control study. 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 any ocular disease such as retinopathy, cataract was known 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 acquity, 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.

4.4.2. Methodology

4.4.2.1. Polymerase chain reaction

Genomic DNA was extracted from whole blood samples using standard protocols. PCR reactions were carried out in a total volume of 50 µl, Containing 50-100 ng genomic DNA, 2-6 pmole of each primer, 1x PCR buffer (Sigma Aldrich) and 0.5 units of Taq DNA polymerase (Sigma Aldrich). The following primer sequences were used for amplification: 5'-GCC TCC CCACCA CAC CAG-3' (sense) and 5'-GCC GCA GCT TGG ACAGGA T-3' (antisense) (Lin, *et al.,* 2006). Expected PCR product of 237 bp was generated successfully (Supplementary data, Fig. S3i). The PCR cycling conditions involved: one cycle of denaturation at 95 °C for 5 min, 30 cycles of

denaturation at 95 °C for 45 s, annealing at 59 °C for 45 s, and extension at 72°C for 45 s, and one final 6 min elongation cycle at 72°C. PCR products were then purified using purification kit or NaI.

4.4.2.2. Conformation sensitive gel electrophoresis (CSGE)

Purified PCR products were subjected to denaturation and renaturation procedures for generation of potential heteroduplexes and analyzed by CSGE strictly as described by Ganguly, *et al.*, 1993. This mutation detection technique has many advantages over other techniques like SSCP and PTT. Samples with unusual mobility during these assays were finally sequenced to confirm the presence of sequence variations along with controls (Fig. 4.1). CSGE conditions described by Ganguly, *et al.*, for amplicons ranging from 200 to 800 bp were able to detect 60 out of 63 single-base mismatches. Still, the migrating bands in this method are sometimes less clear and could lead to human error in reporting results. Therefore, sequencing of the samples screened with CSGE is relied on to provide accurate results, (Blesa, *et al.*, 2004).

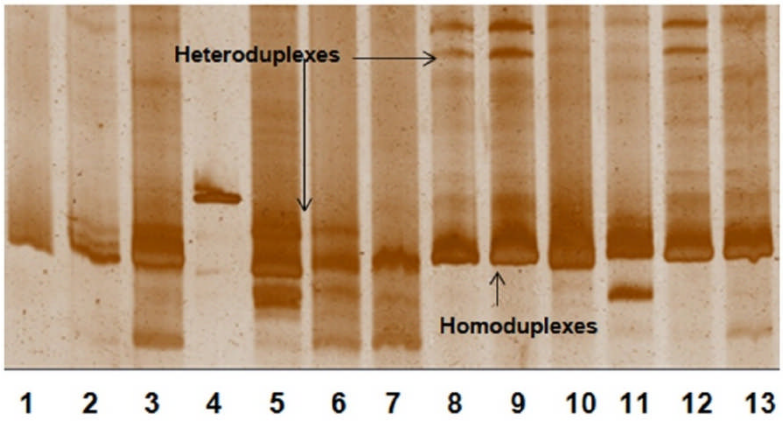


Figure 4.1. Heteroduplex analysis of TGFβ1 amplicons (237bp) by conformation sensitive gel electrophoresis. Heteroduplexes were seen as shown in the above fig; wells (3, 5, 6) show samples with G>C variation while lanes (8, 9, 12) show samples with C>T variation as confirmed by sequencing. Lane 4 shows the separation pattern of 1kb DNA marker with first band corresponding to 250bp. Samples loaded in rest of the lanes donot show any base variation on sequencing.

4.4.2.3. Sequence analysis

Sequence results obtained in fasta and pdf formats were analyzed using ClustalX version 2 software (Thompson, *et al.*, 1997; Larkin, *et al.*, 2007) and by Chromas Pro

version 1.49 beta 2 software for the detailed inspection of individual chromatograms (Supplementary data, Fig. S5V-S5Y).

4.4.2.4. Statistical analysis

Genotypes were obtained by direct counting with subsequent calculation of allele frequencies. Statistical analysis was undertaken using the χ^2 test and significance value (p). A p value of <0.05was considered significant. Adherence to the Hardy-Weinberg equilibrium constant was tested using the χ^2 test with one degree of freedom. Odds ratio and confidence interval were also calculated.

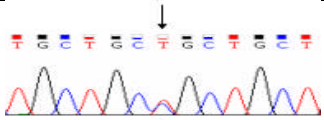
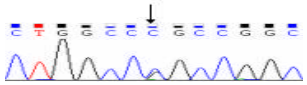
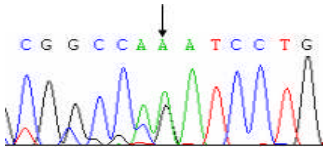
4.4.2.5. Insilico analysis

The amino acid sequence of the protein in fasta format obtained from (NCBI) (www.ncbi.nlm.nih.gov) was submitted to an automated server (I-TASSER) (zhang.bioinformatics.ku.edu/I-TASSER) for 3D structure prediction (Zhang, 2007b; Zhang, 2008). The server furnishes predicted 3D structure in a pdb format. Swiss PDB Viewer was used for viewing pdb files and computing the free energy of the predicted 3D structures (Camacho, *et al.*, 2000; Camacho & Gatchell, 2003; Comeau, *et al.*, 2004).

4.5. Results

Two missense variants C/T (rs1982073) and G/C (rs1800471) at codons 10 and 25 respectively corresponded with previously reported SNPs in public databases. A silent variation G/A at codon 52 observed in the study population appeared to be novel (Table 4.1). Genotype analysis of individual variants revealed the presence of both heterozygous and homozygous genotypes.

Table 4.1. Polymorphism detected in Exon 1 of TGFβ1 gene in ethnic Kashmiri Population

Codon Position	rs no	wild type nucleotide	Observed base pair change	Amino acid change	Chromatogram
Codon 10	1982073	C	T	Pro to Leu	
Codon 25	1800471	G	C	Arg to Pro	
Codon 52	Novel	G	A	Silent	

Subtle and statistically significant (p allele = 0.001, p genotype = 0.003; χ^2allele = 10.36, χ^2genotype = 11.451; OR = 1.59; CI (95%) = 1.9-2.11; Table 4.2) difference in the genotypic frequency for codon 10 variant was indicative of its possible association with high myopia, while the relative frequency of occurrence of variants at codon 25 (p allele = 0.107, p genotype = 0.17; χ^2allele = 2.59, χ^2genotype = 3.46; OR = 0.78; CI (95%) = 0.59-1.05; Table 4.3) and codon 52 (p allele = 0.310, p genotype = 0.629 ; χ^2allele = 1.032, χ^2genotype = 0.928; OR = 1.16 ; CI (95%) = 0.86-1.58; Table 4.4) for high myopes was found to be statistically insignificant, when compared to their occurrence in healthy controls.

4.5.1. Insilco prediction results

TGFβ1 was modeled by I-TASSER to obtain its PDB structure and analysis (energy calculations) was done using PDB Viewer (Supplementary data, Fig. S6.4, Table S6.4).

4.6. Discussion

Diverse populations have presented inconsistent profile of association data owing largely to heterogeneous nature of the subject populations while TGFβ1codon 10 (rs 1800470) polymorphism has been found to associate with high myopia in Taiwanese Chinese showing strong association of CC genotype with high myopia (Lin, *et al.*, 2006).

Table 4.2. Comparison of the distribution of alleles and genotypes of TGFβ1 gene polymorphism at codon 10 in healthy and high myopic subjects

Gene allele & genotype variants	Control (n= 176)		Affected (n=247)		χ^2	p value	OR	CI (95%)
	n	%	n	%				
C	116	(33)	217	(44)	10.36	0.001*		
T	236	(67)	277	(56)				
CC	15	(8.52)	41	(16.60)			1.59	1.9-2.11
CT	86	(48.86)	135	(54.66)	11.451	0.003*		
TT	75	(42.62)	71	(28.74)				

*****Statistically significant

Table 4.3. Comparison of the distribution of alleles and genotypes of TGFβ1 gene polymorphism at codon 25 in healthy and high myopic subjects.

Gene allele & genotype variants	Control (n= 176)		Affected (n=247)		χ^2	p value	OR	CI (95%)
	n	%	n	%				
G	237	(67)	306	(62)	2.59	0.107		
C	115	(33)	188	(38)				
GG	77	(43.75)	96	(38.87)			0.78	0.59-1.05
GC	83	(47.16)	114	46.15)	3.46	0.17		
CC	16	(9.09)	37	(14.98)				

Table 4.4. Comparison of the distribution of alleles and genotypes of TGFβ1 gene polymorphism at codon 52 in healthy and high myopic subjects.

Gene allele & genotype variants	Control (n= 176)		Affected (n=247)		χ^2	p value	OR	CI (95%)
	n	%	n	%				
G	246	(70)	361	(73)	1.032	0.310		
A	106	(30)	133	(27)				
GG	90	(51.14)	136	(55.06)			1.16	0.86-1.58
GA	66	(37.50)	91	(36.03)	0.928	0.629		
AA	20	(11.36)	22	(8.91)				

A later study by (Hayashi, *et al.*, 2007) on TGFβ1 gene polymorphism in high myopia revealed no significant association with high myopia excluding TGFβ1 as a candidate gene for myopia in Japanese population. However a recent study of TGFβ1 polymorphism in high myopia affected Chinese subjects of Hong Kong revealed the association of 4 SNPs in the 5' half of the TGFβ1 locus with high myopia. This study could successfully replicate the positive finding of Lin, *et al.*, 2006, supporting the association of TGFβ1 gene with myopia susceptibility (Zha, *et al.*, 2009; Sandhya, *et al.*, 2011). Kashmiri population representing a homogeneous cohort of common ethnicity provided an opportunity to revalidate the significance of TGFβ1 sequence variants (if any) for defining their relevance in the pathogenesis of the disease.

Genetic polymorphisms have widely been in use to test the association of a gene with a commonly seen and multifactorial disease instead of single gene disease. Since nucleotide polymorphism is not strong enough to result in a lethal phenotype, this allele will not eventually disappear or reach frequency equilibrium without any selective disadvantage for individuals. Since ethnic differences do exit, it is imperative to substantiate or dispute the relevance of such polymorphism in genetically purer cohorts. To date, variations in several genes have been reported to associate with high myopia but only a few studies have been replicated successfully. Our study is a kind of replication study (Lin, *et al.*, 2006; Zha, *et al.*, 2009)

associating TGFβ1 codon 10 polymorphism with high myopia in a population wherein heterogeneity effects seen in other populations are neutralized to a large extent.

Investigating the genetics of common and complex disorders such as myopia remains one of the great challenges in human genetics. Myopia is considered to be a complex and multigenic condition involving several overlapping signaling pathways, each mediated by a group of distinct genes. Therefore, studying the genetic polymorphisms of myopia-related genes can further clarify the relationship between genetics and myopia. This association has helped increase our knowledge of prevention and treatment of myopia. The relationship between TGFβ1and sclera remodeling during the development of myopia is well established (Honda, *et al.,* 1996; Kusakari, *et al.,* 2001). TGFβ1 has been analyzed as a candidate gene because of its differential expression in experimental chicken myopia (Jobling, *et al.,* 2004) and its functional relation with TGIF (Chen, *et al.,* 2003). In the earlier study, only one (rs1982073 at codon 10, 29T/C, Leu10Pro) was analyzed and reported to be associated with high myopia l in a Chinese population living in Taiwan, P = 0.001 (Lin, *et al.,* 2006). At the same time, 10 SNPs (rs1982073 not included) and related haplotypes in TGFβ1 were analyzed in 330 Japanese patients with high myopia and 330 control subjects, but none of them was associated with high myopia (Hayashi, *et al.,* 2007) and a further study on TGFβ1 was suggested (Wang, *et al.,* 2009).

Our study adds support to the idea that the codon 10 polymorphism of the TGF β1 gene contributes to the pathogenesis of myopia. Further investigation is needed to establish the precise role played by TGFβ1 in the development of high myopia especially in the context of codon 10 polymorphism. Insilico predictions show higher energy states for both codon 10 (-8931.029kj/mol) and codon 25 (-8102.402kj/mol) variants as compared to wild type protein (-9573.964kj/mol) and protein that has both variations together (-9501.950kj/mol), which may affect the stability of the protein. Since these SNPs change the energy state of the protein, an interference with the functional properties and stability of the protein may be possible. Further studies are needed to elucidate the actual affect of these changes on protein structure and function. Genes further up- and downstream of TGFβ1 also need to be investigated, as

it is likely that a number of genes will form the genetic background in individuals with myopia, upon which environmental factors will act, to give rise to myopia.

In conclusion, we observed that the frequency of the C allele at codon 10 of TGFβ1 was higher in the high myopia group than in the control group. People who have CC/CT genotypes at codon 10 may be at greater risk for developing high myopia (Table 4.2). Therefore, we conclude that TGFβ1 codon 10 polymorphism is associated with high myopia and is a candidate genetic marker of the disease.

Acknowledgements

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Section 2

4.7. Intronic variants of TGIF1 (variant-008) have a potential to associate with high myopia in ethnic kashmiri population

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4.8. Abstract

This study aims to look at novel variants of TG1F1 gene and explore their potential association with High Myopia in an ethnic population from kashmir (India). 52 Kashmiri subjects (from India) with high myopia and ethnically matched 18 healthy controls were enrolled. Genomic DNA was prepared from peripheral blood. Allele frequencies were tested for Hardy–Weinberg disequilibrium. The genotype and allele frequencies were evaluated using the $\chi 2$ tests or the Fisher exact tests. Mutational screening of TGIF1 in 52 high myopia affected and 18 normal controls from Kashmir revealed a total of three novel, heterozygous and adjacent sequence variations; T>C/A, T>G & G>C in the intronic region immediately after exon 2 boundry of this variant.

Key words: Myopia, Ethnicity, Polymorphism, CSGE, TGIF1

4.9. Introduction

Myopia is the most common eye disorder which remains a significant ocular health problem associated with increased risk of visual loss around the world (Fredrick, 2002) . High myopia considered as more advanced type of myopia may lead to degenerative changes in the eye (degenerative myopia) leading to blindness and often afflicts, people earlier in life when they may still be active professionally (Jacobia, 2005). The wide spectrum of myopia-associated disorders strongly argues for an etiologically heterogeneous nature of myopic refractive errors, where multiple factors with genetic and epigenetic effects contribute at different stages during development (Feldkamper & Schaeffel, 2003). The concept that environmental factors influence

ocular development has been well established in epidemiological and experimental animal studies (Saw, 2002). Despite recognized importance of visual experience in the development of myopia there is abundant evidence for genetic factors determining refractive development (Francois, 1961). First, higher myopia prevalence in developed Asian countries compared to the western world suggests a genetic susceptibility to myopia development. Further, myopic parents are more likely to give rise to offsprings with myopia than non-myopic parents (Goldschmidt, 1981). Linkage studies have mapped at least eight loci (MYP1, MYP2, MYP3, MYP4, MYP5, MYP11, MYP12, and MYP13) responsible for high myopia with Mendelian inheritance (Young, 2004). TGIF1 is expressed in sclera, retina, cornea, and optic nerve and competitively inhibits binding of the retinoic acid receptor to a retinoid-responsive promoter (Young, 1998a).

It is possible that mutations in TGIF1 gene may alter its function and hence the phase of eye development, thus making it a potential candidate gene to study High Myopia. One study has disregarded TGIF1 as potential contributor to the disease (Scavello, *et al*., 2004) although it has been associated with high myopia wherein six single nucleotide polymorphisms (SNPs) appear to associate with the disease in a Chinese cohort (Lam *et al*., 2003). However, association could not be replicated in a second Chinese case control study of high myopia individuals (Li, *et al*., 2003). Another study with Japanese subjects failed to identify association of this gene with high myopia (Hasumi, *et al*., 2006). This discrepancy has largely been attributed to ethnic variations in the genetics of this disease.

4.10. Methodology

Kashmiri population being a pure ethnic group provides an ideal scenario to substantiate the contribution of TGIF1 (if any) in the development of high myopia. 52 high myopic and 18 normal controls of Kashmiri ethnicity were recruited for TGIF1 polymorphism studies (Annexure II). DNA was isolated from venous blood samples and amplified by polymerase chain reaction (Supplementary data, Fig. S3g). PCR products were purified and screened for mutations by heteroduplex assay employing CSGE (conformation sensitive gel electrophoresis). Samples showing differential mobility on CSGE were sent out for commercial sequencing. Sequences were

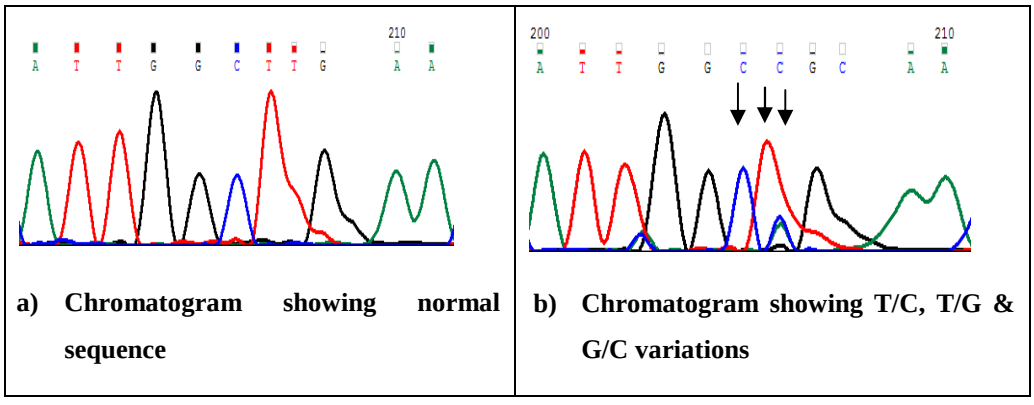
analysed using different software programmes like Chromas pro and Cluatal X2. After analyzing the data, we observed three novel and adjacent intronic SNPs with potential to have a bearing on the etiology of the disease.

4.11. Results and Discussion

Mutational screening of TGIF1 (variant-008, Transcript ID-ENST00000552383) in 52 high myopia affected and 18 normal controls from Kashmir revealed a total of three novel and adjacent sequence variations; T>C/A, T>G & G>C (table 4.5) in the intronic region immediately after exon 2 boundry of this variant.

Table 4.5. Polymorphism found in intronic region of TGIF1 in ethnic High Myopia affected Kashmiri population

S.No	Wild nucleotide	SNP	SNP type	Codon Position	Location
1.	T	T/C or A	Novel	Intronic	Chr.18-3418281
2.	T	T/G	Novel	Intronic	Chr.18-3418281
3.	G	G/C	Novel	Intronic	Chr.18-3418281



a) Chromatogram showing normal sequence

b) Chromatogram showing T/C, T/G & G/C variations

Figure 4.2. Representative chromatograms indicating a) normal sequence b) sequence variants T>C, T>G & G>C in TGIF1 gene.

All the three variations found in heterozygous state (Fig. 4.2., Supplementary data Fig.S5P-S5R). First variation T>C/A (T changed to either C or A depicted by dual peaks) was observed at a frequency of 4/18 in normal controls and 26/52 affected samples. Second variation T>G was also present in both control and affected samples at a frequency of 8/18 in controls and 38/52 in affected samples. Sequence variation G>C was not observed in normal controls but was found in affected samples only at a frequency of 14/52. All the three adjacent variations were found to be present together in 10 samples, which were all affected.

4.11.1. Statistical analysis

The relative frequency of occurrence of variants for SNP T>C/A (**p** allele = 0.08, **p genotype = 0.04;** χ^2allele **= 3.06,** χ^2genotype = 4.07; OR = 2.66; CI (95%) = 0.86-8.23) shows statistical significance of genotype (TC). Likewise the frequency of occurrence for T>G variant (**p** allele = 0.11, **p genotype = 0.02;** χ^2allele **= 2.48,** χ^2genotype = 4.80; OR = 0.49; CI (95%) = 0.20-1.19) also shows significance of genotype (TG) and for G>C variant only p value could be calculated (**p** allele = 0.02; **p** genotype = 0.01) which is statistically significant (Chisquare is calculated only if all the expected cell frequencies are equal to or greater than 5)**.**

TGIF has been implicated to be the candidate gene for high myopia by Single Nucleotide Polymorphism (SNP) studies. We examined the hypothesis that polymorphisms within TGIF1 may influence the susceptibility of ethnic Kashmiri subjects to high myopia. The polymorphisms in TGIF1 studied here reveal significant association with an increased risk of having high myopia in Kashmiri patients when compared with control group. These results suggest that there exists high complexity of genetic background for our high myopic population and TGIF has a considerable effect on myopia onset and severity. Future work is needed to investigate other variants of TGIF and the recently reported candidate genes like TGFβ1 and HGF.

4.11.2. Conclusion

Although p values for these intronic variations come out to be significant sample size needs to be increased further to establish the potential of these variants in the etiology of high myopia.

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Section 3

4.12. A novel G26A mutation in 5' half of TGIF1 gene associates with high myopia in ethnic Kashmiri population from India

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4.13. Abstract

This study aims to look at novel variations of TG1F1 gene and explore their potential association with High Myopia in an ethnic population from Kashmir (India). 120 Kashmiri subjects (from India) with high myopia and ethnically matched 114 healthy controls were enrolled. Genomic DNA was prepared from peripheral blood. Allele frequencies were tested for Hardy–Weinberg disequilibrium. The genotype and allele frequencies were evaluated using the $\chi 2$ tests or the Fisher exact tests. In this study, we found a novel mutation G26A (**GAT to AAT**) in 5' half of TGIF1 gene (p. aspartic acid > asparagine) at a frequency of 62% (74/120, p = <0.0001). Mutation appear to associate with high myopia significantly (p = <0.001) as it happens to be present only in myopic samples, it shows statistical significance for its association with gender and degree of myopia (p = <0.05). Additionally, in Silico predictions show that mutation likely has an impact on structure and functional properties of protein.

Key words: Myopia, Ethnic, mutation, CSGE, TG1F1, Novel

4.14. Introduction

Development of myopia is a consequence of mismatch between the power of optical components and the axial length of the eye (Wensor, *et al*., 1999). Lower grades of myopia (<-6 diopters) are not associated with blinding conditions but higher or pathological grades with a refractive error of <-6 diopters often associates with blinding conditions like macular degeneration, retinal detachment and glaucoma (Lin, *et al*., 2006). Ethnic diversity plays a great role in the development of myopia reaching as high as 70-90% in some parts of Asia, 30-40% in Americans and Europeans and upto 20% in Africans (Lin, *et al*., 2006; Chow, *et al*., 1990).

Comparative prevalence rates of high myopia from diverse parts of the world show considerable variability, but still confirm that it affects a significant proportion of the population in different countries (Wang, *et al*., 1994; Curtin, 1970; Tokoro, 1982; Lin, *et al*., 1988; Wilson & Woo, 1989; Fledelius, 1988; Paluru, *et al*., 2005). The prevalence in India is found to be 19% with 4% in Kashmir (Ahmed, *et al*., 2008). Ahmed *et al*, reported effect of age, gender and socioeconomic conditions on myopia prevalence and showed an increase in its prevalence with increased age (3.76% in the age group of 6-10, 4.9% and 6.16% in age groups 11-15 and 16-22). Additionally Girls on average were 1.52 times more likely to have myopia than boys. The prevalence of myopia among girls was 5.54% compared with 3.6% in boys. Socioeconomic conditions also had an impact on the prevalence of myopia with only 3.23% students from medium and high socioeconomic strata having cb cr567tcb cr567t myopia, it was about three times more in students from low socioeconomic strata (8.60 %) (Ahmed, *et al*., 2008).

Despite many decades of research there is scarce knowledge about the precise molecular defects and abnormal biochemical pathways that result in myopia progression. It is a highly prevalent and complex disorder involving both genetic and environmental factors or it may be interplay of both genetic and environmental factors (Ibay, *et al*., 2004).

Recent mapping studies have identified 24 chromosomal loci suspected of harboring genes for myopia progression (Ng, *et al*., 2009). Among them, 11 have been implicated in high myopia viz., MYP1– MYP5, MYP11, MYP12, MYP13, MYP15, MYP16, MYP18 (Nallasamy, *et al*., 2007; Zhang, *et al*., 2005; Zhang, *et al*., 2006; Wojciechowski, *et al* 2006; Naiglin, *et al*., 2002; Paluru, *et al*., 2003; Paluru, *et al*., 2005; Young, *et al*., 1998a; Young, *et al*., 1998b; Young, *et al*., 2001; Nishizaki, *et al*., 2009; Lam, *et al*., 2008) and seven in myopia viz., MYP6–MYP10, MYP14, MYP17 (Hammond, *et al*., 2004; Stambolian, *et al*., 2004; Ciner, *et al*., 2008). Five of these loci viz., MYP2, MYP3, MYP6, MYP10, MYP13 have been confirmed through replication analysis in independent family studies (Zhang, *et al*., 2007; Lam, *et al*., 2003; Stambolian, *et al*., 2006; Klein, *et al*., 2007; Nurnberg, *et al*., 2008). A high heritability of myopia does not mean that environmental factors have no effect on the development of myopia. Near work activities in childhood have been hypothesized as

environmental risk factors for myopia progression (Saw, *et al*., 2001). Initially one of the study indicated a strong association between myopia and nightlight exposure (Quinn, *et al*., 1999) but recent research has reported contradictory results (Saw, *et al*., 2001; Zadnik, *et al*., 2000; Gwiazda, *et al*., 2000; Guggenheim, *et al*., 2003).

Myopia develops mainly because of excessive elongation in axial dimension rather than changes in corneal or lens power in humans (Zadnik, 1997). Whereas in animal models of myopia, active remodeling of sclera plays a crucial role in axial elongation (McBrien & Gentle, 2003; Rada, *et al*., 2006). Scleral remodeling involves reduced production of extracellular matrix resulting from reduced production of collagen and proteoglycans and from increased degradation of collagen along with concomitant increased activity of matrix metalloproteinase 2 (MMP2) and a reduction in the activity of tissue inhibitors of MMP. Transforming growth factor β (TGFβ) together with its receptor expressed in eye tissues (Saika, 2006) also regulates the proliferation of fibroblasts and production of collagen, MMP2, and tissue inhibitors of MMP (Overall, *et al*., 1989).

MYP2 is a candidate locus of the nonsyndromic autosomal dominant high myopia first identified by Young, Ronan, Drahozal *et al.* (1998) who performed a genome-wide linkage analysis for myopia susceptibility loci in 8 multigenerational families with an autosomal dominant mode of myopia of more than −6.00 diopters, and showed a significant linkage to 18p region and its functional role in ocular development (Yamane, *et al*., 2007). There are 9 known and 6 hypothetical genes considered to be candidates based on mapped position within the MYP2 interval which include clusterin-like 1(CLUL1), elastin microfibril interfacer 2 (EMILIN2), lipin 2 (LPIN2), myomesin 1 (MYOM1), myosin regulatory light chain 3 (MRCL3), myosin regulatory light chain 2 (MRLC2), transforming growth β-induced factor (TGIF1), large Drosophila homolog associated protein 1 (DLGAP1), and zinc finger protein 161 homolog (ZFP161) (Scavello, *et al*., 2005). The genes belonging to this locus may also be expressed in retina and influence the growth of sclera (Wallman, 1993).

TGIF1 is expressed in sclera, retina, cornea, and optic nerve and competitively inhibits binding of the retinoic acid receptor to a retinoid-responsive promoter

(Young, *et al*; 1998; Young, 2004; Scavello, *et al*., 2004; Bertolino, *et al*., 1995; Pertile, *et al*., 2008). It is possible that mutations in TGIF1 gene may alter its function and hence the phase of eye development, thus making it a potential candidate gene to study High Myopia.

4.15. Materials and Methods

4.15.1. Subjects

A total of 234 subjects (120 with high myopia of > -6D and 114 healthy control subjects; Annexure 3) were recruited from local hospital (Ophthalmology unit) as well from our ophthalmologist's clinic. Although authors were interested in doing a Familial kind of study but it happened to be very difficult to find ample number of families with high myopia making authors to design case control study. 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 acquity, 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.

4.15.2. Methodology

4.15.2.1. Polymerase chain reaction

Genomic DNA was extracted from whole blood samples using standard protocols. PCR reactions were carried out in a total volume of 50µl, Containing 50-100ng genomic DNA, 2-6 pmole of each primer, 1x PCR buffer (Sigma Aldrich) and 0.5 units of Taq DNA polymerase (Sigma Aldrich). Following primer sequences were

used for amplification: 5'-GGGAATAAGTGAGGGGCTCT -3' (sense) and 5'-CCTGAACCAGTCGCAAAGTT -3' (antisense). Expected PCR product of 472 bp was generated successfully (Supplementary data, Fig. S3h). The PCR cycling conditions involved: one cycle of denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 45 s, and one final 6 min elongation cycle at 72°C. PCR products were then purified using purification kit or NaI.

4.15.2.2. Conformation sensitive gel electrophoresis (CSGE)

Purified PCR products were subjected to denaturation and renaturation procedures for generation of potential heteroduplexes (Fig 4.3) and analyzed by CSGE (Ganguly, *et al*., 1993). Samples that showed unusual mobility during these assays were finally sequenced to confirm the presence of sequence variations along with controls (Scigenom, Cochin). This mutation detection technique has many advantages over other techniques like SSCP and PTT, Samples with unusual mobility during these assays were finally sequenced to confirm the presence of sequence variations along with controls (Macrogen, Korea). CSGE conditions described by Ganguly *et al*. for amplicons ranging from 200 to 800 bp were able to detect 60 out of 63 single-base mismatches. But still the migrating bands in this method are sometimes less clear and could lead to human error in reporting results, a serious disadvantage for an accurate identification of novel mutations. So sequencing is a must for the samples screened with CSGE to provide accurate results, although it definitely lowers the sequencing expenses (Blesa, *et al*., 2004).

4.15.2.3. Sequencing

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

4.15.2.4. Sequence analysis

Sequence results obtained in fasta and pdf formats were analysed using ClustalX version 2 software (Thompson, *et al*., 1997; Larkin, *et al*., 2007) and by Chromas Pro version 1.49 beta 2 software for the detailed inspection of individual chromatograms (Supplementary data, Fig. S5S-S5U).

4.15.2.5. Statistical analysis

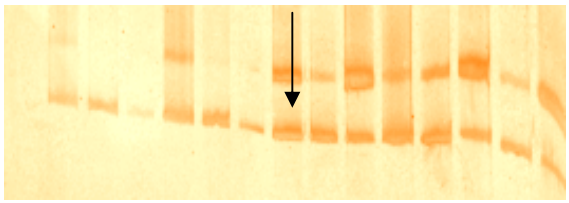
Genotypes were obtained by direct counting with subsequent calculation of allele frequencies. Statistical analysis was undertaken using the χ^2 test and significance value (p). A p value of <0.05 was considered significant. Adherence to the Hardy-Weinberg equilibrium constant was tested using the χ^2 test with one degree of freedom. Odds ratio and confidence interval was also calculated.

4.15.2.6. Insilico analysis

The amino acid sequence of the protein in fasta format obtained from (NCBI) (www.ncbi.nlm.nih.gov) was submitted to an automated server (I-TASSER) (zhang.bioinformatics.ku.edu/I-TASSER) for 3D structure prediction (Zhang, 2007; Zhang, 2008). The server furnishes predicted 3D structure in a pdb format. Swiss PDB Viewer was used for viewing pdb files and computing the free energy of the predicted 3D structures (Supplementary data, Fig. S6.3 & Table S6.3).

4.16. Results

In this study, finally DNA sequencing was used to confirm the results of heteroduplex assays (Fig. 4.3). No previously reported mutations were detected in this study. A novel mis-sense G>A mutation (Fig. 4.4) at codons 26 was identified in 5' half of TGIF1 gene (variant-003, ensemble). An interesting finding of our study was novelty of the mutation. The mutation G26A (p. aspartic acid > asparagine) which has not been reported till date was present at a frequency of 62% (74/120).



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 4.3. Representative CSGE gel showing heteroduplex bands in all lanes except lanes, 1,2 3,4,6 and 7.

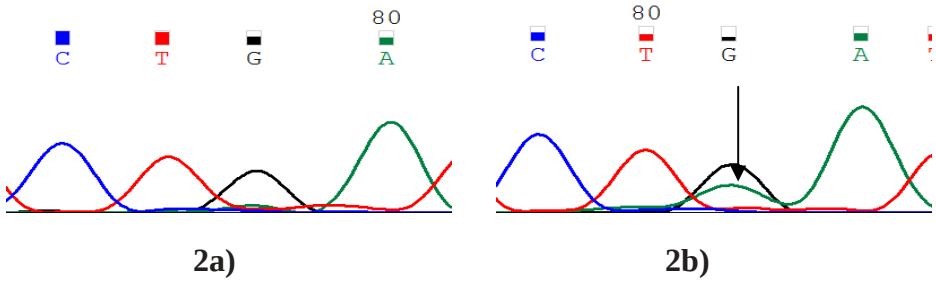


Figure 4.4. Representative partial chromatogram of 2a) Normal sample 2b) affected sample showing G>A mutation at codons 26 in TGIF1 as indicated by arrow.

A subtle and statistically significant **(p = <0.001**; Table 4.6) difference in the allelic frequency for this mutation was indicative of its possible association with high myopia, Furthermore it could be associated with gender and degree of myopia (p = 0.01 and p = < 0.0001, Table 4.7) with the frequency of GA genotype significantly higher in females with degree of myopia more than -6 diopters.

Insilico prediction results show that calculated energy for wild type protein is more (-5820.186 kj/mol) compared to mutant protein (-6595.593kj/mol). This change in energy of mutant protein is suggestive of affecting the protein tertiary structure which may in turn have some impact on protein function. Therefore, further studies are needed to elucidate the actual role of this mutation on protein structure and function.

Table 4.6. Genotype & allele frequencies of TGIF1 gene mutation in cases and controls Pearson's chisquare

variation	Genotype & allele	Cases (%) n= 120	Controls (%) n=114	P value	χ^2
g. 429G>A	GG	46 (38%)	114 (100%)	<0.001	83.5
	GA	74 (62%)	0 (0%)		
	AA	0 (0%)	0 (0%)		
	G	166 (69%)	228 (100%)		
	A	74 (31%)	0 (0%)		

Table 4.7. Association of TGIF1 gene alterations with clinical variables in high myopia affected patients

Parameters	TGIF126 G>A		P value (χ^2)	OR (95% CI)
	GG	GA+AA		
Age				
≤30 years	85	39	1.0	0.9832
>30 years	75	35	(0)	(0.5661-1.7075)
Sex				
Male	94	30	<0.01	0.4787
Female	66	44	(6.74)	(0.2732-0.8387)
Degree of myopia				
<-6	114	0	<0.0001 (102)	NA
-6 to -12	22	38		
>-12	24	36		

NA: Not applicable

4.17. Discussion

Diverse populations have presented inconsistent profile of association data owing largely to heterogeneous nature of the subject populations. Genetic polymorphisms have widely been in use to test the association of a gene with a commonly seen and multifactorial disease instead of single gene disease. Since nucleotide polymorphism is not strong enough to result in a lethal phenotype, this allele will not eventually disappear or reach frequency equilibrium without any selective disadvantage for individuals. Since ethnic difference do exit, it is imperative to substantiate or dispute the relevance of such polymorphism in genetically purer cohorts (Lin, *et al*., 2006; Zha, *et al*., 2009).

Investigating the genetics of common and complex disorders such as myopia remains one of the great challenges in human genetics. Myopia is considered to be a complex and multigenic condition involving several overlapping signaling pathways, each one mediated by a group of distinct genetic profiles. Therefore, studying the genetic polymorphisms of myopia-related genes can further clarify the relationship between genetics and myopia. The association between myopia and various genetic markers has helped increase our knowledge of prevention and treatment of myopia (Honda, *et al*., 1996; Kusakari, *et al*., 2001).

One of the studies has disregarded TGIF1 as potential contributor to the disease (Scavello, *et al*., 2004) although it has been associated with high myopia in a Chinese population where six SNPs showed statistically significant association (Lam, *et al*., 2003) but the association could not be replicated in a second Chinese case control

study of high myopia polpulace (Li, *et al*., 2003). A Japanese study failed to identify association of this gene with high myopia (Hasumi, *et al*., 2006). This inconsistency has largely been attributed to ethnic variations in the genetics of high myopia. Kashmiri population being a pure ethnic group provides an ideal scenario to substantiate the contribution of TGIF1 (if any) in the development of high myopia. 234 high myopic and 114 normal controls of Kashmiri ethnicity were recruited for TGIF1 polymorphic studies. Two genotypes GG & GA for codon 26 mutation occurred at a frequency of 100%: 0.00% in the control group vs 38%:74%, in high myopia group. A subtle and statistically significant **(**$p = <0.001$; Table 4.6) difference in the allelic frequency was indicative of its possible association with high myopia. Furthermore mutation was significantly associated with gender and degree of myopia ($p = 0.01$ and $p = < 0.0001$, table 4.7) with the frequency of GA genotype significantly higher in females with degree of myopia more than -6 diopters. The calculated energy for wild type protein is more (-5820.186 kj/mol) compared to mutant protein (-6595.593kj/mol). This change in energy of mutant protein is suggestive of affecting the protein tertiary structure which may in turn have some impact on protein function. Therefore, further studies are needed to elucidate the actual role of this mutation on protein structure and function.

Additionally the mutation is present in coding sequence of the gene affecting the physico-chemical properties of the protein causing change from polar negatively charged aspartic acid to polar & neutral amino acid asparagines. Further studies are however, needed to rule out the actual affect of the mutation on protein structure and function. Focused investigation is needed to establish the precise role played by TG1F1 in the high myopia development especially in the context of the above observed mutation.

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Section 4

4.18. Polymorphic variants of candidate genes in the MYP2 locus have potential to associate with high myopia in ethnic Kashmiris

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4.19. Abstract

This study aims to look at polymorphic variations in MYP2 candidate genes DLGAP1, MYOM1 and EMILIN2 in a pure ethnic High Myopia affected population from Kashmir (India). This is in continuation with our recent analysis of TGFβ1 and TGIF1 genes to screen all the MYP2 locus genes for their relevance to high myopia. 115 Indian Kashmiri subjects with high myopia and ethnically matched 112 healthy controls were recruited. Genomic DNA was extracted from whole blood samples using standard protocols, followed by PCR, CSGE and sequence analysis of DLGAP1, MYOM1 and EMILIN2 present in MYP2 locus. Genotype frequencies were tested for Hardy–Weinberg disequilibrium. Total of 8 polymorphisms were observed represented by 2 missense, 3 silent and 3 intronic variants. A novel G507A (P=1) was observed in DLGAP1in addition to one reported polymorphic variation G517A with a significant (P=<0.001) occurrence in affected population. A previously reported variant T451C observed in EMILIN2 gene did not appear to associate with disease phenotype. MYOM1 showed five polymorphic variations; two in coding region (G333A; P=<0.0001: G341C; P=0.003) and three intronic (G>A; P=< 0.0001: G>T & C>G; P= < 0.001). Insilico predictions show energy changes in variant proteins that are indicative of their affect on protein function.

Conclusions

Candidate genes present in MYP2 locus have a potential to associate with high myopia.

Key words: Myopia, Ethnic, Polymorphism, CSGE, EMILIN2, DLGAP1, MYOM1, Novel

4.20. Introduction

Myopia is a prevalent multifactorial ocular disorder worldwide, characterized by spherical error of refraction (RE) and retinal defocus that results in decreased visual acuity. The prevalence of myopia has been increasing in recent decades, especially in East Asian countries such as Japan, Singapore, Taiwan and China (Saw SM, 2003; Xu, *et al.*, 2005). High or pathological myopia (RE > 6D) is a progressive form with increased risk for serious complications such as glaucoma, macular degeneration, retinal detachment, and choroidal neovascularization, which when left untreated, may lead to permanent vision loss (Young, 2009). In Asia, the prevalence of high myopia is 1% - 5%, even ranging to 9.1% in some regions (Wong, *et al.*, 2000). Laser refractive surgery as a myopia-related cost was estimated to be 4.6 billion dollars for United States alone in 1990 (Javitt & Chiang, 1994). It is considered to be a complex, multifactorial condition in which several nongenetic/environmental components such as near work, excess illumination, nutritional deficiencies, mechanical stress and mental stress, along with the genetic components influence normal emmetropisation mechanisms of the eye contributing to ocular refraction in myopia (Feldkamper & Schaeffel, 2003).

There is a long-standing dispute on the relative role of genetic versus environmental factors in the development of myopia (Saw, *et al.*, 2000). The concept that environmental factors influence ocular development has been well established in epidemiological and experimental animal studies (Saw, 2002; Schaeffel, 1988). The frequent manifestation of myopia during school and college years, as well as in some occupations requiring intense and prolonged near work, has suggested the critical role of a near vision stimulus in the development of myopia.

There is abundant evidence for genetic factors determining refractive development (Francois, 1961; Zadnik, *et al.*, 1994). First, higher myopia prevalence in developed Asian countries compared with the Western world suggests a genetic susceptibility to myopia development. Further, myopic parents are more likely to give rise to offspring with myopia than non-myopic parents (Goldschmidt, 1981). This finding has been confirmed by recent large-scale epidemiological studies, according to which heritable factors account for 80% of juvenile myopia development (Mutti, *et al.*, 2002). Strong

evidence for the role of inheritance is also provided by twin studies (Teikari, *et al.*, 1991; Hammond, *et al.*, 2001), where in identical twins display a higher similarity in their refractive status than fraternal twins (Jacobi, *et al.*, 2005).

Genetic mapping studies have identified at least 24 chromosomal loci suspected of harboring genes for myopia progression (Ng, *et al.*, 2009). Among them, 11 have been implicated in high myopia viz., MYP1– MYP5, MYP11, MYP12, MYP13, MYP15, MYP16, MYP18 (Nallasamy, *et al.*, 2007; Zhang, *et al.*, 2005; Zhang, *et al.*, 2006; Wojciechowski, *et al.*, 2006; Naiglin, *et al.*, 2002; Paluru, *et al.*, 2003; Paluru, *et al.*, 2005; Young, *et al.*, 1998a; Young, *et al.*, 1998b; Young, *et al.*, 2001; Nishizaki, *et al.*, 2009; Lam, *et al.*, 2008) and seven in myopia viz., MYP6–MYP10, MYP14, MYP17 (Hammond, *et al.*, 2004; Stambolian, *et al.*, 2004; Ciner, *et al.*, 2008). Five of these loci viz., MYP2, MYP3, MYP6, MYP10, MYP13 have been confirmed through replication analysis in independent family studies (Zhang, *et al.*, 2007; Lam, *et al.*, 2003; Stambolian, *et al.*, 2006; Klein, *et al.*, 2007; Nurnberg, *et al.*, 2008).

MYP2 is a candidate locus of the nonsyndromic autosomal dominant high myopia first identified by Young, Ronan, Drahozal *et al.* (1998) who performed a genome-wide linkage analysis for myopia susceptibility loci in 8 multigenerational families with an autosomal dominant mode of myopia of more than −6.00 diopters, and showed a significant linkage to 18p. The maximum lod score was 9.59, with marker D18S481. Haplotype analysis further refined this myopia locus to a 7.6 centi-morgan interval between markers D18S59 and D18S1138 on 18p11.31. Afterwards Young *et al.* (2001) narrowed the candidate region to the interval of 0.8 cM between markers D18S63 and D18S52. This locus on chromosome 18p11.31 is believed to harbor the genes involved in sclera formation or regulation thereby making it most preferential locus with potential to harbor the candidate genes for the disease (Young, 2004; Yamane, *et al.*, 2007). This locus has been screened and multiple candidate genes for high myopia identified within MYP2 critical region and within the other mapped loci (Young, 2004).

Genes that map to MYP2 critical region include clusterin-like 1 (CLUL1), elastin microfibril interfacer 2 (EMILIN2), lipin 2 (LPIN2), myomesin 1 (MYOM1), myosin

regulatory light chain 3 (MRCL3), myosin regulatory light chain 2 (MRLC2), transforming growth β-induced factor (TGIF), large Drosophila homolog associated protein 1 (DLGAP1), and zinc finger protein 161 homolog (ZFP161) (Scavello, *et al.*, 2005).

The role of these genes stands established in sclera remodeling (Honda, *et al.*, 1996; Kusakari, *et al.*, 2001) for their influence in the growth and maintenance of sclera (Wallman, 1993). Several studies produced conflicting results for association of single-nucleotide polymorphisms (SNPs) in MYP2 locus genes and high myopia (Young, 2004; Scavello, *et al.*, 2005; Heath, *et al.*, 2001). Our study serves to clarify this relationship with a case-control design and ethnic purity of our population. We focus to analyze specific fragments of candidate genes such as EMILIN2, DLGAP1 and MYOM1 in our ethnic population.

4.21. Materials and Methods

4.21.1 Study design

This study was conducted at the University of Kashmir, Srinagar (India) between 2010 and 2012. The Ethical Committee has approved the study. All patients signed the written informed consent.

4.21.2. Participants

115 Kashmiri subjects (from India) with high myopia and ethnically matched 112 healthy controls were enrolled for the study from local hospital (Ophthalmology unit) as well from our ophthalmologist's clinic (Annexures IV-VII). 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 acquity, 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.

4.21.3 Methodology

4.21.3.1. DNA extraction from blood

Deoxyribonucleic acid (DNA) extraction of Samples of both high myopia affected and normals was carried out by standard procedures like phenol-chloroform extraction and salting out. Extracted DNA was dissolved in tris–EDTA buffer for further use.

4.21.3.2. Polymerase chain reaction

PCR reactions were carried out in a total volume of 50µl, Containing 50-100ng genomic DNA, 2-6 pmole of each primer (primers listed in table 3.2.methodology section), 1x PCR buffer (Sigma Aldrich) and 0.5 units of Taq DNA polymerase (Sigma Aldrich). The PCR cycling conditions involved: one cycle of denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 45 s, annealing at *t°C (* = different annealing temperatures for different primer sets)for 45s, and extension at 72°C for 45 s, and one final 6 min elongation cycle at 72°C. PCR products of the expected sizes generated (Supplementary data, Fig. S3a-S3f) were then purified using purification kit or NaI.

4.21.3.3. Conformation sensitive gel electrophoresis (CSGE)

Purified PCR products were subjected to denaturation and renaturation procedures for generation of potential heteroduplexes and analyzed by CSGE (Ganguly, *et al.*, 1993). Samples with unusual mobility during these assays were finally sequenced to confirm the presence of sequence variations along with controls (Scigenom, Kerala).

4.21.3.4. Sequencing

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

4.21.3.5. Sequence analysis

Sequence results obtained in fasta and pdf formats were analysed using ClustalX version 2 software (Thompson, *et al.*, 1997) and by Chromas Pro version 1.49 beta 2

software for the detailed inspection of individual chromatograms (Supplementary data, Fig. S5A-S5O).

4.21.3.6. Statistical analysis

Statistical analysis was undertaken using the χ^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 χ^2 test with one degree of freedom. Odds ratio and confidence interval was also calculated.

4.21.3.7. Insilico analysis

The amino acid sequence of the protein in fasta format obtained from (NCBI) (www.ncbi.nlm.nih.gov) was submitted to an automated server (I-TASSER) (zhang.bioinformatics.ku.edu/I-TASSER) for 3D structure prediction (Zhang, 2007; Zhang, 2008). The server furnishes predicted 3D structure in a pdb format. Swiss PDB Viewer was used for viewing pdb files and computing the free energy of the predicted 3D structures (Camacho, *et al.*, 2000; Camacho & Gatchell, 2003; Comeau, *et al.*, 2004).

4.22. Results

This study identifies sequence variants in DLGAP1, EMILIN2 & MYOM1 in a pure ethnic kashmiri population. Prior to DNA sequencing samples were screened for the presence of mutations by Conformation Sensitive Gel Electrophoresis (CSGE) and only those samples were sent out for commercial sequencing that showed differential migration on heteroduplex assay by CSGE (Supplementary data, Fig. S4). Heteroduplex analysis was done to minimize the sequencing load although the results are not 100% but still huge sequencing burden is definitely relaxed to a great extent.

Mutational screening revealed a total of 8 polymorphic variations five of which were exonic and three were intronic. DLGAP1 gene revealed a total of two polymorphic variations. Out of which one G>A variation at codon 507 (Fig.4.5a) happens to be novel and mis-sense changing polar negatively charged amino acid Glutamic acid (Glu; E.) to polar and positively charged amino acid Lysine(K). It was present in all the samples, controls (109/109; (100%) and affected (115/115; 100%) in heterozygous state (GA). Second reported (rs3745051) synonymous & heterozygous

polymorphic variation G>A at codon 517 (Fig. 4.5b) was present only in affected samples at a frequency of 38/109 (35%). None of the normal controls (115) showed this polymorphism. A subtle and statistically significant **(p = <0.001**; Table 4.8) difference in the allelic frequency for codon 517 variation indicates its potential association with high myopia, While the relative frequency of occurrence of variation at codon 507 **(p = 1**; Table 4.8) in high myopes was found to be statistically insignificant, when compared to their occurrence in healthy controls. The DLGAP1 codon 507 polymorphism was not associated with age, gender and the degree of myopia and the frequency of GA genotype was almost equally distributed between cases and controls of all age groups among both genders (Table 4.9). G>A polymorphism at codon 517 however, showed significant p values for gender and degree of myopia with the distribution of GA genotype significantly higher in high myopic females with ≤ 30 years of age (p = < 0.0001, Table 4.9).

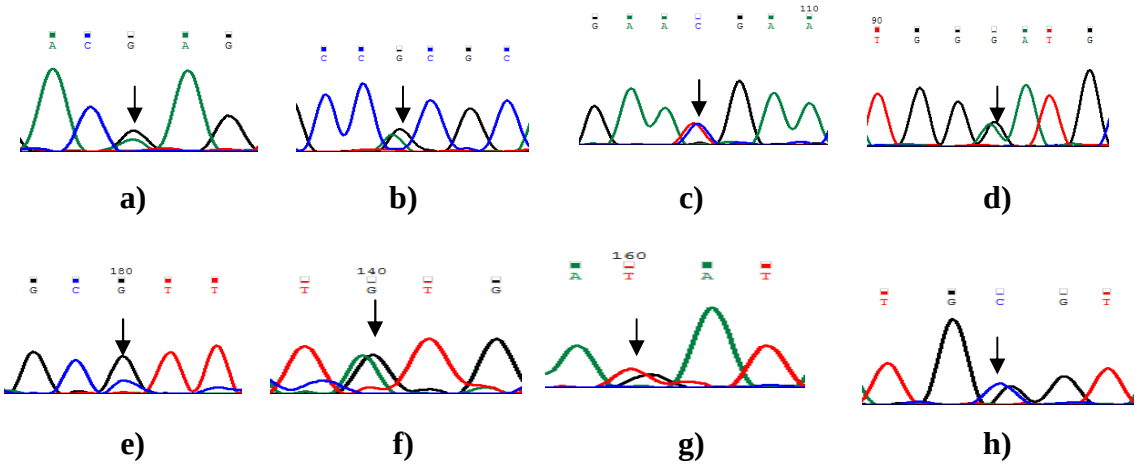

Fig. 4.5. Representative partial chromatograms of affected samples showing sequence variations in DLGAP1 (a) and (b), EMILIN2 (c) and MYOM1 (d, e, f, g, h) indicated by arrows

Polymorphic analysis of exon 4 of EMILIN2 revealed a total of one reported (rs3810067) synonymous polymorphic variation, (T>C) at codon 451. It was observed in heterozygous state (Fig. 4.5c) in 22/115(19%) high myopia affected samples and 28/107 (26%) controls. The observed allele frequency of this variation was not in Hardy–Weinberg equilibrium (P = 0.24, Table 4.8). However the polymorphism showed statistical significance (p = 0.03, Table 4.10) for gender with occurrence of

heterozygous genotype TC more among females than males, yet did not seem to associate with age and degree of myopia (p = > 0.05, Table 4.10).

Mutational screening of specific fragments of MYOM1 in 112 high myopia affected and 110 normal controls revealed a total of two reported polymorphic variations in coding region. Codon 333G>A (Fig. 4.5d) synonymous variation with rs2230162 was present in 28/112 (25%) affected samples in both homozygous 14/112 (12.5%) and heterozygous states in 14/112 (12.5%). In controls only heterozygous state (GA) was observed in 46/110 (42%) samples evaluated. The G>C non-synonymous variation at codon 341 (Fig.4.5e) was observed in all samples 222/222 (100%; controls and cases) in both homozygous and heterozygous states. The homozygous state in affected samples was found in 96/112 (86%) and heterozygous in 16/112 (14%). In controls homozygous state was present in 76/110 (69%) while heterozygous state was observed in 34/110 (31%) samples.

The observed genotype frequency of both the polymorphic variations at codon 333 and 341 was in Hardy–Weinberg equilibrium (P = <0.0001 & p = 0.003, Table 4.8). The MYOM1 G>A polymorphism at codon 333 shows a statistically significant association with gender (p = 0.01, Table 4.11) and degree of myopia (p = 0.0005, Table 4.11) with the frequency of GA genotype significantly higher in males with degree of myopia above -6 diopters. The observed genotype frequency of all the three intronic variations of MYOM1 was in Hardy–Weinberg equilibrium (P = <0.0001, Table 4.8). Intronic variation G>A rs17177479 (Fig.4.5f) does not associate with any of the clinical parameters (p>0.05, Table 4.12), however other two intronic polymorphisms G>T rs55779127 (Fig.4.5g) and C>G rs8096379 (Fig.4.5h) show statistical significance (p < 0.0001, Table 4.12) for the degree of myopia.

Table 4.8. Genotype and allele frequencies of MYP2 locus gene polymorphisms in cases and controls

Gene/ variation	Genotype	Cases (%) n= 112	Controls (%) n=110	P value	χ^2
MYOM1					
rs2230162 g.44094G>A	GG	84 (75%)	64 (58%)	<0.0001	33.75
	GA	14 (12.5%)	46 (42%)		
	AA	14 (12.5%)	0 (0%)		
	G	182 (81%)	174 (79%)	0.56	0.33
	A	42 (19%)	46 (21%)		
rs8099021 g.44117G>C	GG	0 (0%)	0 (0%)	0.005	7.67
	GC	16 (14%)	34 (31%)		
	CC	96 (86%)	76 (69%)		
	G	16 (7%)	34 (16%)		
	C	208 (93%)	186 (84%)		
rs17177479 g.44140G>A	GG	98 (87%)	84 (76%)	<0.0001	41.06
	GA	0 (0%)	26 (24%)		
	AA	14 (13%)	0 (0%)		
	G	196 (87%)	194 (88%)	0.82	0.05
	A	28 (13%)	26 (12%)		
		n= 113	**n= 112**		
rs55779127 g. 107905G>T	GG	0 (0%)	112 (100%)	<0.001	124.1
	GT	15 (13%)	0 (0%)		
	TT	98 (87%)	0 (0%)		
	G	128 (57%)	224 (100%)		
	T	98 (43%)	0 (0%)		
rs8096379 g.107926C>G	CC	15 (13%)	112 (100%)	0.001	124.1
	CG	98 (87%)	0 (0%)		
	GG	0 (0%)	0 (0%)		
	C	128 (57%)	224 (100%)		
	G	98 (43%)	0 (0%)		
		n= 115	**n= 107**		
EMILIN2 rs3810067 g. 44502 T>C	TT	93 (81%)	79 (74%)	0.24	1.37
	TC	22 (19%)	28 (26%)		
	CC	0 (0%)	0 (0%)		
	T	208 (90%)	186 (87%)		
	C	22 (10%)	28 (13%)		
		n=109	**n=115**		
DLGAP1 g.726179G>A	GG	0 (0%)	0 (0%)	1.0	0
	GA	109 (100%)	115 (100%)		
	AA	0 (0%)	0 (0%)		
	G	109 (50%)	115 (50%)		
	A	109 (50%)	115 (50%)		
rs3745051 g.726211 G>A	GG	71 (65%)	115 (100%)	<0.001	43.8
	GA	38 (35%)	0 (0%)		
	AA	0 (0%)	0 (0%)		
	G	180 (82%)	230 (100%)		
	A	38 (18%)	0 (0%)		

Table 4.9. Association of DLGAP1 gene alterations with clinical variables in high myopia affected patients

Parameters	DLGAP1 507G>A		P value (χ^2)	OR (95% CI)	DLGAP1 517G>A		P value (χ^2)	OR (95% CI)
	GG	GA+AA			GG	GA+AA		
Age								
≤30 years	0	121	**NA**	**NA**	99	22	0.07	2.1481
>30 years	0	103			87	9	(3.39)	(0.9392-4.9131)
Sex								
Male	0	115	**NA**	**NA**	109`	6	<0.0001	0.1325
Female	0	109			**77**	32	(23.15)	(0.0528-0.3322)
Degree of myopia								
<-6	0	115			115	0	<0.0001 (48.4)	NA
-6 to -12	0	58	**NA**	**NA**	37	21		
>-12	0	51			34	17		

NA: Not applicable

Table 4.10. Association of EMILIN2 gene alterations with clinical variables in high myopia affected patients

Parameters	EMILIN2 451T>C		P value (χ^2)	OR (95% CI)
	TT	TC+CC		
Age				
≤30 years	91	26	1.0 (0.01)	0.9643 (0.5133-1.8114)
>30 years	81	24		
Sex				
Male	99	20	0.03 (4.8)	0.4916 (0.2588-0.9337)
Female	73	30		
Degree of myopia				
<-6	79	28	0.35 (2.04)	NA
-6 to -12	47	13		
>-12	46	9		

NA: Not applicable

Table 4.11. Association of MYOM1 gene alterations (coding region) with clinical variables in high myopia affected patients

Parameters	MYOM1 333 G>A		P value (χ^2)	OR (95% CI)	MYOM1 341 G>C		P value	OR (95%CI)
	GG	GA+AA			GG	GC+CC		
Age								
≤30 years	82	38	0.56 (0.33)	0.8496 (0.4856-1.4864)	0	120	NA	NA
>30 years	66	36			0	102		
Sex								
Male	69	48	2.1137	(1.1879-3.7611)	0	117	NA	NA
Female	79	26			0	105		
Degree of myopia								
<-6	64	46	0.01 (8.52)	NA	0	110	NA	NA
-6 to -12	42	18			0	60		
>-12	42	10			0	52		

NA: Not applicable

Table 4.12. Association of intronic MYOM1 gene alterations with clinical variables in high myopia affected patients

Parameters	MYOM1 rs55779127 G>T		P value (χ^2)	OR (95% CI)	MYOM1 rs8096379C>G		P value (χ^2)	OR (95% CI)	MYOM1 rs17177479 G>A		P value (χ^2)	OR (95% CI)
	GG	GT+TT			CC	CG+GG			GG	GA+AA		
Age												
≤30 years	69	52	0.84	0.9502	70	51	0.64	0.8836	96	24	0.40	1.438
>30 years	58	46	(0.04	(0.56021.6119)	57	47	(0.21)	(0.5209-1.4989)	86	16		(0.6698-2.696)
Sex												
Male	72	46	0.11	0.6544 (0.3851-1.112)	68	50	0.71	0.9038	94	23	0.80	1.2666
Female	55	52	(2.47)		59	48	(0.14)	(0.5332-1.532)	88	17	(0.45)	(0.6346-2.5278)
Degree of myopia												
<-6	112	0			112	0			84	26		
-6 to -12	8	52	<0.0001	NA	7	53	<0.001	NA	52	8	0.10(4.72)	NA
>-12	7	46	(172)		8	45	(172)		46	6		

NA: Not applicable

4.22.1. Insilico prediction results

MYP2 locus genes were modeled by I-TASSER to obtain the PDB structures and analysis (energy calculations) was done using PDB Viewer. The assessment of the I-TASSER predicted protein structure showed higher energy for mutant protein (20206.113 kj/mol) compared to wild type protein (23265.684kj/mol) in DLGAP1 (Supplementary data, Fig. S6.1 & Table S6.1). While in case of MYOM1 wild protein showed higher energy (-9702.442 kj/mol) as compared to mutant (-11496.317kj/mol) for codon 341G>C variation (Supplementary data, Fig. S6.2 & Table S6.2).

4.23. Discussion

Despite compelling evidence about environmental contribution, genetic predisposition remains a strong ally for myopia. Relevance of genetic factors in myopia has been substantiated by various twin and familial studies indicating correlations between refractive error in parents and siblings (Teikari, *et al.*, 1989). Various autosomal and X-linked loci have been found to be associated with pathological myopia. However, the contribution of the genes on these loci has not been established with regard to the development of myopia. The genes in these loci cannot be solely responsible for the development of myopia in different ethnic groups with wide variability of the prevalence of myopia, difficulty here is the uncertainty surrounding environmental influences and genetic factors in the equation. Ideally, one set of genetic factors will interact with one set of environmental influences to produce identical outcomes, but it is unknown whether this is always the case. Candidate genes that map to MYP2 locus show expression in eye tissues (Lam, *et al.*, 2003) and are important for constituent organization and maintenance of connective tissue function. The genes belonging to this locus may also be expressed in retina and influence the growth of sclera (Wallman, 1993). This retinal hypothesis emanates mainly from animal studies of experimental myopia. The induction of myopia in juvenile animals by deprivation of form vision demonstrates a visual feedback mechanism in eye growth control. Experimental work indicates that this neural control mechanism is at least partly localized to the retina itself, but how retinal signals directly control the growth of the outer coats of the eye is presently unknown (Scavello, *et al.*, 2005).

Diverse populations have presented inconsistent profile of associating data owing largely to heterogeneous nature of the populations studied. Previous mutational screens for MYP2 locus candidate genes like MYOM1, EMILIN2, TGIF, DLGAP1, CLUL1, LPIN2, MRCL3, MRLC2, ZFP161 did identify polymorphic variations in all these genes but none of the mutations segregated with the affected status (Scavello, *et al.*, 2005).Numerous other studies did however; indicate the association of series of SNPs in these genes with high myopia in populations like Chinese and Italian Sardinian cohorts (Heath, *et al.*, 2001), whereas certain other studies have found no such association (Young, 2004; Scavello, *et al.*, 2005). Surprisingly the locus has been shown to have significant association with high myopia in two Chinese families (Lam, *et al.*, 2002) and Consistent association of this locus with high myopia is also reported in an Italian population (Heath, *et al.*, 2001; Lam, *et al.*, 2003). 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.

DLGAP1 (DISCS large associated protein 1) functions as a channel associated proteins. It is known to be highly enriched in synaptosomal preparations of the brain, and is present in the post synaptic density (Entrez Gene).The novel polymorphic variation G507A in DLGAP1 observed in our study group which is apparently population specific, does not however segregate with the disease phenotype while an additional reported sequence variant G517A appeared to associate significantly ($p = 0.0001$).

EMILIN2 an elastic fiber interacting protein confers elasticity to the extracellular matrix (Doliana, *et al.*, 2001). Broadly expressed in connective tissues with cell adhesion promoting functions, it is deposited extracellularly and is abundant in blood vessels, skin, heart, lung, kidney, and cornea (Bressan, *et al.*, 1983; Colombatti, *et al.*, 1988). The expression profile, pro-adhesive functions, and the domain characteristics suggest its fundamental role in the process of elastogenesis in association with other extracellular matrix constituents (Bressan, *et al.*, 1983) providing an important association in scleral wall elasticity seen in high myopia with elongated axial lengths (Scavello, *et al.*, 2005). A previously reported EMILIN2 variant T451C observed in our cohort failed to associate with the disease in agreement with general contention.

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 (Wang, 1985; Maruyama, 1986; Trinick, 1991). Two reported polymorphic variations observed in the coding sequence of MYOM1 G333A & G341C segregated with the affected phenotype (P=<0.0001 & P=0.003) alongside other reported intronic variations observed in MYOM1 (G>A; P=< 0.0001: G>T & C>G; P= < 0.001). Insilico predictions show change in energy state of variant proteins that are indicative of their affect on protein stability and function.

Our study adds support to the idea that the MYP2 locus candidate gene polymorphism contributes to the pathogenesis of myopia. It would however, need focused investigation to establish the precise role played by these genes in the development of high myopia. Since these SNPs appear to change the energy state of protein indicated by insilico analysis, a biological corroboration would be needed to elucidate the actual affect of these changes on the function of these proteins. Genes further up- and downstream also need to be investigated, to present a cumulative genetic profile influenced by genetics and environment in the genesis of high myopia.

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Bibliography

Aaiji C & Borst P. The gel electrophoresis of DNA. *Biochim Biophys Acta*. 1972; 269:192.

Ahmed I, Mian S, Mudasir S, Andrabi KI. Prevalence of myopia in students of Srinagar City of Kashmir, India. *Intl J Health Sci.* 2008; 2(1):77-81.

Alexander GF. Spasm of accommodation. *Trans Ophthalmol Soc UK*. 1940; 60:207-12.

Alphen V. On emmetropia and ametropia. *Ophthalmologica*. 1961; 142:1-92.

Andrew IJ, Nguyen M, Gentle A. Isoform-specific changes in scleral transforming growth factor-expression and the regulation of collagen synthesis during myopia progression. *J Biol Chem*. 2004; 18:18121–18126.

Angle J & Wissmann DA. Age, reading, and myopia. *Am J Optom Physiol Opt*. 1978; 55: 302 -8.

Angle J & Wissmann DA. The epidemiology of myopia. *Am J Epidemiol*. 1980; 111: 220-8.

Araki M. Studies on refractive components of human eye by means of ultrasonic echogram. Report III. The correlation among refractive components. *Acta Soc Ophthalmol Jpn.* 1962; 128-47.

Bassam BJ & Gresshoff PM. Silver staining DNA in polyacrylamide gels. *Nat Protoc*. 2007; 2(11):2649-54.

Bear JC. Epidemiology and genetics of refractive anomalies. In: Grosvenor T, Flom MC, eds. Refractive anomalies: research and clinical applications. Stoneham, MA: Butterworth-Heinemann, 1991.

Bertolino E, Reimund B, Wildt-Perinic D, Clerc RG. A novel homeobox protein which recognizes a TGT core and functionally interferes with a retinoid-responsive motif. *J Biol Chem*. 1995; 270:31178-88.

Bilgihan K, Hondur A, Hasanreisoglu B. Laser subepithelial keratomileusis for myopia of -6 to -10 diopters with astigmatism with the MEL60 laser. *J. Refract Surg*. 2004; 20(2):121-126.

Blesa JR, Prieto-Ruiz JA, Hernandez-Yago J. Conformation-Sensitive Gel Electrophoresis as an ideal high-throughput strategy for accurate detection of sequence variations in DNA: Screening hTomm and hTimm genes. *J Biomol Screening*. 2004; 9:7.

Blin N & Stafford DW. A general method for isolation of high molecular weight DNA from eukaryotes. *Nucleic Acids Res*. 1976; 3: 2303-8.

Bressan GM, Castellani I, Colombatti A, Volpin D. Isolation and characterization of a 115,000-dalton matrix-associated glycoprotein from chick aorta. *J Biol Chem.* 1983; 258:13262-7.

Burton TC. The influence of refractive error and lattice degeneration on the incidence of RD. *Trans Am Ophthalmol Soc*. 1990; 87:143-155.

Camacho CJ, Gatchell DW, Kimura SR, Vajda S. Scoring docked conformations generated by rigid body docking, Proteins: Structure, Function, and Genetics. 2000; 40:525-537.

Camacho CJ & Gatchell DW. Successful Discrimination of protein interactions, Proteins: Structure, Function and Genetics. 2003; 52:92-97.

Celorio JM & Pruett RC. Prevalence of lattice degeneration and its relation to axial length in severe myopia. *Am J Ophthalmol*. 1991; 111:20-3.

Chen CP, Chern SR, Du SH, Wang W. Molecular diagnosis of a novel heterozygous 268C—>T (R90C) mutation in TGIF gene in a fetus with holoprosencephaly and premaxillary agenesis. *Prenat Diagn.* 2002; 22:5-7.

Chen CY, Stankovich J, Scurrah KJ, Garoufalis P, Dirani M, Pertile KK, Richardson AJ, Baird PN. Linkage Replication of the MYP12 Locus in Common Myopia. *Invest Ophthalmol Vis Sci*. 2007; 48: 10.

Chen F, Ogawa K, Nagarajan RP, Zhang M, Kuang C, Chen Y. Regulation of TG-interacting factor by transforming growth factor-beta. *Biochem J.* 2003; 371:257–263.

Chow YC, Dhillon B, Chew PT, Chew SJ. Refractive errors in Singapore medical students. *Singapore Med J*. 1990; 31: 472-3.

Ciner E, Wojciechowski R, Ibay G, Bailey-Wilson JE, Stambolian D. Genomewide scan of ocular refraction in African-American families shows significant linkage to chromosome 7p15. *Genet Epidemiol*. 2008; 32:454-63.

Cline D, Hofstetter HW, Griffin JR. Dictionary of Visual Science, 4th ed, Boston: Butterworth-Heinemann, 1997; ISBN 0-7506-9895-0.

Colombatti A, Bonaldo P, Volpin D, Bressan GM. The elastin associated glycoprotein gp115. Synthesis and secretion by chick cells in culture. *J Biol Chem*. 1988; 263:17534-40.

Colombatti A, Doliana R, Bot S, Canton A, Mongiat M, Mungiguerra G, Paron-Cilli S, Spessotto P. The EMILIN protein family. *Matrix Biol*. 2000; 19:289-301.

Comeau SR, Gatchell DW, Vajda S, Camacho CJ. ClusPro: an automated docking and discrimination method for the prediction of protein complexes. *Bioinformatics*. 2004; 20:45-50.

Curtin BJ. Myopia: a review of its etiology, pathogenesis, and treatment. *Surv Ophthalmol*. 1970; 15:1-17.

Curtin BJ & Karlin DB. Axial length measurements and fundus changes of the myopic eye. *Am J Ophthalmol.* 1971; 71:42–53.

Curtin BJ. The posterior staphyloma of pathologic myopia. Trans Am Ophthalmol Soc. 1977; 75:67-86.

Curtin BJ. Posterior staphyloma development in pathologic myopia. *Ann Ophthalmol.* 1982; 14:655-8.

Curtin BJ. The Myopias: Basic Science and Clinical Management. New York: Harper & Rowe, 1985; 237-245.

Crawford JS, Shagass C, Pashby TJ. Relationship between visual acuity and refractive error in myopia. *Am J Ophthalmol*. 1945; 28:1220-5.

Dandona R, Dandona L, Naduvilath TJ, Srinivas M, McCarty CA, Rao GN. Refractive errors in an urban population in Southern India: The Andhra Pradesh Eye Disease Study. *Invest Ophthalmol Vis Sci*. 1999; 40:2810-8.

Dandona R & Dandona L. Refractive error blindness. Bull World Health Organ. 2001; 79:237–43.

Daubs JE & Crick RP. Effect of refractive error on the risk of ocular hypertension and open angle glaucoma. *Trans Ophthalmol Soc*. 1981; 101:121-6.

Diamond MS, Staunton DE, Marlin SD, Springer TA. Binding of the integrin Mac-1 (CD11b/CD18) to the third immunoglobulin- like domain of ICAM-1 (CD54) and its regulation by glycosylation. *Cell*. 1991; 65:961-71.

Doliana R, Bot S, Mungiguerra G, Canton A, Cilli SP, Colombatti A. Isolation and characterization of EMILIN-2, a new component of the growing EMILINs family and a member of the EMI domain-containing superfamily. *J Biol Chem.* 2001; 276:12003-11.

Duffey RJ & Leaming D. Trends in refractive surgery in the United States. *J Cataract Refract Surg*. 2004; 30(8): 1781-1785.

Edwards MH. The development of myopia in Hong Kong children between the ages of 7 and 12 years: a five-year longitudinal study. *Ophthal Physiol Opt*. 1999; 19: 286-294.

El Danasoury MA. Prospective bilateral study of night glares after laser in situ keratomileusis with single zone and transition zone ablation. *J Refract Surg.* 1998; 14(5):512-516.

Epstein D. Accommodation as the primary cause of lowluminance myopia--experimental evidence. *Acta Ophthalmol*. 1983; 61:424-30.

Fallon FL. Myopia encyclopedia of nursing and allied health, 2007

Feldkamper M & Schaeffel F. Interactions of genes and environment in myopia. *Dev Ophthalmol*. 2003; 37: 34-49.

Fernandez-Vega L, Alfonso JF, Villacampa T. Clear lens extraction for the correction of high myopia. *Ophthalmology*. 2003; 110(12): 2349-2354.

Flaxel CJ, Jay M, Thiselton DL, Nayudu M, Hardcastle AJ, Wright A, Bird AC. Difference between RP2 and RP3 phenotypes in X linked retinitis pigmentosa. *Br. J. Ophthalmol*. 1999; 83: 1144–1148.

Fledelius HC. Myopia prevalence in Scandinavia: a survey, with emphasis on factors of relevance for epidemiological refraction studies in general. *Acta Ophthalmol*. 1988; 185: 44–50.

Fledelius H, Zak M, Pedersen FK. Refraction in juvenile chronic arthritis: a long-term follow-up study, with emphasis on myopia. *Acta Ophthalmol Scand*. 2001; 79:237–239.

Francois J. Heredity in Ophthalmology, Mosby, St Louis, 1961.

Francois J & Goes F. Ultrasonographic study of 100 emmetropic eyes. *Ophthalmologica*. 1977; 175:321-7.

Fredrick DR. Myopia. BMJ. 2002; 324: 1195-99.

Ganguly A, Rock MJ, Prockop DJ. Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in double-stranded PCR products and DNA fragments: evidence for solvent-induced bends in DNA heteroduplexes. *Proc. Natl. Acad. Sci*. 1993; 90:10325-9.

Goldschmidt E. The importance of heredity and environment in the aetiology of low myopia. *Acta Ophthalmol*. 1981; 59: 759–762.

Goldschmidt E, Fledelius HC, Fuchs J, Nissen KR. High myopia in Denmark with emphasis on visual loss and fundus changes. *Acta Ophthalmol*. 1990; 68:95-7.

Goss DA & Eskridge JB. Myopia. In: Amos JF, ed. Diagnosis and management in vision care. Boston: *Butterworths.* 1987; 121-71.

Goss DA, Grosvenor TP, Keller JT, Marsh-Tootle W, Norton TT, Zadnik K. Optometric Clinical Practice Guideline Care of the Patient with myopia. American Optometric Association. 1997; 243 N. Lindbergh Blvd., St. Louis, MO 63141-7881.

Gripp KW, Wotton D, Edwards MC, Roessler E, Ades L, Meinecke P, Richieri-Costa A, Zackai EH, Massague J, Muenke M, Elledge SJ. Mutations in TGIF cause

holoprosencephaly and link NODAL signalling to human neural axis determination. *Nat Genet*. 2000; 25:205-8.

Grosvenor T. A review and a suggested classification system for myopia on the basis of age-related prevalence and age of onset. *Am J Optom Physiol Opt*. 1987; 64(7): 545-54.

Guggenheim JA & McBrien NA. Form-deprivation myopia induces activation of scleral matrix metalloproteinase-2 in tree shrew. *Invest Ophthalmol Vis Sci*. 1996; 37:1380-95.

Guggenheim JA, Hill C, Yam TF. "Myopia, genetics, and ambient lighting at night in a UK sample". *Br J Ophthalmol*. 2003; 87 (5): 580–2.

Gwiazda J, Ong E, Held R, Thorn F. Vision: Myopia and ambient night-time lighting. *Nature*. 2000; 404 (6774): 144.

Gwiazda J. Treatment Options for Myopia. *Optom Vis Sci*. 2009; 86(6): 624–628.

Hamilton DR, Hardten DR, Lindstrom RL. Demographics of refractive surgery: the role of phakic intraocular lenses. In: Hardten DR, Lindstrom RL, Davis EA, eds. Phakic Intraocular Lenses: Principles and Practice. Thorofare, NJ: Slack Inc; 2004.

Hammond CJ, Snieder H, Gilbert CE, Spector TD. Genes and environment in refractive error: the twin eye study. *Invest Ophthalmol Vis Sci*. 2001; 42: 1232–1236.

Hammond CJ, Andrew T, Mak YT, Spector TD. A susceptibility locus for myopia in the normal population is linked to the PAX6 gene region on chromosome 11: a genome wide scan of dizygotic twins. *Am J Hum Genet*. 2004; 75: 294-304.

Han W, Yap MK, Wang J, Yip SP. Family-based association analysis of hepatocyte growth factor (HGF) gene polymorphisms in high myopia. *Invest Ophthalmol Vis Sci*. 2006; 47: 2291-9.

Hasumi Y, Inoko H, Mano S, Ota M, Okada E, Kulski JK, Nishizaki R, Mok J, Oka A, Kumagai N, Nishida T, Ohno S, Mizuki N. Analysis of single nucleotide polymorphisms at 13 loci within the transforming growth factorinduced

factorinduced factor gene shows no association with high myopia in Japanese subjects. *Immunogenetics*. 2006; 58(12):947–953.

Hatfield EM. Why are they blind? Sight-Saving Rev. 1975; 45:3-22.

Hayashi T, Inoko H, Nishizaki R, Ohno S, Mizuki N. Exclusion of transforming growth factor-β1 as a candidate gene for myopia in the Japanese. *Jpn J Ophthalmol*. 2007; 51(2):96-99.

Hayward CP, Hassell JA, Denomme GA, Rachubinski RA, Brown C, Kelton JG. The cDNA sequence of human endothelial cell multimerin. A unique protein with RGDS, coiled-coil, and epidermal growth factor-like domains and a carboxyl terminus similar to the globular domain of complement C1q and collagens type VIII and X. *J Biol Chem*. 1995; 270:18246-18251.

Heath S, Robeldo R, Beggs W, Feola G, Parodo C, Rinaldi A, Contu L, Dana D, Stambolian D, Siniscalco M. A novel approach to search for identity by descent in small samples of patients and controls from the same Mendelian breeding unit: a pilot study on myopia. *Hum Hered*. 2001; 52:183-90.

Hersh PS, Herse P, Gothwal VK. Need for vision rehabilitation in India. *Acta Ophthalmol Scand.* 1998; 76: 606–609.

Hirsch MJ. Relation of visual acuity to myopia. *Arch Ophthalmol*. 1945; 34:418-21.

Hoffman DJ & Heath DA. Staphyloma and other risk factors in axial myopia. *J Am Optom Assoc*. 1987; 58: 907-13.

Honda S, Fujii S, Sekiya Y, Yamamoto M. Retinal control on the axial length mediated by transforming growth factor-beta in chick eye. *Invest Ophthalmol Vis Sci.* 1996; 37: 2519-26.

Hope GM, Rubin ML. Night myopia. *Surv Ophthalmol*. 1984; 29:129-36.

Hung GK, Fiedler AJ, Ciuffreda KJ. Homeomorphic Matlab Model of Myopia Development. *J Comput Sci Syst Biol*. 2010; 3: 030-039.

Hui-Min W, Yap BS, Peng-Huat E, Seang-Mei S, Tock-Han LC, Kee-Seng. Does education explain ethnic differences in myopia prevalence? A population-

based study of young adult males in Singapore. *Optom Vis Sci*. 2001; 78 (4): 234–239.

Ibay G, Doan B, Reider L, Dana D, Schlifka M, Hu H, Holmes T, O'Neill J, Owens R, Ciner E, Bailey–Wilson JE, Stambolian D. Candidate high myopia loci on chromosomes 18p and 12q do not play a major role in susceptibility to common myopia. *BMC Med Genet*. 2004; 5:20.

Inamori Y, Ota M, Inoko H, Okada E, Nishizaki R, Shiota T, Mok J, Oka A, Ohno S, Mizuki N. The COL1A1 gene and high myopia susceptibility in Japanese. *Hum Genet*. 2007; 122: 151–157.

Irving EL, Callendar MG, Sivak JG. Inducing myopia, hyperopia, and astigmatism in chicks. *Optom Vis Sci*. 1991; 68: 364-368.

Irving EL, Callender MG, Sivak JG. Inducing ametropias in hatchling chicks by defocusaperture effects and cylindrical lenses. *Vision Res*. 1995; 35: 1165-1174.

Iwase A, Araie M, Tomidokoro A, Yamamoto T, Shimizu H, Kitazawa Y. Prevalence and causes of low vision and blindness in a Japanese adult population: the Tajimi Study. *Ophthalmology*. 2006; 113:1354-62.

Jacobi FK, Zrenner E, Broghammer M, Pusch CM. A genetic perspective on myopia., *Cell Mol Life Sci*. 2005; 62: 800-808.

Jansson F. Measurement of intraocular distances by ultrasound and comparison between optical and ultrasonic determinations of the depth of the anterior chamber. *Acta Ophthalmol*. 1963; 41: 25-61.

Javitt JC & Chiang YP. The socioeconomic aspects of laser refractive surgery. *Arch Ophthalmol.* 1994; 112: 1526–1530.

Jobling AI, Nguyen M, Gentle A, McBrien NA. Isoform-specific changes in scleral transforming growth factor-beta expression and the regulation of collagen synthesis during myopia progression. *J Biol Chem*. 2004; 279:18121–18126.

Jones LA, Sinnott LT, Mutti DO, Mitchell GL, Moeschberger ML, Zadnik K. Parental History of Myopia, Sports and Outdoor Activities, and Future Myopia. *Invest Ophthalmol Vis Sci*. 2007; 48: 3524-3532 8.

Karlin DB & Curtin BJ. Peripheral chorioretinal lesions and axial length of the myopic eye. *Am J Ophthalmol*. 1976; 81:625-35.

Katz J, Tielsch JM, Sommer A. Prevalence and risk factors for refractive errors in an adult inner city population. *Invest Ophthalmol Vis Sci*. 1997; 334–340.

Kempen JH, Mitchell P, Lee KE, Tielsch JM, Broman AT, Taylor HR, Ikram MK, Congdon NG, O'Colmain BJ. Eye Diseases Prevalence Research Group. The prevalence of refractive errors among adults in the United States, Western Europe, and Australia. *Arch Ophthalmol*. 2004; 122: 495-505.

Kim JK, Kim SS, Lee HK, Lee IS, Seong GJ, Kim EK, Han SH. Laser in situ keratomileusis versus laser-assisted subepithelial keratectomy for the correction of high myopia. *J Cataract Refract Surg*. 2004; 30(7): 1405-1411.

Kim E, Naisbitt S, Hsueh YP, Rao A, Rothschild A, Craig AM, Sheng M. GKAP, a novel synaptic protein that interacts with the guanylate kinase-like domain of the PSD-95/SAP90 family of channel clustering molecules. *J Cell Biol*. 1997; 136:669-78.

Klein AP, Duggal P, Lee KE, Klein R, Bailey-Wilson JE, Klein BE. Confirmation of linkage to ocular refraction on chromosome 22q and identification of a novel linkage region on 1q. *Arch Ophthalmol*. 2007; 125:80-5.

Kubalogclu A, Yazicioglu, Tacer S. Small incision clear lens extraction for correction of high myopia. *Eur J Ophthalmol*. 2004; 14(1): 1-6.

Kusakari T, Sato T, Tokoro T. Visual deprivation stimulates the exchange of the fibrous sclera into the cartilaginous sclera in chicks. *Exp Eye Res*. 2001; 73: 533-46.

Lakhotia S & Somasundaram K. Conformation-Sensitive Gel Electrophoresis for Detecting BRCA1 Mutations. *Methods Mol Biol*. 2003; 223: 403-412.

Lam CSY & Edwards MH. Myopia - prevalence and risk factors. *Optician*. 1999; 217: 28-31.

Lam CSY & Goh WSH. The incidence of refractive errors among school children in Hong Kong and its relationship with the optical components. *Clin Exp Optom*. 1991; 74: 97-103.

Lam CY, Tam PO, Fan DS, Fan BJ, Wang DY, Lee CW, Pang CP, Lam DS. Genome-wide Scan Maps a Novel High Myopia Locus to 5p15. *Invest Ophthalmol Vis Sci.* 2008; 49:3768-78.

Lam D, Leung Y, Fan D, Baum L, Tam P, Pang C. To locate a gene for familial myopia by linkage analysis. *Clin Exp Ophthalmol.* 2002; 30:480.

Lam DS, Tam PO, Fan DS, Baum L, Leung YF, Pang CP. Familial high myopia linkage to chromosome 18p. *Ophthalmologica* 2003a; 217:115-8.

Lam DS, Lee WS, Leung YF, Tam PO, Fan DS, Fan BJ, Pang CP. TGF beta-induced factor: a candidate gene for high myopia. *Invest Ophthalmol Vis Sci*. 2003b; 44: 1012-51.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. Clustal W and Clustal X version 2.0. *Bioinformatics*. 2007; 23: 2947-2948.

Larsen JS. Axial length of the emmetropic eye and its relation to the head size. *Acta Ophthalmol*. 1979; 57: 76-83.

Leibowitz HW & Owens DA. Night myopia and the intermediate dark focus of accommodation. *J Opt Soc Am*. 1975; 65:1121-8.

Leibowitz HM, Krueger DE, Maunder LR. The Framingham eye study monograph. *Surv Ophthalmol.* 1980; 24: 472–479.

Levy JH, Pollock HM, Curtin BJ. The Fuchs' spot. *Ann Ophthalmol*. 1977; 9:1433-43.

Li CY, Lin KK, Lin YC, Lee JS. Low vision and methods of rehabilitation: a comparison between the past and present. *Chang Gung Med J*. 2002; 25(3): 153-61

Lin HJ, Wan L, Tsai Y, Tsai YY, Fan SS, Tsai CH, Tsai FJ. The TGF β1 gene codon 10 polymorphism contributes to the genetic predisposition to high myopia. *Mol Vis.* 2006; 12: 698-703.

Lin HJ, Wan L, Tsai Y, Su-Ching L, Wen-Chi C, Shih-Wei T, Fuu-Jen T. Sclera-related gene polymorphisms in high myopia. *Mol Vis*. 2009; 15:1655-1663.

Li J, Zhang QJ, Xiao XS, Li JZ, Zhang FS, Li SQ, Li W, Li T, Jia XY, Guo L, Guo XM. The SNPs analysis of encoding sequence of interacting factor gene in Chinese population. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi*. 2003; 20(5):454–456.

Lin LLK, Tsai CB, Lieu JC, Shih YF, Hung PT. Correlation between ocular refractions with longitudinal study among school children in Tiawan. In: Myopia Updates. Springer-Verlag, Tokyo. 1998; 53-57.

Lin LL, Shih YF, Tsai CB, Chen CJ, Lee LA, Hung PT, Hou PK. Epidemiologic study of ocular refraction among school children in Taiwan in 1995. *Optom Vis Sci.* 1999; 76: 275-81.

Lin LL, Shih YF, Hsiao CK, Chen CJ. Prevalence of myopia in Taiwanese schoolchildren: 1983 to 2000. *Ann Acad Med Singapore.* 2004; 33:27-33.

Lin LL, Chen CJ, Hung PT, Ko LS. Nation-wide survey of myopia among school children in Taiwan. *Acta Ophthalmol*. 1988; 66: 29–33.

MacDonald AE. Causes of blindness in Canada. Can Med Assoc J. 1965; 92:264-79.

Mantyjarvi MI. Changes in refraction in schoolchildren. *Arch Ophthalmol.* 1985; 103: 790-792.

Maruyama K. Connectin, an elastic filamentous protein of striated muscle. *Int Rev Cytol.* 1986; 104:81-114.

Matsumura H & Hirai H. Prevalence of myopia and refractive changes in students from 3 to 17 years of age. *Surv Ophthalmol*. 1999; 44: 109-115.

McBrien NA & Norton TT. The development of experimental myopia and ocular component dimensions in monocularly lid-suture tree shrews Tupaia belangeri). *Vision Res*. 1992; 32: 843-852.

McBrien NA & Gentle A. Role of the sclera in the development and pathological complications of myopia. *Progress in Retinal and Eye Research*. 2003; 22(3): 307-38.

Morgan IG. The biological basis of myopic refractive error. *Clin Exp Optom*. 2003; 86: 276–288.

Muenke M & Beachy PA. Genetics of ventral forebrain development and holoprosencephaly. *Curr Opin Genet Dev*. 2000; 10:262-9.

Mutti DO, Cooper ME, O'Brien S, Jones LA, Marazita ML, Murray JC, Zadnik K. Candidate gene and locus analysis of myopia. *Mol Vis*. 2007; 13: 1012-9.

Mutti DO, Mitchell GL, Moeschberger ML, Jones LA, Zadnik K. Parental myopia, near work, school achievement, and children's refractive error. *Invest Ophthalmol Vis Sci*. 2002; 43: 3633–3640.

Mutti DO, Zadnik K, Adams AJ. Myopia : The nature versus nurture debate goes on. *Invest Ophthalmol Vis Sci*. 1996; 37: 952-7.

Naiglin L, Gazagne C, Dallongeville F, Thalamas C, Idder A, Rascol O, Malecaze F, Calvas P. A genome wide scan for familial high myopia suggests a novel locus on chromosome 7q36. *J Med genet.* 2002; 39: 118-124.

Nallasamy S, Paluru P, Devoto M, Wasserman NF, Zhou J, Young TL. Genetic linkage of high-grade myopia in a Hutterite population from South Dakota. *Mol Vis.* 2007; 13:229-36.

Ng TK, Lam CY, Lam DSC, Chiang SWY, Tam POS, Wang DY, Fan BJ, Yam GHF, Fan DSP, Pang CP. AC and AG dinucleotide repeats in the PAX6 P1 promoter are associated with high myopia. *Mol Vis*. 2009; 15: 2239-48.

Nishizaki R, Ota M, Inoko H, Meguro A, Shiota T, Okada E, Mok J, Oka A, Ohno S, Mizuki N. New susceptibility locus for high myopia is linked to the uromodulin-like 1(UMODL1) gene region on chromosome 21q22.3. *Eye*. 2009; 23:222-9.

Norton TT. Animal models of myopia: learning how vision controls the size of the eye. *ILAR J*. 1999; 40:59-77.

Nurnberg G, Jacobi FK, Broghammer M, Becker C, Blin N, Nurnberg P, Stephani U, Pusch CM. Refinement of the MYP3 locus on human chromosome 12 in a German family with Mendelian autosomal dominant high-grade myopia by SNP array mapping. *Int J Mol Med*. 2008; 21:429-38.

O'Brart DP, Al-Attar M, Hussein B, Angunawela R, Marshall J. Laser subepithelial keratomileusis for the correction of high myopia with the Schwind ESIRIS scanning spot laser. *J Refract Surg*. 2006; 22(3): 253-262.

O'Brien TP & Awwad ST. Phakic intraocular lenses and refractory lensectomy for myopia. *Curr Opin Ophthalmol.* 2002; 13(4): 264-270.

Oshika T, Klyce SD, Applegate RA, Howland HC, El Danasoury MA. Comparison of corneal wavefront aberrations after photorefractive keratectomy and laser in situ keratomileusis. *Am J Ophthalmol.* 1999; 127(1): 1-7.

Overall CM, Wrana JL & Sodek, J. Independent regulation of collagenase, 72-kDa progelatinase, and metalloendoproteinase inhibitor expression in human fibroblasts by transforming growth factor β. *J Biol Chem.* 1989; 264(3), 1860-1869.

Overhauser J, Mitchell HF, Zackai EH, Tick DB, Rojas K, Muenke M. Physical mapping of the holoprosencephaly critical region in 18p11.3. *Am J Hum Genet*. 1995; 57:1080-5.

Owens DA & Leibowitz HW. Night myopia: cause and a possible basis for amelioration. *Am J Optom Physiol Opt.* 1976; 53:709- 17.

Paget S, Julia S, Vitezica ZG, Soler V, Malecaze F, Calvas P. Linkage analysis of high myopia susceptibility locus in 26 families. *Mol Vis*. 2008; 14: 2566-2574.

Paluru PC, Nallasamy S, Devoto M, Young T. Novel locus maps to chromosome 2q37 for autosomal dominant high myopia. *Invest Ophthalmol Vis Sci*. 2005; 46: 2300-2307.

Paluru P, Ronan SM, Heon E, Devoto M, Wildenberg SC, Scavello G, Holleschau A, Makitie O, Cole WG, King RA, Young TL. New locus for autosomal

dominant high myopia maps to the long arm of chromosome 17. *Invest Ophthalmol Vis Sci.* 2003; 44: 1830-1836.

Palmer EA, Patz A, Phelps DL, Spencer R. Retinopathy of prematurity. *In: Retina,* 1994; 2: 1473–1498.

Pararajasegaram R. The right to sight: from strategies to action. *Am J Ophthalmol*. 1999; 182: 359-60.

Patel A, Scott WR, Lympany PA, Rippin JD, Gill GV, Barnett AH, Bain SC. The TGFbeta 1 gene codon 10 polymorphism contributes to the genetic predisposition to nephropathy in Type 1 diabetes. *Diabet Med.* 2005; 22(1):69-73.

Perkins ES. Glaucoma in the younger age groups. *Arch Ophthalmol*. 1960a; 64:882-91.

Perkins ES & Jay BS. Pigmentary glaucoma. *Trans Ophthalmol Soc*. 1960b; 80:153-67.

Perkins ES. Morbidity from myopia. Sight-Saving Rev. 1979; 49:11-9.

Perkins ES & Phelps CD. Open angle glaucoma, ocular hypertension, low tension glaucoma and refraction. *Arch Ophthalmol.* 1982; 100:1464-7.

Pertile KK, Schache MF, Islam MA, Chen CY, Dirani M, Mitchell P, Baird PN. Assessment of TGIF as Candidate Gene for Myopia. *Invest Ophthalmol Vis Sci.* 2008; 49:1

Peters HB. The relationship between refractive error and visual acuity at three age levels. *Am J Optom.* 1961; 38:194 -8.

Pop M & Payette Y. Risk factors for night vision complaints after LASIK for myopia. *Ophthalmology*. 2004; 111(1): 3-10.

Price MG & Gomer RH. Skelemin, a cytoskeletal M-disc periphery protein, contains motifs of adhesion/recognition and intermediate filament proteins. *J Biol Chem*. 1993; 268:21800-10.

Pusch CM, Zeitz C, Brandau O, Pesch K, Achatz H, Feil S, Scharfe C, Maurer J, Jacobi FK, Pinckers A, Andreasson S, Hardcastle A, Wissinger B, Berger W,

Meindl A. The complete form of X-linked congenital stationary night blindness is caused by mutations in a gene encoding a leucine-rich repeat protein. *Nat Genet.* 2000; 26: 324–327.

Quinn GE, Shin CH, Maguire MG, Stone RA. Myopia and ambient lighting at night. *Nature*. 1999; 399 (6732): 113–4.

Rada JA, Shelton S, Norton TT. The sclera and myopia. *Experimental Eye Res*. 2006; 82(2): 185-200.

Raviola E & Wiesel TN. An animal model of myopia. *N Engl J Med.* 1985; 312: 1609-1615.

Richler A & Bear JC. Refraction, nearwork and education. A population study in Newfoundland. *Acta Ophthalmol.* 1980; 58: 468-78.

Risch N & Merikangas K. The future of genetic studies of complex human diseases. *Science*. 1996; 273:1516-7.

Saika, S. TGFβ pathobiology in the eye. Lab Invest. 2006; 86(2): 106-115.

Sandhya A, Bindu CH, Reddy KP, Vishnupriya S. TGFB1 codon 10 polymorphism and its association with the development of myopia: a case-control study. *Biol and Med*. 2011; 3 (4): 18-24.

Saw SM, Chua WH, Wu HM, Yap E, Chia KS, Stone RA. Myopia: gene-environment interaction. *Ann Acad Med Singapore*. 2000; 29: 290-7.

Saw SM, Hong CY, Chia KS, Stone RA, Tan D. Nearwork and myopia in young children. *Lancet*. 2001; 357(9253):390.

Saw SM, Chua WH, Hong CY, Wu HM, Chan WY, Chia KS, Stone RA, Tan D. Nearwork in early-onset myopia. *Invest Ophthalmol Vis Sci*. 2002; 43, 332–339.

Saw SM. A synopsis of the prevalence rates and environmental risk factors for myopia. *Clin Exp Optom*. 2003; 86(5): 289-94.

Saw SM, Gazzard G, Shih-Yen EC, Chua WH. Myopia and associated pathological complications. *Ophthal Physiol Opt*. 2005; 25: 381.

Sa Q & Hoover-Plow JL. EMILIN2 (Elastin microfibril interface located protein), potential modifier of thrombosis. *Thrombosis*. 2011; 9:9.

Scavello GS, Paluru PC, Ganter WR, Young TL. Sequence variants in the transforming growth beta-induced factor (TGIF) gene are not associated with high myopia. *Invest Ophthalmol Vis Sci*. 2004; 45(7):2091–2097.

Scavello GS, Paluru PC, Zhou J, White PS, Rappaport EF, Young TL. Genomic structure and organization of the high grade Myopia-2 locus (MYP2) critical region: mutation screening of 9 positional andidate genes. *Mol Vis*. 2005; 11: 97-110.

Schache M, Chen CY, Pertile KK, Richardson AJ, Dirani M, Mitchell P, Baird PN. Fine mapping linkage analysis identifies a novel susceptibility locus for myopia on chromosome 2q37 adjacent to but not overlapping MYP12. *Mol Vis*. 2009; 15:722-730.

Schaeffel F, Simon P, Feldkaemper M, Ohngemach S, Williams RW. Molecular biology of myopia. *Clin Exp Optom*. 2003; 86: 295-307.

Schaeffel F, Glasser A, Howland HC. Accommodation, refractive error and eye growth in chickens. *Vis Res*. 1988; 28: 639–657.

Schor C. The influence of interactions between accommodation and convergence on the lag of accommodation. *Ophthalmic Physiol.* 1998; 19: 1–17.

Schwartz M, Haim M, Skarscholm D. X-linked myopia: Bornholm eye diseases-linkage to DNA markers on the distal part on Xq. *Clinical Genet*. 1990; 38: 281-286.

Seko Y, Shimokawa H, Tokoro T. Expression of bFGF and TGFbeta 2 in experimental myopia in chicks. *Invest Ophthalmol Vis Sci*. 1995; 36:1183–1187.

Seiler T & Quurke AW. Iatrogenic keratectasia after LASIK in a case of forme fruste keratoconus. *J Cataract Refract Surg*, 1998; 24(7): 1007-1009.

Shapiro M, Chandra SR. Evolution of lacquer cracks in high myopia. *Ann Ophthalmol*. 1985; 17:231-5.

Sherman SM, Norton TT, Casagrande VA. Myopia in the lid-sutured tree shrew (Tupaia glis). *Brain Res*. 1977; 124: 154-157.

Siegwart JT & Norton TT. Refractive and ocular changes in tree shrews raised with plus or minus lenses. *Invest Ophthalmol Vis Sci*. 1993; 34: 1208.

Silva RA, Jain A, Manche EE. Prospective Long-term Evaluation of the Efficacy,Safety, and Stability of the Phakic Intraocular Lens for High Myopia. *Arch Ophthalmol*. 2008; 126(6): 775-781.

Song QH, Singh RP, Richardson TP, Nugent MA, Trinkaus-Randall V. Transforming growth factor-beta 1 expression in cultured corneal fibroblasts in response to injury. *J Cell Biochem*. 2000; 77: 186–199.

Sorsby A, Benjamin B, Davey JB, et al. Emmetropia and its aberrations. Medical Research Council Special Report Series no 293. London: Her Majesty's Stationery Office, 1957.

Sorsby A. Department of Health and Social Security. Reports on public health and medical subjects, no. 128. The incidence and causes of blindness in England and Wales 1963-68, with an appendix on services available for incipient blindness. London: Her Majesty's Stationery Office. 1972; 128:1-72.

Sorsby A, Sheridan M, Leary G. Refraction and its components in twins. MRC Report No 303, 1962a.

Sorsby A, Leary GA, Richards MJ. Correlation ametropia and component ametropia. *Vision Res*. 1962b; 2: 309–313.

Speel EJ, van der Ven PF, Albrechts JC, Ramaekers FC, Furst DO, Hopman AH. Assignment of the human gene for the sarcomeric M-band protein myomesin (MYOM1) to 18p11.31- p11.32. *Genomics*. 1998; 54:184-6.

Sperduto RD, Seigel D, Roberts J, Rowland M. Prevalence of myopia in the United States. *Arch Ophthalmol.* 1983; 101: 405–407.

Stambolian D, Ibay G, Reider L, Dana D, Moy C, Schlifka M, Holmes T, Ciner E, Bailey-Wilson JE. Genomewide Linkage Scan for Myopia Susceptibility Loci

among Ashkenazi Jewish Families Shows Evidence of Linkage on Chromosome 22q12. *Am J Hum Genet*. 2004; 75:448-59.

Stambolian D, Ciner EB, Reider LC, Moy C, Dana D, Owens R, Schlifka M, Holmes T, Ibay G, Bailey-Wilson JE. Genome-wide scan for myopia in the Old Order Amish. *Am J Ophthalmol. 2005*; 140:469-76.

Stambolian D, Ibay G, Reider L, Dana D, Moy C, Schlifka M, Holmes TN, Ciner E, Bailey-Wilson JE. Genome-wide scan of additional Jewish families confirms linkage of a myopia susceptibility locus to chromosome 22q12. *Mol Vis*. 2006; 12: 1499-505.

Stenstrom S. Investigation of the variation and the correlation of the optical elements of human eyes. *Am J Optom*. 1948; 25:340-50.

Stenson SM & Raskind RH. Pseudomyopia: etiology, mechanisms and therapy. *J Pediatr Ophthalmol*. 1970; 7:110-5.

Strom TM, Nyakatura G, Apfelstedt-Sylla E, Hellebrand H, Lorenz B, Weber BH, Wutz K, Gutwillinger N, Ruther K, Drescher B, Sauer C, Zrenner E, Meitinger T, Rosenthal A, Meindl A. An L-type calciumchannel gene mutated in incomplete X-linked congenital stationary night blindness. *Nat Genet.* 1998; 19: 260–263.

Stulting RD, Carr JD, Thompson KP, Waring GO, Wiley WM, Walker JG. Complications of laser in situ keratomileusis for the correction of myopia. *Ophthalmology*, 1999; 106 (1): 13-20.

Su J, Wall ST, Healy KE, Wildsoet CF. Scleral Reinforcement Through Host Tissue Integration with Biomimetic Enzymatically Degradable Semi-Interpenetrating Polymer Network. *Tissue Engineering*, 2010; 16: 3.

Tanihara H, Yoshida M, Matsumoto M, Yoshimura N. Identification of transforming growth factor-beta expressed in cultured human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 1993; 34(2): 413–419.

Tahzib NG, Nuijts RM, Wu WY, Budo CJ. Long-term study of Artisan phakic intraocular lens implantation for the correction of moderate to high myopia. *Ophthalmology*. 2007; 114(6): 1133-1142.

Tan DTH. The future is near: focus on myopia Singapore. Med J, 2004; 45(10): 451.

Tan NW, Saw SM, Lam DS, Cheng HM, Rajan U, Chew SJ. Temporal variations in myopia progression in Singaporean children within an academic year. *Optom Vis Sci*. 2000; 77: 465-472.

Tang WC, Yap MK, Yip SP. A review of current approaches to identifying human genes involved in myopia. *Clin Exp Optom*. 2008; 91: 4-22.

Teikari J, O'Donnell JJ, Kaprio J, Koskenvuo M. Genetic and environmental effects on oculometric traits. *Optom Vis Sci.* 1989; 66: 594-599.

Teikari JM, O'Donnell J, Kaprio J, Koskenvuo M. Impact of heredity in myopia. Hum Hered. 1991; 41: 151–156.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*. 1997; 25: 4876-4882.

Tokoro T & Sato A. Results of Investigation of Pathologic Myopia in Japan. Report of Myopic Chorioretinal Atrophy. Tokyo: Ministry of Health and Welfare, 1982; 32–35.

Trinick J. Elastic filaments and giant proteins in muscle. *Curr Opin Cell Biol*. 1991; 3:112-9.

Troilo D & Wallman J. The regulation of eye growth and refractive state: an experimental study of emmetropization. *Vision Res.* 1991; 31: 1237–1250.

Verma A & Singh D. Myopia, Phakic IOL.eMedicine.com, 2005.

Vukojevic N, Sikic J, Curkovic T, Juratovac Z, Katusic D, Saric B, Jukic T. Axial eye length after retinal detachment surgery. *Collegium antropologicum*. 2005; 25–27.

Wallman J & Adams JI. Developmental aspects of experimental myopia in chicks: susceptibility, recovery and relation to emmetropization. *Vision Res*. 1987; 27: 1139-1163.

Wallman J. Retinal control of eye growth and refraction. *Prog Ret Res*. 1993; 12:134-53.

Wallman J & Mc Fadden S. Monkey eyes grow into focus. *Nat Med*. 1995; 1:737-739.

Wallman J, Wildsoet C, Xu A, Gottlieb MD, Nickla DL, Marran L, Krebs W, Christensen AM. Moving the retina: choroidal modulation of refractive state. *Vision Res*. 1995; 35: 37-50.

Wang K. Sarcomere-associated cytoskeletal lattices in striated muscle. Review and hypothesis. *Cell Muscle Motil*. 1985; 6:315-69.

Wang Q, Klein BE, Klein R, Moss SE. Refractive status in the Beaver Dam Eye Study. *Invest Ophthalmol Vis Sci*. 1994; 35, 4344–4347.

Wang P, Li S, Xiao X, Jia X, Jiao X, Guo X, Zhang Q. High myopia is not associated with the SNPs in the TGIF, Lumican, TGFB1, and HGF genes. *Invest Oph Vis Sci*. 2008; 08-2537.

Wang P, Li S, Xiao X, Jia X, Jiao X, Guo X, Qingjiong Zhang Q. High myopia is not associated with SNPs in the TGIF, Lumican, TGFB1 and HGF Genes. *Invest Ophthalmol Vis Sci*. 2009; 50: 4.

Wensor M, McCarty CA, Taylor HR. Prevalence and Risk Factors of Myopia in Victoria, Australia**.** *Arch Ophthalmol.* 1999; 117: 658-663.

Wildsoet CF. Active emmetropization: evidence for its existence and ramifications for clinical practice. *Ophthalmic Physiol Opt*. 1997; 17: 279-290.

Wilson A & Woo G. A Review of the prevalence and causes of myopia. *Singapore Med J.* 1989; 30: 479-484.

Wojciechowski R, Moy C, Ciner E, Ibay G, Reider L, Bailey-Wilson JE, Stambolian D. Genomewide scan in Ashkenazi Jewish families demonstrates evidence of

linkage of ocular refraction to a QTL on chromosome 1p36. *Hum Genet.* 2006; 119: 389-399.

Wong TY, Foster PJ, Hee J, Ng TP, Tielsch JM, Chew SJ, Johnson GJ, Seah SK. Prevalence and risk factors for refractive errors in adult Chinese in Singapore. *Invest Ophthalmol Vis Sci*. 2000; 41:2486-94.

Wotton D, Lo RS, Swaby LA, Massague J. Multiple modes of repression by the Smad transcriptional corepressor TGIF. *J Biol Chem.* 1999(a); 274:37105-10.

Wotton D, Lo RS, Lee S, Massague J. A Smad transcriptional corepressor. *Cell*. 1999(b); 97:29-39.

Xu L, Li J, Cui T, Hu A, Fan G, Zhang R, Yang H, Sun B, Jonas JB. Refractive error in urban and rural adult Chinese in Beijing. *Ophthalmology*. 2005; 112(10): 1676–83.

Yamane T, Mok J, Oka A, Okada E, Nishizaki R, Meguro A, Yonemoto J, Kulski JK, Ohno S, Inoko H, Mizuki N. Lack of association with high myopia and the MYP2 locus in the Japanese population by high resolution microsatellite analysis on chromosome 18. *Clin Ophthalmol*. 2007; 1(3): 311–316.

Yap M, Wu M, Liu ZM, Lee FL, Wang SH. Role of heredity in the genesis of myopia. *Ophthalmic Physiol Opt.* 1993; 13: 316-9.

Yokoyama A, Maruiwa F, Hayakawa M, Kanai A, Vervoort R, Wright AF, Yamada K, Niikawa N, Naoi N. Three novel mutations of the RPGR gene exon ORF15 in three Japanese families with X-linked retinitis pigmentosa. *Am J Med Genet.* 2001; 104 (3): 232–238.

Young TL, Ronan SM, Drahozal LA, Wildenberg SC, Alvear AB, Oetting WS, Atwood LD, Wilkin DJ, King RA. Evidence that a locus for familial high myopia maps to chromosome 18p.*Am J Hum Genet.* 1998a; 63: 109-119.

Young TL, Ronan SM, alvear AB, Wildenberg SC, Oetting WS, Atwood LD, Wilkin DJ, King RA. A second locus for familial high myopia maps to chromosome 12q. *Am J Hum Genet*. 1998b; 63: 1419-1424.

Young TL, Atwood LD, Ronan SM, Dewan AT, Alvear AB, Peterson J, Holleschau A, King RA. Further refinement of the MYP2 locus for autosomal dominant high myopia by linkage disequilibrium analysis. *Ophthalmic Genet*. 2001; 22:69-75.

Young TL. Dissecting the Genetics of human high myopia: a molecular biologic approach. *Trans Am Ophthalmol Soc.* 2004; 102: 423-446.

Young TL. Metlapally R, Shay AE. Complex trait genetics of refractive error. *Arch Ophthalmol*. 2007; 125: 38-48.

Young TL. Molecular Genetics of Human Myopia: An Update. *Optom Vis Sci*. 2009; 86:1.

Zadnik K, Satariano WA, Mutti DO, Sholtz RI, Adams AJ. The effect of parental history of myopia on children's eye size. *JAMA*. 1994; 271: 1323-7.

Zadnik, K. Myopia development in childhood. *Optom Vis Sci*. 1997; 74(8): 603-608.

Zadnik K, Jones LA, Irvin BC, Kleinstein RN, Manny RE, Shin JA, Mutti DO. Myopia and ambient night-time lighting. CLEERE Study Group. Collaborative Longitudinal Evaluation of Ethnicity and Refractive Error. *Nature*. 2000; 404 (6774): 143–4.

Zha Y, Leung KH, Lo KK, Fung WY, Ng PW, Shi MG, Yap MK, Yip SP. TGFβ1 as a Susceptibility Gene for High Myopia; A Replication study with new findings. *Arch ophthalmol*. 2009; 127(4): 541-8.

Zhang Q, Guo X, Xiao X, Jia X, Li S, Hejtmancik JF. A new locus for autosomal dominant high myopia maps to 4q22–q27 between D4S1578 and D4S1612. *Mol Vis*. 2005; 11: 554–560.

Zhang Q, Guo X, Xiao X, Jia X, Li S, Hejtmancik JF. Novel locus for X linked recessive high myopia maps to Xq23-q25 but outside MYP1. *J Med Genet.* 2006; 43 e20; doi: 10.1136.

Zhang Q, Li S, Xiao X, Guo X. Confirmation of a genetic locus for X-linked recessive high myopia outside MYP1. *J Hum Genet*. 2007a; 52: 469-72.

Zhang Y. Template-based modeling and free modeling by I-TASSER in CASP7. *Proteins*. 2007b; 8:108-117.

Zhang Y. I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics*. 2008; 9:40.

Zhou J, Rappaport EF, Tobias JW, Young TL. Differential Gene Expression in Mouse Sclera during Ocular Development. *Invest Ophthalmol Vis Sci*. 2006; 47: 5.

Website references

- www.ncbi.nlm.nih.gov
- zhang.bioinformatics.ku.edu/I-TASSER
- Entrez Gene: DLGAP1 discs, large (Drosophila) homolog-associated protein 1
- Entrez Gene: MYOM1 myomesin 1 (skelemin) 185kDa

Supplementary

S1. Chemicals & Reagents

S1.1. Chemicals

Chemical Name	Company
Absolute ethanol	Jiangsu Huaxi International
Acrylamide	Sisco Research Laboratories (SRL)
Acetic Acid	Spectrochem
Agarose	SRL
Ammonium Chloride	Qualigens
Ammonuim persulphate	SRL
Ammonium acetate	Qualigens
Bisacrylamide	Qualigens
Betaine	Sigma
Bromophenol Blue	Sigma
Chloroform	Qualigens
Dimethyl sulfoxide	Biogene
Dithiothritol	Sigma
Ethidium Bromide	Spectrochem
Ethylene diamine tetra acetate	Himedia
Ethylene Glycol	Qualigens
Formamide	Amresco
Formaldehyde	BDH (MERCK, India)
Glycerol	Qualigens
Isoamyl alcohol	Qualigens
Magnesium Acetate	SRL
Phenol	Sigma, SD Fine
Pottassium bicarbonate	Qualigens
Sodium acetate	CDH
Sodium bisulphite	CDH
Sodium chloride	SD Fine
Sodium dodecyl sulphate	CDH
Sodium hydroxide	SD Fine
Silver nitrate	CDH
Sucrose	Qualigens
Taurine	Fluka (Sigma Aldrich)
TEMED (Tetramethylethylenediamine)	Hi Media
Tris Base	Qualigens
Xylene cyanol FF	Bangalore GENEI
Enzymes:	
Taq Polymerase	Fermentas
Proteinase K	Biogene
Miscelllaneous Materials	
100 bp DNA Ladder	Fermentas
dNTP mix	Fermentas
Taq Buffer	Fermentas
HindIII digest DNA marker	Fermentas

S1.2. Reagents

S1.3. Reagents for DNA Extraction

1X Tris EDTA (TE) (pH 8).
Lysis buffer.

Proteinase K.
TE saturated phenol.
TE saturated phenol-chloroform-isoamylalcohol (25:24:1).
Chloroform-isoamylalcohol (24:1).
3 M Sodium acetate solution (pH 5.2).
Absolute ethanol.
70 % Ethanol.
96% Ethanol
6M NaCl
Detergent

S1.4. Reagents for Agarose Gel Electrophoresis

1.5 % Agarose.
1X Tris Acetae EDTA (TAE).
Ethidium bromide.

S1.5. Reagents for DNA Amplification

dNTP mix (dATP, dTTP, dGTP, dCTP in equal molar concentrations)
Primers (Forward and Reverse)
Taq polymerase
Taq Buffer (Tris-HCl, KCl, $(NH_4)_2SO_4$, 1.5mM $MgCl_2$) (pH 8.7)

S1.6. Reagents for PCR Purification

2 % Agarose.
Tris Acetae EDTA (TAE)
Sodium iodide solution (Sodium iodide, sodium bisulphate).
Glass milk (Nitric acid treated Glass beads).
Wash buffer (Tris HCl pH 7.4 and Absolute Ethanol in 1:1 ratio).

S1.7. Reagents for Conformation Sensitive Gel Electrophoresis

40 % Acrylamide Mix (99:1).
Ethylene Glycol.
Formamide.
20X Tris Taurine EDTA (TTE) (44 mM Tris, 14.5 mM Taurine,
0.1mM EDTA buffer, pH 9.0).
10% Ammonium persulphate.
TEMED.
10X Stock loading buffer (10X stock solution of 30%, Glycerol, 0.25%, Bromophenol blue, 0.25% Xylene cyanol FF).
0.5X TTE as Electrode buffer.

S1.8. Reagents for Silver Staining

Fixing Solution (10% Absolute Ehanol, 1% Acetic acid).
Staining Solution (0.4 % Silver Nitrate).
Developing Solution (1.2% Sodium hydroxide, 1µl/ml Formaldehyde).
Reaction Stop Solution (10% Acetic acid)

S1.9.Instruments

Slit Lamp, Autorefractor/autokeratometer, A-ultrasonography, Streak retinoscope, Fundus camera Centrifuge, Eppendorf, Microfuge, Thermocycler, Electrophoresis set, Wealter Corp Taiwan, Gel-doc,ImageMaster VDS, Incubator Memmert Germany, UV-Vis spectrophotometer Scimadzu Japan, Vortex-2 GENIE Science Industries Inc., USA,White/Ultraviolet Transilluminator Bio Doc ITTM system USA, Waterbath

S1.10. Computer software used

Chromas.MFC. Version 2.22, Technelysium Pty Ltd, USA.
Chromas Pro. Clustel IX, Primer 3.

S2. Genomic DNA

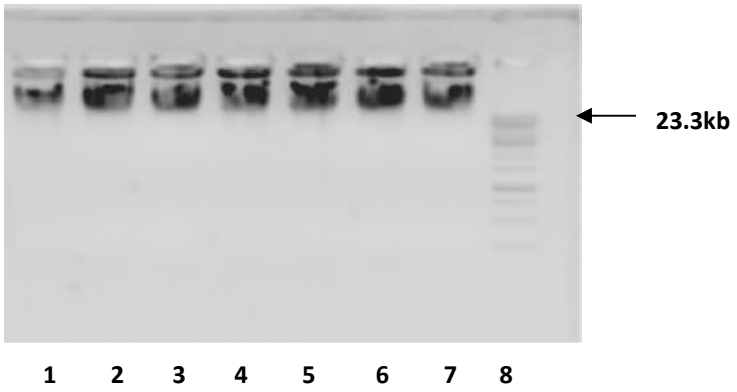
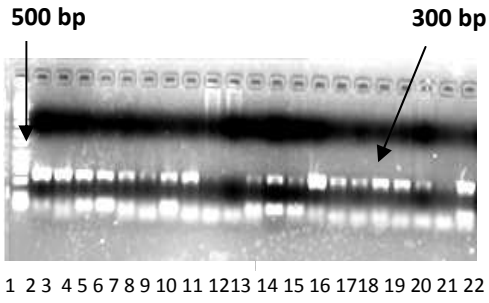
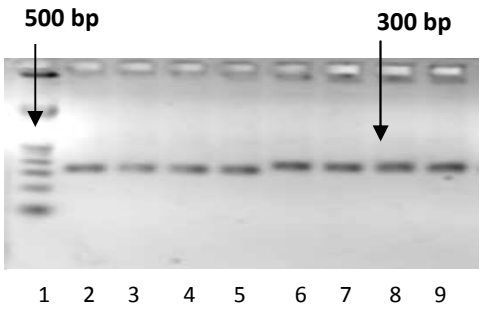


Figure S2. Genomic DNA extracted from blood samples run on 0.8% agarose gel. Lane 8 shows separation pattern of Hind III digested λ DNA and sample DNA was run in the remaining lanes.

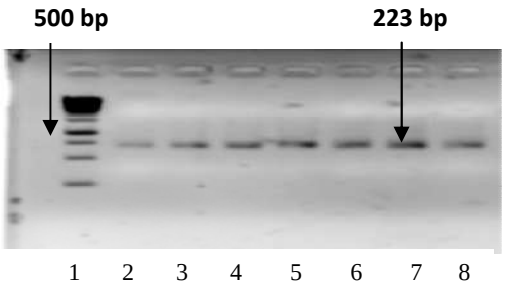
S3. Amplification



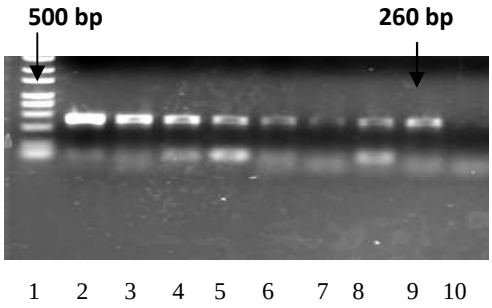
S3a



S3b



S3c



S3d

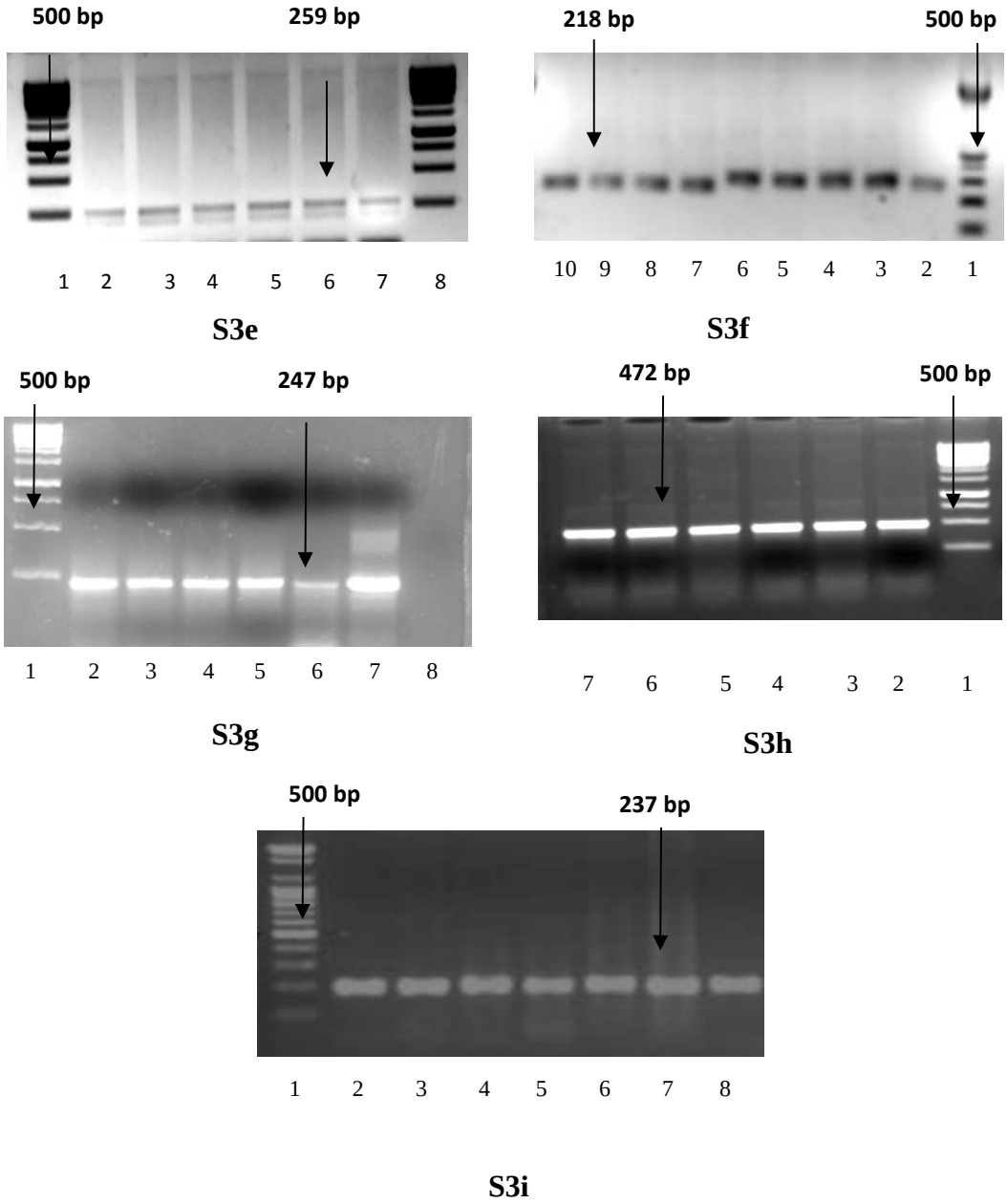
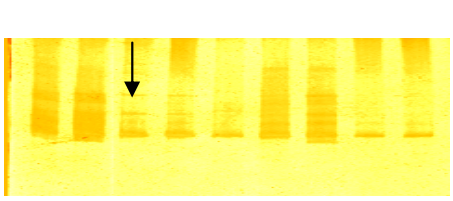


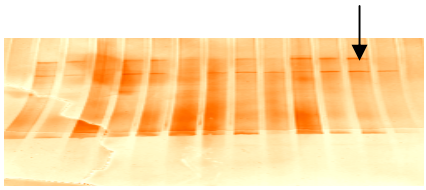
Figure S3. DLGAP1 (S3a), EMILIN2 (S3b), MYOM1 Exons 2 (S3c), 4 (S3d), 19 (S3e) and 29 (S3f), TGIF1 exon2 (S3g) & 6 (S3h) and TGFβ1 exon1 (S3i) amplification products with 100 bp/1kb DNA ladder as marker. Lane 1 shows the separation pattern of DNA ladder in all the gel pictures while rest of the lanes in each gel show analysis of 5µl aliquot of PCR product.

S4. Heteroduplex analysis



1 2 3 4 5 6 7 8 9

S4a)

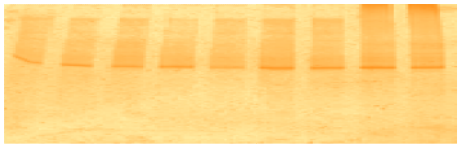


1 2 3 4 5 6 7 8 9 10 11 12 13

S4b)

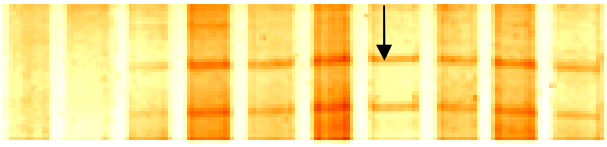
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

S4c)



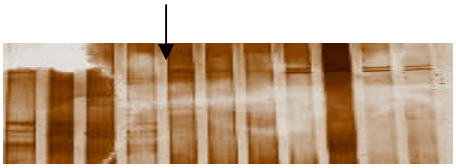
1 2 3 4 5 6 7 8 9

S4d)



1 2 3 4 5 6 7 8 9 10

S4e)



1 2 3 4 5 6 7 8 9 10 11

S4f)

1 2 3 4 5 6 7 8 9

S4g)

Figure S4. Heteroduplex analysis of different amplicons by conformation sensitive gel electrophoresis. Different heteroduplex patterens (indicated by arrows in the above fig.'s) were obtained which were suggestive of the presence of variation. Fig. S4c & S4d shows the CSGE pattern of normal controls while Fig. S4a, S4b, S4e, S4f & S4g show the heteroduplex analysis of myopia samples for DLGAP1, EMILIN2 & MYOM1.

S5. Sequencing

1) DLGAP1

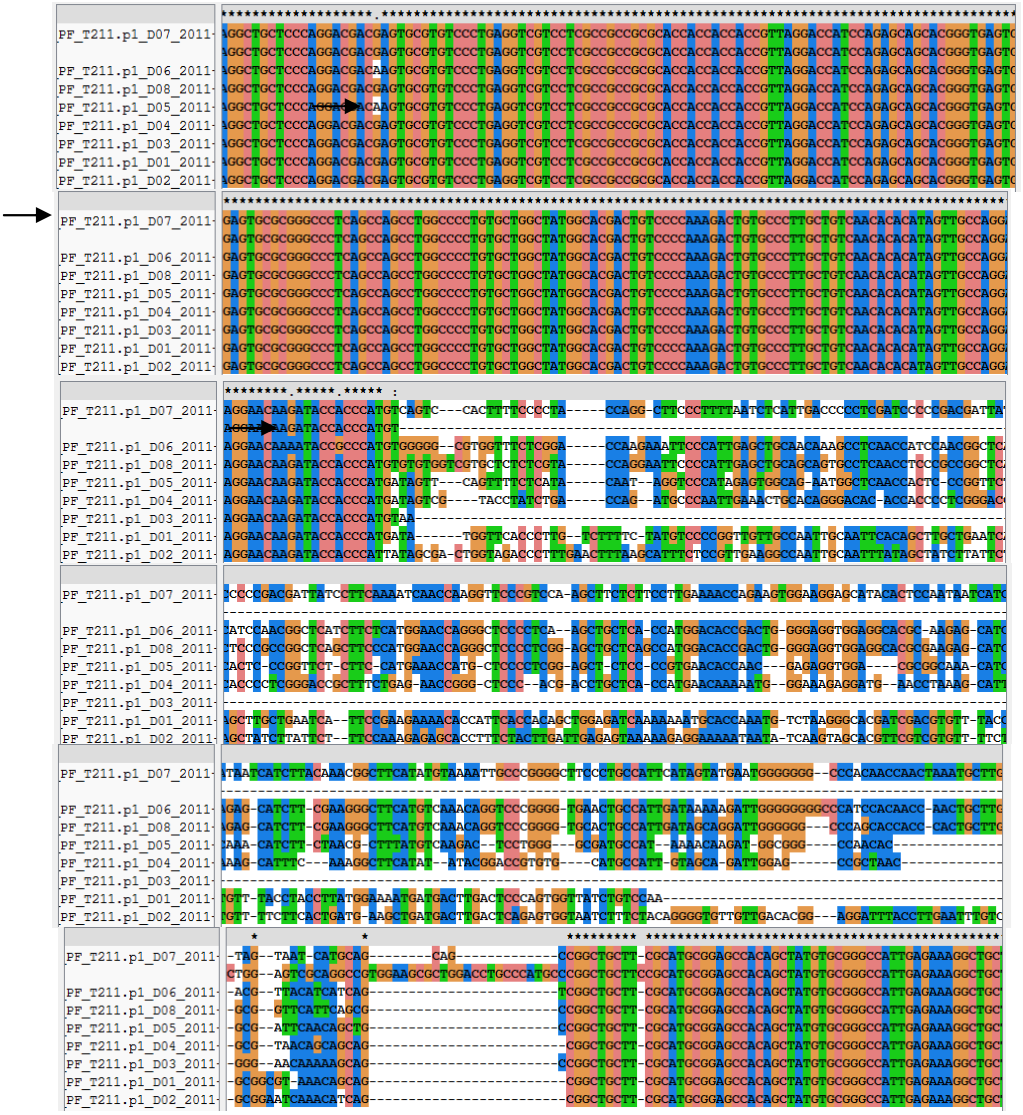


Figure S5A. Representing the multiple sequence alignment of samples generated from Exon 4 (DLGAP1) amplification. The fasta sequences of samples were aligned with the reference sequence (pointed out by black arrow head), using ClustalX software, SNPs are indicated by arrows.

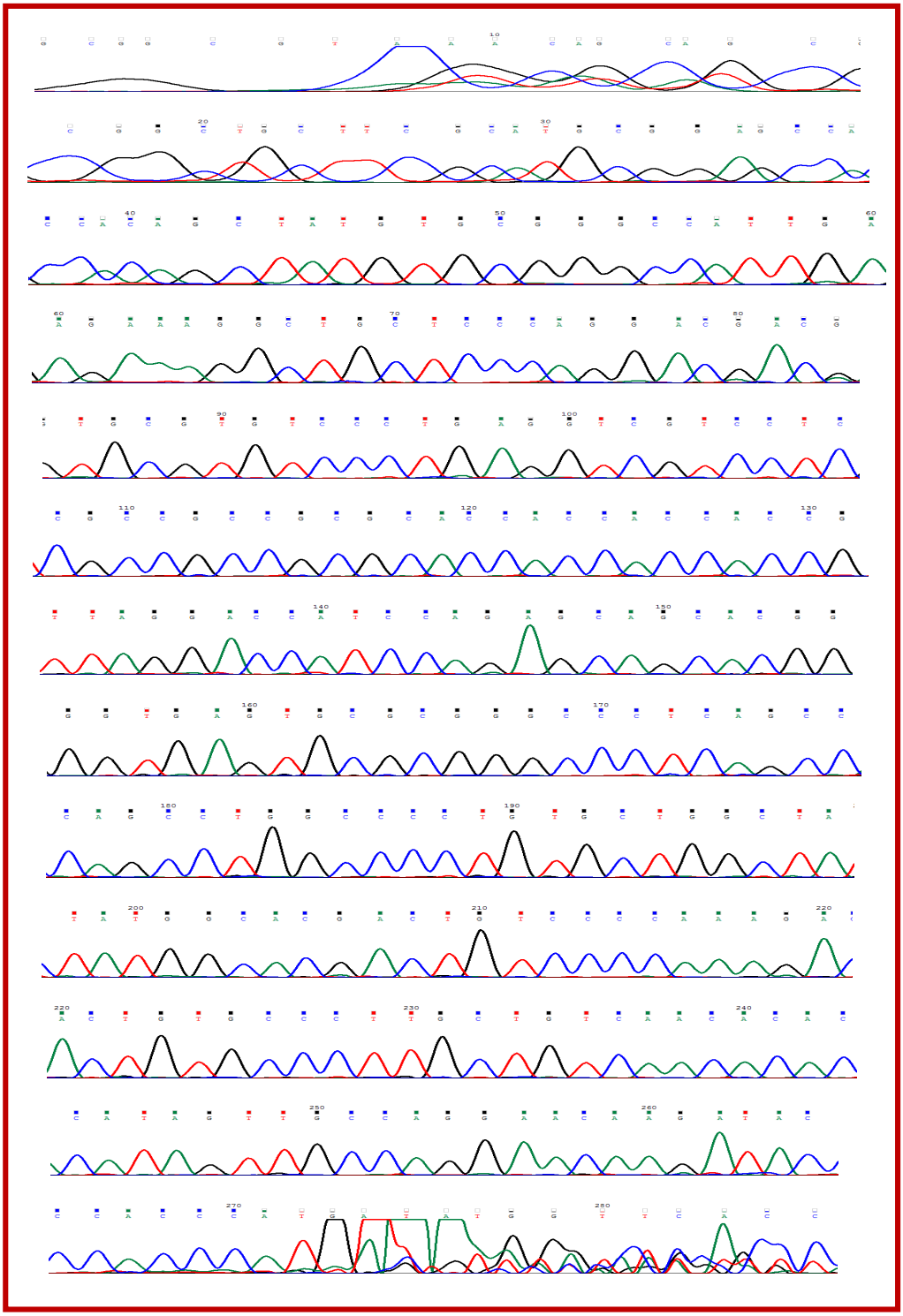


Figure S5B. Representative chromatogram of a normal sample for DLGAP1.

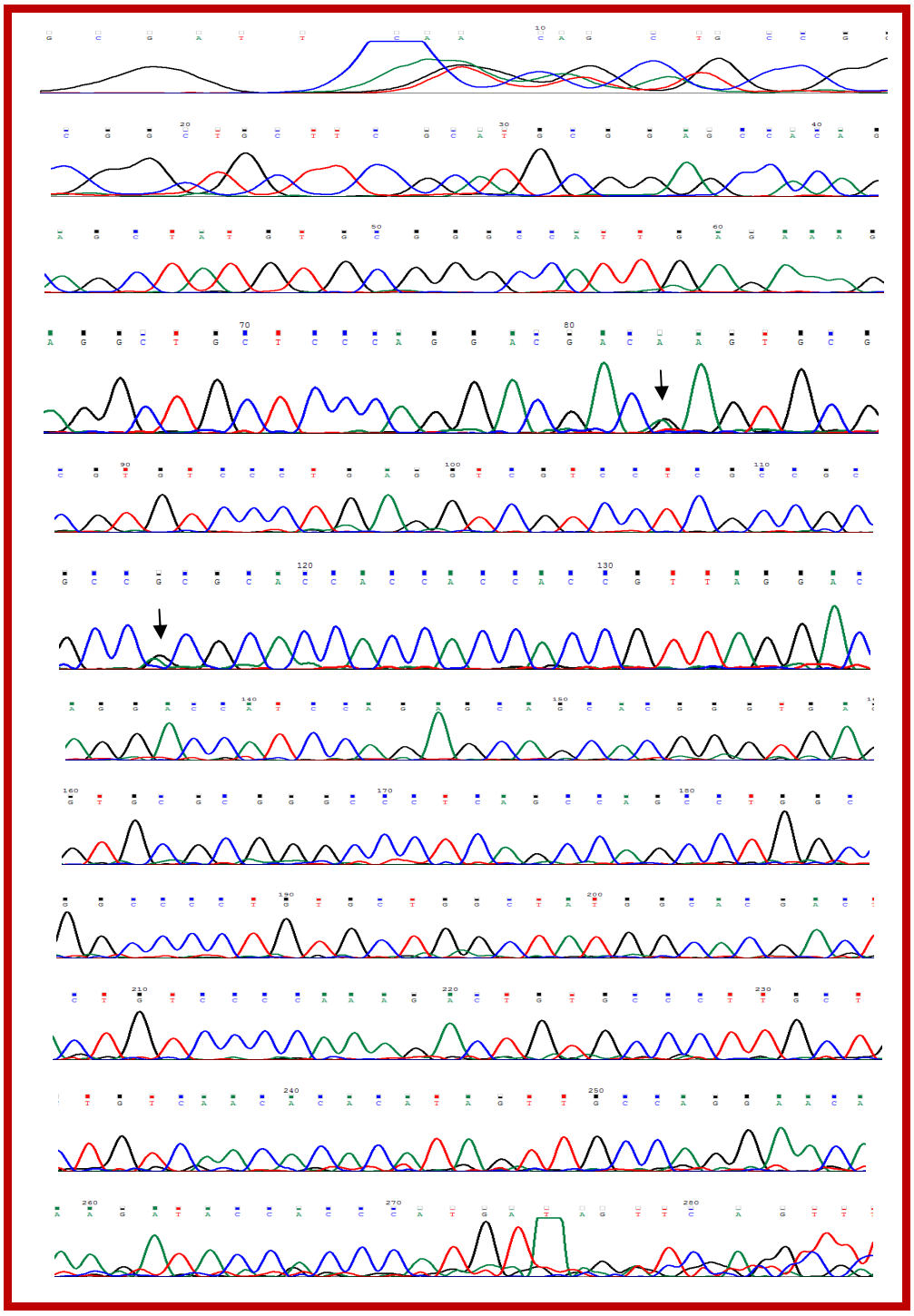


Figure S5C. Representative chromatogram of affected sample, showing novel SNP at codon 507 & reported SNP with rs3745051 at codon 517 in heterozygous state in exon4 of DLGAP1.

2) EMILIN2

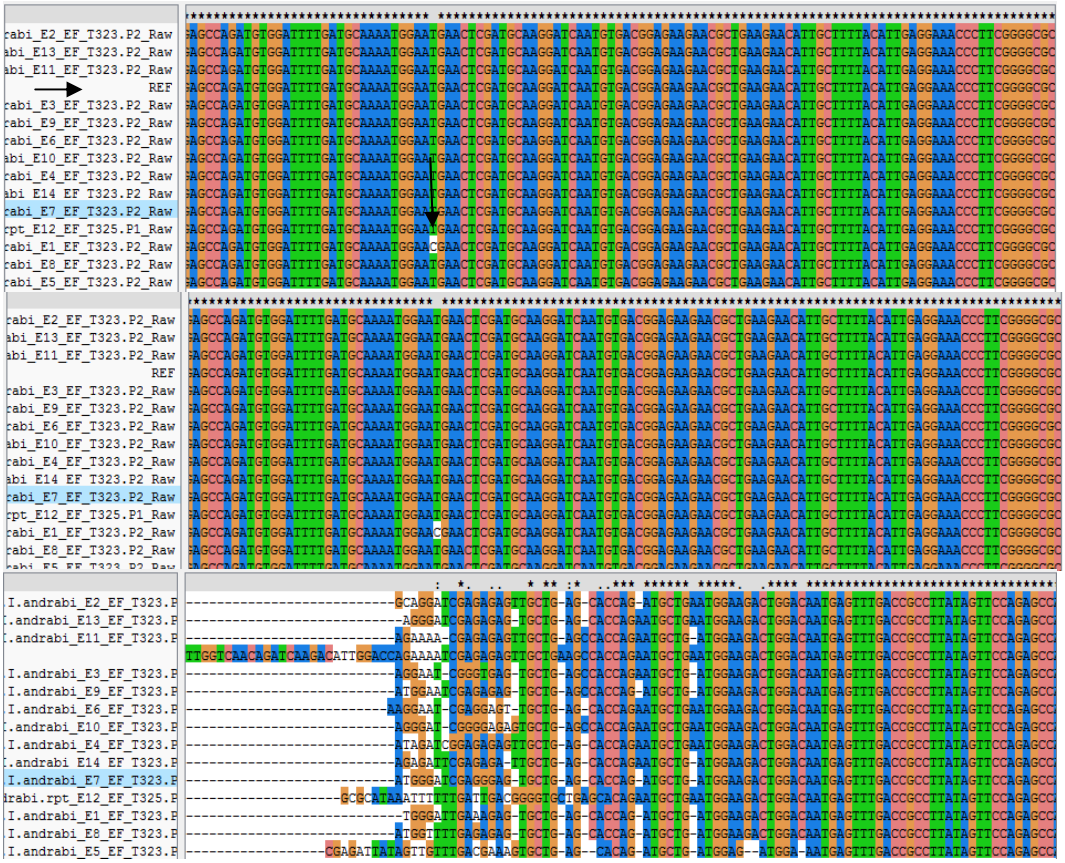


Figure S5D. Representing the multiple sequence alignment of samples generated from Exon 4 (EMILIN2) amplification. The fasta sequences of samples were aligned with the reference sequence (pointed out by black arrow head), using ClustalX software, SNPs are also indicated by black arrows

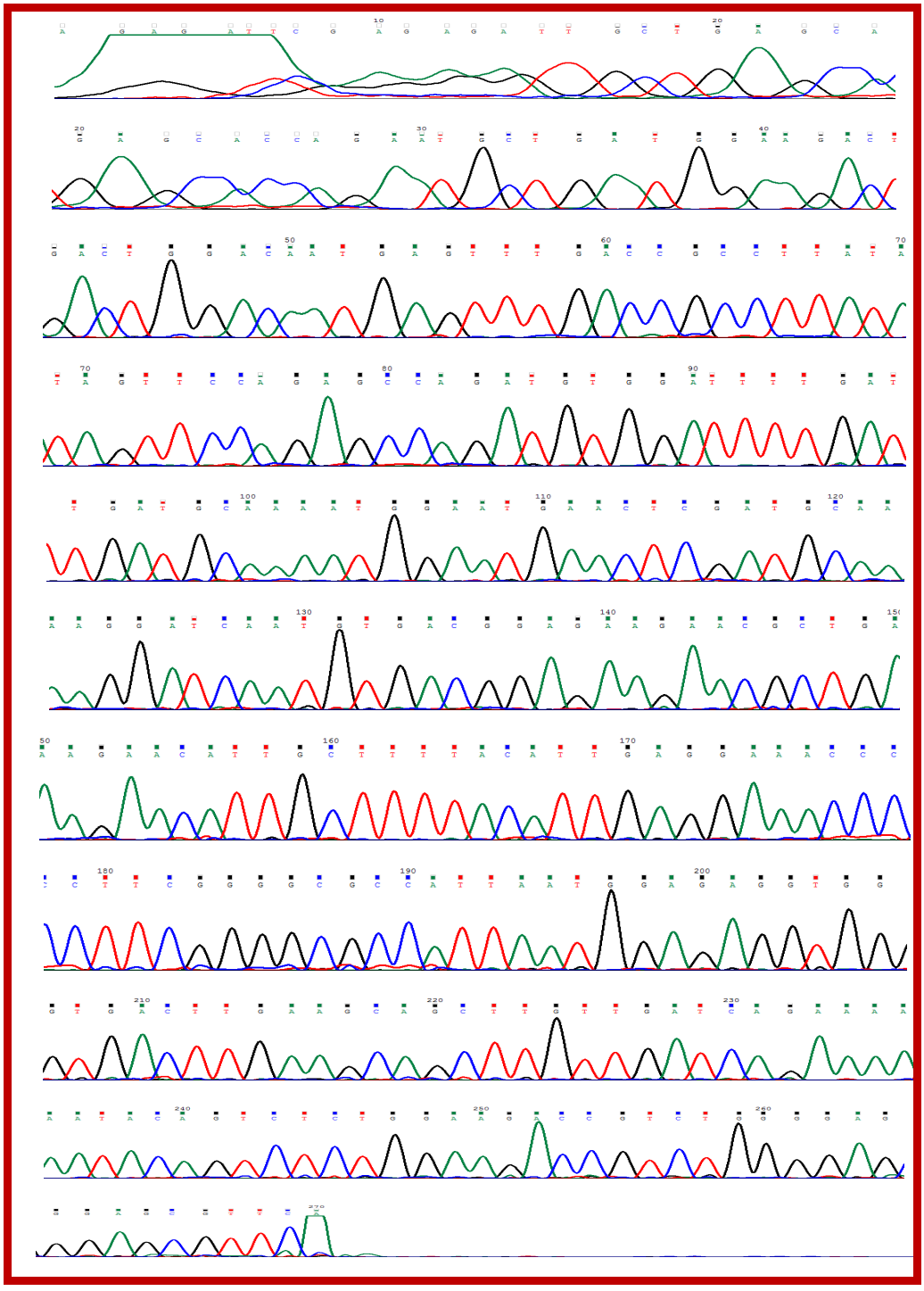


Figure S5E. Representative chromatogram of normal sample, exon 4 EMILIN2.

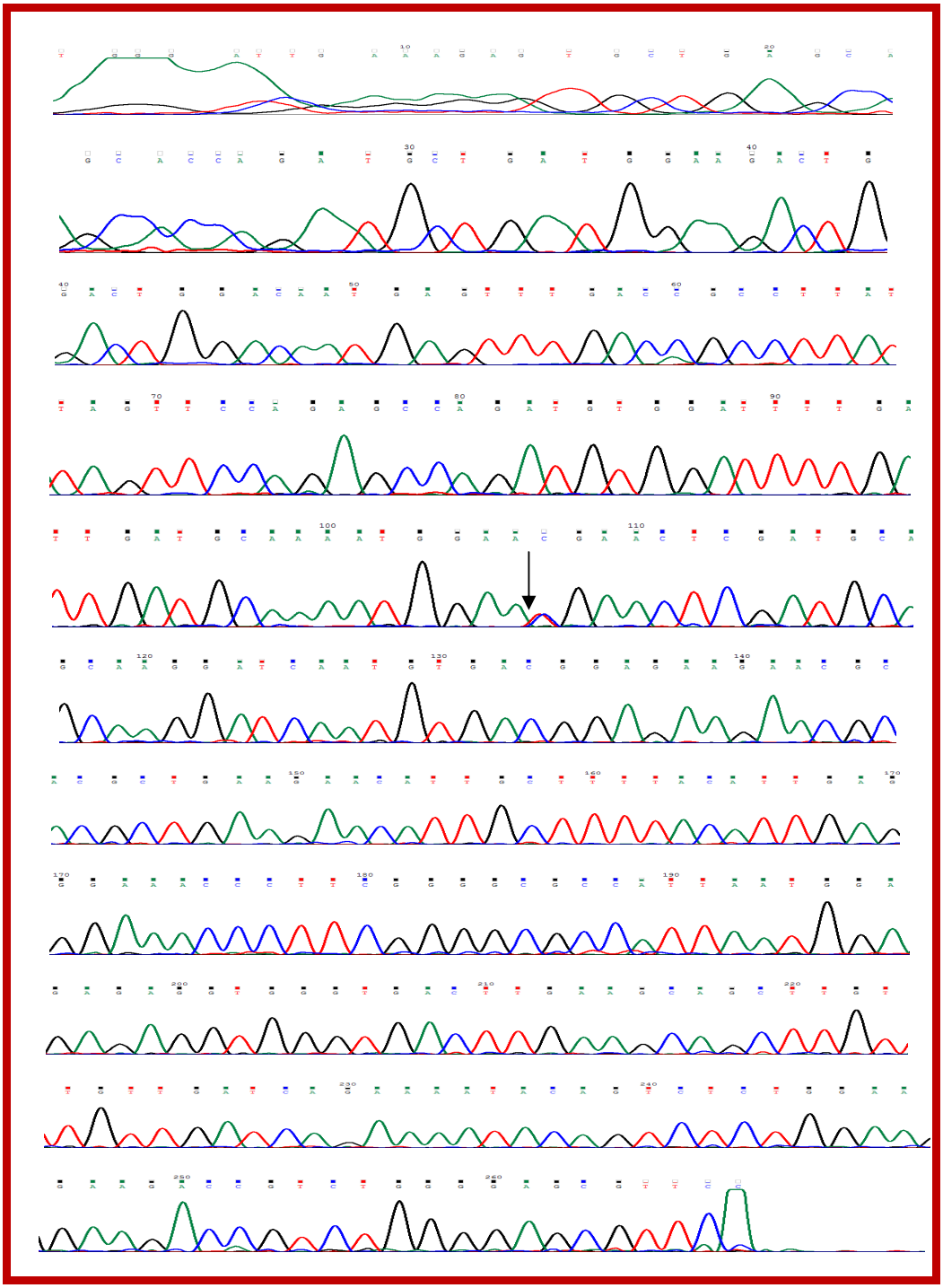


Figure S5F. Representative chromatogram of an affected sample showing synonymous SNP T>C at codon451 (rs rs381006) in EMILIN2 exon 4.

3) MYOM1

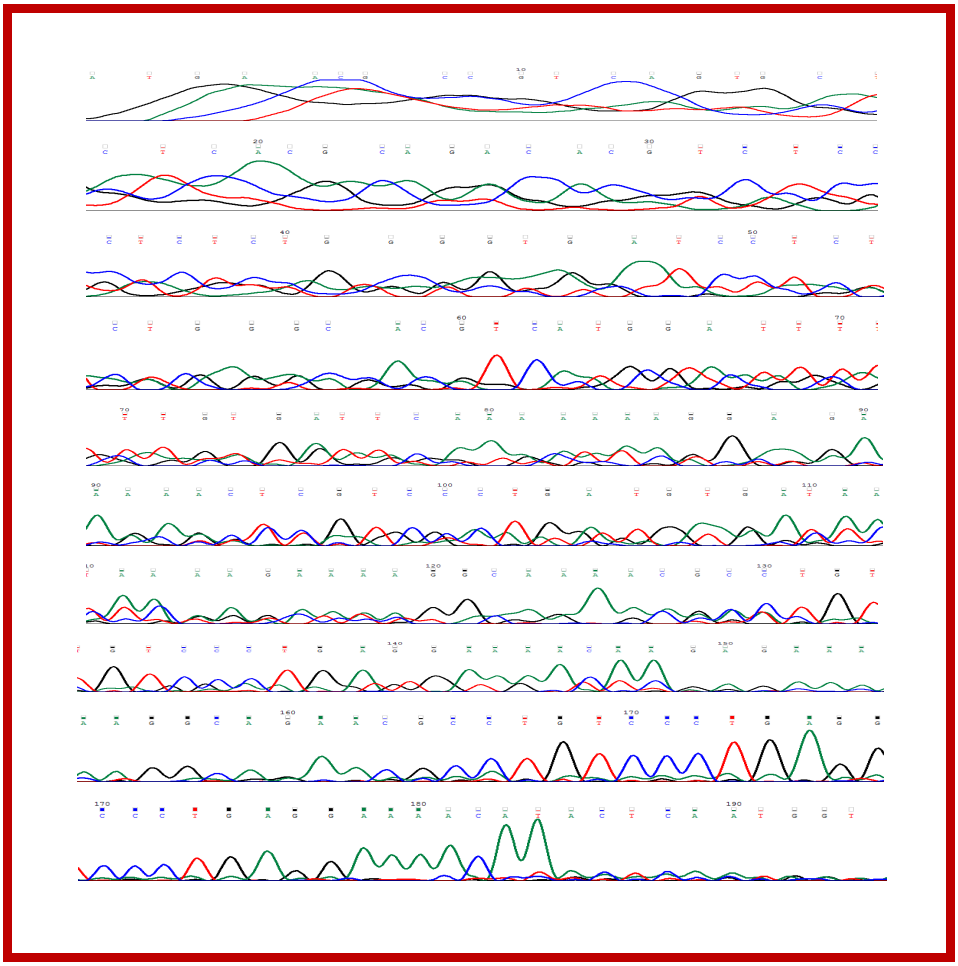


Figure S5G. Representative chromatogram of affected sample for exon 2 of MYOM1 showing sequences in bad format

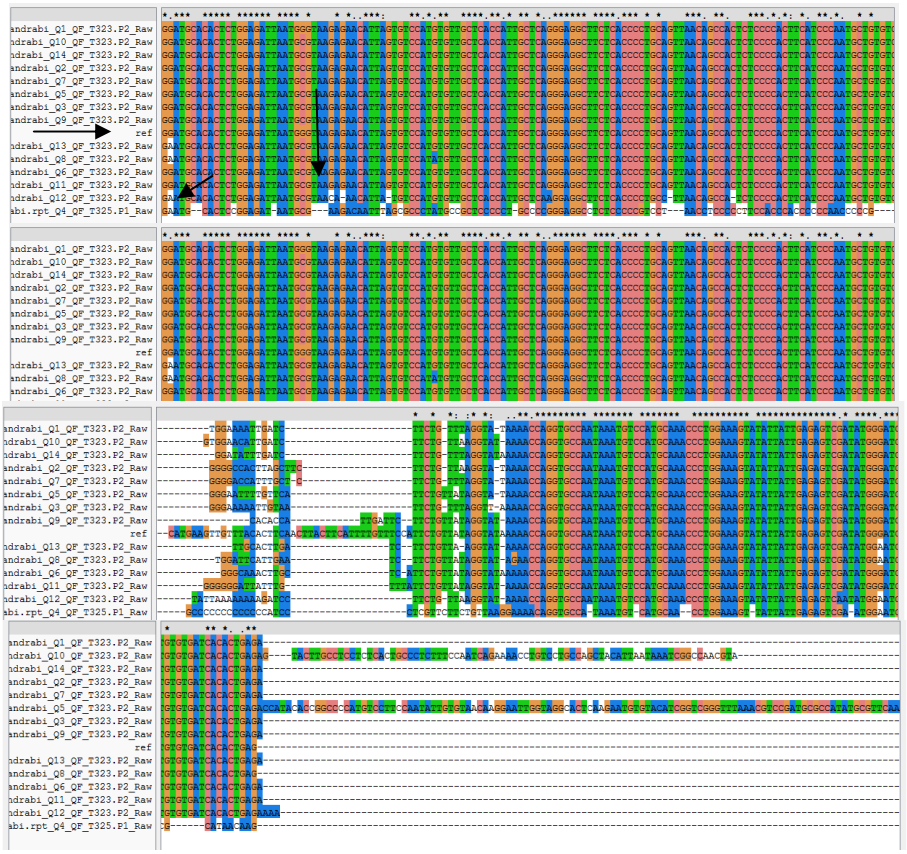


Figure S5H. Representing the multiple sequence alignment of samples generated from Exon 4 (MYOM1) amplification. The fasta sequences of samples were aligned with the reference sequence (pointed out by black arrow head), using ClustalX software, SNPs are also indicated by black arrows**.**

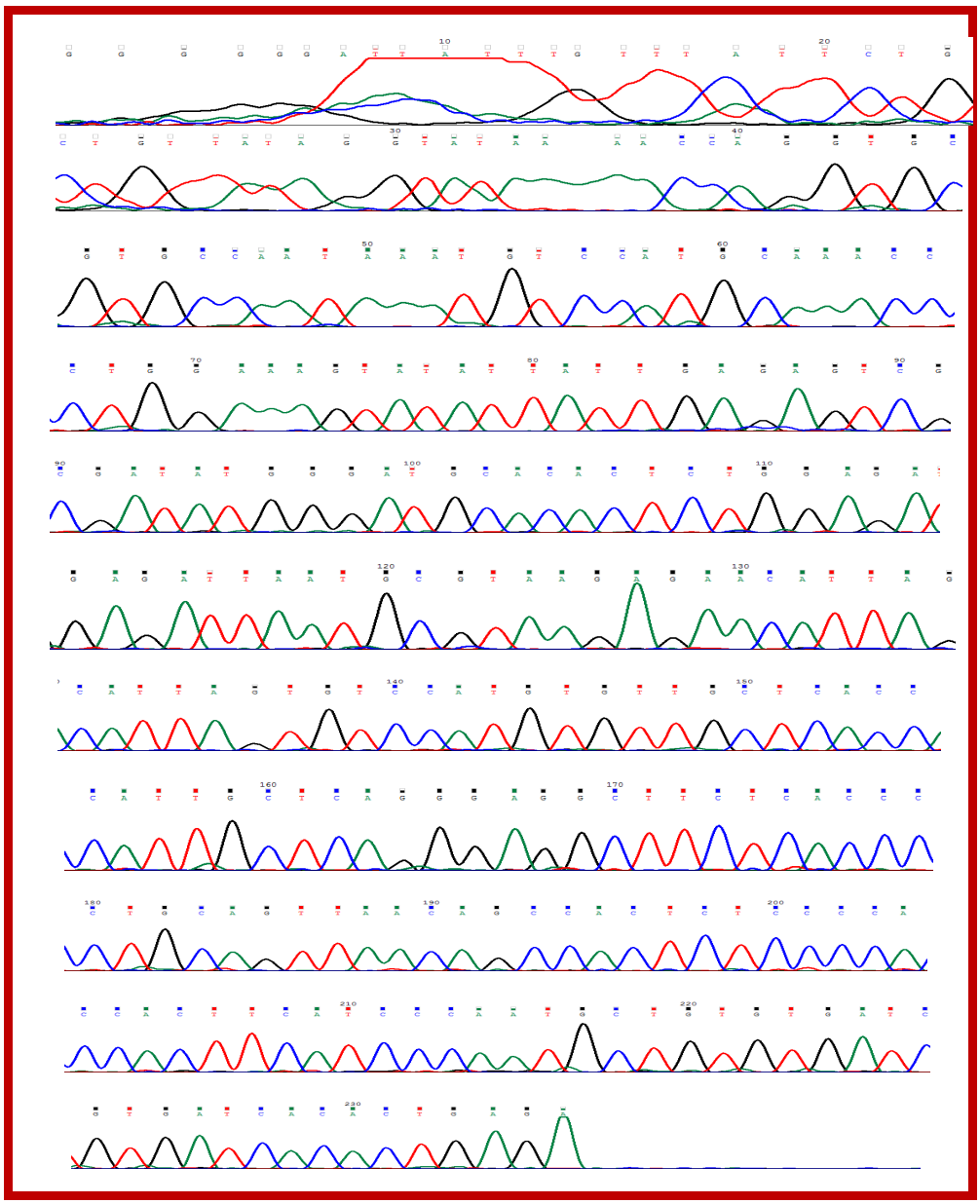


Figure S5I. Representative chromatogram of exon 4 MYOM1 for a Normal sample under Evaluation

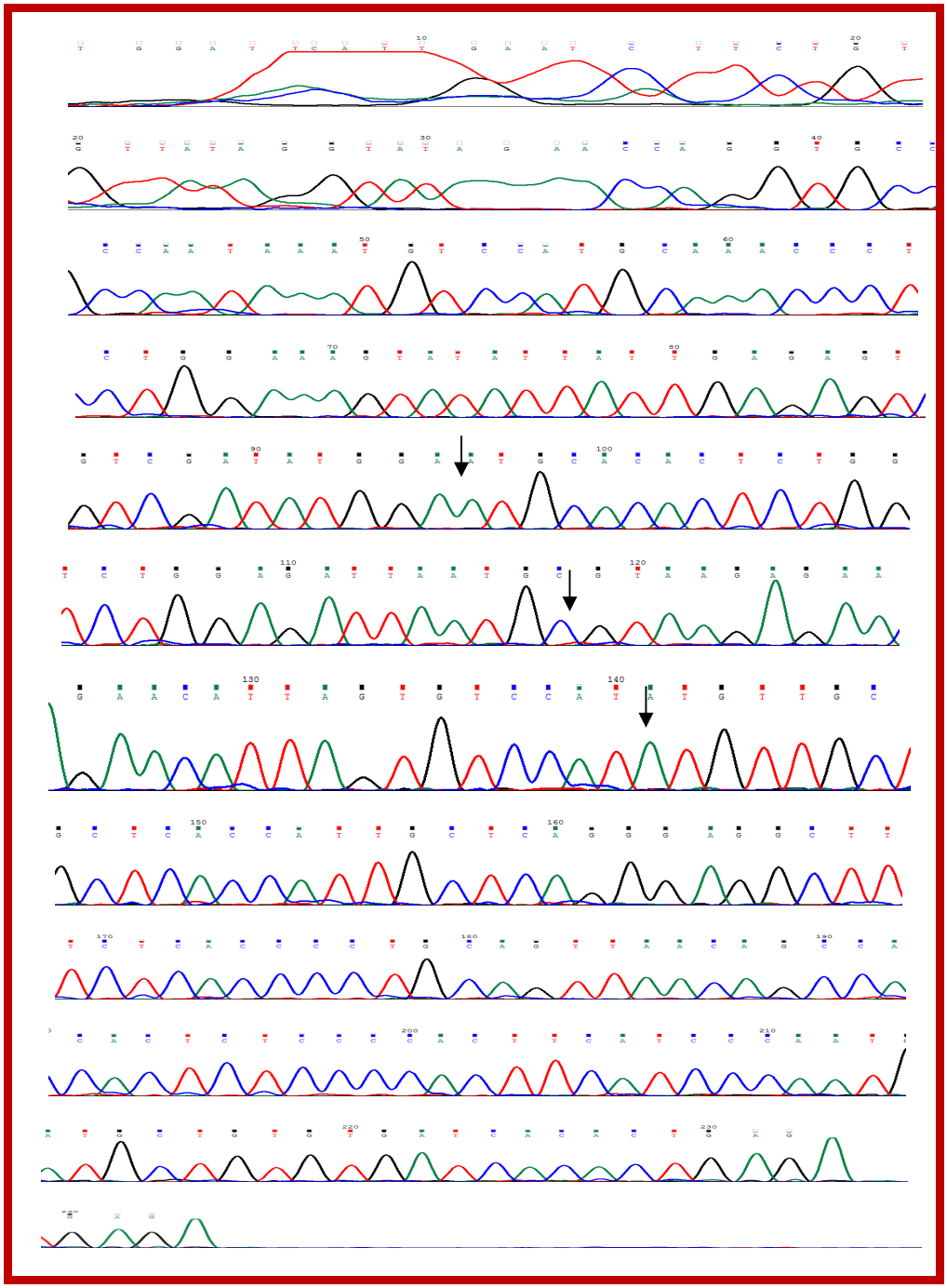


Figure S5J. Representative chromatogram of affected sample, showing reported SNPs at codons 333 & 341in exon 4 MYOM1 & reported intronic SNP immediately after exon 4 of MYOM1.

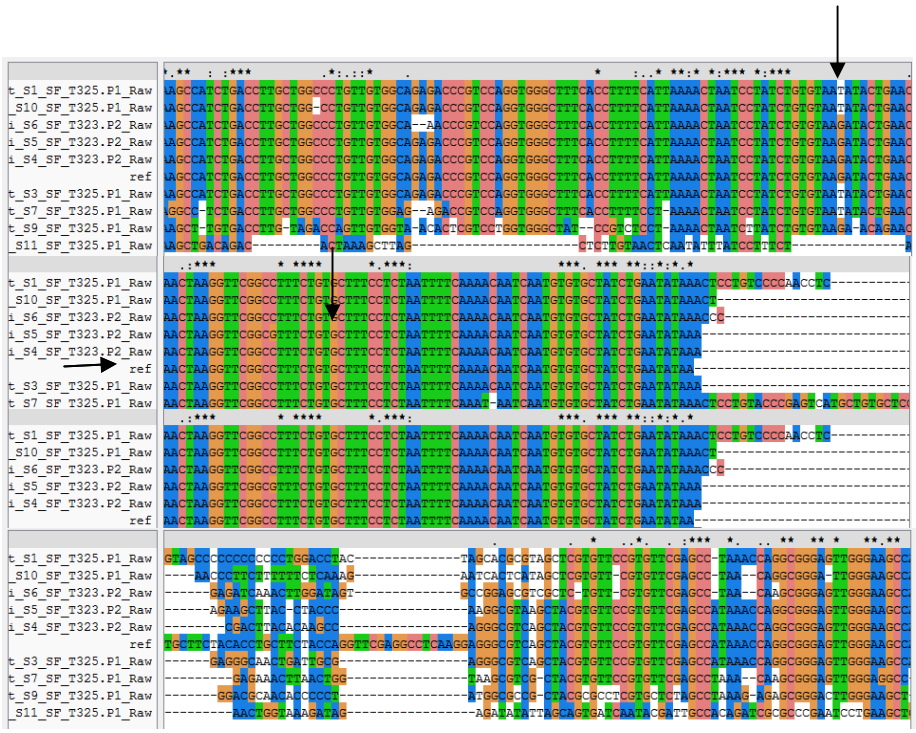


Figure S5K. Representing the multiple sequence alignment of samples generated from Exon 19 (MYOM1) amplification. The fasta sequences of samples were aligned with the reference sequence (pointed out by black arrow head), using ClustalX software, SNPs are also indicated by black arrows.

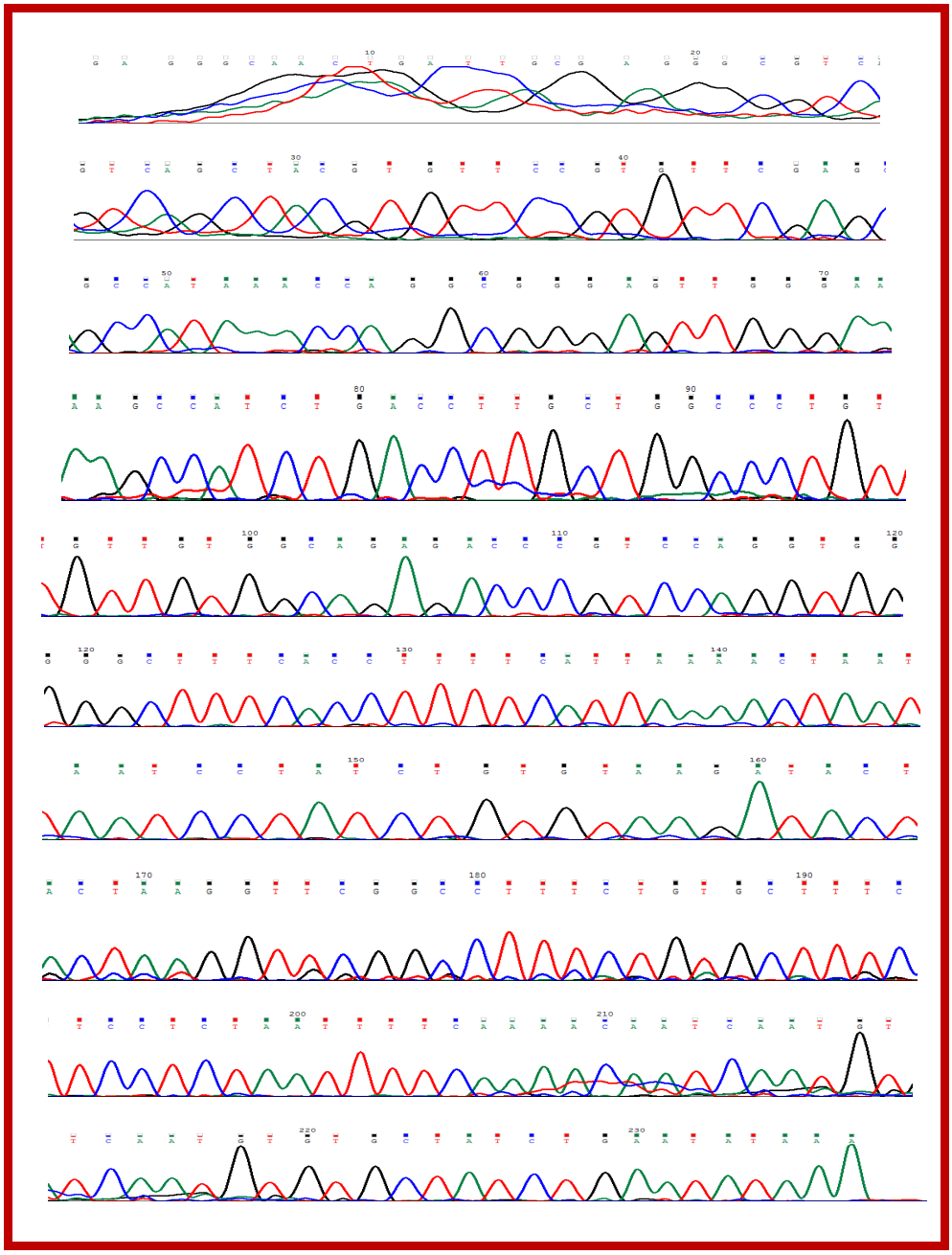


Figure S5L. Representative chromatogram of exon 19 MYOM1 for a Normal sample under evaluation.

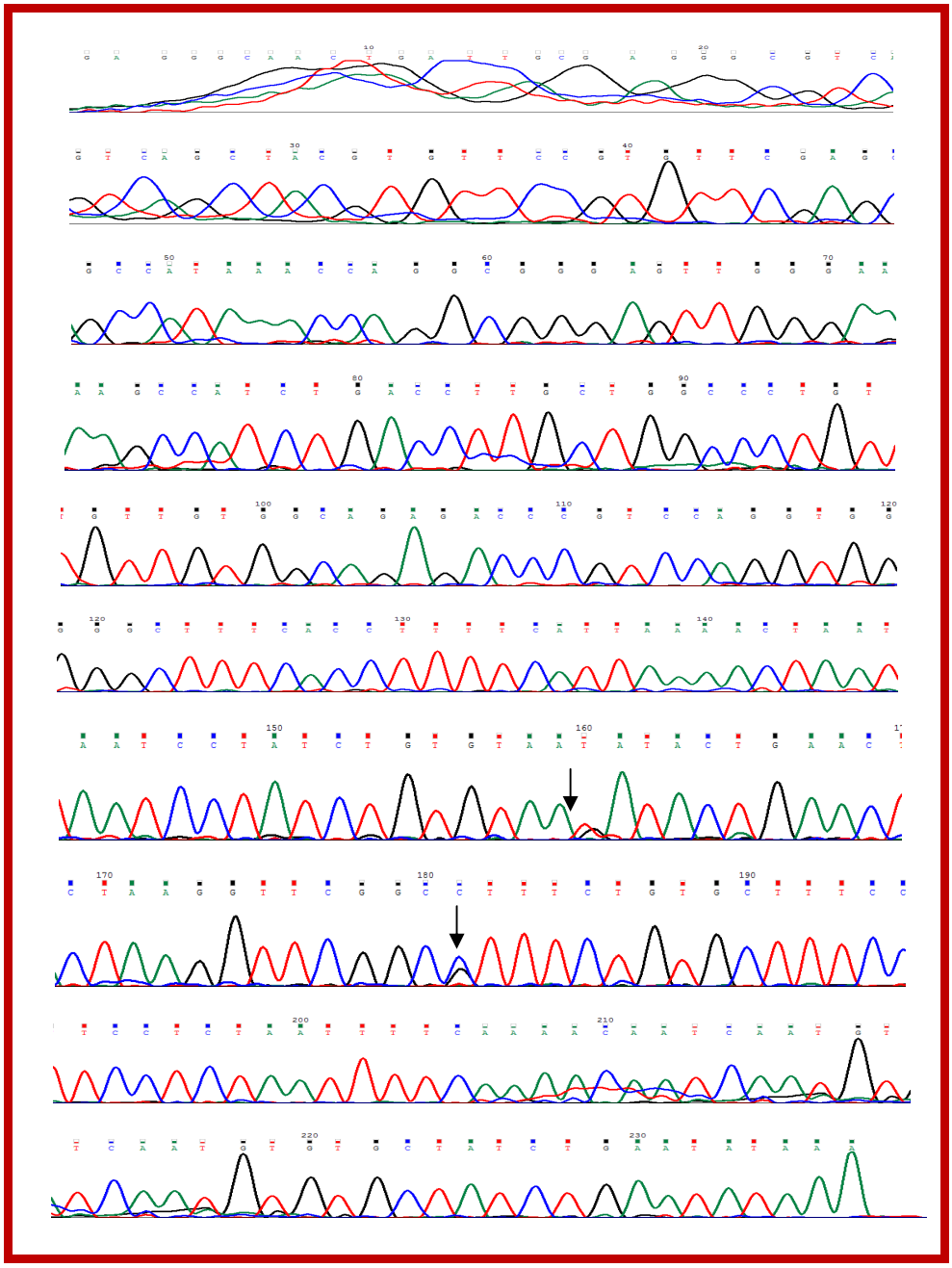


Figure S5M. Representative chromatogram of affected sample, showing two intronic SNPs with rs55779127 and rs8096379 immediately after exon 19 boundry in MYOM1

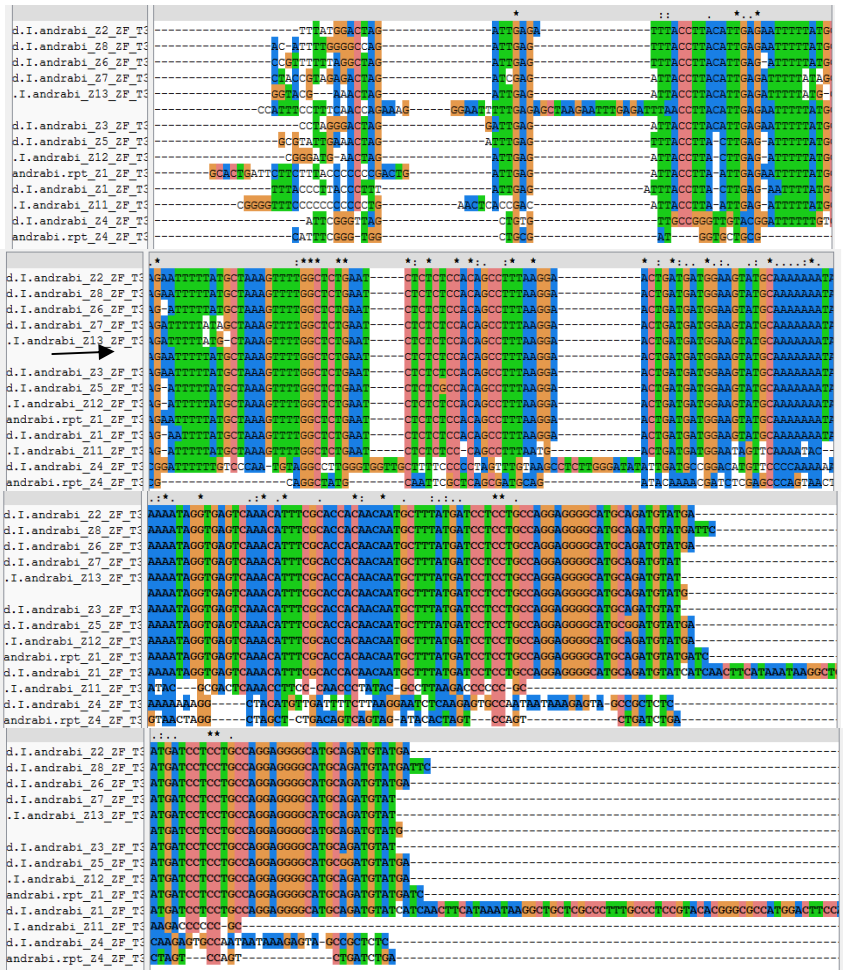


Figure S5N. Representing the multiple sequence alignment of samples generated from Exon 29 (MYOM1) amplification. The fasta sequences of samples were aligned with the reference sequence (pointed out by black arrow head), using ClustalX software.

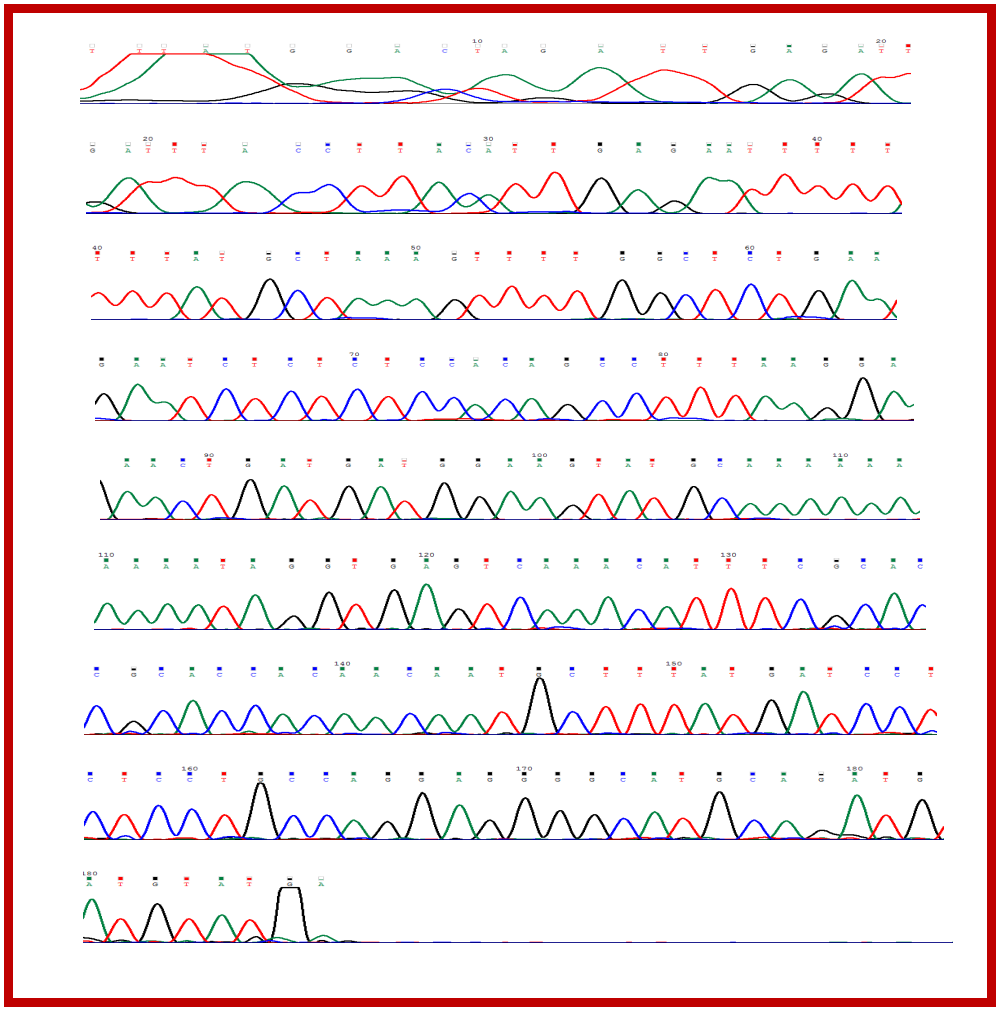


Figure S5O. Representative chromatogram of affected sample, no SNP or mutation found in this exon (MYOM1, exon 29)

TGIF1

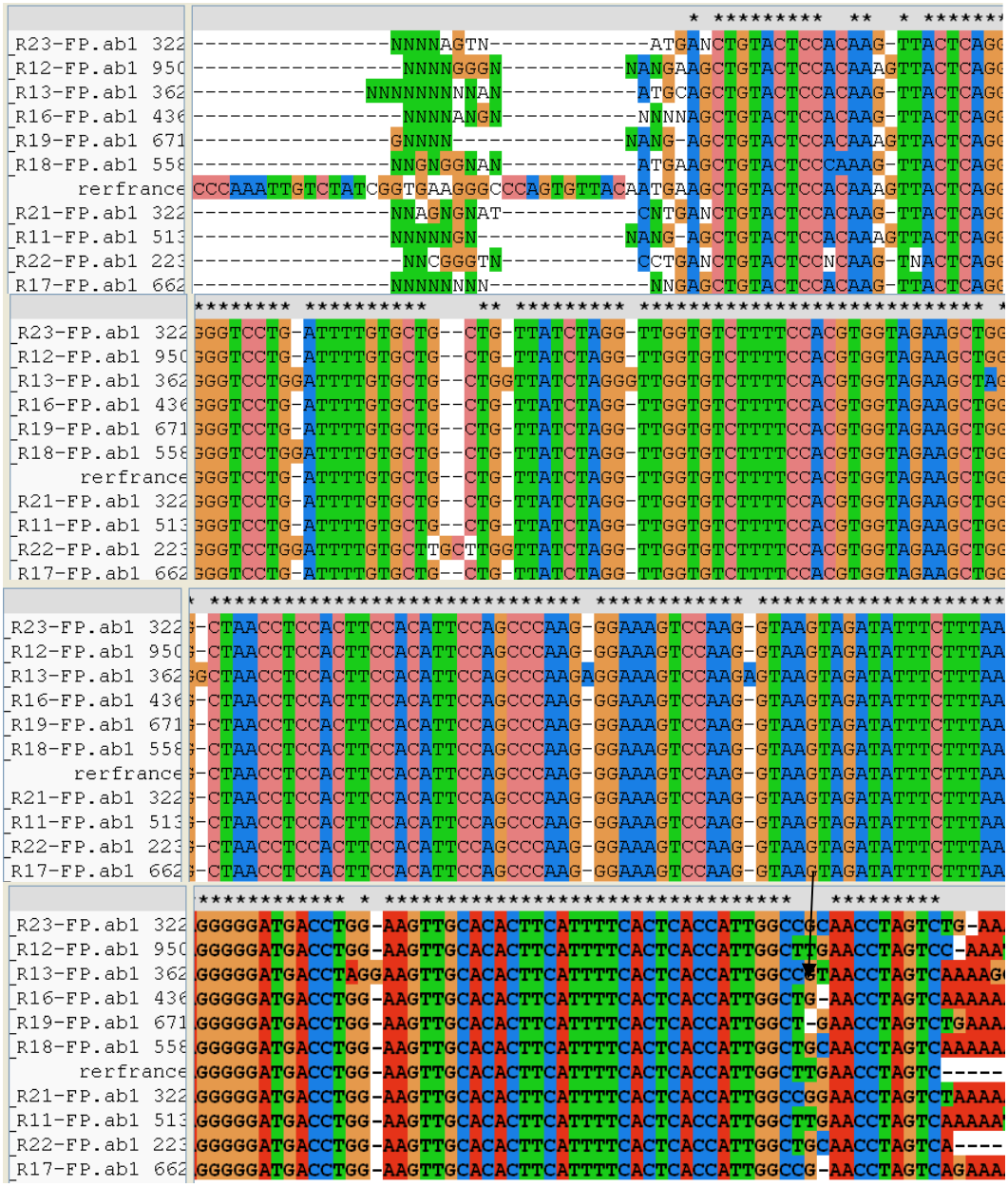


Figure S5P. Representing the multiple sequence alignment of samples generated from Exon 2 amplification. The fasta sequences of samples were aligned with the reference sequence from NCBI indicated by black arrow using ClustalX software. SNPSs indicated by black arrows.

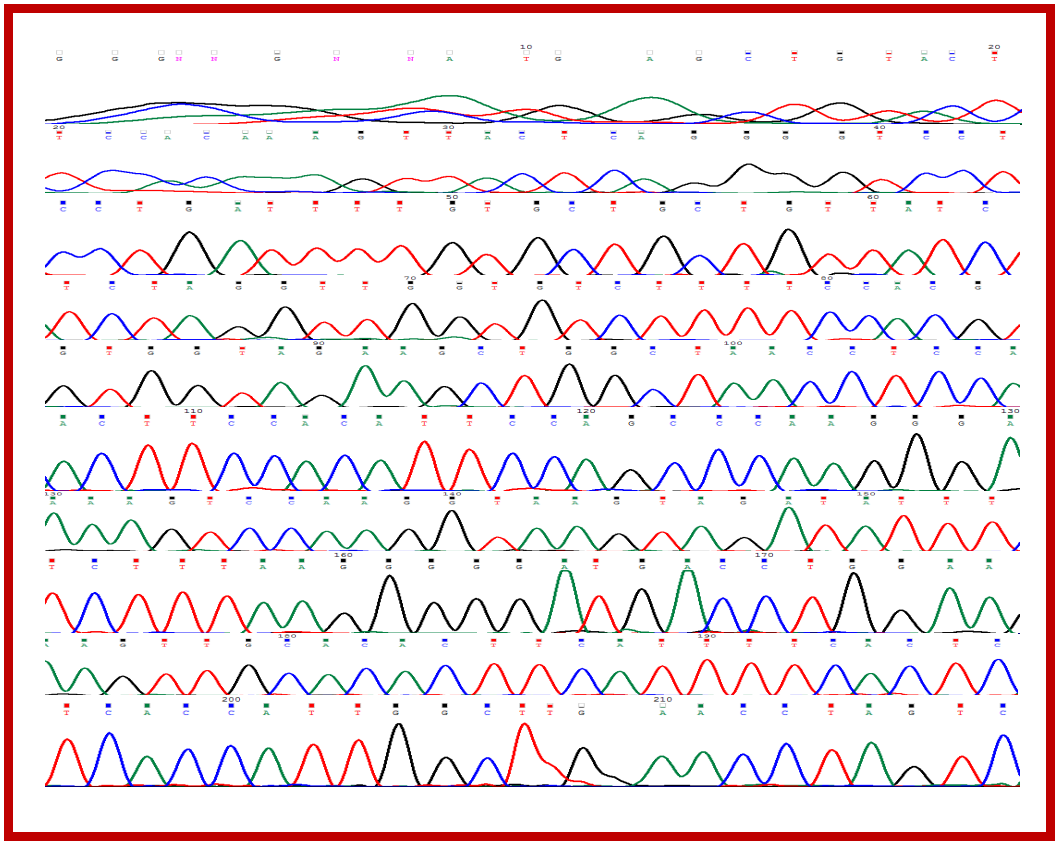


Figure S5Q. Representative chromatogram of a normal sample under evaluation for exon 2 TGIF1.

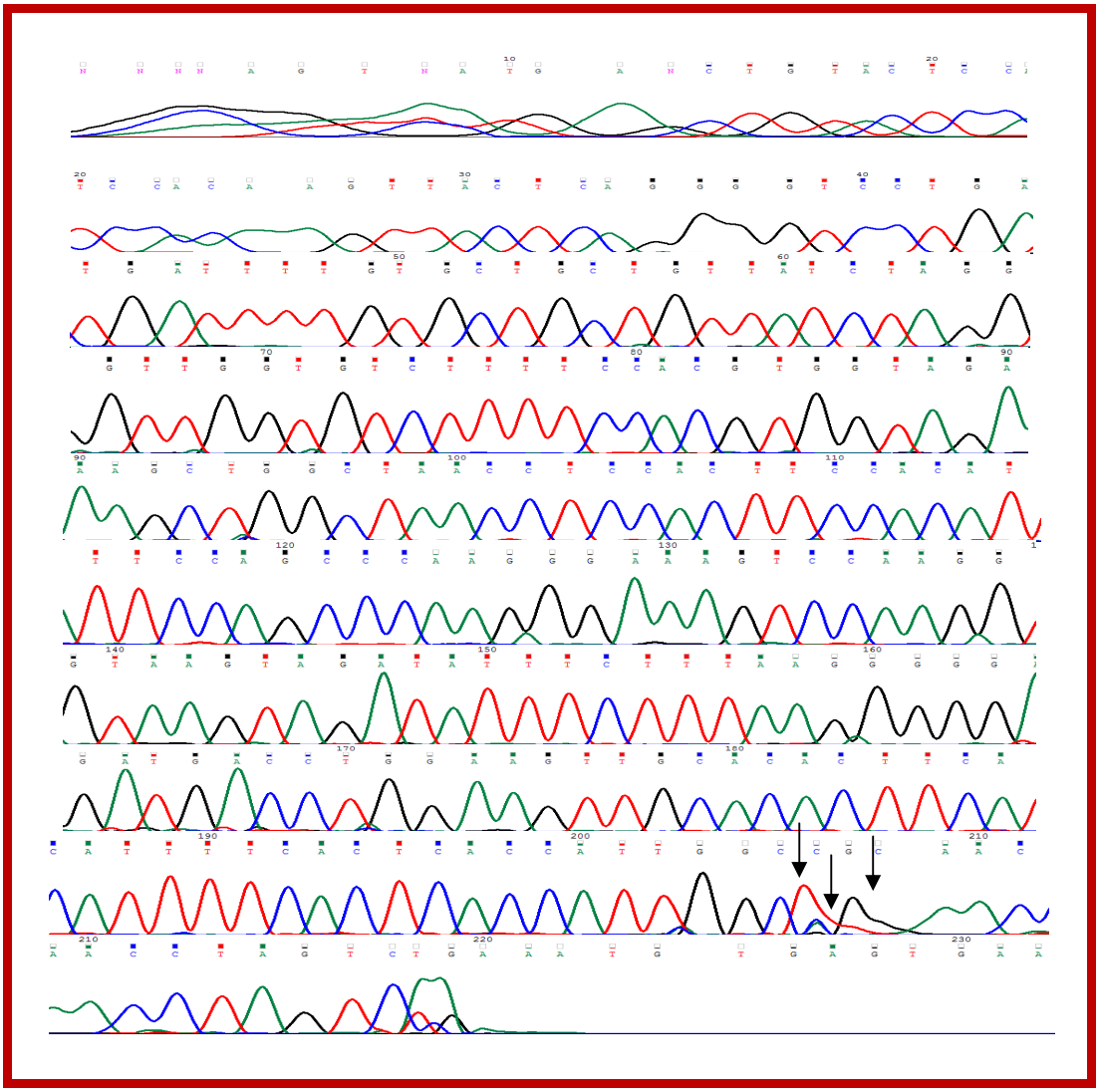


Figure S5R. Representative chromatogram of affected sample, showing three adjacent novel intronic variations after exon 2 junction TGIF1, indicated by black arrow.

Figure S5S. Representing the multiple sequence alignment of samples generated from Exon 6 amplification. The fasta sequences of samples were aligned with the reference sequence from NCBI indicated byblack arrow using ClustalX software. SNPs are also indicated by black arrows.

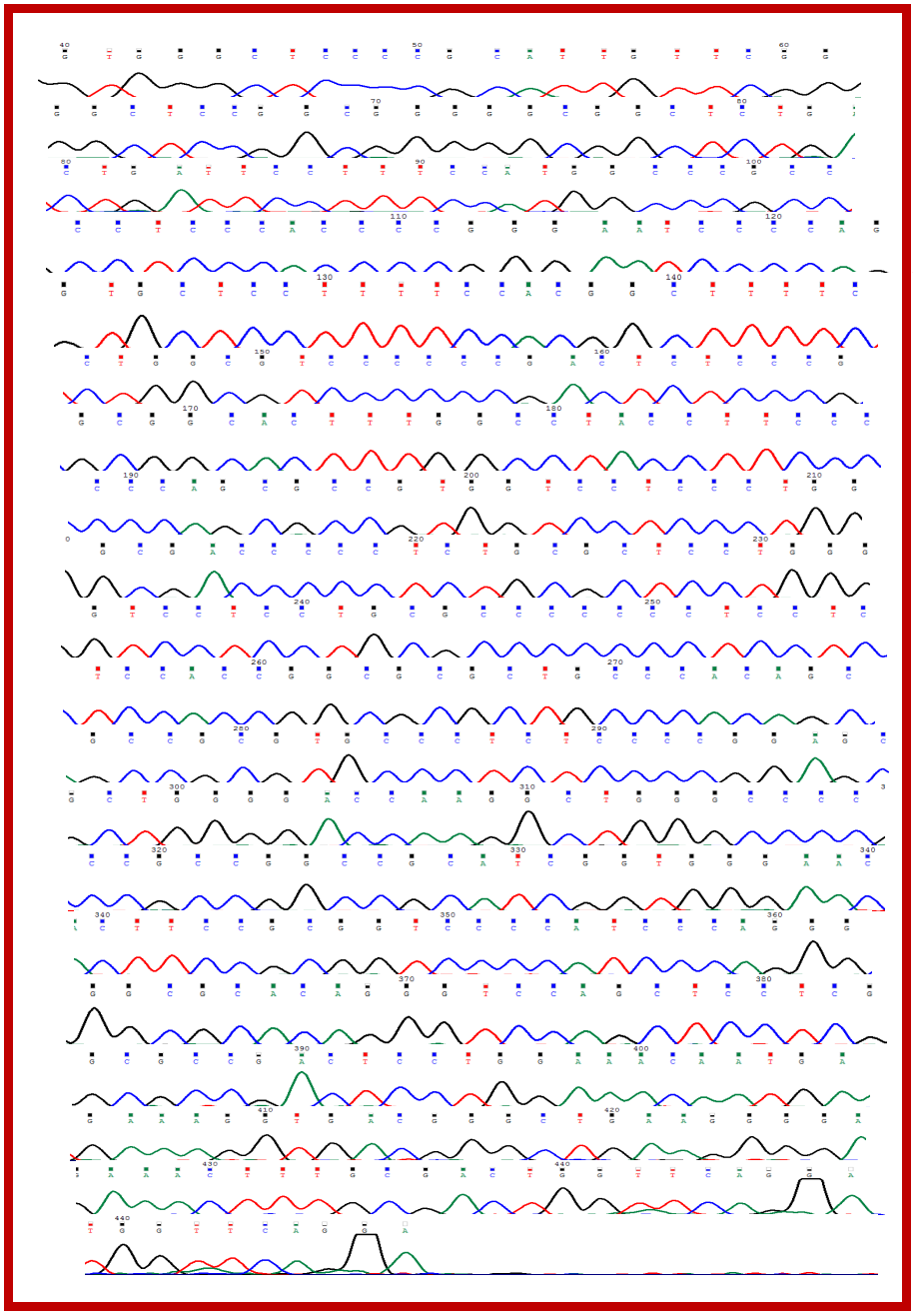


Figure S5T. Representative chromatogram of a Normal sample, TGIF1 exon 6 under evaluation

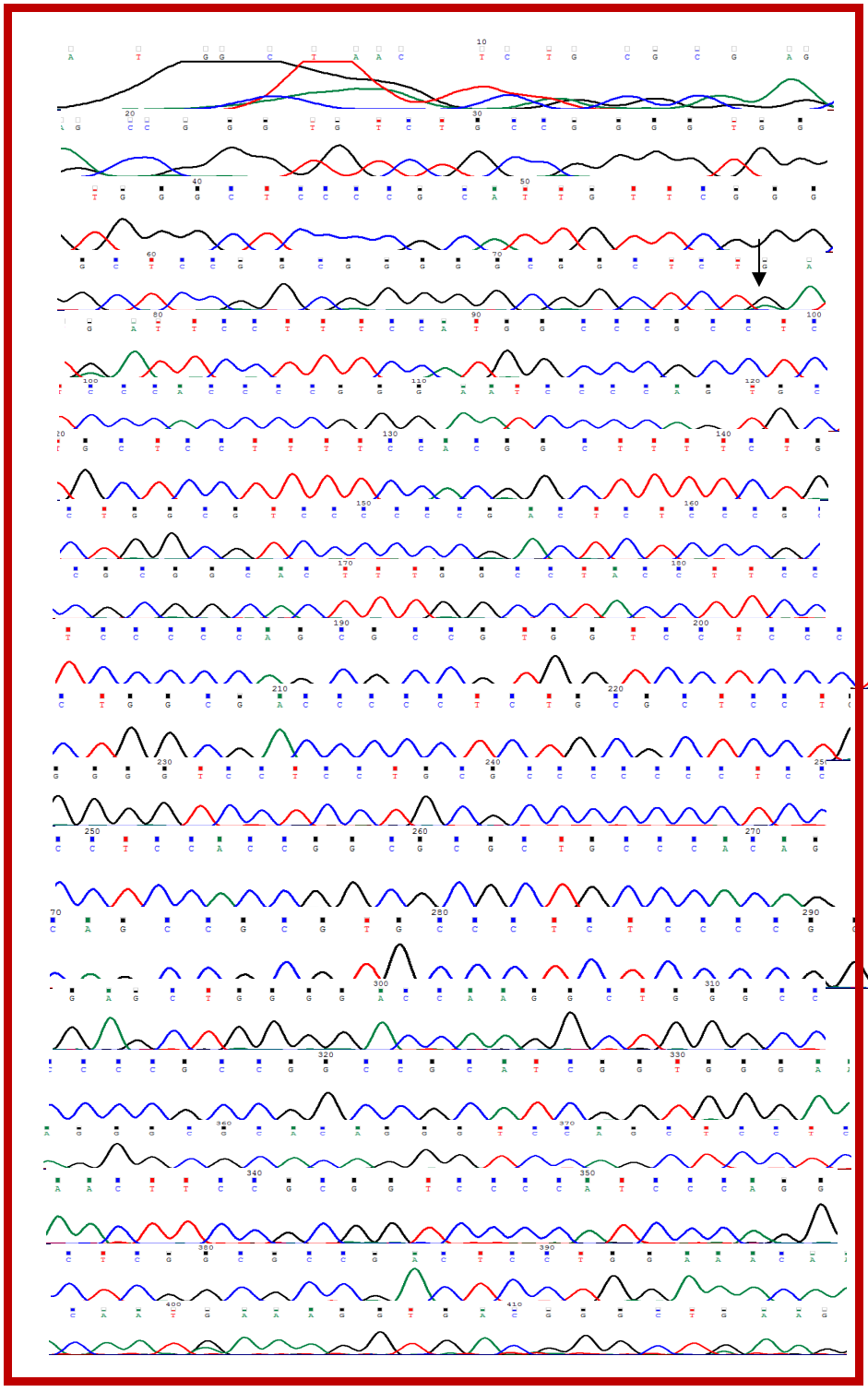


Figure S5U. Representative chromatogram of affected sample, showing a novel mutation G>A at codon 26 of TGIF1 in heterozygous state.

TGFβ1

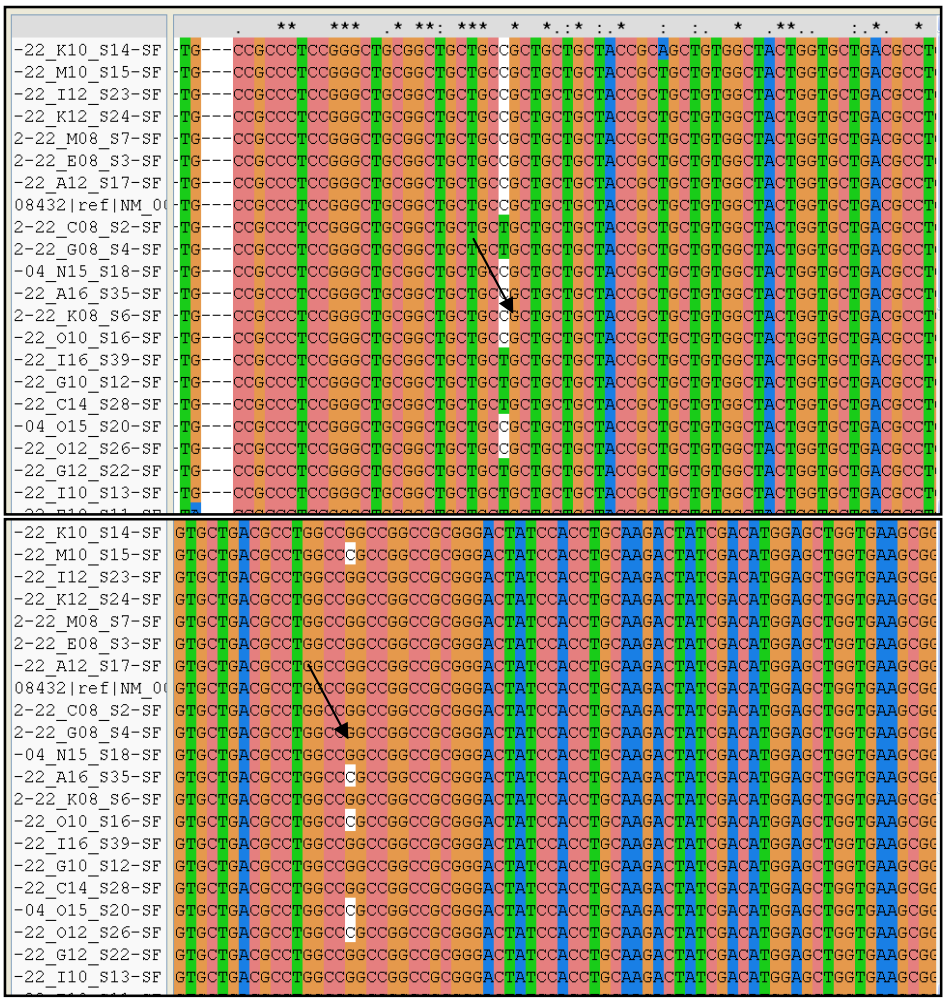


Figure S5V. Representing the multiple sequence alignment of samples generated from TGFβ1 Exon 1 (amplification. The fasta sequences of samples were aligned with the reference sequence available sample (pointed out by black arrow head), using ClustalX software, SNPs are indicated by red arrows.

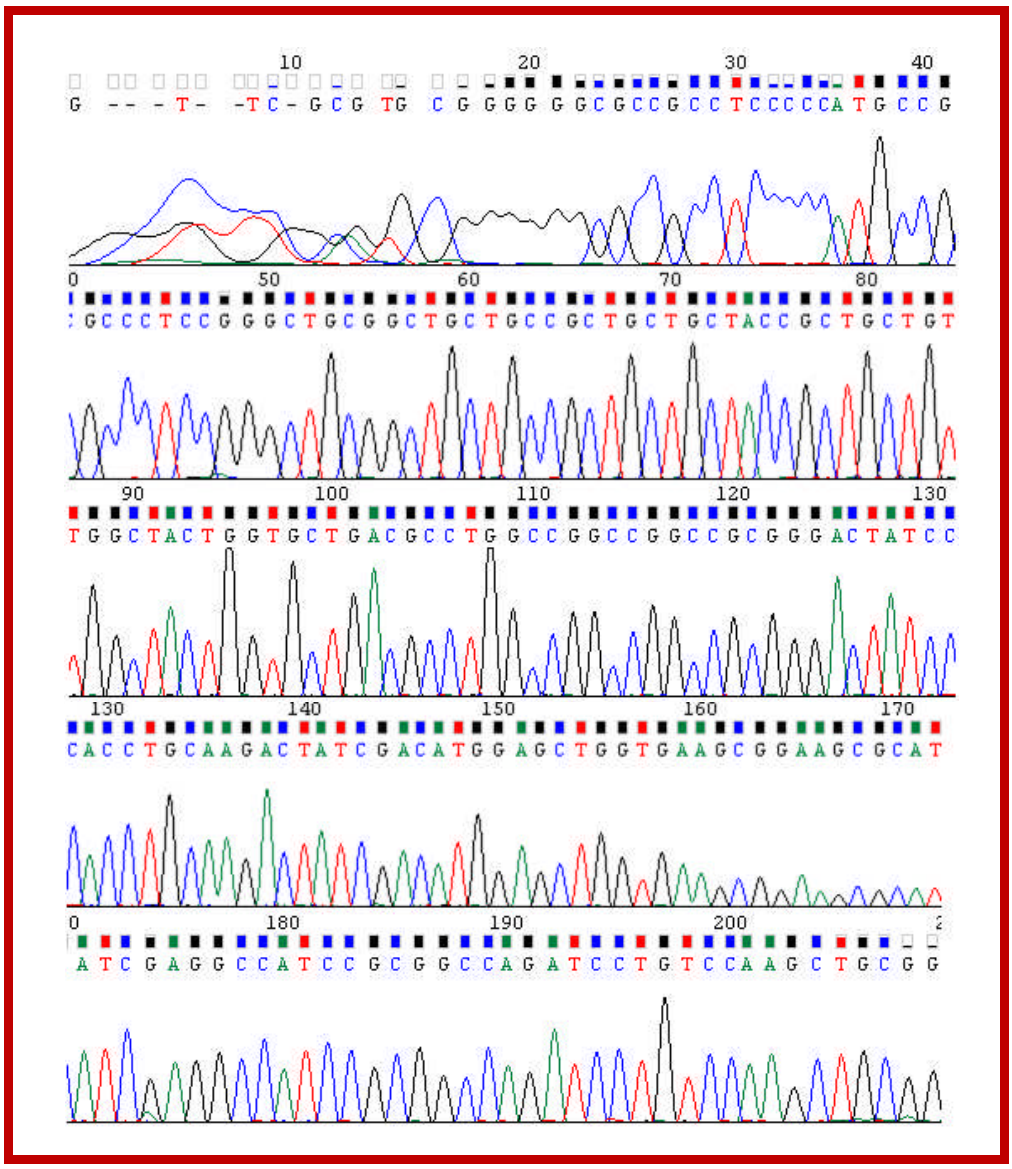


Figure S5W. Representative chromatogram for normal sample under evaluation (TGFβ1, exon1)

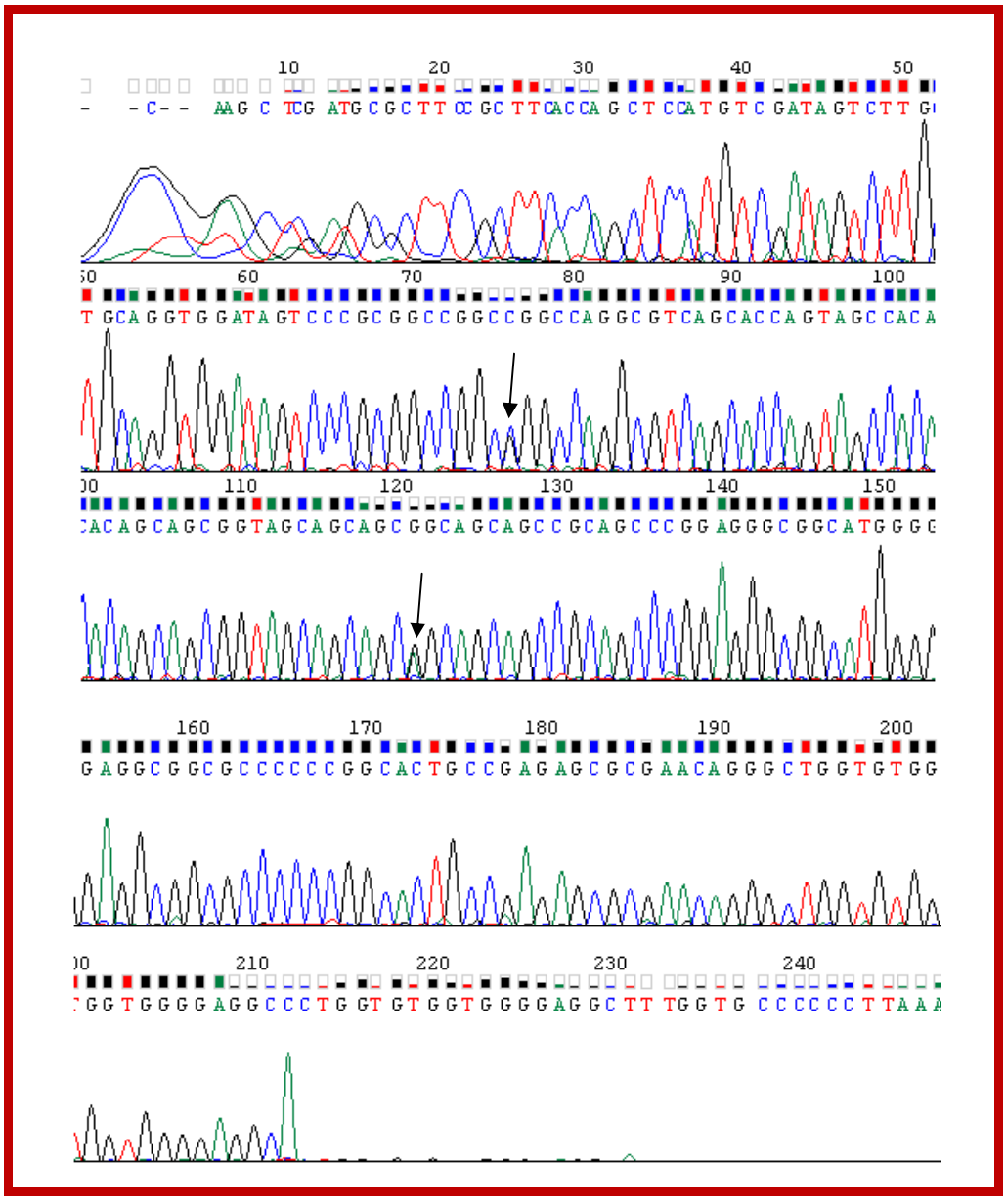


Figure S5X. Representative chromatogram of affected sample showing SNP at codon 10 and 25, TGFβ1 exon 1.

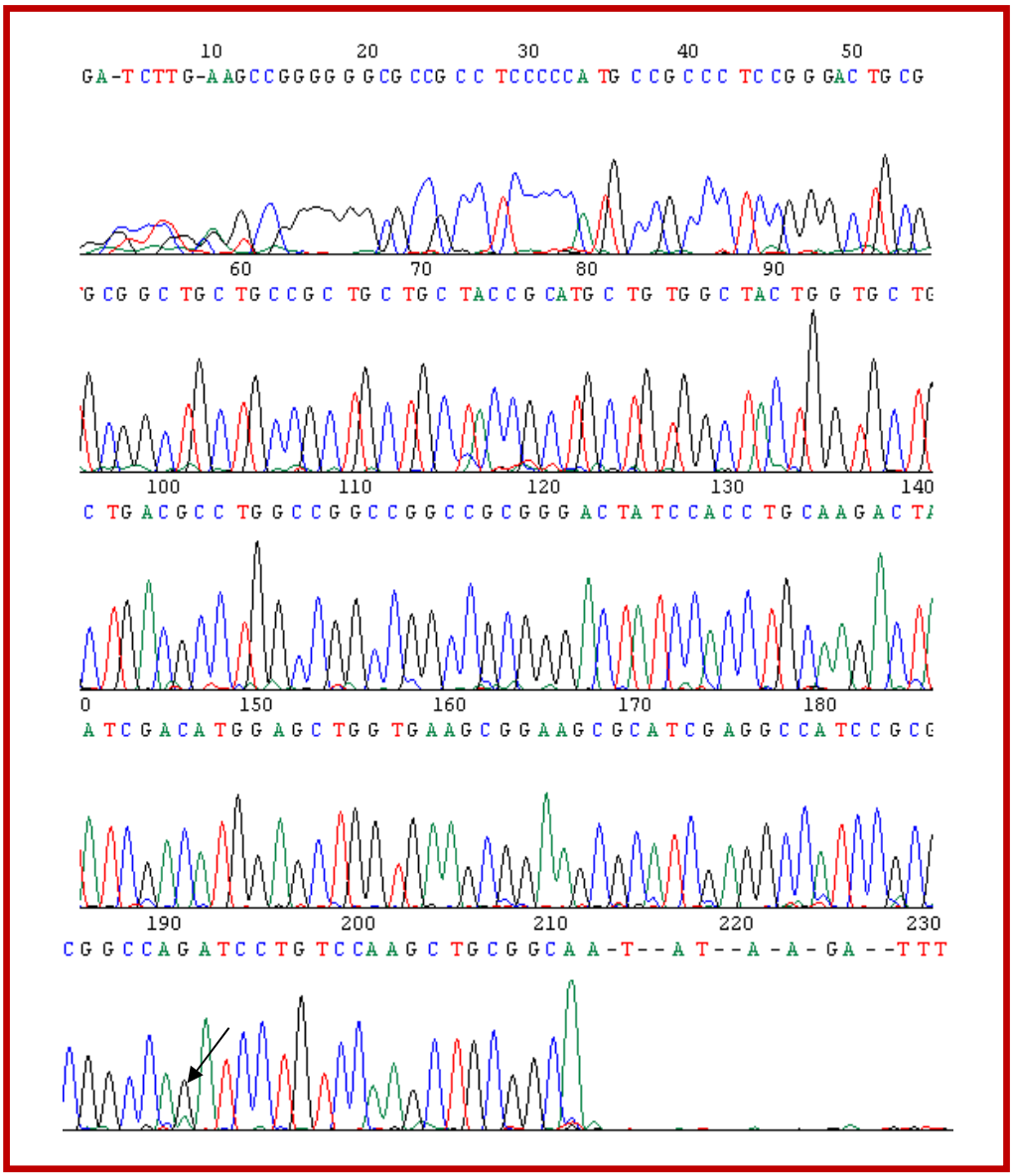
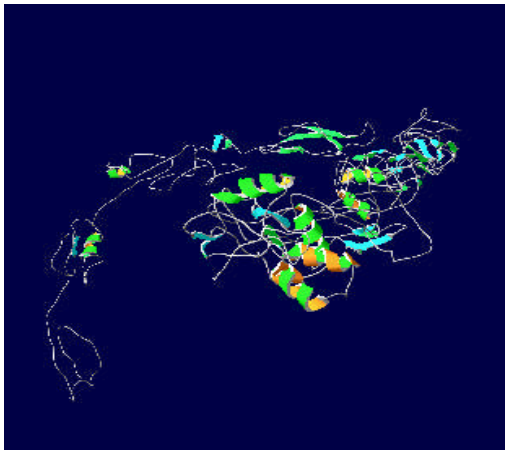
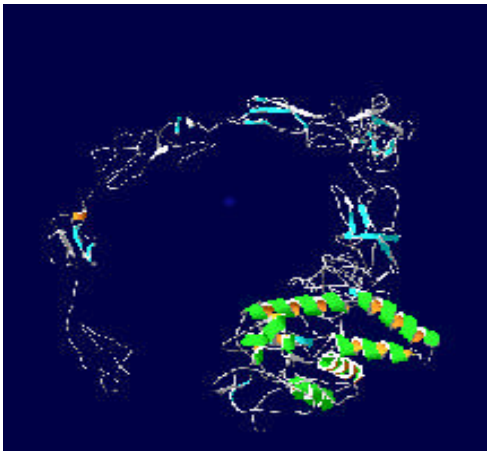


Figure S5Y. Representative chromatogram of affected sample showing heterozygous G>A SNP at codon 52 as indicated by arrow.

S6. Insilco predictions



S6a)

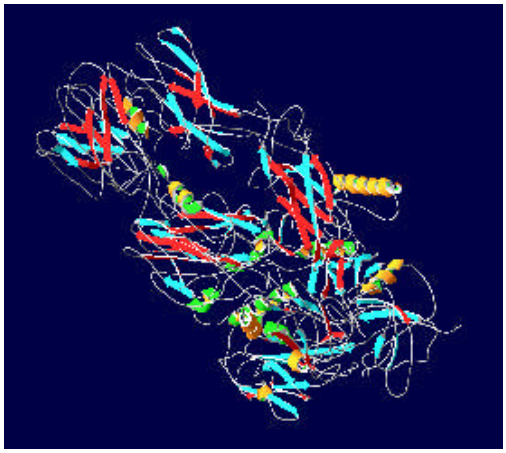


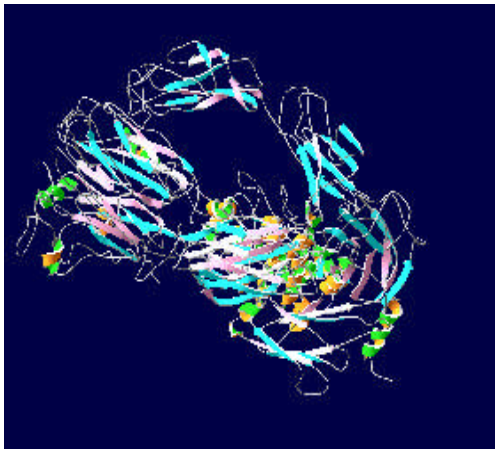
S6b)

Figure S6.1. S6a) Wild type and S6b) mutant protein models of DLGAP1 predicted by I-TASSER

Table S6.1. Table shows the total energy of the I-TASSER predicted DLGAP1 tertiary structures calculated by Swiss PDB Viewer. Model mutant 1 has higher energy compared to wild type 1of DLGAP1.

S.No.	Protein model Name	C-score	Energy
1	Wildtype 1	-1.65	-23265.684
2	Mutant 1	-1.49	-20206.113



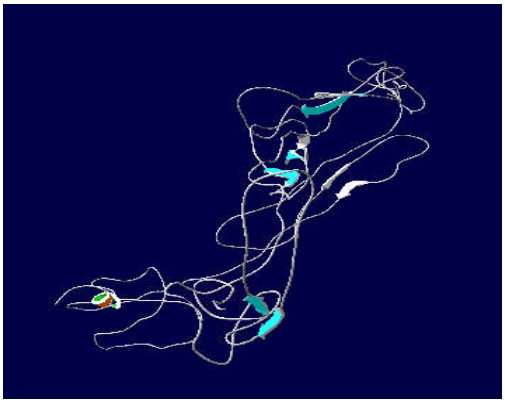


S6c) **S6d)**

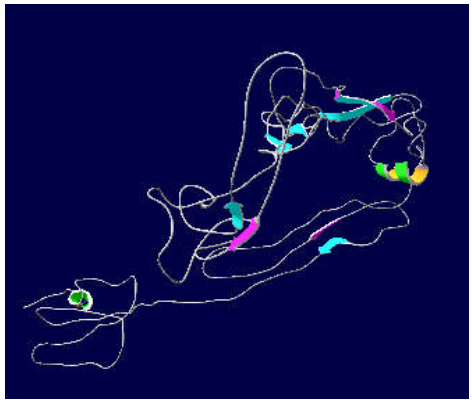
Figure S6.2. S6c) Wild type and S6d) mutant protein models of MYOM11 predicted by I-TASSER

Table S6.2. Table shows the total energy of the I-TASSER predicted MYOM1 (Exon 4) tertiary structures calculated by Swiss PDB Viewer. Model mutant 1 has lower energy compared to wild type 1.

S. No.	Protein model Name	C-score	Energy kj/mol
1	Wildtype 1	-0.04	-9702.442
2	Mutant 1	-1.37	-11496.317



S6e)



S6f)

Figure S6.3. S6e) Wild type and S6f) mutant protein models of TGIF1 (exon 6) predicted by I-TASSER

Table S6.3. Table shows the total energy of the I-TASSER predicted TGIF (Exon 6) tertiary structures calculated by Swiss PDB Viewer.

S. No.	Protein Model	C-Score	Energy kj/mol
1	Wildtype 1	-0.42	-5820.186
2	Mutant	-0.47	-6595.593

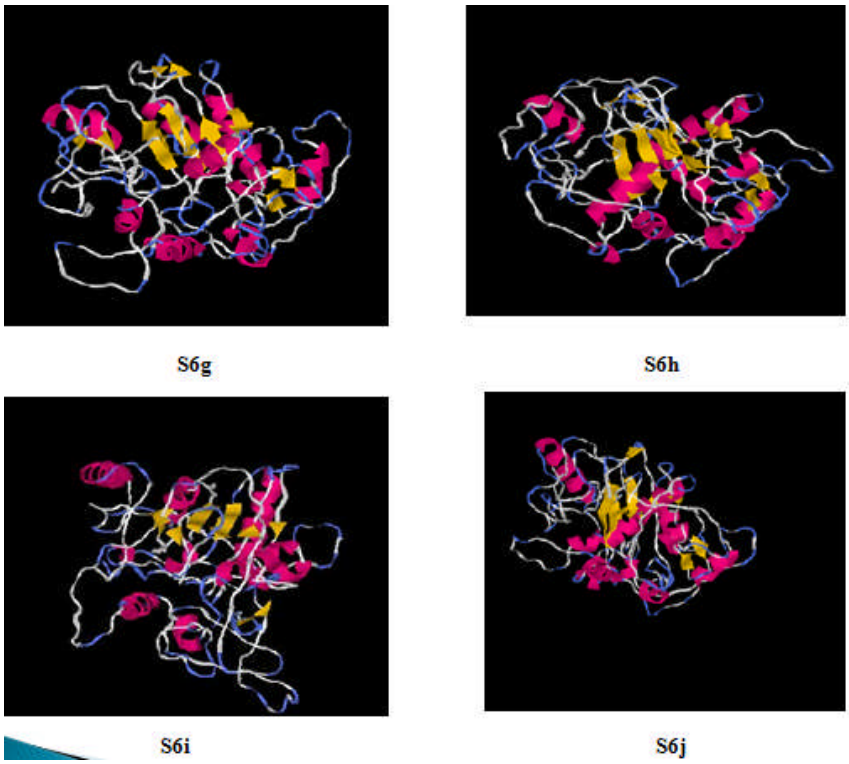


Figure S6.4. S6g) Wild type and mutant protein models of TGFβ1 (exon 1) predicted by I-TASSER. Structures with incorporated SNP C>T (S6h), G>C (S6i) & with both SNPs C>T & G to C (S6J).

Table S6.4. Table shows the total energy of the I-TASSER predicted TGFβ1 (exon 1) tertiary structures calculated by Swiss PDB Viewer.

S. No.	Protein Model	C-Score	Energy kj/mol
1	Wildtype 1	-2.64	- 9573.964kj/mol
2	Mutant, C>T	-2.64	-8931.029kj/mol
3	Mutant, G>C	-2.75	-8102.402kj/mol
4	Mutant, C>T + G>C	-2.21	-9501.950kj/mol

APPENDICES

Annexure - I

Sample ID	Age (Yrs)	Status	Degree	SNP1	SNP2	SNP3
F1S2	45	Case	-21	C/C	G/C	G/G
F2S6	13	Case	-11	C/C	G/C	G/G
F2S3	20	Case	-6	C/C	G/C	G/G
F3F3	15	Case	-6	C/C	G/C	G/G
F3S4	11	Case	-6	C/C	G/C	G/G
F4S2	32	Case	-6.5	C/C	C/C	G/G
F4S5	15	Case	-7.75	C/C	G/G	G/G
F5S5	35	Case	-10	C/C	G/G	G/G
F7S5	8	Case	-25	C/C	G/G	G/G
F9S4	18	Case	-9	C/C	G/G	G/G
F10S2	47	Case	-10	C/C	G/G	G/G
F3S1	32	Case	-13	C/C	G/G	G/A
F4S3	18	Case	-12	C/C	G/G	G/A
F5S1	62	Case	>-6	C/C	G/G	G/A
F5S10	5	Case	-13.5	C/C	G/G	G/A
F5S11	5	Case	-6	C/C	G/G	G/A
F6S6	35	Case	-16.25	C/C	G/G	G/A
F6S7	33	Case	-23	C/C	G/G	G/A
F7S3	14	Case	-28	C/C	G/G	G/A
F7S6	6	Case	-24	C/C	G/G	G/A
F8S1	60	Case	-7	C/C	G/G	G/A
F8S3	25	Case	-19	C/C	G/G	G/A
F8S4	29	Case	-10	C/C	G/G	G/A
F8S5	27	Case	-12	C/C	G/G	G/A
F9S1	45	Case	-6.5	C/C	G/G	G/A
F10S4	25	Case	-6	C/C	G/G	G/A
M1	30	Case	-9	C/C	G/G	G/A
M2	17	Case	-8	C/C	G/G	G/A
M3	22	Case	-10	C/C	G/G	G/A
M4	35	Case	-8	C/C	G/G	G/A
M5	48	Case	-13	C/C	G/G	G/A
M6	65	Case	-7	C/C	G/G	G/A
M7	25	Case	-9	C/C	G/G	G/A
M8	40	Case	-16	C/C	G/G	G/A
M9	32	Case	-10	C/C	G/G	G/A
M10	20	Case	-6.5	C/C	G/G	G/A
M11	22	Case	-6	C/C	G/G	G/A
M12	38	Case	-6	C/C	G/G	G/A
M13	57	Case	-7	C/C	G/G	G/A
M14	16	Case	-20	C/C	G/G	G/A
M15	13	Case	-18	C/C	G/G	G/A
M16	40	Case	-11	C/T	G/G	G/A
M17	21	Case	-13	C/T	G/G	G/A
M18	8	Case	-12	C/T	G/G	G/A
M19	22	Case	-15	C/T	G/G	G/A
M20	40	Case	-10	C/T	G/G	G/A
M21	35	Case	-11	C/T	G/G	G/A
M22	20	Case	-18	C/T	G/G	G/A
M23	18	Case	-8	C/T	G/G	G/A
M24	25	Case	-10	C/T	G/G	G/A
M25	18	Case	-20	T/T	G/G	G/A
M26	58	Case	-8	T/T	G/G	G/A
M27	13	Case	-13	T/T	G/G	G/A
M28	25	Case	-6	T/T	G/G	G/A
M29	65	Case	-6	T/T	G/G	G/A
M30	35	Case	-16	T/T	G/G	G/A
M31	24	Case	-14	T/T	G/G	G/A
M32	27	Case	-10	T/T	G/G	G/A
M33	22	Case	-11	T/T	C/C	G/A
M34	40	Case	-12	T/T	C/C	G/A
M35	42	Case	-11	T/T	C/C	G/A
M40	30	Case	-14	T/T	C/C	G/A
M41	29	Case	-8	T/T	C/C	G/G
M43	32	Case	-7	T/T	C/C	G/G
M46	17	Case	-9	T/T	C/C	G/G
M48	26	Case	-15	T/T	C/C	G/G
M51	25	Case	-18	T/T	C/C	A/A
M54	70	Case	-11	T/T	C/C	A/A
M57	16	Case	-19	T/T	C/C	A/A
M58	15	Case	-12	T/T	C/C	A/A
M64	35	Case	-10	T/T	C/C	A/A
M65	12	Case	-10	T/T	C/C	A/A
M66	21	Case	-13	T/T	C/C	A/A
M67	53	Case	-11	T/T	C/C	G/G
M68	64	Case	-22	T/T	G/G	G/G
M69	22	Case	-14	T/T	G/G	G/G
M70	23	Case	-7	T/T	G/G	G/G
M71	62	Case	-9	T/T	G/G	G/G
M72	19	Case	-14	T/T	G/G	G/A
M73	54	Case	-20	T/T	G/G	G/A
M74	43	Case	-14	T/T	G/G	G/A
M75	27	Case	-18	T/T	G/G	G/A
M76	32	Case	-7	T/T	G/G	G/A
M77	37	Case	-8.5	T/T	G/G	G/A
M78	45	Case	-9	T/T	G/G	G/A
M79	48	Case	-14	T/T	G/G	G/A
M80	14	Case	-24.4	C/T	G/G	G/A
M81	34	Case	-16	C/T	G/G	G/A
M82	66	Case	-17	C/T	G/G	G/A
M84	22	Case	-15	C/T	G/G	G/A
M85	17	Case	-27	C/T	G/G	G/A
M86	13	Case	-22	C/T	G/G	G/A
M87	42	Case	-16	C/T	G/G	G/A
M88	25	Case	-9	C/T	G/G	G/A
M89	35	Case	-8	C/T	G/G	G/A
M90	60	Case	-13	C/T	G/G	G/A
M91	51	Case	-25	C/T	G/G	G/A
M92	45	Case	-6.3	C/T	G/G	G/A
M93	32	Case	-17	C/T	G/G	G/A
M94	16	Case	-21	C/T	G/G	G/A
M95	8	Case	-32	C/T	G/G	G/A
M96	56	Case	-19.8	C/T	G/G	G/A
M97	17	Case	-6	T/T	G/G	G/A
M98	27	Case	-7	T/T	G/G	G/A
M99	33	Case	-10	T/T	G/G	G/A
M100	64	Case	-15	T/T	G/G	G/A
M101	10	Case	-12	T/T	G/G	G/A
M102	19	Case	-13	T/T	G/G	G/A
M103	32	Case	-19	T/T	G/G	G/A
M104	24	Case	-20.5	T/T	G/G	G/A
M105	55	Case	-16	T/T	G/G	G/A
M106	47	Case	-8	T/T	G/G	G/A
M107	26	Case	-6	T/T	G/G	G/A
M108	33	Case	-9	T/T	G/G	G/A
M109	22	Case	-10	T/T	G/G	G/A
M110	19	Case	-13	T/T	G/G	G/A
M111	41	Case	-11	T/T	G/G	G/A
M112	27	Case	-15	T/T	G/G	G/A
M113	33	Case	-25	T/T	G/C	G/G
M114	37	Case	-32	T/T	G/C	G/G
M115	42	Case	-18	T/T	G/C	G/G
M116	45	Case	-22	T/T	G/C	G/G
M117	25	Case	-15	T/T	G/C	G/G
M118	23	Case	-20	T/T	G/C	G/G
M119	18	Case	-26	T/T	G/C	A/A
M120	40	Case	-30	T/T	G/C	A/A
M121	67	Case	-12	T/T	C/C	A/A
M122	63	Case	-21	T/T	C/C	A/A
M123	44	Case	-6	T/T	C/C	A/A
M124	12	Case	-10	T/T	C/C	A/A
M125	21	Case	-13	T/T	C/C	A/A
M126	53	Case	-11	T/T	C/C	A/A
M127	64	Case	-22	T/T	C/C	A/A
M128	22	Case	-14	T/T	C/C	A/A
M129	23	Case	-7	T/T	C/C	A/A
M130	25	Case	-10	T/T	C/C	A/A
M131	18	Case	-20	T/T	C/C	A/A
M132	58	Case	-8	C/T	C/C	A/A
M133	13	Case	-13	C/T	C/C	A/A
M134	27	Case	-18	C/T	C/C	G/G
M135	32	Case	-7	C/T	C/C	G/G
M136	37	Case	-8.5	C/T	C/C	G/G
M137	45	Case	-9	C/T	C/C	G/G
M138	48	Case	-14	C/T	C/C	G/G
M139	14	Case	-24.4	C/T	C/C	G/G
M140	34	Case	-16	C/T	C/C	G/G
M141	66	Case	-17	C/T	G/C	G/G
M142	30	Case	-6.8	C/T	G/C	G/G
M143	22	Case	-15	C/T	G/C	G/G
M144	17	Case	-27	C/T	G/C	G/G
M145	13	Case	-22	C/T	G/C	G/G
M146	42	Case	-16	C/T	G/C	G/G
M147	25	Case	-9	C/T	G/C	G/G
M148	35	Case	-8	C/T	G/C	G/G
M149	60	Case	-13	C/T	G/C	G/G
M150	51	Case	-25	C/T	G/C	G/G
M151	45	Case	-6.3	C/T	G/C	G/G
M152	32	Case	-17	C/T	G/C	G/G

M153	16	Case	-21	C/T	G/C	G/G
M154	8	Case	-32	C/T	G/C	G/G
M155	56	Case	-19.8	C/T	G/C	G/G
M156	17	Case	-6	C/T	G/C	G/G
M157	27	Case	-7	C/T	G/C	G/G
M158	33	Case	-10	C/T	G/C	G/G
M159	64	Case	-15	C/T	G/C	G/G
M160	10	Case	-12	C/T	G/C	G/G
M161	19	Case	-13	C/T	G/C	G/G
M162	32	Case	-19	C/T	G/C	G/G
M163	24	Case	-20.5	C/T	G/C	G/G
M164	55	Case	-16	C/T	G/C	G/G
M165	47	Case	-8	C/T	G/C	G/G
M166	26	Case	-6	C/T	G/C	G/G
M167	33	Case	-9	C/T	G/C	G/G
M168	67	Case	-12	C/T	G/C	G/G
M169	54	Case	-9	C/T	G/C	G/G
M170	22	Case	-24	C/T	G/C	G/G
M171	28	Case	-13	C/T	G/C	G/G
M172	16	Case	-7	C/T	G/C	G/G
M173	12	Case	-10	C/T	G/C	G/G
M174	9	Case	-6	C/T	G/C	G/G
M175	17	Case	-23	C/T	G/C	G/G
M176	48	Case	-8	C/T	G/C	G/G
M177	23	Case	-23	C/T	G/C	G/G
M178	54	Case	-14	C/T	G/C	G/G
M179	37	Case	-7	C/T	G/C	G/G
M180	28	Case	-12	C/T	G/C	G/G
M181	32	Case	-17	C/T	G/C	G/G
M182	61	Case	-13	C/T	G/C	G/G
M183	40	Case	-9	C/T	G/C	G/G
M184	31	Case	-8.8	C/T	G/C	G/G
M185	15	Case	-25	C/T	G/C	G/G
M186	62	Case	-6	C/T	G/C	G/G
M187	60	Case	-27	C/T	G/C	G/G
M188	45	Case	-9	C/T	G/C	G/G
M189	20	Case	-6	C/T	G/C	G/G
M190	13	Case	-12	C/T	G/C	G/G
M191	43	Case	-25	C/T	G/C	G/G
M192	26	Case	-23	C/T	G/C	G/G
M193	32	Case	-12	C/T	G/C	G/G
M194	25	Case	-10	C/T	G/C	G/G
M195	9	Case	-16	C/T	G/C	G/G
M196	61	Case	-11	C/T	G/C	G/G
M197	22	Case	-7	C/T	G/C	G/G
M198	56	Case	-24	C/T	G/C	G/G
M199	28	Case	-15	C/T	G/C	G/G
M200	12	Case	-21	C/T	G/C	G/G
M201	21	Case	-22	C/T	G/C	G/G
M202	15	Case	-14	C/T	G/C	G/G
M203	14	Case	-8	C/T	G/C	G/G
M204	18	Case	-6	C/T	G/C	G/G
M205	13	Case	-10	C/T	G/C	G/G
M206	21	Case	-11	C/T	G/C	G/G
M207	43	Case	-11	C/T	G/C	G/G
M208	44	Case	-13	C/T	G/C	G/G
M209	23	Case	-30	C/T	G/C	G/G
M210	16	Case	-22	C/T	G/C	G/G
M211	20	Case	-12	C/T	G/C	G/G
M212	22	Case	-13	C/T	G/C	G/G
M214	35	Case	-10	C/T	G/C	G/G
M215	38	Case	-25	C/T	G/C	G/G
M230	40	Case	-27	C/T	G/C	G/G
M231	45	Case	-14	C/T	G/C	G/G
M232	60	Case	-19	C/T	G/C	G/G
M233	32	Case	-20	C/T	G/C	G/G
M234	25	Case	-23	C/T	G/C	G/G
M235	22	Case	-22	C/T	G/C	G/G
M236	65	Case	-18	C/T	G/C	G/G
M237	54	Case	-13	C/T	G/C	G/G
M238	23	Case	-17	C/T	G/C	G/G
M239	19	Case	-11	C/T	G/C	G/G
M240	32	Case	-18	C/T	G/C	G/G
M241	58	Case	-10	C/T	G/C	G/G
M242	44	Case	-9	C/T	G/C	G/G
M243	21	Case	-8	C/T	G/C	G/G
M244	17	Case	-13	C/T	G/C	G/G
M245	20	Case	-11	C/T	G/C	G/G
M246	15	Case	-13	C/T	G/C	G/G
M247	22	Case	-9	C/T	G/C	G/G

M248	26	Case	-12	C/T	G/C	G/G
M249	27	Case	-25	C/T	G/C	G/G
M250	28	Case	-23	C/T	G/C	G/G
M251	50	Case	-11	C/T	G/C	G/G
M252	44	Case	-14	C/T	G/C	G/G
M253	32	Case	-15	C/T	G/C	G/G
M254	19	Case	-13	C/T	G/C	G/G
M255	20	Case	-11	C/T	G/C	G/G
M256	33	Case	-8	C/T	G/C	G/G
F1S3	21	Control	-0.75	T/T	G/C	G/G
F2S2	39	Control	NIL	T/T	G/C	G/G
F2S4	18	Control	NIL	T/T	G/C	G/G
F2S5	16	Control	NIL	T/T	G/C	G/G
F2S7	10	Control	NIL	T/T	C/C	A/A
F3S2	26	Control	NIL	T/T	C/C	A/A
F3S6	7	Control	NIL	T/T	C/C	A/A
F4S4	20	Control	-1.75	T/T	G/C	A/A
F5S2	58	Control	NIL	T/T	G/C	A/A
F5S7	28	Control	NIL	T/T	G/C	G/G
F5S9	7	Control	NIL	T/T	G/G	G/G
F6S2	55	Control	-1.5	T/T	G/G	G/G
F7S2	33	Control	-0.05	C/C	G/G	G/G
F7S4	12	Control	-0.25	C/C	G/G	G/G
F8S2	52	Control	NIL	C/C	G/G	G/G
F8S7	21	Control	NIL	C/C	G/G	G/G
F9S2	39	Control	-0.5	C/C	G/G	G/G
N1	45	Control	NIL	C/C	G/G	G/G
N2	20	Control	NIL	C/C	G/G	G/G
N3	19	Control	NIL	C/C	G/G	G/G
N4	34	Control	NIL	C/C	G/G	G/G
N5	30	Control	NIL	C/C	G/G	G/G
N6	45	Control	NIL	C/C	G/G	G/G
N7	38	Control	NIL	C/C	G/G	G/G
N8	24	Control	NIL	C/C	G/G	G/G
N9	32	Control	NIL	C/C	G/G	A/A
N10	40	Control	NIL	C/C	G/G	A/A
N11	56	Control	NIL	T/T	G/G	A/A
N12	50	Control	NIL	T/T	G/G	A/A
N13	17	Control	NIL	T/T	G/G	A/A
N14	35	Control	NIL	T/T	G/G	A/A
N15	18	Control	NIL	T/T	G/G	G/G
N16	42	Control	NIL	T/T	G/G	G/G
N17	22	Control	NIL	T/T	G/G	G/G
N18	36	Control	NIL	T/T	G/G	G/G
N19	33	Control	NIL	C/T	G/G	G/G
N20	21	Control	NIL	C/T	G/G	G/G
N21	40	Control	NIL	C/T	G/G	G/G
N22	42	Control	NIL	C/T	G/G	A/A
N23	28	Control	NIL	C/T	G/G	A/A
N24	30	Control	NIL	C/T	G/G	A/A
N25	22	Control	NIL	C/T	G/G	A/A
N26	27	Control	NIL	C/T	G/G	A/A
N27	16	Control	NIL	C/T	G/G	A/A
N28	26	Control	NIL	C/T	G/G	A/A
N29	31	Control	NIL	C/T	G/G	A/A
N30	19	Control	NIL	C/T	G/G	A/A
N31	24	Control	NIL	C/T	G/G	G/G
N32	37	Control	NIL	C/T	G/G	G/A
N33	18	Control	NIL	C/T	G/G	G/A
N34	45	Control	NIL	C/T	G/G	G/A
N35	31	Control	NIL	C/T	G/G	G/A
N36	25	Control	NIL	C/T	G/G	G/A
N37	31	Control	NIL	C/T	G/G	G/A
N38	14	Control	NIL	C/T	G/G	G/A
N39	8	Control	NIL	C/T	G/G	G/A
N40	8	Control	NIL	C/T	G/G	G/A
N41	19	Control	NIL	C/T	G/G	G/A
N42	26	Control	NIL	C/T	G/G	G/A
N43	25	Control	NIL	C/T	G/G	G/A
N44	57	Control	NIL	C/T	G/G	G/A
N45	28	Control	NIL	C/T	G/G	G/A
N46	37	Control	NIL	C/T	G/C	G/A
N47	65	Control	NIL	C/T	G/C	G/G
N48	19	Control	NIL	C/T	G/C	G/G
N49	21	Control	NIL	C/T	C/C	G/G
N50	19	Control	NIL	C/T	C/C	G/G
N51	21	Control	NIL	C/T	C/C	G/G
N52	26	Control	NIL	C/T	C/C	G/G
N53	13	Control	NIL	C/T	C/C	G/G
N54	17	Control	NIL	C/T	C/C	G/G

N55	25	Control	NIL	C/T	C/C	G/G
N56	27	Control	NIL	C/T	C/C	G/G
N57	43	Control	NIL	C/T	C/C	G/G
N58	11	Control	NIL	C/T	C/C	G/A
N59	55	Control	NIL	C/T	G/C	G/A
N60	46	Control	NIL	C/T	G/G	G/A
N61	30	Control	NIL	C/T	G/G	G/A
N62	22	Control	NIL	C/T	G/G	G/A
N63	15	Control	NIL	C/T	G/G	G/A
N64	42	Control	NIL	C/T	G/G	G/A
N65	60	Control	NIL	C/T	G/G	G/A
N66	46	Control	NIL	C/T	G/G	G/A
N67	30	Control	NIL	C/T	G/G	G/A
N68	22	Control	NIL	C/T	G/G	G/A
N69	15	Control	NIL	C/T	G/G	G/A
N70	52	Control	NIL	C/T	G/G	G/A
N71	44	Control	NIL	C/T	G/G	G/A
N72	26	Control	NIL	C/T	G/G	G/A
N73	32	Control	NIL	C/T	G/G	G/A
N74	24	Control	NIL	C/T	G/G	G/A
N75	56	Control	NIL	C/T	G/G	G/A
N76	27	Control	NIL	C/T	G/G	G/A
N77	12	Control	NIL	C/T	G/G	G/A
N78	14	Control	NIL	C/T	G/G	G/A
N79	21	Control	NIL	C/T	G/G	G/A
N80	19	Control	NIL	C/T	G/G	G/A
N81	40	Control	NIL	C/T	G/G	G/A
N82	36	Control	NIL	C/T	G/G	G/A
N83	22	Control	NIL	C/T	G/G	G/A
N84	33	Control	NIL	C/T	G/G	G/A
N85	47	Control	NIL	C/T	G/C	G/A
N86	62	Control	NIL	C/T	G/C	G/A
N87	20	Control	NIL	C/T	G/C	G/A
N88	69	Control	NIL	C/T	G/C	G/G
N89	67	Control	NIL	C/T	G/C	G/G
N90	51	Control	NIL	C/T	G/C	G/G
N91	49	Control	NIL	C/T	G/C	G/G
N92	25	Control	NIL	C/T	C/C	G/G
N93	32	Control	NIL	C/T	C/C	G/G
N94	19	Control	NIL	C/T	C/C	G/G
N95	20	Control	NIL	C/T	G/C	G/G
N96	16	Control	NIL	C/T	G/C	G/G
N97	67	Control	NIL	C/T	G/C	G/G
N98	55	Control	NIL	C/T	G/C	G/G
N99	25	Control	NIL	C/T	G/C	G/G
N100	20	Control	NIL	C/T	G/C	G/G
N101	49	Control	NIL	C/T	G/C	G/A
N102	23	Control	NIL	C/T	G/C	G/A
N103	32	Control	NIL	C/T	G/C	G/A
N104	52	Control	NIL	C/T	G/C	G/A
N105	30	Control	NIL	T/T	G/C	G/A
N106	41	Control	NIL	T/T	G/C	G/A
N107	63	Control	NIL	T/T	G/C	G/A
N108	14	Control	NIL	T/T	G/C	G/A
N109	61	Control	NIL	T/T	G/C	G/A
N110	55	Control	NIL	T/T	G/C	G/A
N111	23	Control	NIL	T/T	G/C	G/A
N112	18	Control	NIL	T/T	G/C	G/A
N113	17	Control	NIL	T/T	G/C	G/A
N114	33	Control	NIL	T/T	G/C	G/A
N115	41	Control	NIL	T/T	G/C	G/A
N116	24	Control	NIL	T/T	G/C	G/A
N117	50	Control	NIL	T/T	G/C	G/A
N118	25	Control	NIL	T/T	G/C	G/A
N119	19	Control	NIL	T/T	G/C	G/A
N120	22	Control	NIL	T/T	G/C	G/A
N121	48	Control	NIL	T/T	G/C	G/A
N122	67	Control	NIL	T/T	G/C	G/G
N123	31	Control	NIL	T/T	G/C	G/G
N124	47	Control	NIL	T/T	G/C	G/G
N125	28	Control	NIL	T/T	G/C	G/G
N126	38	Control	NIL	T/T	G/C	G/G
N127	42	Control	NIL	T/T	G/C	G/G
N128	19	Control	NIL	T/T	G/C	G/G
N129	47	Control	NIL	T/T	G/C	G/G
N130	31	Control	NIL	T/T	G/C	G/G
N131	20	Control	NIL	T/T	G/C	G/G
N132	46	Control	NIL	T/T	G/C	G/G
N133	54	Control	NIL	T/T	G/C	G/G
N134	39	Control	NIL	T/T	G/C	G/G
N135	56	Control	NIL	T/T	G/C	G/G
N136	28	Control	NIL	T/T	G/C	G/G
N137	22	Control	NIL	T/T	G/C	G/G
N138	11	Control	NIL	T/T	G/C	G/G
N139	9	Control	NIL	T/T	G/C	G/G
N140	43	Control	NIL	T/T	G/C	G/G
N141	56	Control	NIL	T/T	G/C	G/G
F1S1	50	Control	0.25	T/T	G/C	G/G
F1S4	18	Control	NIL	T/T	G/C	G/G
F2S1	45	Control	-0.75	T/T	G/C	G/G
F3S5	9	Control	NIL	T/T	G/C	G/G
F4S1	37	Control	NIL	T/T	G/C	G/G
F5S3	40	Control	NIL	T/T	G/C	G/G
F5S6	31	Control	NIL	T/T	G/C	G/G
F5S8	27	Control	NIL	T/T	G/C	G/G
F6S1	58	Control	NIL	T/T	G/C	G/G
F6S3	40	Control	NIL	T/T	G/C	G/G
F6S4	38	Control	NIL	T/T	G/C	G/G
F6S5	37	Control	NIL	T/T	G/C	G/G
F7S1	33	Control	-0.75	T/T	G/C	G/G
F8S6	24	Control	NIL	T/T	G/C	G/G
F9S3	21	Control	NIL	T/T	G/C	G/G
F10S1	53	Control	-0.5	T/T	G/C	G/G
F10S3	29	Control	NIL	T/T	G/C	G/G
F10S5	22	Control	NIL	T/T	G/C	G/G

Annexure - II							
Sample ID	Age (Yrs)	Gender	Status	Degree	SNP1	SNP2	SNP3
M48	26	F	Cases	-15	T/C,T/A	T/T	G/C
M51	25	M	Cases	-18	T/C,T/A	T/T	G/C
M54	70	F	Cases	-11	T/C,T/A	T/T	G/C
M57	16	M	Cases	-19	T/C,T/A	T/T	G/C
M58	15	M	Cases	-12	T/C,T/A	T/T	G/G
M64	35	M	Cases	-10	T/C,T/A	T/T	G/G
F1S2	45	F	Cases	-21	T/C,T/A	T/T	G/G
F2S3	20	F	Cases	-6	T/C,T/A	T/T	G/G
F2S6	13	F	Cases	-11	T/C,T/A	T/T	G/G
F5S10	5	M	Cases	-13.5	T/C,T/A	T/T	G/G
F5S11	5	M	Cases	-6	T/C,T/A	T/T	G/G
F7S5	8	F	Cases	-25	T/C,T/A	T/T	G/G
F8S3	25	M	Cases	-19	T/C,T/A	T/T	G/G
F9S1	45	M	Cases	-6.5	T/C,T/A	T/T	G/G
F9S4	18	F	Cases	-9	T/C,T/A	T/G	G/G
M1	30	F	Cases	-9	T/C,T/A	T/G	G/G
M2	17	F	Cases	-8	T/C,T/A	T/G	G/C
M3	22	M	Cases	-10	T/C,T/A	T/G	G/C
M4	35	F	Cases	-8	T/C,T/A	T/G	G/C
M5	48	M	Cases	-13	T/C,T/A	T/G	G/C
M6	65	M	Cases	-7	T/C,T/A	T/G	G/C
M7	25	F	Cases	-9	T/C,T/A	T/G	G/C
M8	40	F	Cases	-16	T/C,T/A	T/G	G/C
M9	32	M	Cases	-10	T/C,T/A	T/G	G/C
M10	20	M	Cases	-6.5	T/C,T/A	T/G	G/C
M11	22	M	Cases	-6	T/C,T/A	T/G	G/C
M12	38	M	Cases	-6	T/T	T/G	G/G
M13	57	F	Cases	-7	T/T	T/G	G/G
M14	16	M	Cases	-20	T/T	T/G	G/G
M15	13	M	Cases	-18	T/T	T/G	G/G
M16	40	M	Cases	-11	T/T	T/G	G/G
M17	21	M	Cases	-13	T/T	T/G	G/G
M18	8	F	Cases	-12	T/T	T/G	G/G
M19	22	M	Cases	-15	T/T	T/G	G/G
M20	40	F	Cases	-10	T/T	T/G	G/G
M21	35	F	Cases	-11	T/T	T/G	G/G
M22	20	F	Cases	-18	T/T	T/G	G/G
M23	18	M	Cases	-8	T/T	T/G	G/G
M24	25	M	Cases	-10	T/T	T/G	G/G
M25	18	M	Cases	-20	T/T	T/G	G/G
M26	58	M	Cases	-8	T/T	T/G	G/G
M27	13	M	Cases	-13	T/T	T/G	G/G
M28	25	M	Cases	-6	T/T	T/G	G/G
M29	65	F	Cases	-6	T/T	T/G	G/G
M30	35	F	Cases	-16	T/T	T/G	G/G
M31	24	M	Cases	-14	T/T	T/G	G/G
M32	27	F	Cases	-10	T/T	T/G	G/G
M33	22	F	Cases	-11	T/T	T/G	G/G
M34	40	F	Cases	-12	T/T	T/G	G/G
M35	42	M	Cases	-11	T/T	T/G	G/G
M40	30	M	Cases	-14	T/T	T/G	G/G
M41	29	M	Cases	-8	T/T	T/G	G/G
N1A	18	F	Controls	-0.25	T/T	T/T	G/G
F2S1	45	M	Controls	-0.75	T/T	T/T	G/G
F2S2	39	F	Controls	NIL	T/T	T/T	G/G
F4S1	37	M	Controls	NIL	T/T	T/G	G/G
F4S4	20	F	Controls	-0.75	T/T	T/G	G/G
F5S3	40	M	Controls	NIL	T/T	T/G	G/G
F6S2	55	F	Controls	-0.5	T/C,T/A	T/G	G/G
F6S4	38	M	Controls	NIL	T/C,T/A	T/G	G/G
F7S1	33	M	Controls	-0.75	T/C,T/A	T/G	G/G
F7S2	33	F	Controls	-0.05	T/C,T/A	T/G	G/G
F7S4	12	F	Controls	-0.25	T/T	T/G	G/G
F9S3	21	M	Controls	N.A	T/T	T/T	G/G
F10S3	29	M	Controls	NIL	T/T	T/T	G/G
F10S5	22	M	Controls	NIL	T/T	T/T	G/G
F2S4	18	F	Controls	NIL	T/T	T/T	G/G
F2S5	16	F	Controls	NIL	T/T	T/T	G/G
F2S7	10	F	Controls	NIL	T/T	T/T	G/G
F3S2	26	F	Controls	NIL	T/T	T/T	G/G

F = Female, M = Male

Annexure III

ID	Age (Yrs)	Gender	Status	Degree	SNP 1
M48	26	F	case	-15	G/A
M51	25	M	case	-18	G/A
M54	70	F	case	-11	G/A
M57	16	M	case	-19	G/A
M58	15	M	case	-12	G/A
M64	35	M	case	-10	G/A
F1S2	45	F	case	-21	G/A
F2S3	20	F	case	-6	G/A
F2S6	13	F	case	-11	G/A
F5S10	5	M	case	-13.5	G/A
F5S11	5	M	case	-6	G/A
F7S5	8	F	case	-25	G/A
F8S3	25	M	case	-19	G/A
F9S1	45	M	case	-6.5	G/A
F9S4	18	F	case	-9	G/A
M1	30	F	case	-9	G/A
M2	17	F	case	-8	G/A
M3	22	M	case	-10	G/A
M4	35	F	case	-8	G/A
M5	48	M	case	-13	G/A
M6	65	M	case	-7	G/A
M7	25	F	case	-9	G/A
M8	40	F	case	-16	G/A
M9	32	M	case	-10	G/A
M10	20	M	case	-6.5	G/A
M11	22	M	case	-6	G/A
M12	38	M	case	-6	G/A
M13	57	F	case	-7	G/A
M14	16	M	case	-20	G/A
M15	13	M	case	-18	G/A
M16	40	M	case	-11	G/A
M17	21	M	case	-13	G/A
M18	8	F	case	-12	G/A
M19	22	M	case	-15	G/A
M20	40	F	case	-10	G/A
M21	35	F	case	-11	G/A
M22	20	F	case	-18	G/G
M23	18	M	case	-8	G/G
M24	25	M	case	-10	G/G
M25	18	M	case	-20	G/G
M26	58	M	case	-8	G/G
M27	13	M	case	-13	G/G
M28	25	M	case	-6	G/G
M29	65	F	case	-6	G/G
M30	35	F	case	-16	G/G
M31	24	M	case	-14	G/G
M32	27	F	case	-10	G/G
M33	22	F	case	-11	G/G
M34	40	F	case	-12	G/G
M35	42	M	case	-11	G/G
M40	30	M	case	-14	G/G
M41	29	M	case	-8	G/G
M43	32	F	case	-7	G/G
M46	17	F	case	-9	G/G
M49	17	F	case	-23	G/A
M52	14	F	case	-12	G/A
M55	22	F	case	-14	G/A
M50	38	F	case	-7	G/A
M59	61	F	case	-18	G/A
M60	55	F	case	-9	G/A
M61	43	F	case	-17	G/A
M62	63	F	case	-21	G/A
M63	44	F	case	-6	G/A
M65	12	F	case	-10	G/A
M66	21	F	case	-13	G/A
M67	53	F	case	-11	G/A
M68	64	F	case	-22	G/A
M69	22	F	case	-14	G/A
M70	23	F	case	-7	G/A
M71	62	F	case	-9	G/A
M72	19	F	case	-14	G/A
M73	54	F	case	-20	G/A
M74	43	F	case	-14	G/A
M75	27	F	case	-18	G/A
M76	32	F	case	-7	G/A
M77	37	F	case	-8.5	G/A
M78	45	F	case	-9	G/A
M79	48	F	case	-14	G/A
M80	14	F	case	-24.4	G/A
M81	34	F	case	-16	G/A
M82	66	M	case	-17	G/A
M83	30	M	case	-6.8	G/A
M84	22	M	case	-15	G/A
M85	17	M	case	-27	G/A
M86	13	M	case	-22	G/A
M87	42	M	case	-16	G/A
M88	25	M	case	-9	G/A
M89	35	M	case	-8	G/A
M90	60	M	case	-13	G/A
M91	51	M	case	-25	G/A
M92	45	M	case	-6.3	G/G
M93	32	M	case	-17	G/G
M94	16	M	case	-21	G/G
M95	8	M	case	-32	G/G
M96	56	M	case	-19.8	G/G
M97	17	M	case	-6	G/G
M98	27	M	case	-7	G/G
M99	33	M	case	-10	G/G
M100	64	M	case	-15	G/G
M101	10	M	case	-12	G/G
M102	19	M	case	-13	G/G
M103	32	M	case	-19	G/G
M104	24	M	case	-20.5	G/G
M105	55	M	case	-16	G/G
M106	47	M	case	-8	G/G
M107	26	M	case	-6	G/G
M108	33	M	case	-9	G/G
M109	22	F	case	-10	G/A
M110	19	F	case	-13	G/A
M111	41	F	case	-11	G/G
M112	27	M	case	-15	G/G
M113	33	M	case	-25	G/G
M114	37	M	case	-32	G/G
M115	42	M	case	-18	G/G
M116	45	F	case	-22	G/G
M117	25	F	case	-15	G/G
M118	23	F	case	-20	G/G
M119	18	F	case	-26	G/G
M120	40	F	case	-30	G/G
M121	67	M	case	-12	G/G
N1A	18	F	control	-0.25	G/G
F2S1	45	M	control	-0.75	G/G
F2S2	39	F	control	NIL	G/G
F4S1	37	M	control	NIL	G/G
F4S4	20	F	control	-1.75	G/G
F5S3	40	M	control	NIL	G/G
F6S2	55	F	control	-1.5	G/G
F6S4	38	M	control	NIL	G/G
F7S1	33	M	control	-0.75	G/G
F7S2	33	F	control	-0.05	G/G
F7S4	12	F	control	-0.25	G/G
F9S3	21	M	control	N.A	G/G
F10S3	29	M	control	NIL	G/G
F10S5	22	M	control	NIL	G/G
F2S4	18	F	control	NIL	G/G
F2S5	16	F	control	NIL	G/G
F2S7	10	F	control	NIL	G/G
F3S2	26	F	control	NIL	G/G
F3S6	7	F	control	NIL	G/G
F5S2	58	F	control	NIL	G/G
F5S7	28	F	control	NIL	G/G
F5S9	7	F	control	NIL	G/G
FISI	50	M	control	-0.25	G/G
F1S4	18	M	control	NIL	G/G
F3S5	9	M	control	NIL	G/G

F5S6	31	M	control	NIL	G/G
F5S8	27	M	control	NIL	G/G
F6S1	58	M	control	NIL	G/G
F6S3	40	M	control	NIL	G/G
N1	45	F	control	NIL	G/G
N2	20	M	control	NIL	G/G
N5	30	F	control	NIL	G/G
N7	38	F	control	NIL	G/G
N10	40	M	control	NIL	G/G
N21	40	F	control	NIL	G/G
N24	30	F	control	NIL	G/G
N3	19	M	control	NIL	G/G
N4	34	M	control	NIL	G/G
N6	45	M	control	NIL	G/G
N8	24	M	control	NIL	G/G
N9	32	M	control	NIL	G/G
N12	50	F	control	NIL	G/G
N13	17	F	control	NIL	G/G
N14	35	F	control	NIL	G/G
N16	42	F	control	NIL	G/G
N23	28	F	control	NIL	G/G
N25	22	M	control	NIL	G/G
N26	27	M	control	NIL	G/G
N29	31	M	control	NIL	G/G
N30	19	F	control	NIL	G/G
N31	24	F	control	NIL	G/G
N32	37	F	control	NIL	G/G
N33	18	F	control	NIL	G/G
N34	45	F	control	NIL	G/G
N35	31	F	control	NIL	G/G
N36	25	F	control	NIL	G/G
N38	14	F	control	NIL	G/G
N39	8	F	control	NIL	G/G
N41	19	F	control	NIL	G/G
N42	26	F	control	NIL	G/G
N43	25	F	control	NIL	G/G
N44	57	F	control	NIL	G/G
N45	28	F	control	NIL	G/G
N46	37	M	control	NIL	G/G
N47	65	M	control	NIL	G/G
N50	19	M	control	NIL	G/G
N51	21	M	control	NIL	G/G
N52	26	M	control	NIL	G/G
N53	13	M	control	NIL	G/G
N54	17	M	control	NIL	G/G
N55	25	M	control	NIL	G/G
N56	27	M	control	NIL	G/G
N57	43	M	control	NIL	G/G
N58	11	M	control	NIL	G/G
N59	55	M	control	NIL	G/G
N64	42	M	control	NIL	G/G
N65	60	M	control	NIL	G/G
N66	46	F	control	NIL	G/G
N67	30	F	control	NIL	G/G
N68	22	F	control	NIL	G/G
N69	15	F	control	NIL	G/G
N70	52	F	control	NIL	G/G
N71	44	M	control	NIL	G/G
N72	26	M	control	NIL	G/G
N73	32	M	control	NIL	G/G
N74	24	M	control	NIL	G/G
N75	56	M	control	NIL	G/G
N76	27	M	control	NIL	G/G
N77	12	M	control	NIL	G/G
N78	14	M	control	NIL	G/G
N79	21	M	control	NIL	G/G
N80	19	M	control	NIL	G/G
N81	40	M	control	NIL	G/G
N82	36	M	control	NIL	G/G
N83	22	M	control	NIL	G/G
N84	33	M	control	NIL	G/G
N85	47	M	control	NIL	G/G
N86	62	M	control	NIL	G/G
N88	69	M	control	NIL	G/G
N90	51	M	control	NIL	G/G
N91	49	M	control	NIL	G/G
N92	25	F	control	NIL	G/G
N93	32	F	control	NIL	G/G
N94	19	F	control	NIL	G/G
N95	20	M	control	NIL	G/G
N96	16	M	control	NIL	G/G
N97	67	M	control	NIL	G/G
N98	55	M	control	NIL	G/G
N99	25	F	control	NIL	G/G
N100	20	F	control	NIL	G/G
N101	49	F	control	NIL	G/G
N102	23	F	control	NIL	G/G
N103	32	F	control	NIL	G/G
N104	52	F	control	NIL	G/G

F = Female; M = Male

Annexure IV

Sample ID	Age (Yrs)	Gender	Status	Degree	SNP1	SNP2
M48	26	F	Case	-15	G/A	G/G
M51	25	M	Case	-18	G/A	G/G
M54	70	F	Case	-11	G/A	G/G
M57	16	M	Case	-19	G/A	G/G
M58	15	M	Case	-12	G/A	G/G
M64	35	M	Case	-10	G/A	G/G
F1S2	45	F	Case	-21	G/A	G/G
F2S3	20	F	Case	-6	G/A	G/G
F2S6	13	F	Case	-11	G/A	G/G
F5S10	5	M	Case	-13.5	G/A	G/G
F5S11	5	M	Case	-6	G/A	G/G
F7S5	8	F	Case	-25	G/A	G/G
F8S3	25	M	Case	-19	G/A	G/G
F9S1	45	M	Case	-6.5	G/A	G/G
F9S4	18	F	Case	-9	G/A	G/G
M1	30	F	Case	-9	G/A	G/G
M2	17	F	Case	-8	G/A	G/G
M3	22	M	Case	-10	G/A	G/G
M4	35	F	Case	-8	G/A	G/G
M5	48	M	Case	-13	G/A	G/G
M6	65	M	Case	-7	G/A	G/G
M7	25	F	Case	-9	G/A	G/G
M8	40	F	Case	-16	G/A	G/G
M9	32	M	Case	-10	G/A	G/G
M10	20	M	Case	-6.5	G/A	G/G
M11	22	M	Case	-6	G/A	G/G
M12	38	M	Case	-6	G/A	G/G
M13	57	F	Case	-7	G/A	G/G
M14	16	M	Case	-20	G/A	G/G
M15	13	M	Case	-18	G/A	G/G
M16	40	M	Case	-11	G/A	G/G
M17	21	M	Case	-13	G/A	G/G
M18	8	F	Case	-12	G/A	G/G
M19	22	M	Case	-15	G/A	G/G
M20	40	F	Case	-10	G/A	G/G
M21	35	F	Case	-11	G/A	G/G
M22	20	F	Case	-18	G/A	G/A
M23	18	M	Case	-8	G/A	G/A
M130	25	F	Case	-10	G/A	G/A
M131	18	F	Case	-20	G/A	G/A
M132	58	F	Case	-8	G/A	G/A
M133	13	F	Case	-13	G/A	G/A
M28	25	M	Case	-6	G/A	G/A
M29	65	F	Case	-6	G/A	G/A
M30	35	F	Case	-16	G/A	G/A
M31	24	M	Case	-14	G/A	G/A
M32	27	F	Case	-10	G/A	G/A
M33	22	F	Case	-11	G/A	G/A
M34	40	F	Case	-12	G/A	G/A
M35	42	M	Case	-11	G/A	G/A
M40	30	M	Case	-14	G/A	G/A
M41	29	M	Case	-8	G/A	G/A
M43	32	F	Case	-7	G/A	G/A
M46	17	F	Case	-9	G/A	G/A
M49	17	F	Case	-23	G/A	G/A
M52	14	F	Case	-12	G/A	G/A
M55	22	F	Case	-14	G/A	G/A
M50	38	F	Case	-7	G/A	G/A
M59	61	F	Case	-18	G/A	G/A
M60	55	F	Case	-9	G/A	G/A
M61	43	F	Case	-17	G/A	G/A
M62	63	F	Case	-21	G/A	G/A
M63	44	F	Case	-6	G/A	G/A
M65	12	F	Case	-10	G/A	G/A
M66	21	F	Case	-13	G/A	G/A
M67	53	F	Case	-11	G/A	G/A
M68	64	F	Case	-22	G/A	G/A
M69	22	F	Case	-14	G/A	G/A
M70	23	F	Case	-7	G/A	G/A
M71	62	F	Case	-9	G/A	G/A
M72	19	F	Case	-14	G/A	G/A
M73	54	F	Case	-20	G/A	G/A
M74	43	F	Case	-14	G/A	G/G
M75	27	F	Case	-18	G/A	G/G
M76	32	F	Case	-7	G/A	G/G
M77	37	F	Case	-8.5	G/A	G/G
M78	45	F	Case	-9	G/A	G/G
M79	48	F	Case	-14	G/A	G/G
M80	14	F	Case	-24.4	G/A	G/G
M81	34	F	Case	-16	G/A	G/G
M82	66	M	Case	-17	G/A	G/G
M83	30	M	Case	-6.8	G/A	G/G
M84	22	M	Case	-15	G/A	G/G
M85	17	M	Case	-27	G/A	G/G
M86	13	M	Case	-22	G/A	G/G
M87	42	M	Case	-16	G/A	G/G
M88	25	M	Case	-9	G/A	G/G
M89	35	M	Case	-8	G/A	G/G
M90	60	M	Case	-13	G/A	G/G
M91	51	M	Case	-25	G/A	G/G
M92	45	M	Case	-6.3	G/A	G/G
M93	32	M	Case	-17	G/A	G/G
M94	16	M	Case	-21	G/A	G/G
M95	8	M	Case	-32	G/A	G/G
M96	56	M	Case	-19.8	G/A	G/G
M97	17	M	Case	-6	G/A	G/G
M98	27	M	Case	-7	G/A	G/G
M99	33	M	Case	-10	G/A	G/G
M100	64	M	Case	-15	G/A	G/G
M101	10	M	Case	-12	G/A	G/G
M102	19	M	Case	-13	G/A	G/G
M103	32	M	Case	-19	G/A	G/G
M104	24	M	Case	-20.5	G/A	G/G
M105	55	M	Case	-16	G/A	G/G
M106	47	M	Case	-8	G/A	G/G
M107	26	M	Case	-6	G/A	G/G
M108	33	M	Case	-9	G/A	G/G
M109	22	F	Case	-10	G/A	G/A
M110	19	F	Case	-13	G/A	G/A
N1A	18	F	control	-0.25	G/A	G/G
F2S1	45	M	control	-0.75	G/A	G/G
F2S2	39	F	control	NIL	G/A	G/G
F4S1	37	M	control	NIL	G/A	G/G
F4S4	20	F	control	-0.75	G/A	G/G
F5S3	40	M	control	NIL	G/A	G/G
F6S2	55	F	control	-0.5	G/A	G/G
F6S4	38	M	control	NIL	G/A	G/G
F7S1	33	M	control	-0.75	G/A	G/G
F7S2	33	F	control	-0.05	G/A	G/G
F7S4	12	F	control	-0.25	G/A	G/G
F9S3	21	M	control	N.A	G/A	G/G
F10S3	29	M	control	NIL	G/A	G/G
F10S5	22	M	control	NIL	G/A	G/G
F2S4	18	F	control	NIL	G/A	G/G
F2S5	16	F	control	NIL	G/A	G/G
F2S7	10	F	control	NIL	G/A	G/G
F3S2	26	F	control	NIL	G/A	G/G
F3S6	7	F	control	NIL	G/A	G/G
F5S2	58	F	control	NIL	G/A	G/G
F5S7	28	F	control	NIL	G/A	G/G
F5S9	7	F	control	NIL	G/A	G/G
FISI	50	M	control	-0.25	G/A	G/G
F1S4	18	M	control	NIL	G/A	G/G
F3S5	9	M	control	NIL	G/A	G/G
F5S6	31	M	control	NIL	G/A	G/G
F5S8	27	M	control	NIL	G/A	G/G
F6S1	58	M	control	NIL	G/A	G/G
F6S3	40	M	control	NIL	G/A	G/G
N1	45	F	control	NIL	G/A	G/G
N2	20	M	control	NIL	G/A	G/G
N5	30	F	control	NIL	G/A	G/G
N7	38	F	control	NIL	G/A	G/G
N10	40	M	control	NIL	G/A	G/G
N21	40	F	control	NIL	G/A	G/G
N24	30	F	control	NIL	G/A	G/G
N3	19	M	control	NIL	G/A	G/G
N4	34	M	control	NIL	G/A	G/G
N6	45	M	control	NIL	G/A	G/G
N8	24	M	control	NIL	G/A	G/G
N9	32	M	control	NIL	G/A	G/G
N12	50	F	control	NIL	G/A	G/G
N13	17	F	control	NIL	G/A	G/G
N14	35	F	control	NIL	G/A	G/G
N16	42	F	control	NIL	G/A	G/G
N23	28	F	control	NIL	G/A	G/G
N25	22	M	control	NIL	G/A	G/G
N26	27	M	control	NIL	G/A	G/G

N29	31	M	control	NIL	G/A	G/G
N30	19	F	control	NIL	G/A	G/G
N31	24	F	control	NIL	G/A	G/G
N32	37	F	control	NIL	G/A	G/G
N33	18	F	control	NIL	G/A	G/G
N34	45	F	control	NIL	G/A	G/G
N35	31	F	control	NIL	G/A	G/G
N36	25	F	control	NIL	G/A	G/G
N38	14	F	control	NIL	G/A	G/G
N39	8	F	control	NIL	G/A	G/G
N41	19	F	control	NIL	G/A	G/G
N42	26	F	control	NIL	G/A	G/G
N43	25	F	control	NIL	G/A	G/G
N44	57	F	control	NIL	G/A	G/G
N45	28	F	control	NIL	G/A	G/G
N46	37	M	control	NIL	G/A	G/G
N47	65	M	control	NIL	G/A	G/G
N50	19	M	control	NIL	G/A	G/G
N51	21	M	control	NIL	G/A	G/G
N52	26	M	control	NIL	G/A	G/G
N53	13	M	control	NIL	G/A	G/G
N54	17	M	control	NIL	G/A	G/G
N55	25	M	control	NIL	G/A	G/G
N56	27	M	control	NIL	G/A	G/G
N57	43	M	control	NIL	G/A	G/G
N58	11	M	control	NIL	G/A	G/G
N59	55	M	control	NIL	G/A	G/G
N64	42	M	control	NIL	G/A	G/G
N65	60	M	control	NIL	G/A	G/G
N66	46	F	control	NIL	G/A	G/G
N67	30	F	control	NIL	G/A	G/G
N68	22	F	control	NIL	G/A	G/G
N69	15	F	control	NIL	G/A	G/G
N70	52	F	control	NIL	G/A	G/G
N71	44	M	control	NIL	G/A	G/G
N72	26	M	control	NIL	G/A	G/G
N73	32	M	control	NIL	G/A	G/G
N74	24	M	control	NIL	G/A	G/G
N75	56	M	control	NIL	G/A	G/G
N76	27	M	control	NIL	G/A	G/G
N77	12	M	control	NIL	G/A	G/G
N78	14	M	control	NIL	G/A	G/G
N79	21	M	control	NIL	G/A	G/G
N80	19	M	control	NIL	G/A	G/G
N81	40	M	control	NIL	G/A	G/G
N82	36	M	control	NIL	G/A	G/G
N83	22	M	control	NIL	G/A	G/G
N84	33	M	control	NIL	G/A	G/G
N85	47	M	control	NIL	G/A	G/G
N86	62	M	control	NIL	G/A	G/G
N88	69	M	control	NIL	G/A	G/G
N90	51	M	control	NIL	G/A	G/G
N91	49	M	control	NIL	G/A	G/G
N92	25	F	control	NIL	G/A	G/G
N93	32	F	control	NIL	G/A	G/G
N94	19	F	control	NIL	G/A	G/G
N95	20	M	control	NIL	G/A	G/G
N96	16	M	control	NIL	G/A	G/G
N97	67	M	control	NIL	G/A	G/G
N98	55	M	control	NIL	G/A	G/G
N99	25	F	control	NIL	G/A	G/G
N100	20	F	control	NIL	G/A	G/G
N101	49	F	control	NIL	G/A	G/G
N102	23	F	control	NIL	G/A	G/G
N103	32	F	control	NIL	G/A	G/G
N104	52	F	control	NIL	G/A	G/G
N105	30	F	control	NIL	G/A	G/G

F = Female; M = Male

Annexure V

Sample ID	Age (Yrs)	Gender	Status	Degree	SNP1
M48	26	F	Case	-15	T/T
M51	25	M	Case	-18	T/T
M54	70	F	Case	-11	T/T
M57	16	M	Case	-19	T/T
M58	15	M	Case	-12	T/T
M64	35	M	Case	-10	T/T
F1S2	45	F	Case	-21	T/T
F2S3	20	F	Case	-6	T/T
F2S6	13	F	Case	-11	T/T
F5S10	5	M	Case	-13.5	T/T
F5S11	5	M	Case	-6	T/T
F7S5	8	F	Case	-25	T/T
F8S3	25	M	Case	-19	T/T
F9S1	45	M	Case	-6.5	T/T
F9S4	18	F	Case	-9	T/T
M1	30	F	Case	-9	T/T
M2	17	F	Case	-8	T/T
M3	22	M	Case	-10	T/T
M4	35	F	Case	-8	T/T
M5	48	M	Case	-13	T/T
M6	65	M	Case	-7	T/C
M7	25	F	Case	-9	T/C
M8	40	F	Case	-16	T/C
M9	32	M	Case	-10	T/C
M10	20	M	Case	-6.5	T/C
M11	22	M	Case	-6	T/C
M12	38	M	Case	-6	T/C
M13	57	F	Case	-7	T/C
M14	16	M	Case	-20	T/C
M15	13	M	Case	-18	T/C
M16	40	M	Case	-11	T/T
M17	21	M	Case	-13	T/T
M18	8	F	Case	-12	T/T
M19	22	M	Case	-15	T/T
M20	40	F	Case	-10	T/T
M21	35	F	Case	-11	T/T
M22	20	F	Case	-18	T/T
M23	18	M	Case	-8	T/T
M130	25	F	Case	-10	T/T
M131	18	F	Case	-20	T/T
M132	58	F	Case	-8	T/T
M133	13	F	Case	-13	T/T
M28	25	M	Case	-6	T/T
M29	65	F	Case	-6	T/T
M30	35	F	Case	-16	T/T
M31	24	M	Case	-14	T/T
M32	27	F	Case	-10	T/T
M33	22	F	Case	-11	T/T
M34	40	F	Case	-12	T/T
M35	42	M	Case	-11	T/T
M40	30	M	Case	-14	T/T
M41	29	M	Case	-8	T/T
M43	32	F	Case	-7	T/T
M46	17	F	Case	-9	T/T
M49	17	F	Case	-23	T/C
M52	14	F	Case	-12	T/C
M55	22	F	Case	-14	T/C
M50	38	F	Case	-7	T/C
M59	61	F	Case	-18	T/C
M60	55	F	Case	-9	T/C
M61	43	F	Case	-17	T/C
M62	63	F	Case	-21	T/C
M63	44	F	Case	-6	T/C
M65	12	F	Case	-10	T/C
M66	21	F	Case	-13	T/T
M67	53	F	Case	-11	T/T
M68	64	F	Case	-22	T/T
M69	22	F	Case	-14	T/T
M70	23	F	Case	-7	T/T
M71	62	F	Case	-9	T/T
M72	19	F	Case	-14	T/T
M73	54	F	Case	-20	T/T
M74	43	F	Case	-14	T/T
M75	27	F	Case	-18	T/T
M76	32	F	Case	-7	T/T
M77	37	F	Case	-8.5	T/T
M78	45	F	Case	-9	T/T
M79	48	F	Case	-14	T/T
M80	14	F	Case	-24.4	T/T
M81	34	F	Case	-16	T/T
M82	66	M	Case	-17	T/T
M83	30	M	Case	-6.8	T/T
M84	22	M	Case	-15	T/T
M85	17	M	Case	-27	T/T
M86	13	M	Case	-22	T/T
M87	42	M	Case	-16	T/T
M88	25	M	Case	-9	T/T
M89	35	M	Case	-8	T/T
M90	60	M	Case	-13	T/T
M91	51	M	Case	-25	T/T
M92	45	M	Case	-6.3	T/T
M93	32	M	Case	-17	T/T
M94	16	M	Case	-21	T/T
M95	8	M	Case	-32	T/T
M96	56	M	Case	-19.8	T/T
M97	17	M	Case	-6	T/T
M98	27	M	Case	-7	T/T
M99	33	M	Case	-10	T/T
M100	64	M	Case	-15	T/T
M101	10	M	Case	-12	T/T
M102	19	M	Case	-13	T/T
M103	32	M	Case	-19	T/T
M104	24	M	Case	-20.5	T/T
M105	55	M	Case	-16	T/T
M106	47	M	Case	-8	T/T
M107	26	M	Case	-6	T/T
M108	33	M	Case	-9	T/T
M109	22	F	Case	-10	T/C
M110	19	F	Case	-13	T/C
M111	41	F	Case	-11	T/T
M112	27	M	Case	-15	T/T
M113	33	M	Case	-25	T/T
M114	37	M	Case	-32	T/T
M115	42	M	Case	-18	T/T
M121	67	M	Case	-12	T/T
N1A	18	F	control	-0.25	T/C
F2S1	45	M	control	-0.75	T/C
F2S2	39	F	control	NIL	T/C
F4S1	37	M	control	NIL	T/C
F4S4	20	F	control	-0.75	T/C
F5S3	40	M	control	NIL	T/C
F6S2	55	F	control	-0.5	T/C
F6S4	38	M	control	NIL	T/C
F7S1	33	M	control	-0.75	T/C
F7S2	33	F	control	-0.05	T/C
F7S4	12	F	control	-0.25	T/C
F9S3	21	M	control	N.A	T/C
F10S3	29	M	control	NIL	T/C
F10S5	22	M	control	NIL	T/C
F2S4	18	F	control	NIL	T/T
F2S5	16	F	control	NIL	T/T
F2S7	10	F	control	NIL	T/T
F3S2	26	F	control	NIL	T/T
F3S6	7	F	control	NIL	T/T
F5S2	58	F	control	NIL	T/T
F5S7	28	F	control	NIL	T/T
F5S9	7	F	control	NIL	T/T
FISI	50	M	control	-0.25	T/T
F1S4	18	M	control	NIL	T/T
F3S5	9	M	control	NIL	T/T
F5S6	31	M	control	NIL	T/T
F5S8	27	M	control	NIL	T/T
F6S1	58	M	control	NIL	T/T
F6S3	40	M	control	NIL	T/T
N1	45	F	control	NIL	T/T
N2	20	M	control	NIL	T/T
N5	30	F	control	NIL	T/T
N7	38	F	control	NIL	T/T
N10	40	M	control	NIL	T/T

N21	40	F	control	NIL	T/T
N24	30	F	control	NIL	T/T
N3	19	M	control	NIL	T/T
N4	34	M	control	NIL	T/T
N6	45	M	control	NIL	T/T
N8	24	M	control	NIL	T/T
N9	32	M	control	NIL	T/T
N12	50	F	control	NIL	T/T
N13	17	F	control	NIL	T/T
N14	35	F	control	NIL	T/T
N16	42	F	control	NIL	T/T
N23	28	F	control	NIL	T/T
N25	22	M	control	NIL	T/T
N26	27	M	control	NIL	T/T
N29	31	M	control	NIL	T/T
N30	19	F	control	NIL	T/T
N31	24	F	control	NIL	T/T
N32	37	F	control	NIL	T/T
N33	18	F	control	NIL	T/T
N34	45	F	control	NIL	T/T
N35	31	F	control	NIL	T/C
N36	25	F	control	NIL	T/C
N38	14	F	control	NIL	T/C
N39	8	F	control	NIL	T/C
N41	19	F	control	NIL	T/C
N42	26	F	control	NIL	T/C
N43	25	F	control	NIL	T/C
N44	57	F	control	NIL	T/C
N45	28	F	control	NIL	T/C
N46	37	M	control	NIL	T/C
N47	65	M	control	NIL	T/C
N50	19	M	control	NIL	T/C
N51	21	M	control	NIL	T/C
N52	26	M	control	NIL	T/C
N53	13	M	control	NIL	T/T
N54	17	M	control	NIL	T/T
N55	25	M	control	NIL	T/T
N56	27	M	control	NIL	T/T
N57	43	M	control	NIL	T/T
N58	11	M	control	NIL	T/T
N59	55	M	control	NIL	T/T
N64	42	M	control	NIL	T/T
N65	60	M	control	NIL	T/T
N66	46	F	control	NIL	T/T
N67	30	F	control	NIL	T/T
N68	22	F	control	NIL	T/T
N69	15	F	control	NIL	T/T
N70	52	F	control	NIL	T/T
N71	44	M	control	NIL	T/T
N72	26	M	control	NIL	T/T
N73	32	M	control	NIL	T/T
N74	24	M	control	NIL	T/T
N75	56	M	control	NIL	T/T
N76	27	M	control	NIL	T/T
N77	12	M	control	NIL	T/T
N78	14	M	control	NIL	T/T
N79	21	M	control	NIL	T/T
N80	19	M	control	NIL	T/T
N81	40	M	control	NIL	T/T
N82	36	M	control	NIL	T/T
N83	22	M	control	NIL	T/T
N84	33	M	control	NIL	T/T
N85	47	M	control	NIL	T/T
N86	62	M	control	NIL	T/T
N88	69	M	control	NIL	T/T
N90	51	M	control	NIL	T/T
N91	49	M	control	NIL	T/T
N92	25	F	control	NIL	T/T
N93	32	F	control	NIL	T/T
N94	19	F	control	NIL	T/T
N95	20	M	control	NIL	T/T
N96	16	M	control	NIL	T/T
N97	67	M	control	NIL	T/T

F = Female; M = Male

Annexure VI

Sample ID	Age (Yrs)	Gender	Status	Degree	SNP1	SNP2	SNP3
M48	26	F	Case	-15	G/G	C/C	G/G
M51	25	M	Case	-18	G/G	C/C	G/G
M54	70	F	Case	-11	G/G	C/C	G/G
M57	16	M	Case	-19	G/G	C/C	G/G
M58	15	M	Case	-12	G/G	C/C	G/G
M64	35	M	Case	-10	G/G	C/C	G/G
F1S2	45	F	Case	-21	G/G	C/C	G/G
F2S3	20	F	Case	-6	G/G	C/C	G/G
F2S6	13	F	Case	-11	G/G	C/C	G/G
F5S10	5	M	Case	-13.5	G/G	C/C	G/G
F5S11	5	M	Case	-6	G/G	C/C	G/G
F7S5	8	F	Case	-25	G/G	C/C	G/G
F8S3	25	M	Case	-19	G/G	C/C	G/G
F9S1	45	M	Case	-6.5	G/G	C/C	G/G
F9S4	18	F	Case	-9	G/G	C/C	G/G
M1	30	F	Case	-9	G/G	C/C	G/G
M2	17	F	Case	-8	G/G	C/C	G/G
M3	22	M	Case	-10	G/G	C/C	G/G
M4	35	F	Case	-8	G/G	C/C	G/G
M5	48	M	Case	-13	G/G	C/C	G/G
M6	65	M	Case	-7	G/G	C/C	G/G
M7	25	F	Case	-9	G/G	C/C	G/G
M8	40	F	Case	-16	G/G	C/C	G/G
M9	32	M	Case	-10	G/G	C/C	G/G
M10	20	M	Case	-6.5	G/G	C/C	G/G
M11	22	M	Case	-6	G/G	C/C	G/G
M12	38	M	Case	-6	G/G	C/C	G/G
M13	57	F	Case	-7	G/G	C/C	G/G
M14	16	M	Case	-20	G/G	C/C	G/G
M15	13	M	Case	-18	G/G	C/C	G/G
M16	40	M	Case	-11	G/G	C/C	G/G
M17	21	M	Case	-13	G/G	C/C	G/G
M18	8	F	Case	-12	G/G	C/C	G/G
M19	22	M	Case	-15	G/G	C/C	G/G
M20	40	F	Case	-10	G/G	C/C	G/G
M21	35	F	Case	-11	G/G	G/C	G/G
M22	20	F	Case	-18	G/G	G/C	G/G
M23	18	M	Case	-8	G/G	C/C	G/G
M130	25	F	Case	-10	G/G	C/C	G/G
M131	18	F	Case	-20	G/G	C/C	G/G
M132	58	F	Case	-8	G/G	C/C	G/G
M133	13	F	Case	-13	G/G	C/C	G/G
M28	25	M	Case	-6	A/A	C/C	A/A
M29	65	F	Case	-6	A/A	C/C	A/A
M30	35	F	Case	-16	A/A	C/C	A/A
M31	24	M	Case	-14	A/A	C/C	A/A
M32	27	F	Case	-10	G/A	C/C	A/A
M33	22	F	Case	-11	G/A	C/C	A/A
M34	40	F	Case	-12	A/A	C/C	G/G
M35	42	M	Case	-11	A/A	G/C	G/G
M40	30	M	Case	-14	G/A	G/C	G/G
M41	29	M	Case	-8	G/A	G/C	G/G
M43	32	F	Case	-7	G/A	G/C	G/G
M46	17	F	Case	-9	G/A	G/C	G/G
M49	17	F	Case	-23	G/G	C/C	G/G
M52	14	F	Case	-12	G/G	C/C	G/G
M55	22	F	Case	-14	G/G	C/C	G/G
M50	38	F	Case	-7	G/G	C/C	G/G
M59	61	F	Case	-18	G/G	C/C	G/G
M60	55	F	Case	-9	G/G	C/C	G/G
M61	43	F	Case	-17	G/G	C/C	G/G
M62	63	F	Case	-21	G/G	C/C	G/G
M63	44	F	Case	-6	G/G	C/C	G/G
M65	12	F	Case	-10	G/G	C/C	G/G
M66	21	F	Case	-13	G/G	C/C	G/G
M67	53	F	Case	-11	G/G	C/C	G/G
M68	64	F	Case	-22	G/G	C/C	G/G
M69	22	F	Case	-14	G/G	C/C	G/G
M70	23	F	Case	-7	G/G	C/C	G/G
M71	62	F	Case	-9	G/G	C/C	G/G
M72	19	F	Case	-14	G/G	C/C	G/G
M73	54	F	Case	-20	G/G	C/C	G/G
M74	43	F	Case	-14	G/G	C/C	G/G
M75	27	F	Case	-18	G/G	C/C	G/G
M76	32	F	Case	-7	G/G	C/C	G/G
M77	37	F	Case	-8.5	G/G	C/C	G/G
M78	45	F	Case	-9	G/G	C/C	G/G
M79	48	F	Case	-14	G/G	C/C	G/G
M80	14	F	Case	-24.4	G/G	C/C	G/G
M81	34	F	Case	-16	G/G	C/C	G/G
M82	66	M	Case	-17	G/G	C/C	G/G
M83	30	M	Case	-6.8	G/G	C/C	G/G
M84	22	M	Case	-15	G/G	C/C	G/G
M85	17	M	Case	-27	G/G	C/C	G/G
M86	13	M	Case	-22	G/G	C/C	G/G
M87	42	M	Case	-16	G/G	C/C	G/G
M88	25	M	Case	-9	G/G	C/C	G/G
M89	35	M	Case	-8	G/G	C/C	G/G
M90	60	M	Case	-13	G/G	C/C	G/G
M91	51	M	Case	-25	G/G	G/C	G/G
M92	45	M	Case	-6.3	G/G	G/C	G/G
M93	32	M	Case	-17	G/G	C/C	G/G
M94	16	M	Case	-21	G/G	C/C	G/G
M95	8	M	Case	-32	G/G	C/C	G/G
M96	56	M	Case	-19.8	G/G	C/C	G/G
M97	17	M	Case	-6	G/G	C/C	G/G
M98	27	M	Case	-7	A/A	C/C	A/A
M99	33	M	Case	-10	A/A	C/C	A/A
M100	64	M	Case	-15	A/A	C/C	A/A
M101	10	M	Case	-12	A/A	C/C	A/A
M102	19	M	Case	-13	G/A	C/C	A/A
M103	32	M	Case	-19	G/A	C/C	A/A
M104	24	M	Case	-20.5	A/A	C/C	G/G
M105	55	M	Case	-16	A/A	G/C	G/G
M106	47	M	Case	-8	G/A	G/C	G/G
M107	26	M	Case	-6	G/A	G/C	G/G
M108	33	M	Case	-9	G/A	G/C	G/G
M109	22	F	Case	-10	G/A	C/C	G/G
M110	19	F	Case	-13	G/A	C/C	G/G
M111	41	F	Case	-11	A/A	G/C	A/A
M112	27	M	Case	-15	A/A	G/C	A/A
M121	67	M	Case	-12	G/A	G/C	G/G
N1A	18	F	control	-0.25	G/G	C/C	G/G
F2S1	45	M	control	-0.75	G/G	C/C	G/G
F2S2	39	F	control	NIL	G/G	C/C	G/G
F4S1	37	M	control	NIL	G/G	C/C	G/G
F4S4	20	F	control	-1.75	G/G	C/C	G/G
F5S3	40	M	control	NIL	G/G	C/C	G/G
F6S2	55	F	control	-1.5	G/G	C/C	G/G
F6S4	38	M	control	NIL	G/G	C/C	G/G
F7S1	33	M	control	-0.75	G/G	C/C	G/G
F7S2	33	F	control	-0.05	G/G	C/C	G/G
F7S4	12	F	control	-0.25	G/G	C/C	G/G
F9S3	21	M	control	N.A	G/G	G/C	G/G
F10S3	29	M	control	NIL	G/G	G/C	G/G
F10S5	22	M	control	NIL	G/G	G/C	G/G
F2S4	18	F	control	NIL	G/G	G/C	G/G
F2S5	16	F	control	NIL	G/G	G/C	G/G
F2S7	10	F	control	NIL	G/G	G/C	G/G
F3S2	26	F	control	NIL	G/G	G/C	G/G
F3S6	7	F	control	NIL	G/G	G/C	G/G
F5S2	58	F	control	NIL	G/G	G/C	G/G
F5S7	28	F	control	NIL	G/G	G/C	G/G
F5S9	7	F	control	NIL	G/G	G/C	G/G
FISI	50	M	control	-0.25	G/G	G/C	G/G
F1S4	18	M	control	NIL	G/G	G/C	G/G
F3S5	9	M	control	NIL	G/G	G/C	G/G
F5S6	31	M	control	NIL	G/G	G/C	G/G
F5S8	27	M	control	NIL	G/G	G/C	G/G
F6S1	58	M	control	NIL	G/G	G/C	G/G
F6S3	40	M	control	NIL	G/G	C/C	G/G
N1	45	F	control	NIL	G/A	C/C	G/A
N2	20	M	control	NIL	G/A	C/C	G/A
N5	30	F	control	NIL	G/A	C/C	G/A
N7	38	F	control	NIL	G/A	C/C	G/A
N10	40	M	control	NIL	G/A	C/C	G/A
N21	40	F	control	NIL	G/A	C/C	G/A
N24	30	F	control	NIL	G/A	C/C	G/A
N3	19	M	control	NIL	G/A	C/C	G/G
N4	34	M	control	NIL	G/A	C/C	G/G
N6	45	M	control	NIL	G/A	C/C	G/G
N8	24	M	control	NIL	G/A	C/C	G/G
N9	32	M	control	NIL	G/A	C/C	G/G
N12	50	F	control	NIL	G/A	C/C	G/G
N13	17	F	control	NIL	G/A	C/C	G/G
N14	35	F	control	NIL	G/A	C/C	G/G
N16	42	F	control	NIL	G/A	C/C	G/G

N23	**28**	**F**	**control**	**NIL**	**G/A**	**C/C**	**G/G**
N25	**22**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/G**
N26	**27**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/A**
N29	**31**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/A**
N30	**19**	**F**	**control**	**NIL**	**G/A**	**C/C**	**G/A**
N31	**24**	**F**	**control**	**NIL**	**G/A**	**C/C**	**G/A**
N32	**37**	**F**	**control**	**NIL**	**G/A**	**C/C**	**G/A**
N33	**18**	**F**	**control**	**NIL**	**G/G**	**C/C**	**G/A**
N34	**45**	**F**	**control**	**NIL**	**G/G**	**C/C**	**G/G**
N35	**31**	**F**	**control**	**NIL**	**G/G**	**C/C**	**G/G**
N36	**25**	**F**	**control**	**NIL**	**G/G**	**C/C**	**G/G**
N38	**14**	**F**	**control**	**NIL**	**G/G**	**C/C**	**G/G**
N39	**8**	**F**	**control**	**NIL**	**G/G**	**C/C**	**G/G**
N41	**19**	**F**	**control**	**NIL**	**G/G**	**C/C**	**G/G**
N42	**26**	**F**	**control**	**NIL**	**G/G**	**C/C**	**G/G**
N43	**25**	**F**	**control**	**NIL**	**G/G**	**C/C**	**G/G**
N44	**57**	**F**	**control**	**NIL**	**G/G**	**C/C**	**G/G**
N45	**28**	**F**	**control**	**NIL**	**G/G**	**C/C**	**G/G**
N46	**37**	**M**	**control**	**NIL**	**G/G**	**C/C**	**G/G**
N47	**65**	**M**	**control**	**NIL**	**G/G**	**C/C**	**G/G**
N50	**19**	**M**	**control**	**NIL**	**G/G**	**G/C**	**G/G**
N51	**21**	**M**	**control**	**NIL**	**G/G**	**G/C**	**G/G**
N52	**26**	**M**	**control**	**NIL**	**G/G**	**G/C**	**G/G**
N53	**13**	**M**	**control**	**NIL**	**G/G**	**G/C**	**G/G**
N54	**17**	**M**	**control**	**NIL**	**G/G**	**G/C**	**G/G**
N55	**25**	**M**	**control**	**NIL**	**G/G**	**G/C**	**G/G**
N56	**27**	**M**	**control**	**NIL**	**G/G**	**G/C**	**G/G**
N57	**43**	**M**	**control**	**NIL**	**G/G**	**G/C**	**G/G**
N58	**11**	**M**	**control**	**NIL**	**G/G**	**G/C**	**G/G**
N59	**55**	**M**	**control**	**NIL**	**G/G**	**G/C**	**G/G**
N64	**42**	**M**	**control**	**NIL**	**G/G**	**G/C**	**G/G**
N65	**60**	**M**	**control**	**NIL**	**G/G**	**G/C**	**G/G**
N66	**46**	**F**	**control**	**NIL**	**G/G**	**G/C**	**G/G**
N67	**30**	**F**	**control**	**NIL**	**G/G**	**G/C**	**G/G**
N68	**22**	**F**	**control**	**NIL**	**G/G**	**G/C**	**G/G**
N69	**15**	**F**	**control**	**NIL**	**G/G**	**G/C**	**G/G**
N70	**52**	**F**	**control**	**NIL**	**G/G**	**G/C**	**G/G**
N71	**44**	**M**	**control**	**NIL**	**G/G**	**C/C**	**G/G**
N72	**26**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/A**
N73	**32**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/A**
N74	**24**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/A**
N75	**56**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/A**
N76	**27**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/A**
N77	**12**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/A**
N78	**14**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/A**
N79	**21**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/G**
N80	**19**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/G**
N81	**40**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/G**
N82	**36**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/G**
N83	**22**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/G**
N84	**33**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/G**
N85	**47**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/G**
N86	**62**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/G**
N88	**69**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/G**
N90	**51**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/G**
N91	**49**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/G**
N92	**25**	**F**	**control**	**NIL**	**G/A**	**C/C**	**G/A**
N93	**32**	**F**	**control**	**NIL**	**G/A**	**C/C**	**G/A**
N94	**19**	**F**	**control**	**NIL**	**G/A**	**C/C**	**G/A**
N95	**20**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/A**
N96	**16**	**M**	**control**	**NIL**	**G/A**	**C/C**	**G/A**
N97	**67**	**M**	**control**	**NIL**	**G/G**	**C/C**	**G/A**
N98	**55**	**M**	**control**	**NIL**	**G/G**	**C/C**	**G/G**
N99	**25**	**F**	**control**	**NIL**	**G/G**	**C/C**	**G/G**
N100	**20**	**F**	**control**	**NIL**	**G/G**	**C/C**	**G/G**

F = Female; M = Male

Annexure VII

Sample ID	Age (Yrs)	Gender	Status	Degree	SNP1	SNP2
M48	26	F	Case	-15	G/T	C/G
M51	25	M	Case	-18	G/T	C/G
M54	70	F	Case	-11	G/T	C/G
M57	16	M	Case	-19	G/T	C/G
M58	15	M	Case	-12	G/T	C/G
M64	35	M	Case	-10	G/T	C/G
F1S2	45	F	Case	-21	G/T	C/G
F2S3	20	F	Case	-6	G/T	C/G
F2S6	13	F	Case	-11	G/T	C/C
F5S10	5	M	Case	-13.5	G/T	C/C
F5S11	5	M	Case	-6	G/T	C/C
F7S5	8	F	Case	-25	G/T	C/C
F8S3	25	M	Case	-19	G/T	C/C
F9S1	45	M	Case	-6.5	G/T	C/C
F9S4	18	F	Case	-9	G/T	C/G
M1	30	F	Case	-9	G/T	C/G
M2	17	F	Case	-8	G/T	C/G
M3	22	M	Case	-10	G/T	C/G
M4	35	F	Case	-8	G/T	C/G
M5	48	M	Case	-13	G/T	C/G
M6	65	M	Case	-7	G/T	C/G
M7	25	F	Case	-9	G/T	C/G
M8	40	F	Case	-16	G/T	C/G
M9	32	M	Case	-10	G/T	C/G
M10	20	M	Case	-6.5	G/T	C/G
M11	22	M	Case	-6	G/T	C/G
M12	38	M	Case	-6	G/T	C/G
M13	57	F	Case	-7	G/T	C/G
M14	16	M	Case	-20	G/T	C/G
M15	13	M	Case	-18	G/T	C/G
M16	40	M	Case	-11	G/T	C/G
M17	21	M	Case	-13	G/T	C/G
M18	8	F	Case	-12	G/T	C/G
M19	22	M	Case	-15	G/T	C/G
M20	40	F	Case	-10	G/T	C/G
M21	35	F	Case	-11	G/T	C/G
M22	20	F	Case	-18	G/T	C/G
M23	18	M	Case	-8	G/T	C/G
M130	25	F	Case	-10	G/T	C/G
M131	18	F	Case	-20	G/T	C/G
M132	58	F	Case	-8	G/T	C/G
M133	13	F	Case	-13	G/T	C/G
M28	25	M	Case	-6	G/G	C/G
M29	65	F	Case	-6	G/G	C/G
M30	35	F	Case	-16	G/G	C/G
M31	24	M	Case	-14	G/G	C/G
M32	27	F	Case	-10	G/G	C/G
M33	22	F	Case	-11	G/G	C/G
M34	40	F	Case	-12	G/T	C/G
M35	42	M	Case	-11	G/T	C/G
M40	30	M	Case	-14	G/T	C/G
M41	29	M	Case	-8	G/T	C/G
M43	32	F	Case	-7	G/T	C/G
M46	17	F	Case	-9	G/T	C/G
M49	17	F	Case	-23	G/T	C/G
M52	14	F	Case	-12	G/T	C/G
M55	22	F	Case	-14	G/T	C/G
M50	38	F	Case	-7	G/T	C/G
M59	61	F	Case	-18	G/T	C/G
M60	55	F	Case	-9	G/T	C/G
M61	43	F	Case	-17	G/T	C/G
M62	63	F	Case	-21	G/T	C/G
M63	44	F	Case	-6	G/T	C/C
M65	12	F	Case	-10	G/T	C/C
M66	21	F	Case	-13	G/T	C/C
M67	53	F	Case	-11	G/T	C/C
M68	64	F	Case	-22	G/T	C/C
M69	22	F	Case	-14	G/T	C/C
M70	23	F	Case	-7	G/T	C/G
M71	62	F	Case	-9	G/T	C/G
M72	19	F	Case	-14	G/T	C/G
M73	54	F	Case	-20	G/T	C/G
M74	43	F	Case	-14	G/T	C/G
M75	27	F	Case	-18	G/T	C/G
M76	32	F	Case	-7	G/T	C/G
M77	37	F	Case	-8.5	G/T	C/G
M78	45	F	Case	-9	G/T	C/G
M79	48	F	Case	-14	G/T	C/G
M80	14	F	Case	-24.4	G/T	C/G
M81	34	F	Case	-16	G/T	C/G
M82	66	M	Case	-17	G/T	C/G
M83	30	M	Case	-6.8	G/T	C/G
M84	22	M	Case	-15	G/T	C/G
M85	17	M	Case	-27	G/T	C/G
M86	13	M	Case	-22	G/T	C/G
M87	42	M	Case	-16	G/T	C/G
M88	25	M	Case	-9	G/T	C/G
M89	35	M	Case	-8	G/T	C/G
M90	60	M	Case	-13	G/T	C/G
M91	51	M	Case	-25	G/T	C/G
M92	45	M	Case	-6.3	G/T	C/G
M93	32	M	Case	-17	G/T	C/G
M94	16	M	Case	-21	G/T	C/G
M95	8	M	Case	-32	G/T	C/G
M96	56	M	Case	-19.8	G/T	C/G
M97	17	M	Case	-6	G/T	C/G
M98	27	M	Case	-7	G/G	C/G
M99	33	M	Case	-10	G/G	C/G
M100	64	M	Case	-15	G/G	C/G
M101	10	M	Case	-12	G/G	C/G
M102	19	M	Case	-13	G/G	C/G
M103	32	M	Case	-19	G/G	C/G
M104	24	M	Case	-20.5	G/T	C/G
M105	55	M	Case	-16	G/T	C/G
M106	47	M	Case	-8	G/T	C/G
M107	26	M	Case	-6	G/T	C/G
M108	33	M	Case	-9	G/T	C/G
M109	22	F	Case	-10	G/T	C/G
M110	19	F	Case	-13	G/T	C/G
M111	41	F	Case	-11	G/G	C/C
M112	27	M	Case	-15	G/G	C/C
M113	33	M	Case	-25	G/G	C/C
M121	67	M	Case	-12	G/T	C/G
N1A	18	F	control	0.25	G/G	C/C
F2S1	45	M	control	-0.75	G/G	C/C
F2S2	39	F	control	NIL	G/G	C/C
F4S1	37	M	control	NIL	G/G	C/C
F4S4	20	F	control	-1.75	G/G	C/C
F5S3	40	M	control	NIL	G/G	C/C
F6S2	55	F	control	-1.5	G/G	C/C
F6S4	38	M	control	NIL	G/G	C/C
F7S1	33	M	control	-0.75	G/G	C/C
F7S2	33	F	control	-0.05	G/G	C/C
F7S4	12	F	control	-0.25	G/G	C/C
F9S3	21	M	control	N.A	G/G	C/C
F10S3	29	M	control	NIL	G/G	C/C
F10S5	22	M	control	NIL	G/G	C/C
F2S4	18	F	control	NIL	G/G	C/C
F2S5	16	F	control	NIL	G/G	C/C
F2S7	10	F	control	NIL	G/G	C/C
F3S2	26	F	control	NIL	G/G	C/C
F3S6	7	F	control	NIL	G/G	C/C
F5S2	58	F	control	NIL	G/G	C/C
F5S7	28	F	control	NIL	G/G	C/C
F5S9	7	F	control	NIL	G/G	C/C
FISI	50	M	control	-0.25	G/G	C/C
F1S4	18	M	control	NIL	G/G	C/C
F3S5	9	M	control	NIL	G/G	C/C
F5S6	31	M	control	NIL	G/G	C/C
F5S8	27	M	control	NIL	G/G	C/C
F6S1	58	M	control	NIL	G/G	C/C
F6S3	40	M	control	NIL	G/G	C/C
N1	45	F	control	NIL	G/G	C/C
N2	20	M	control	NIL	G/G	C/C
N5	30	F	control	NIL	G/G	C/C
N7	38	F	control	NIL	G/G	C/C
N10	40	M	control	NIL	G/G	C/C
N21	40	F	control	NIL	G/G	C/C
N24	30	F	control	NIL	G/G	C/C
N3	19	M	control	NIL	G/G	C/C
N4	34	M	control	NIL	G/G	C/C
N6	45	M	control	NIL	G/G	C/C
N8	24	M	control	NIL	G/G	C/C
N9	32	M	control	NIL	G/G	C/C
N12	50	F	control	NIL	G/G	C/C
N13	17	F	control	NIL	G/G	C/C
N14	35	F	control	NIL	G/G	C/C

N16	**42**	**F**	**control**	**NIL**	**G/G**	**C/C**
N23	**28**	**F**	**control**	**NIL**	**G/G**	**C/C**
N25	**22**	**M**	**control**	**NIL**	**G/G**	**C/C**
N26	**27**	**M**	**control**	**NIL**	**G/G**	**C/C**
N29	**31**	**M**	**control**	**NIL**	**G/G**	**C/C**
N30	**19**	**F**	**control**	**NIL**	**G/G**	**C/C**
N31	**24**	**F**	**control**	**NIL**	**G/G**	**C/C**
N32	**37**	**F**	**control**	**NIL**	**G/G**	**C/C**
N33	**18**	**F**	**control**	**NIL**	**G/G**	**C/C**
N34	**45**	**F**	**control**	**NIL**	**G/G**	**C/C**
N35	**31**	**F**	**control**	**NIL**	**G/G**	**C/C**
N36	**25**	**F**	**control**	**NIL**	**G/G**	**C/C**
N38	**14**	**F**	**control**	**NIL**	**G/G**	**C/C**
N39	**8**	**F**	**control**	**NIL**	**G/G**	**C/C**
N41	**19**	**F**	**control**	**NIL**	**G/G**	**C/C**
N42	**26**	**F**	**control**	**NIL**	**G/G**	**C/C**
N43	**25**	**F**	**control**	**NIL**	**G/G**	**C/C**
N44	**57**	**F**	**control**	**NIL**	**G/G**	**C/C**
N45	**28**	**F**	**control**	**NIL**	**G/G**	**C/C**
N46	**37**	**M**	**control**	**NIL**	**G/G**	**C/C**
N47	**65**	**M**	**control**	**NIL**	**G/G**	**C/C**
N50	**19**	**M**	**control**	**NIL**	**G/G**	**C/C**
N51	**21**	**M**	**control**	**NIL**	**G/G**	**C/C**
N52	**26**	**M**	**control**	**NIL**	**G/G**	**C/C**
N53	**13**	**M**	**control**	**NIL**	**G/G**	**C/C**
N54	**17**	**M**	**control**	**NIL**	**G/G**	**C/C**
N55	**25**	**M**	**control**	**NIL**	**G/G**	**C/C**
N56	**27**	**M**	**control**	**NIL**	**G/G**	**C/C**
N57	**43**	**M**	**control**	**NIL**	**G/G**	**C/C**
N58	**11**	**M**	**control**	**NIL**	**G/G**	**C/C**
N59	**55**	**M**	**control**	**NIL**	**G/G**	**C/C**
N64	**42**	**M**	**control**	**NIL**	**G/G**	**C/C**
N65	**60**	**M**	**control**	**NIL**	**G/G**	**C/C**
N66	**46**	**F**	**control**	**NIL**	**G/G**	**C/C**
N67	**30**	**F**	**control**	**NIL**	**G/G**	**C/C**
N68	**22**	**F**	**control**	**NIL**	**G/G**	**C/C**
N69	**15**	**F**	**control**	**NIL**	**G/G**	**C/C**
N70	**52**	**F**	**control**	**NIL**	**G/G**	**C/C**
N71	**44**	**M**	**control**	**NIL**	**G/G**	**C/C**
N72	**26**	**M**	**control**	**NIL**	**G/G**	**C/C**
N73	**32**	**M**	**control**	**NIL**	**G/G**	**C/C**
N74	**24**	**M**	**control**	**NIL**	**G/G**	**C/C**
N75	**56**	**M**	**control**	**NIL**	**G/G**	**C/C**
N76	**27**	**M**	**control**	**NIL**	**G/G**	**C/C**
N77	**12**	**M**	**control**	**NIL**	**G/G**	**C/C**
N78	**14**	**M**	**control**	**NIL**	**G/G**	**C/C**
N79	**21**	**M**	**control**	**NIL**	**G/G**	**C/C**
N80	**19**	**M**	**control**	**NIL**	**G/G**	**C/C**
N81	**40**	**M**	**control**	**NIL**	**G/G**	**C/C**
N82	**36**	**M**	**control**	**NIL**	**G/G**	**C/C**
N83	**22**	**M**	**control**	**NIL**	**G/G**	**C/C**
N84	**33**	**M**	**control**	**NIL**	**G/G**	**C/C**
N85	**47**	**M**	**control**	**NIL**	**G/G**	**C/C**
N86	**62**	**M**	**control**	**NIL**	**G/G**	**C/C**
N88	**69**	**M**	**control**	**NIL**	**G/G**	**C/C**
N90	**51**	**M**	**control**	**NIL**	**G/G**	**C/C**
N91	**49**	**M**	**control**	**NIL**	**G/G**	**C/C**
N92	**25**	**F**	**control**	**NIL**	**G/G**	**C/C**
N93	**32**	**F**	**control**	**NIL**	**G/G**	**C/C**
N94	**19**	**F**	**control**	**NIL**	**G/G**	**C/C**
N95	**20**	**M**	**control**	**NIL**	**G/G**	**C/C**
N96	**16**	**M**	**control**	**NIL**	**G/G**	**C/C**
N97	**67**	**M**	**control**	**NIL**	**G/G**	**C/C**
N98	**55**	**M**	**control**	**NIL**	**G/G**	**C/C**
N99	**25**	**F**	**control**	**NIL**	**G/G**	**C/C**
N100	**20**	**F**	**control**	**NIL**	**G/G**	**C/C**
N101	**49**	**F**	**control**	**NIL**	**G/G**	**C/C**
N102	**23**	**F**	**control**	**NIL**	**G/G**	**C/C**

F = Female; M = Male