Leber Congenital Amaurosis: Clinical Profiling and Genetic Analysis Using High Throughput Resequencing in an Indian Cohort

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.....Dedicated to my Family

(For their strong support, encouragement and understanding)

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- Ronald T. Azuma

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ABSTRACT

BACKGROUND

Retinal blinding disorders together have a prevalence of 1 in 2000 worldwide. Mutations in genes that are expressed either in retinal pigment epithelium (RPE) cells, photoreceptors or bipolar cells can cause varying forms of degenerative or stationary retinal disorders, as the encoded proteins are crucial for normal function, maintenance and synaptic interaction. Depending on the type of photoreceptor affected, the retinal degenerative diseases (RDDs) are categorised as rod dominated, cone dominated, and generalised retinal degenerations involving both rods and cones. Leber congenital amaurosis (LCA) is an inherited blindness, caused due to degenerations of rods and cones, occurring within the age of one year, characterized by an extinguished electroretinogram (ERG) and manifesting nystagmus, photophobia, hyperopia. Genetically it is heterogeneous, with twenty-nine candidate genes identified so far (*AIPL1*, *ALMS, CABP4*, *CCT2, CEP290*, *CNGA3, CLUAP1*, *CRB1*, *CRX*, *DTHD1, GDF6, GUCY2D*, *IQCB1*, *IMPDH1*, *IFT140, KCNJ13*, *LCA5, LRAT*, *MERTK*, *MYO7A, NMNAT1*, *OTX2, PRPH2, RD3*, *RDH12*, *RPE65*, *RPGRIP1*, *SPATA7* and *TULP1*) and most of them are inherited in an autosomal recessive manner except *CRX, IMPDH1* and *OTX2* which are associated with autosomal dominant inheritance pattern.The conventional method of disease gene discovery in Mendelian disorders includes those based on linkage analysis as well as homozygosity mapping. Recent developments in high-throughput sequence capture methods have made next-generation sequencing technologies more feasible and cost effective. In LCA, the prevalence of mutation in the candidate genes varies in different populations and comprehensive mutation study focused on individual ethnicities in large cohorts are few. The mutation studies conducted on Indian LCA cohorts either had a very small sample size with fewer genes screened or included Indians as a part of multicentre studies or case report. A comprehensive candidate gene study or data on the prevalence of mutations in LCA candidate genes from a larger Indian cohort is still unavailable. This report, for the first time, presents a comprehensive data on the

prevalence of mutations from an Indian cohort of 92 LCA cases using targeted re-sequencing on NGS platform for 20 candidate genes.

AIM

To perform a comprehensive candidate genes screening for LCA from a larger Indian cohort by targeted next generation sequencing technology (NGS) and study genotype-phenotype correlation.

OBJECTIVES

- Clinical examination, complete phenotype documentation and recruitment of around 100 patients with Leber Congenital Amaurosis (LCA).
- Perform high throughput re-sequencing for the candidate genes on NGS platform
- To validate the results obtained by targeted re-sequencing using Sanger sequencing, perform segregation analysis in the families and control screening for the identified mutations to confirm the pathogenicity.
- Analyse the pathogenicity of the identified mutations using various bioinformatics tools.
- To perform genotype-phenotype correlation.

METHODOLOGY:

Subjects were recruited after complete ophthalmic examination and written informed consent was obtained. Apart from ERG, they were examined for finer fundus details with posterior pole fundus photograph, fundus autoflourescence (FAF), retinal optical coherence tomography (OCT) measurements (if possible) for performing Genotype-phenotype correlation. DNA was extracted from the Acid Citrate Dextrose (ACD) anticoagulated blood samples using NucleoSpin® Blood XL kit. Targeted re-sequencing was performed using custom target enrichment probes designed by Agilent Sure Design software. Target enrichment was done using Agilent HaloPlex target enrichment assay and subjected to NGS in IlluminaMiSeq platform. The filtered reads were aligned to the reference genome (hg19) using Burrow-Wheeler Aligner (BWA) program and further called and annotated using Samtools and Genome Analysis Toolkit (GATK), respectively. The identified pathogenic and likely pathogenic variants were validated by Sanger sequencing, segregation analysis

within the family and control screening too was performed. Online bioinformatics tools were used to predict the pathogenicity of the identified mutations.

RESULTS:

The NGS assay generated 75 million paired end reads, 9.7 GB of data with 83% of the data having >Q30 quality. An average of 99.03% sequence coverage across the 20 candidate genes with an average depth of 134X was obtained. The coding regions of all the twenty genes alone had 126 single nucleotide variations (SNVs) and 13 Indels. Data analysis using the standard bioinformatics pipeline and further Sanger validation identified pathogenic or likely pathogenic mutation in 61% (56/92) of the cohort, with 39% (21/53) mutation being novel. These mutations are distributed among 14/20 candidate genes. We also observed digenic and triallelic variantsthat may contribute to the phenotype of the disease.

Two cases of syndromic LCA were also observed in the study. Senior-Loken Syndrome in LRS 92 characterized by combination of LCA and kidney disease was found to be caused due to *IQCB1* mutation and, thiamine responsive megaloblastic anaemia (TRMA) in LRS 73 characterized by triad of megaloblastic anemia, diabetes mellitus (non-type 1) and sensorineural deafness along with LCA was found to be due to *SLC19A2* mutation.

CONCLUSION:

To our knowledge, this study presents the first comprehensive mutation spectrum of LCA in a large Indian cohort of 92 unrelated index cases diagnosed with LCA. Also, this study for the first time reported association of TRMA with LCA as the retinal disease component. Distinct fundus phenotype to genotype was also observed which might aid the clinicians to prognosticate the progression of the disease. This study has aided in patient care through genetic counselling by offering carrier testing, prenatal testing and disease management. In this cohort 38% have no mutation in the twenty known candidate genes thus providing a scope for finding novel candidate gene(s). Molecular diagnosis may also help to offer potential gene therapy trials to patients in future and thus treatment of the disease.

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LIST OF ABBREVIATIONS/ SYMBOLS

ABCA4	*ATP*-Binding Cassette, Subfamily A, Member 4
ABHD12	Abhydrolase Domain-Containing Protein 12
ACD	Acid Citrate Dextrose
AIPL1	Aryl hydrocarbon-Interacting Receptor Protein-Like 1
ALMS1	Alstrom syndrome protein 1
AMD	Age related Macular Degeneration
APEX	Arrayed primer extension
BBS	Bardet Biedl Syndrome
bp	Base pair
CACNA1F 1	Calcium Channel, Voltage-Dependent, Alpha-1F1 Subunit
CCDS	consensus coding sequence
CD	Cone Dystrophy
CNGA3	Cyclic nucleotide gated channel alpha 3
CNGB3	Cyclic nucleotide gated channel beta 3
CRB1	Crumbs, Drosophila, Homolog Of*, 1*
CRD	Cone Rod Dystrophy
CSNB	Congenital Stationary Night Blindness
DHDDS 1	Dehydrodolichyl Diphosphate Synthase
DNA	Deoxyribonucleic acid

EDTA	Ethylenediaminetetraacetic acid
ELOVL4	Elongation of Very Long Chain Fatty Acids-Like 4
ERG	Electroretinography
ExAC	The Exome Aggregation Consortium
ExoSap	Exonuclease and Shrimp alkaline phosphatase
GA	Genome Analyzer
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATK	Genome Analysis Toolkit
Gb	Giga base
GNAT2	Guanine Nucleotide-Binding Protein, Alpha-Transducing 2
GTPase	Guanosine triphosphate hydrolase
GUCY2D	Guanylate Cyclase 2D
HK1	Hexokinase 1
hRPE65v2	Recombinant adeno-associated virus retinal pigment epithelium gene vector
hTERT	Human Telomerase reverse transcriptase
IGV	Integrated genome viewer
Indel	Insertion deletion
LCA	Leber Congenital Amaurosis
LRIT3	Leucine-Rich Repeat, Immunoglobulin-like and Transmembrane Domains-Containing Protein 3
LRS	LCA resequencing
MAF	Minimum Allele Frequency
MYO7A	Myosin VIIA

NAD	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NC	Negative control
NGS	Next Generation Sequencing
NMNAT1	Nicotinamide Nucleotide Adenylyltransferase 1
OCT	Optical coherence tomography
OD	(Latin *oculus dexter*) indicates the right eye.
OMIM	Online Mendelian Inheritance in Man
ONL	Outer nuclear layer
OS	(Latin *oculus sinister*) indicates the left eye.
PCR	Polymerase Chain Reaction
PDE6C	Phosphodiesterase 6C
PDE6H	Phosphodiesterase 6H
Q30	Quality score 30
RAB28	RAS-Associated Protein 28
RD	Retinal Degeneration
RDH12	Retinol Dehydrogenase 12
RNA	Ribonucleic acid
RP	Retinitis Pigmentosa
RPE	Retinal Pigment Epithelium
RPE65	Retinal Pigment Epithelium-Specific Protein, 65- Kilo Dalton
RPGR	Retinitis Pigmentosa GTPase Regulator
RPGRIP1	Retinitis Pigmentosa GTPase Regulator-Interacting Protein

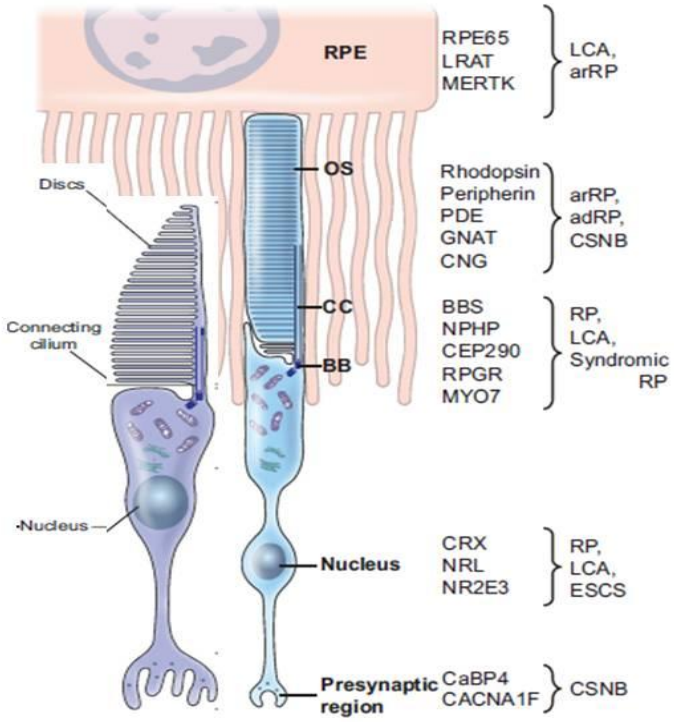
RT-PCR	Reverse transcriptase PCR
SLC19A2	Solute Carrier Family 19 (Thiamine Transporter), Member 2
SNP	Single Nucleotide Polymorphism
TCP	T-complex polypeptide 1
UTR	untranslated region
VUS	Variant of unknown significance
WES	Whole Exome Sequencing

CHAPTER 1

INTRODUCTION

The completion of the human genome project has paved way in providing new avenues and advances in medicine. The knowledge of human genome and significant development in genomic research has substantially improved the understanding of genetic basis of many diseases including Mendelian, mitochondrial and complex disorders, their classification and management.

Retinal degeneration are heterogeneous group of inherited diseases which are currently untreatable and share common pathological features affecting the photoreceptor and retinal pigment epithelial cells of retina causing varying degrees of irreversible vision loss[1]. Retinal degenerative diseases (RDD) together have a worldwide prevalence of 1 in 2000. Photoreceptors, retinal ganglion cells and other second order neurons encode proteins that are vital for normal function, maintenance, synaptic interaction, and signalling (**Figure 1**).



Shobivelleri et al, Disease models and mech.2015

Figure 1: Genes expressed in different layers of retina and RPE, and the spectrum of diseases they cause when mutated

Mutations in these genes cause various types of either degenerative or non-progressive retinal diseases[2].

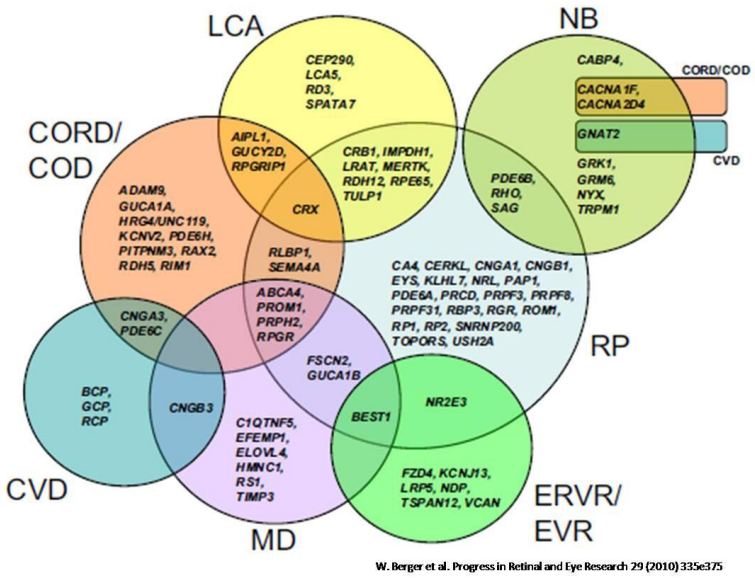
Most RDDs follow Mendelian (monogenic) pattern of inheritance and may be non-syndromic or syndromic forms (**Table 1**) with clinically distinguishable findings. These affect photoreceptor and/or RPE development and function[3] as seen in retinitis pigmentosa, Leber congenital amaurosis etc. Retinal degenerative diseases can also follow multifactorial inheritance like in age related macular degeneration (ARMD) and glaucoma. Visual image is formed by the interaction of many proteins synthesised by different cells of the retina. So far more than 200 different genes have been identified to cause monogenic retinal degenerative diseases[4]. The severity of the disease/s depends on the type of mutation within the gene, degree of damage caused by different mutations that may result in either total absence or presence of a non-functional or potentially toxic protein.

Table 1: A partial list of syndromic and non-syndromic retinal degenerative disease with monogenic inheritance.[4, 5]

Diseases	Affected cell type	Mode of inheritance	Genes associated
Non - syndromic monogenic			
CSNB	Rods more than cones	Dominant	*GNAT1, PDE6B, RHO*
		Recessive	*CABP4, GNAT1, GNB3, GPR179, GRK1,GRM6, LRIT3 , RDH5, SAG, SLC24A1, TRPM1*
		X-linked	*CACNA1F, NYX*
LCA	Rods and cones	Dominant	*CRX, IMPDH1, OTX2*
		Recessive	*AIPL1, ALMS1, CABP4, CCT2, CEP290, CNGA3, CRB1, CRX, CLUAP1, DTHD1, GUCY2D, GDF6, IQCB1, IMPDH1, IFT140, KCNJ13, LCA5, LRAT,MERTK, MYO7A, NMNAT1, OTX2, PRPH2, RD3, RDH12, RPE65, RPGRIP1, SPATA7, TULP1*

RP	Rods, Cones and RPE	Dominant	*ARL3, BEST1, CA4, CRX, FSCN2, GUCA1B,HK1, IMP DH1, KLHL7, NR2E3, NRL, PRPF3,PRPF4, PRPF6, P RPF8, PRPF31, PRPH2,RDH12, RHO, ROM1, RP1, R P9, RPE65,SEMA4A, SNRNP200, SPP2, TOPORS*
		Recessive	*ABCA4, AGBL5, ARL6, ARL2BP, BBS1,BBS2, BEST1, C2orf71, C8orf37, CERKL,CLRN1, CNGA1, CNGB1, C RB1, CYP4V2,DHDDS, DHX38, EMC1, EYS, FAM161 A,GPR125, HGSNAT, IDH3B, IFT140, IFT172,IMPG2, KIAA1549, KIZ, LRAT, MAK,MERTK, MVK, NEK2, NEU ROD1, NR2E3,NRL, PDE6A, PDE6B, PDE6G, POMG NT1,PRCD, PROM1, RBP3, RGR, RHO, RLBP1,RP1, RP1L1, RPE65, SAG, SLC7A14, SPATA7,TRNT1, TTC 8, TULP1, USH2A, ZNF408,ZNF513*
		X-linked	*OFD1, RP2, RPGR*
CD or CRD	Cones more than rods	Dominant	*AIPL1, CRX, GUCA1A, GUCY2D, PITPNM3,PROM1, P RPH2, RIMS1, SEMA4A, UNC119*
		Recessive	*ABCA4, ADAM9, ATF6, C21orf2, C8orf37,CACNA2D4, CDHR1, CERKL, CNGA3,CNGB3, CNNM4, GNAT2, K CNV2, PDE6C,PDE6H, POC1B, RAB28, RAX2, RDH5, RPGRIP1, TTLL5*
		X-linked	*CACNA1F, RPGR*
Macular degeneration	Rods and cones	Dominant	*BEST1, C1QTNF5, CTNNA1, EFEMP1,ELOVL4, FSCN 2, GUCA1B, HMCN1, IMPG1,OTX2, PRDM13, PROM1 , PRPH2, RP1L1,TIMP3*
		Recessive	*ABCA4, CFH, DRAM2, IMPG1, MFSD8*
		X-linked	*RPGR*
<u>Syndromic</u>			
BBS	Rods and cones	Recessive	*ADIPOR1, ARL6, BBIP1, BBS1, BBS2, BBS4,BBS5, B BS7, BBS9, BBS10, BBS12, C8orf37,CEP290, IFT172, IFT27, INPP5E, KCNJ13,LZTFL1, MKKS, MKS1, NPH P1, SDCCAG8,TRIM32, TTC8*
Joubert syndrome	Rods and cones	Recessive	*INPP5E, TMEM216, AHI1, NPHP1, CEP290 (NPHP6), TMEM67 (MKS3), RPGRIP1L, ARL13B, CC2D2A, TTC21B, KIF7, TCTN1, TCTN2, TMEM237, CEP41, TMEM138, C5orf42, TCTN3, TMEM231, CSPP1, PDE6D*
		Dominant	*ZNF423*
		X-linked	*OFD1*
Senior- loken syndrome	Rods and cones	Recessive	*CEP164, CEP290, INVS, IQCB1, NPHP1, NPHP3, NPHP4, SDCCAG8, TRAF3IP1, WDR19*
Usher syndrome	Rods and cones	Recessive	*ABHD12, ADGRV1, CDH23, CEP250, CIB2,CLRN1, D FNB31, HARS, MYO7A, PCDH15,USH1C, USH1G, US H2A*

Mutations in the same gene cause a range of clinical phenotypes defined as allelic heterogeneity, a common feature of monogenic RDD. For example, mutation in transcription factor, *CRX* (cone-rod homeobox), that plays a vital role in photoreceptor development and homeostasis[6] (**Figure 2**) can cause either Leber congenital amaurosis (LCA) where there is congenital blindness or progressive cone-rod dystrophy (CRD)/ retinitis pigmentosa (RP), where the disease progresses over time. Hereditary retinal degenerations are probably the most genetically heterogeneous group of diseases in humans i.e. they demonstrate locus heterogeneity where a single disease phenotype can be caused by mutations in different genes. Therefore, providing a molecular diagnosis in RDD becomes challenging along with variable expressivity, incomplete penetrance, and frequent clinical and genetic overlap[7].



<u>Figure 2:</u> Overlapping genotypes in non-syndromic monogenic retinal and vitreoretinal degenerative diseases.

1.1. Broad classification of RDDs:

Depending on the type of photoreceptor affected, the RDDs are categorised as rod dominated diseases, cone dominated diseases, and generalised retinal degenerations involving both rods and cones[7].

1.1.1. Rod dominated diseases

In these diseases, the rod photoreceptors are primarily affected followed by cones, like in retinitis pigmentosa (RP). Retinitis pigmentosa is considered the most common form of RD with a frequency of 1 in 3000-7000 individuals[8]. It is a progressive disease and has been associated with many syndromic RDs like Bardet- Biedl syndrome (BBS), Usher syndrome etc. The disease presents with progressive deterioration in the ability to see in dim light causing night blindness, followed by loss of peripheral vision that slowly moves towards the centre resulting in tunnel vision. So far 82 genes and 7 loci have been associated with autosomal dominant, autosomal recessive, and X-linked RP.

1.1.2. Cone dominated diseases

Cone dystrophies have a prevalence of 1/40,000and are caused due to degeneration of the cone cells of the retina. Cone cells are present throughout retina with maximum concentration clustered in the macula. Degeneration of cones leads to reduced visual acuity, central vision loss, reduced ability to see colours and photophobia. Cone dystrophy (CD) can be autosomal dominant, recessive or X-linked and can be stationary or progressive. Till now, 33 genes have been associated with the disease. Achromatopsia is a stationary, congenital, autosomal recessive inherited disorder characterized by reduced visual acuity, pendular nystagmus, increased sensitivity to light (photophobia) and reduced or complete loss of colour discrimination. Mutations in *CNGA3*, *CNGB3*, *GNAT2*, *PDE6C* or *PDE6H* are shown to cause achromatopsia[9].

In the progressive forms, complete blindness may occur in the later stages because the rod photoreceptors also undergo degeneration. Stargardt disease is a juvenile macular degeneration characterised by central vision loss where cone cells are more concentrated. It is associated with mutation in *ABCA4* (autosomal recessive) and *ELOVL4* (autosomal dominant) gene. X-linked cone dystrophy (XLCOD) is a progressive disorder where the affected males have decreased visual acuity, myopia, cone ERG disturbance and colour vision defects. Mutation in *CACNA1F* and *RPGR* genes are shown to cause XLCOD[10].

1.1.3. Generalized RDs

This involves the simultaneous degeneration of both rod and cone photoreceptor functions and is present with progressive, often severe, deterioration of vision. Leber congenital amaurosis is a generalised RD, which is congenital and is considered the most severe. LCA accounts for 5% of inherited retinal degenerative disorders. The reported worldwide prevalence of LCA is 1 in 30,000 to 1 in 81,000 [11].

The only X-linked form of non-syndromic generalised RD is choroideremia (CHM). Affected males develop night blindness in their second decade, followed by progressive loss of peripheral vision and blindness. The incidence of CHM is estimated as 1 in 100,000[12].

Congenital stationary night blindness (CSNB) is a non-progressive retinal disorder characterized by impaired night vision, decreased visual acuity, nystagmus, myopia, and strabismus. Thirteen genes have been associated with the disease so far and are inherited either as autosomal dominant or recessive or X- linked. Based on the ERG wave form CSNB is classified as Riggs and Schubert-Bornschein. The later is further classified as complete form (type 1 CSNB), characterized by the complete absence of rod pathway function, whereas the incomplete form (type 2 CSNB) is due to impaired rod and cone pathway function. Oguchi disease and fundus albipunctatus are forms of CSNB with abnormal fundi. In Oguchi disease, the fundus displays a yellow sheen after exposure to light; this sheen disappears following dark adaptation. In fundus albipunctatus, the retina develops yellow-white dots. The mutated genes code for proteins that are involved either in phototransduction cascade or signalling from photoreceptors to second order neurons[13, 14].

1.2. Identification of Candidate Genes in RDD

Identification of candidate genes and the causal mutation/ variant for monogenic diseases has been mainly through linkage analysis. The segregation of the genetic markers in affected individuals indicate the plausible genomic region of the candidate gene[15]. The distance between the genetic markers (θ=frequency of recombination) helps identify the disease loci. There are two types of linkage

mapping – parametric, where the exact mode of inheritance is a pre requisite and often performed for Mendelian disorders and non-parametric that is independent of inheritance patterns and analysed based on: identical by descent (IBD) or identical by state (IBS). Linkage mapping requires large multigenerational families with many affected and unaffected and it is used to identify candidate genes for all modes of inheritance i.e. autosomal dominant or recessive and X-linked recessive. Homozygosity mapping is a very efficient approach to study recessive disorders in both consanguineous and non-consanguineous families where the information of IBD is used. Here the markers (alleles) are homozygous by descent and are shared between the affected individuals thus indicating possible disease loci[16, 17]. Animal studies and knowledge of allelic heterogeneity have also lead to identification of candidate genes in various forms of RDD.

Over the years, numerous candidate genes have been identified. Sanger sequencing is considered 'gold standard' in terms of both read length and sequencing accuracy. But screening all the genes by Sanger sequencing is both time consuming and expensive. In order to simplify molecular diagnostics in diseases that are genetically heterogeneous, APEX genotyping microarrays (ASPER Ophthalmics, Estonia)[18] were developed in which the PCR products of each amplimer targeting the mutation regions are combined and hybridized to oligonucleotide primers arrayed on the chip. A template-dependent single-nucleotide extension reaction with fluorescently labelled dye terminator nucleotides helps in the detection of variants for both sense and antisense strand. The limitation of this technology is that it allows the detection of only known mutations and novel mutations are missed. These limitations have been overcome by advancement in genotyping technology, i.e. the advent of massively parallel sequencing using NGS.

1.3. Next Generation Sequencing Technologies

Several next generation sequencing (NGS) technology platforms have emerged which includes Roche 454, Illumina GA, Ion torrent, and ABI SOLiD that are based on massive parallel sequencing and generate large data. They are considerably less expensive especially in screening genetically heterogeneous diseases.

NGS and its wide range of applications include chromatin immunoprecipitation coupled to sequencing (ChIP-seq), Bisulphite sequencing, RNA sequencing (RNA-

seq), whole genome sequencing, whole exome sequencing, targeted re-sequencing denovo assembling, and re-assembling of genome[19]. The broadest application of NGS is the re-sequencing of human genomes to understand the genetic differences in health and disease[20].

NGS has helped in comprehensive understanding of the genetic architecture of RDDs. NGS was first employed in retinal disease to study an Ashkenazi Jewish family, where whole exome sequencing of three affected siblings revealed a mutation in a novel gene, *DHDDS*, as a cause of RP [21]. Linkage analysis followed by whole exome sequencing (WES) or targeted sequencing has identified *HK1* as a novel causative gene for adRP, *RAB28* gene in a German family with arCRD[22], *ABHD12* gene in a family clinically diagnosed with Usher syndrome type 3[23], *LRIT3* gene in CSNB[24], *NMNAT1* in Leber congenital amaurosis[25].

Table 2: List of novel genes identified in retinal and macular degeneration using next-generation sequencing approaches[26].

Method	**Diseases**	**Gene(s) identified**
Whole -exome sequencing	Retinitis pigmentosa (RP)	*DHDDS, MAK, GNPTG, EMC1, GPR125, KIAA1549, ARL2BP*
	Leber congenital amaurosis (LCA)	*NMNAT1, KCNJ13, DTHD1, CCT2, CLUAP1, ALMS1, CNGA3 and MYO7A*
	Congenital stationary night blindness	*LRIT3, GPR179,*
	Ciliopathy with skeleton abnormality	*WDR19*
	High myopia	*ZNF644*
	Bardet- Biedl syndrome (BBS)	*LZTFL1*
	Nephronophthisis with retinal degeneration	*ZNF423, CEP164*
	Usher syndrome	*HARS*
	Cone-rod dystrophy	*RAB28, ACBDS, C21orf2*
Targeted sequencing	RP, Cone -rod dystrophy	*C8orf37*
	AMD	*CFH, CF1*
	LCA	*IFT140, PRPH2,*
	Usher syndrome	*ABHD12*
	CSNB	*GPR179*
	X-Linked RP	*OFD1*
	Joubert syndrome	*TMEM237*
	Familial exudative vitreoretinopathy	*TSPAN12*

Targeted NGS or WES has identified the genetic cause in 50-80% of the RDD in various cohorts studied. It has proven to be a robust and cost effective technology for identifying both novel and known mutations in candidate genes by targeted capture [27] or involvement of known or novel genes by exome or whole genome capture [25] (**Table 2**).

1.4. Leber congenital amaurosis

Leber congenital amaurosis is a severe form of retinal degenerative disease diagnosed in children earlier than one year of age. This disease was described initially by Theodore Leber in 1869 as a congenital form of retinitis pigmentosa[28]. The clinically distinguishing features of LCA include severe visual impairment present at birth or shortly thereafter; extinguished or non-recordable ERG, pendular or searching nystagmus, photophobia, and digito-ocular sign (Franceschetti- Leber phenomenon), with progressive retinal degeneration[29].

1.4.1. Associated ocular clinical features of LCA:

Visual acuity (VA) in LCA range widely from 20/200 to little or no light perception. LCA patients often have high refractive errors (from hyperopia to myopia) but most patients are high hyperopes[30]. The oculo- digital sign of Franceschetti is an important feature of LCA. The sign consists of a repetitive, deep pushing of the knuckle or finger into the eye and socket. The oculo- digital phenomenon due to persistent pushing, causes orbital fat atrophy, deep set eyes (enophthalmos) and keratoconus, (the thinning of the cornea)[30]. Cataracts and keratoconus are often associated with LCA[31] as is nyctalopia (night blindness)[32]. Certain features are specific to genotypes such as patients with *RPE65* mutations show mild improvements in their visual function in their second decade, but it is then shown to decline after aperiod of stability[33]. Patients with *CRX* and *GUCY2D* mutations appear to have a very significant loss of vision, but the loss remains stable[34].

1.4.2. Other systemic clinical features of LCA

Apart from these, about 19.8% LCA patients are shown to suffer from mental retardation or seizures or autism[30, 35].

1.4.3. Phenotype documentation in LCA:

- Electroretinogram (ERG)

ERG documentation is the primary diagnostic tool used to confirm LCA. It evaluates the visual function by measuring the electrical response of the entire retina i.e. first order neuron function (a-wave from photoreceptors), and second-order neuron function (b-wave from ON- bipolar and Muller cells). ERG helps in differential diagnosis among retinal dystrophies. In LCA, ERG responses are non-detectable or extinguished within one year of life indicating the severity of the rod and cone degeneration (**Figure 3**)[36].

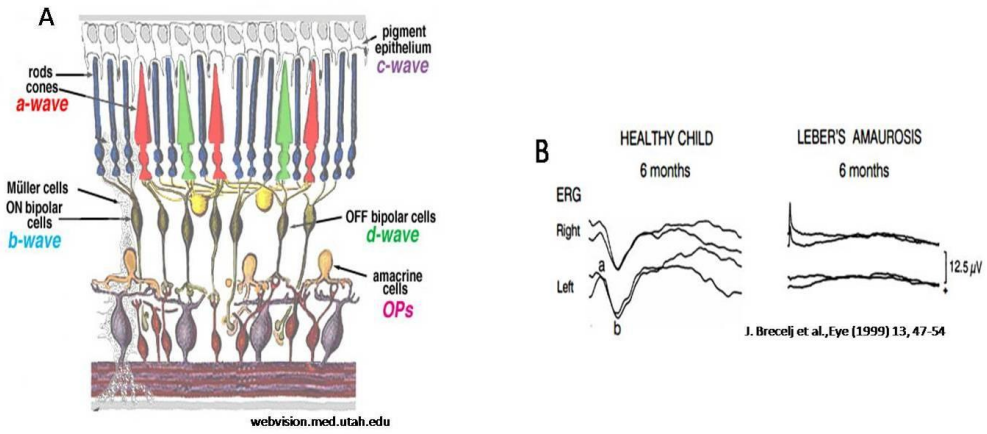


Figure 3: A. Diagrammatic representation of retinal layers and the retinal cells associated with specific ERG wave pattern. B. Shows ERG of Leber congenital amaurosis compared with a normal control.

- Optical coherence tomography

Optical coherence tomography (OCT), is a non-invasive imaging technique used to obtain high resolution cross-sectional images of the retinal architecture. OCT imaging is considered an important phenotypic documentation as it helps in staging the severity of retinal degenerative diseases by calculating the average photoreceptor layer thickness across a wide retinal area (**Figure 4**). Measurable photoreceptor thickness in patients increases the prospect for clinical trials where retrospective or prospective staging could be related to treatment efficacy[37].

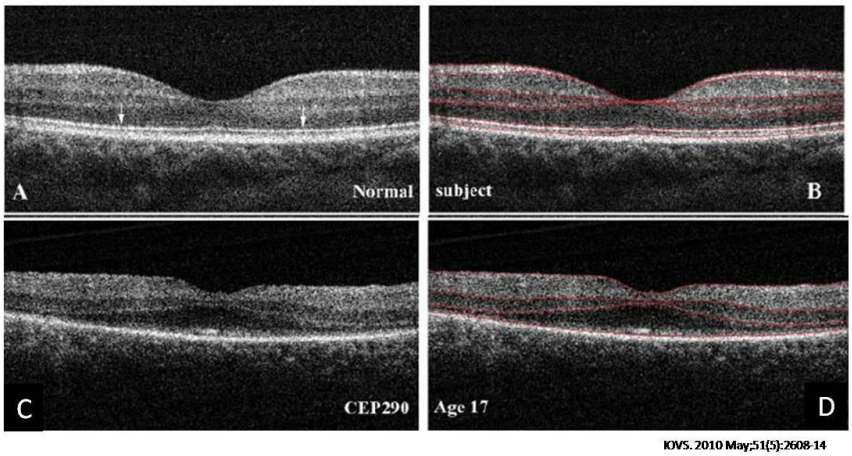


Figure 4: A. OCT image of normal subject with defined photoreceptor inner/ outer segment junction. B. Six lamellar layers – retinal nerve fibre layer, ganglion cell + inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer + photoreceptor inner segment and photoreceptor outer segment (top to bottom) C. and D. OCT image of 17 yr old *CEP290* mutation positive patient showing only three lamellar layers with preservation of ONL centrally.

- Fundus photo and Fundus autoflourescence

Fundus imaging such as colour photographs, fluorescein angiography, and fundus autoflourescence records changes in central and peripheral retina, optic disc and macula. It helps in defining the severity and monitoring the progression of disease. In LCA, the retinal appearances differ considerably from the normal, mild retinal vessel attenuation, pseudopapilledema of the optic disc, maculopathy, macular coloboma, bone spicule pigmentation, nummular pigmentation, salt and pepper pigmentation, yellow confluent peripheral spots, white retinal spots, marbleized retinal changes and preserved para-arteriolar RPE (PPRPE)[30]. Gene specific changes are also reported (**Figure 5**) like peripheral retinal bone spicule - like pigmentation (*RPE65*), Bull's eye maculopathy (*AIPL1*), mild RPE atrophy, mid peripheral

hyperpigmentation (*RDH12*), nummular pigmentation (*CRB1*), tapetal reflex (*CEP290*) and mild foveal atrophy to macular coloboma (*NMNAT1*) [38].

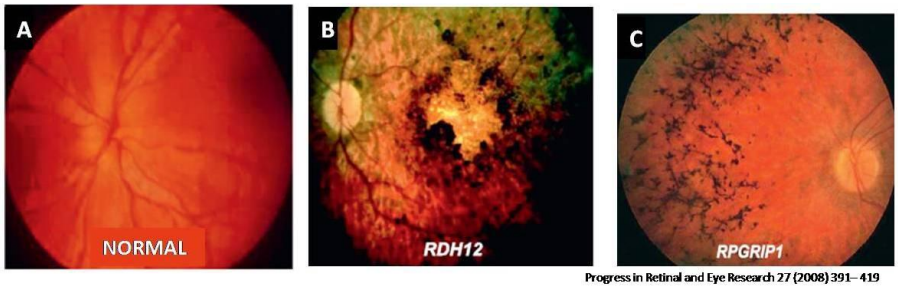


Figure 5: Representative picture of retinal colour fundus photograph with known genotypes. Fundus of A. normal individual B. LCA patient with *RDH12* mutation C. LCA patient with *RPGRIP1* mutation.

1.4.4. Genetics of LCA:

LCA is both clinically and genetically heterogeneous. So far, twenty-nine candidate genes (*AIPL1* (MIM*604392), *ALMS1* (MIM*606844), *CABP4* (MIM *608965), *CCT2* (MIM*605139), *CEP290* (MIM *610142), *CNGA3* (MIM* 600053), *CLUAP1* (MIM* 616787), *CRB1* (MIM *604210), *CRX* (MIM *602225), *DTHD1* (MIM* 616979), *GDF6* (MIM* 601147), *GUCY2D* (MIM *600179), *IQCB1* (MIM *609237), *IMPDH1* (MIM *146690), *IFT140* (MIM* 614620), *KCNJ13* (MIM *603208), *LCA5* (MIM *611408), *LRAT* (MIM *604863), *MERTK* (MIM *604705), *MYO7A* (MIM* 276903), *NMNAT1* (MIM *608700), *OTX2* (MIM* 600037), *PRPH2* (MIM* 179605), *RD3* (MIM *180040), *RDH12* (MIM *608830), *RPE65* (MIM *180069), *RPGRIP1* (MIM *605446), *SPATA7* (MIM *609868) and *TULP1* (MIM 602280)) have been identified and most of them are inherited in an autosomal recessive manner except *CRX, IMPDH1* and *OTX2* which are associated with autosomal dominant inheritance pattern[4, 11].

1.4.4.1. Gene mapping in LCA

Various methods like classical linkage analysis, homozygosity mapping, candidate gene approach based on tissue specific expression or animal models, next generation sequencing and/ or combinations of one or more approaches have been used to identify candidate genes for LCA. Genes such as *RDH12, TULP1, CRB1, CRX, GDF6, IMPDH1, OTX2, RD3, RPE65* and *RPGRIP1* were reported to be

associated with retinal functions or other retinal diseases. These were screened and identified to be associated with LCA too[39,40,41,42,43,44,45,46,47,48]. Using whole genome linkage analysis *CEP290*[49] was identified. *AIPL1* was identified as candidate gene using linkage analysis coupled with *insitu* hybridization[50]. Homozygosity mapping identified *CABP4*[51], *GUCY2D*[52], *IQCB1*[53], *LCA5*[54] *and SPATA7*[55]. *LRAT* and *MERTK* were recognized as candidate genes for LCA while screening many retinal genes in oligonucleotides SNP arrays[56]. With the advent of next generation sequencing technology, targeted re-sequencing of retinal and ciliopathy genes identified *IFT140*[57] and *PRPH2*[58] while whole exome sequencing alone or with homozygosity mapping identified *CCT2, CLUAP1, ALMS1, CNGA3, MYO7A, DTHD1, KCNJ13, and NMNAT1*[29, 59-62].

1.4.4.2. LCA genes and their functions

The candidate genes code for proteins that belong to different families and perform varied functions. Some of these genes code for ciliary proteins – as photoreceptors are modified cilia, they are involved in photoreceptor morphogenesis and stabilization, those that code for signalling molecules and ion channels- as ions are required for synapses, those that are essential in phototransduction pathways and visual cycle and those that perform other functions like phagocytosis, protein trafficking, as molecular chaperones and signalling peptides.

- <u>Ciliary genes:</u>

ALMS1

ALMS1, located on chromosome 2p13 codes for a centrosome and basal body associated protein. The protein localizes to centrosomes and basal bodies of ciliated cells. ALMS1 protein is involved in microtubule organization, cell cycle regulation, intraciliary transport[63], cell migration and extracellular matrix production[64]. As ciliary bodies are present in multiple systems, defect in the protein results in syndromic disease like Alstrom syndrome involving cone-rod retinal dystrophy, hearing loss, childhood obesity, type 2 diabetes mellitus, cardiomyopathy, fibrosis and multiple organ failure[65]. *ALMS1* is also shown to be associated with non-syndromic LCA[29].

CEP290

Centrosomal protein, 290-KD (also known as *NPHP6*) is involved in ciliary assembly and trafficking. *CEP290* is located on 12q21.32 and mutation in this gene is shown to be associated with many diseases, ranging from isolated blindness to various syndromes like Senior-Loken syndrome (SLS), nephronophthisis (NPHP), Joubert syndrome (related disorders) (JS[RD]), Bardet-Biedl syndrome (BBS), to the lethal Meckel-Gruber syndrome (MKS). Knockdown experiments in zebra fish using morpholinos revealed Cep290 deficiency caused defects involving retinal, cerebellar, and optic cavity developmental abnormalities as well as pronephric cyst formation, ectopic brain tissue in the fourth ventricle and an abnormal mid to-hindbrain region associated with hydrocephalus[66].

CLUAP1

Clusterin-associated protein 1 (*CLUAP1*) appears to be involved in assembly and turnaround of intraflagellar transport (IFT) particles at the base and tip of the cilium. Located at 16p13.3 *CLUAP1* transcript is expressed abundantly in testis, thyroid, trachea, eye (connecting cilium of photoreceptor cells), and moderately in spinal cord and adrenal gland [67]. *CLUAP1* has been shown to be a tumor associated antigen[68] and recently is identified as a candidate gene for non-syndromic LCA[60]

IQCB1

Located on 3q13.33, it encodes an IQ motif-containing protein B1 (also known as NPHP5), these proteins localize to connecting cilia of photoreceptors. Present in almost all tissues, *NPHP5* and *CEP290* plays important role in controlling integrity and ciliary trafficking of the BBSome. As a transition zone protein NPHP5 contains two separate BBS-binding sites and interacts with the BBSome to mediate its integrity[69]. Mutations in *IQCB1* are reported in Senior-Loken syndrome, Bardet-Biedl syndrome and non-syndromic LCA[53].

IFT140

Intraflagellar transport 140 (*IFT140*) located on 16p13.3, encodes a subunit of intraflagellar transport complex A (IFTA), which is involved in retrograde ciliary transport. It is shown to be highly expressed in kidney, moderately in ovary, testis, prostate, and lung, and low expression in thymus, brain, heart, placenta, and skeletal muscle. In the eye, IFT proteins are expressed during all stages of

ciliogenesis in photoreceptor cells and are found within the differentiating photoreceptor ciliary shaft during early stages of development[70]. Mutations in *IFT140* were previously reported in skeletal ciliopathies and recently in non-syndromic LCA[71].

LCA5

Located in 6q14.1, encodes Lebercilin (*LCA5*) protein that localizes to the ciliary axoneme, with increased amounts at the base of the primary cilia. It is a ubiquitously expressed protein and in the eye it is found to be present in the connecting cilium and the basal bodies of photoreceptor cells and involved in ciliary microtubule dynamics[54].

MYO7A

Located in 11q13.5 encodes a protein belonging to myosin family. These are motor molecules with structurally conserved heads that move along actin filaments thus enabling them to transport cargo[72], *MYO7A* expression is seen in human liver, kidney, retinal pigment epithelium, and photoreceptor cells of the retina as well as in cochlear and vestibular neuroepithelia. Mutations in *MYO7A* were previously found to be associated with deafness and Usher syndrome type 1B[73].

RPGRIP1

Located on 14q11.2, encodes Retinitis pigmentosa GTPase regulator-interacting protein which is expressed in retina and testis. It localizes specifically in the photoreceptor connecting cilium. RPGRIP anchors RPGR in the connecting cilium and regulates protein transport.[74]. Mutations in *RPGRIP1* also cause cone-rod dystrophy[75].

SPATA7

Spermatogenesis-associated protein 7, a ciliary protein, is expressed only in testis and retina. The gene is located on 14q31.3. In P21 mice, Spata7 protein is observed in multiple layers of the retina, including the ganglion cell, inner nuclear layers, and inner segments of photoreceptors. SPATA7 localizes at the primary cilium and connecting cilium (CC) of photoreceptor cells in hTERT RPE-1 cells.SPATA7 directly interacts with the retinitis pigmentosa GTPase regulator interacting protein 1 (*RPGRIP1*), a key connecting cilium protein that has also

known to cause LCA. This complex plays an important role in protein trafficking across the CC to the outer segments and a malfunction of this result in rhodopsin accumulation in the inner segments and around the nucleus of photoreceptors. This accumulation then likely triggers the apoptosis of rod photoreceptors [76]. Apart from LCA, mutation in *SPATA7* is also associated with autosomal recessive retinitis pigmentosa (RP).

- Photoreceptor morphogenesis and maintenance genes:

CRB1

Crumbs, drosophila, homolog of, 1 (*CRB1*) localize to subdomains of the photoreceptor apical plasma membrane i.e. the inner segment of mammalian photoreceptors. The subdomains support the morphogenesis and orientation of the photosensitive membrane organelles. Located on 1q31.3, it is expressed exclusively in eye and the central nervous system[77]. Mutations in *CRB1* is also associated with pigmented paravenous chorioretinal atrophy and autosomal recessive retinitis pigmentosa[78].

CRX

Cone-rod homeobox-containing gene is located on 19q13.33 and is also associated with cone-rod dystrophy[79]. *CRX* is expressed specifically in the photoreceptor cells of developing and adult retina. It is a photoreceptor-specific transcription factor which plays a role in the differentiation of these cells[80].

CABP4

Located in 11q13.2 encodes a calcium-binding protein 4 (CABP4) that localizes to the synaptic terminals of photoreceptors. It belongs to a subfamily of at least eight calmodulin (CaM)-like neuronal Ca^{2+} binding proteins. It co-localizes and interacts with Cav1.4 voltage-dependent Ca^{2+} channels (VDCCs) and plays an important role in the development and/ or maintenance of the photoreceptor output synapse, probably through modulation of photoreceptor VDCCs and transmitter release[81]. *CABP4* is also a candidate gene for congenital stationary night blindness[82].

GDF6

Growth/ differentiation factor 6 is a member of the transforming growth factor-beta superfamily. Located on 8q22.1, it is expressed in ganglion cell layer, inner plexiform layer, and retinal pigment epithelia in the eye. It has been hypothesised

that *GDF6* may regulate ectoderm patterning and control eye morphogenesis by regulating neural and vascular development. *GDF6* mutations have been associated with eye phenotypes such as microphthalmia, anophthalmia[83] and Klippel–Feil syndrome[84].

KCNJ13

Potassium channel, inwardly rectifying, subfamily J, member 13, is expressed in wide variety of cells with the common role of maintaining resting membrane potential near the potassium equilibrium potential. Located in 2q37.1, *KCNJ13* is expressed in apical RPE and help to maintain potassium homeostasis around the photoreceptor outer segments[85]. *KCNJ13* mutations were first reported in Snowflake vitreoretinal degeneration[86]

OTX2

Located on 14q22.3*,* Orthodenticle, drosophila homolog of 2, is a transcription factor and plays a role in brain, craniofacial and sensory organ development. OTX2 is also required for the development and maintenance of the neural retina. It is found in bipolar and in ganglion cells, while in the outer nuclear layer it regulates the expression of the closely related *CRX* gene that controls the expression of a suite of photoreceptor function genes, including opsins[87]. Mutations in this *OTX2* also cause syndromic microphthalmia 5 (MCOPS5) and combined pituitary hormone deficiency 6 (CPHD6)[88].

PRPH2

Peripherin-2 is tetra spanning membrane proteins localized along the rim of rod and cone photoreceptor outer segment discs. Peripherin-2 is critical to the formation and stabilization of photoreceptor outer segments. It is located in 6p21.1 and mutations in this gene is reported in retinitis pigmentosa and macular dystrophy[89, 90].

RD3

Located on 1q32.3, retinal degeneration 3 (*RD3*) is expressed only in retina. The protein is localised in outer nuclear layer, inner nuclear layer, and the ganglion cell layer. RD3 mediates the export of GC1 (Guanylatecyclase 1) from the endoplasmic reticulum to endosomal vesicles. RD3 co localizes, binds to GC1 and GC2 to play a

crucial role in their stable expression and membrane trafficking in rod and cone photoreceptors[91].

- Molecular chaperones:

AIPL1

Located in 17p13.1, *AIPL1* encodes human aryl hydrocarbon receptor-interacting protein-like 1 (AIPL1) and is similar to (49% identity, 69% positive) human aryl hydrocarbon receptor-interacting protein (AIP), a member of the FK506-binding protein (FKBP) family. This protein contains three tetratricopeptide (TPR) motifs, consistent with nuclear transport or chaperone activity. It is expressed in photoreceptor and pineal-gland[50]. Mutation in *AIPL1* is also reported in cone-rod dystrophy and retinitis pigmentosa[92].

CCT2

Chaperonin containing TCP1, subunit 2, located on 12q15, encodes the molecular chaperone protein, CCTβ. Although expressed ubiquitously, in retina it is expressed in retinal ganglion cell layer and near the connecting cilium in the photoreceptor cells. CCTβ interacts with chaperonin type BBSs, including BBSs 6, 10, and 12 which are ciliary proteins. CCT2 is also shown to bind to G protein subunit Gβ1 which is involved in transducin- associated phototransduction[59]

NMNAT1

Nicotinamide nucleotide adenylyltransferase 1, located on 1p36.22, is a central enzyme in NAD biosynthesis, catalysing the condensation of nicotinamide mononucleotide (NMN) or nicotinic acid mononucleotide (NaMN) with the AMP moiety of ATP to form NAD or NaAD. It is expressed in skeletal muscle, heart, liver, and kidney. In the eye, NMNAT act as a stress-response protein that works as a chaperone for neuronal maintenance and protection[93].

- Phototransduction:

CNGA3

Cyclic nucleotide-gated channel, alpha-3 (*CNGA3),* an achromatopsia-2 gene, is located on 2q11.2. It is found to be expressed in cone photoreceptors, testis, kidney, and heart[94]. The *CNGA3* encodes one of a family of alpha subunits that form CNG ion channels required for sensory transduction in rod photoreceptors and

in olfactory neurons. Localized to the photoreceptor plasma membrane, is essential for the generation of light- evoked electrical responses in the red, green, and blue-sensitive cones.

GUCY2D

Guanylatecyclase 2D, in 17p13.1 region encodes a retina-specific Guanylate cyclase (RetGC), which is a member of the membrane guanylylcyclase family. RetGC mediates Ca^{2+} feedback on cGMP metabolism during photoreceptor light adaptation and/ or recovery following photo excitation[95]. Mutations in this gene are also reported in dominant cone-rod dystrophy[96].

LRAT

Lecithin retinol acyltransferase catalyses the esterification of all-trans-retinol into all-trans-retinyl ester. This reaction is an important step in vitamin A metabolism in the visual system. Located in 4q32.1, it is expressed in several fetal and adult human tissues, including the retinal pigment epithelium (RPE) and liver. Mutation in *LRAT* was also observed in early-onset severe retinal dystrophy[97].

RDH12

Located on 14q24.1, retinol dehydrogenase 12 belongs to a family of dual-specificity retinol dehydrogenases that metabolize both all-trans- and cis-retinols. *RDH12* is expressed predominantly in eye and also in kidney, brain, skeletal muscle and stomach. In eye, it is expressed at the base of photoreceptor inner segments and during visual cycle catalyses the reduction of all-trans-retinal and its 9-cis-, 11-cis-, and 13-cis-retinal isomers in the presence of NADPH[98].

RPE65

Located on 1p31.3 encodes the retinal pigment epithelium- specific protein 65KD, , which catalyses the conversion of all-trans retinyl ester to 11-cis retinol in the RPE[99]. This RPE specific protein acts as the receptor for retinol-binding protein on the surface of the retinal pigment epithelium. Other than LCA, mutations in *RPE65* are also implicated in retinitis pigmentosa.

IMPDH1

Inosine-5-prime-monophosphate dehydrogenase, type I is an enzyme that catalyses the synthesis of xanthine monophosphate (XMP) from inosine-5'-mono-

phosphate (IMP). This is the rate-limiting step in the de novo synthesis of guanine nucleotides. Located on 7q32.1, it is also a candidate gene for autosomal dominant retinitis pigmentosa and is ubiquitously expressed but has a retina specific isoform derived from alternative splicing. In the retina, it is localized to the inner segment and synaptic terminals of photoreceptors[100] and is found to be associated with polyribosomes containing rhodopsin mRNA. Mutations in *IMPDH1* are reported in autosomal dominant LCA as well.

- Signalling pathway, Phagocytosis and protein trafficking

DTHD1

Death domain-containing protein 1 in 4p14 is predicted to function in signalling pathways, formation of signalling complexes, as well as in apoptosis pathway. Localization of these signalling proteins in the eye are yet unknown.

MERTK

Mer tyrosine kinase protooncogene located on 2q13 is expressed in normal peripheral blood monocytes, bone marrow, in tissues of epithelial and reproductive origin. Mer receptor tyrosine kinase is critical for the engulfment and efficient clearance of apoptotic cells. In retina, *MERTK* plays an important role in phagocytosis of outer segments by RPE cells[101]. Mutations in *MERTK* were first implicated in RP[101].

TULP1

Located on 6p21.31, Tubby-like protein 1 is expressed exclusively in retina. TULP1 is localized to the photoreceptor inner segment and synapse. TULP1 is shown to be involved in protein trafficking from inner segment to outer segment where they bind to several cytoskeletal scaffold proteins, as well as members of the phototransduction cascade and in vesicle cycling at the synaptic terminal[102]. Mutations in *TULP1* are also implicated in RP[103].

1.4.5. Syndromic LCA

'LCA like ocular phenotype' is observed as one of the characteristic features in few syndromes. These syndromes initially present as LCA, as it is diagnosed earlier to one year of age and later presents systemic features, which involve various other organs.

1.4.5.1. Alstrom syndrome

Alstrom syndrome is an autosomal recessive disorder characterised by LCA appearing first in infancy and then progressive development of multi-organ pathology which include hearing loss, childhood truncal obesity, insulin resistance and hyperinsulinemia, type 2 diabetes, hypertriglyceridemia, short stature in adulthood, cardiomyopathy, and progressive pulmonary, hepatic, and renal dysfunction. Alstrom syndrome is caused by mutations in *ALMS* gene. ALMS1 protein is found in centrosomes, basal bodies, and cytosol of all tissues affected by the disease[104].

1.4.5.2. Batten disease

Also known as the neuronal ceroid lipofuscinoses (NCL; CLN), is clinically and genetically heterogeneous group of neurodegenerative disorders caused due to intracellular accumulation of autofluorescent lipopigment storage material. Clinically, the phenotypes are categorized according to the age of onset and order of appearance of clinical features; as infantile, late-infantile, juvenile, adult, Northern epilepsy. The genes associated among these phenotypes are also different. The disease is characterized by progressive intellectual and motor deterioration, seizures, and early death. Visual loss is a feature of most form and LCA is reported in the infantile form[105].

1.4.5.3. Cerebello-oculo-renal syndromes

Syndromes involving brain, eye and kidney abnormalities come under Cerebello-oculo-renal syndromes.

- ✓ Senior-Loken syndrome

Senior-Loken syndrome is a ciliopathic autosomal recessive disease with main features being nephronophthisis (an autosomal recessive cystic kidney disease) and progressive eye disease[106]. Both Leber congenital amaurosis and retinitis pigmentosa are reported as ocular components. Six genes and one locus have so far been identified and *IQCB1* (SLSN5) is reported as a candidate gene in non-syndromic LCA as well. Some of the LCA patients with *IQCB1* mutation develop renal abnormality and are thus re-diagnosed as SLSN[53].

✓ Joubert syndrome

Joubert syndrome is an autosomal recessive disease involving defects in cerebellum and common features include developmental delays, ataxia (lack of muscle control), hyperpnoea (abnormal breathing patterns), sleep apnea, abnormal eye and tongue movements, and hypotonia in early childhood. Features of retinal dystrophy like RP or LCA are also reported. Twenty-six genes have been reported so far in Joubert syndrome including *CEP290* which is one of the candidate genes for LCA[107].

1.4.5.4. Peroxisome biogenesis disorders

Peroxisomal disorders are a group of genetically heterogeneous metabolic diseases that share dysfunction of peroxisomes, an organelle important for specific metabolic pathways, such as beta-oxidation of very-long-chain fatty acids (VLCFA), detoxification of hydrogen peroxide and production of cholesterol, bile acids, and plasmalogens. Some common clinical characteristic of Zellweger spectrum of peroxisomal biogenesis defects include neurologic, eye abnormalities (Leber congenital amaurosis, retinitis pigmentosa, optic atrophy, cataracts), hepatorenal defects, rhizomelic limb shortening, and calcific stippling of the patella[108].

1.4.5.5. Thiamine responsive megaloblastic anaemia

Thiamine responsive megaloblastic anaemia (TRMA) is an autosomal recessive disease caused by mutations in *SLC19A2* and is characterized by a triad of megaloblastic anaemia, non-type 1 diabetes mellitus and sensorineural deafness. Ocular abnormalities which include cone rod dystrophy, retinitis pigmentosa and optic atrophy are also reported in few cases. Thiamine transporter protein THTR1, localized in red blood cells, inner hair cells of the cochlea, pancreas, and small intestine is encoded by *SLC19A2* gene. This protein helps in the absorption of thiamine inside the cells. [109].

1.4.6. Gene therapy in LCA

Gene identification, proof-of-concept and safety studies of gene therapy in animals, and detailed human studies on photoreceptor layer integrity have led to treatment trials in the molecular form of LCA caused by mutations in *RPE65*, the gene encoding retinal pigment epithelium-specific protein, 65 kDa[110]. In this gene therapy trial, the researchers packaged a normal version of *RPE65* gene inside a

genetically engineered vector, the adeno-associated virus (AAV). This was injected subretinally to *RPE65* mutated LCA patients. The vector delivers the gene to cells in the retina, where the gene produces the functional enzyme and completes the visual cycle which otherwise is not functional due to mutated *RPE65*.

The results of the phase 1 gene replacement therapy trial conducted at Moorefield's eye hospital, University College London eye gene therapy study group showed sustained improvement in three young adults who received gene therapy and remained healthy maintaining the visual gains one year after treatment with AAV2-hRPE65v2[111, 112]. Early intervention was associated with better results. The immunological effect of antibodies after administration of AAV2-hRPE65v2 viral vector in both eyes in canine and non-human primate animal models suggested that simultaneous treatment of both eyes does not induce an immune response complicating the treatment[113]. Jacobson et al in 2012 reported the safety and efficacy in 15 children and adults followed up to 3 years where visual function improved in all patients to different degrees and improvements were localized to treated areas. Cone and rod sensitivities increased significantly in the study eyes but not in the control eyes. Minor acuity improvements were recorded in many study and control eyes[114]. Issues such as pseudo fovea formation, continued degeneration of photoreceptors, increase in dosage level are now being addressed[115, 116]. Other than the *RPE65* human gene therapy trials, animal models for gene therapy for LCA genes, *AIPL1*[117], *SPATA7*[118], *GUCY2D*[119] and *CEP290*[120] are being conducted and they also show promising results.

Another strategy that is being developed to treat RD is by targeting mutated gene using antisense oligonucleotides (AONs). AONs are small stretch of oligonucleotides that binds complementarily to the target sequence thereby hindering either transcription or translation. By binding to the target sequence, they can mask pre-mRNA splicing or trigger mRNA degradation as a DNA oligo can activate RNAse H. In *CEP290* associated LCA, AONs are used for splice correction where the intronic *CEP290* mutation c.2991+1655A>G creates cryptic splice-site leading to pseudoexon formation and thereby aberrant protein product. By AON-based therapy, the cryptic site is masked and the pseudoexon is skipped leading to normal *CEP290* splicing and wild-type CEP290 protein levels. *Invitro* and *invivo*

studies on mice models are promising [121]. Thus, the AON treatment addressing altered splicing has a therapeutic potential for many genetic diseases.

1.4.7. Targeted genotyping in Leber congenital amaurosis – Need for the study

LCA accounts for 3-5% of childhood blindness and has a prevalence of 1/50,000 - 1/33,000 live births (http://www.orpha.net -last updated July 2015). The genes associated with LCA have so far accounted for approximately 75% of all LCA cases. The frequency of mutations in LCA genes varies in different populations and comprehensive mutation study focused on individual ethnicities in large cohorts are few. Also, the gene that contributes to the highest mutation rate differs among the populations.

The clinical and genetic heterogeneity of LCA hampers its routine molecular diagnosis. Mutation- specific assays that are designed based on data available from LCA patients from one ethnic population are not the most efficient approach for studying patients from different populations and ethnic backgrounds. The mutation studies conducted on Indian LCA cohorts either had a very small sample size with fewer genes screened or included Indians as a part of multicentre studies or case report. Screening *RPE65* in 60 unrelated cases from Asian Indian cohort contributed to only 1.7%[122] whereas it contributes to 6% of LCA among various other populations. Thirty-eight LCA patients from Southern India were tested for the presence of 104 previously reported mutations in eight LCA genes from a Northern American cohort data. Only one proband was found to carry one of these 104 mutations[123].

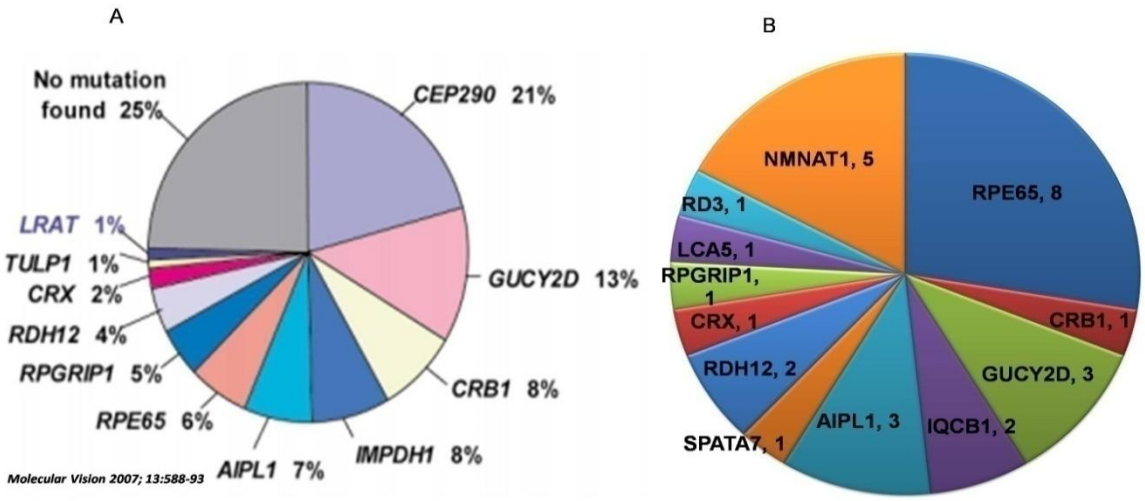


Figure 6: **A.** Pie chart showing distribution of LCA candidate genes mutations in North American cohort from 30 published studies [124] **B.** Distribution of LCA candidate genes mutations in Indian patients from 8 published studies [122, 123, 125-130]. The numbers in the pie diagram show the number of mutations identified in the specific gene in Indian patients.

Considering all the previous publications on gene screening for LCA from Indian cohorts, only 25 mutations in 33 cases are reported[122, 123, 125-131] (**Figure 6**). There is yet no comprehensive study on LCA candidate genes from a larger Indian cohort and the current study addresses this lacuna.

Sanger sequencing approach has been the gold standard for DNA sequencing for the past 35 years[132]. In 2005, the commercial launch of massively parallel pyrosequencing platforms piloted a new era of high throughput genome analysis, the next generation sequencing (NGS). The major advantage of this 'second-generation' or 'massively parallel' sequencing technologies, compared to Sanger sequencing, is their remarkably higher throughput, lower cost per sequenced base and less time consuming[133]. Using NGS technology in genetically heterogeneous diseases helps generate molecular data for a larger cohort at a reasonable time and cost.

1.5. Objectives of the study

Objective 1:

- Clinical examination, complete phenotype documentation and recruitment of 100 patients with Leber congenital amaurosis (LCA).

Objective 2:

- To design a customized probe to capture all exons and 20bp flanking sequences (exon - intron boundary) of the LCA candidate genes.
- Perform high throughput re-sequencing for the candidate genes on NGS platform.

.**Objective 3:**

- To validate the results obtained by targeted re-sequencing using Sanger sequencing, to perform segregation analysis in the families and control screening for the identified mutations to confirm the pathogenicity.
- To perform appropriate bioinformatics analyses for the identified mutations.

Objective 4:

- To perform genotype-phenotype correlation.

CHAPTER 2: MATERIALS AND METHODS

Overview of the methodology

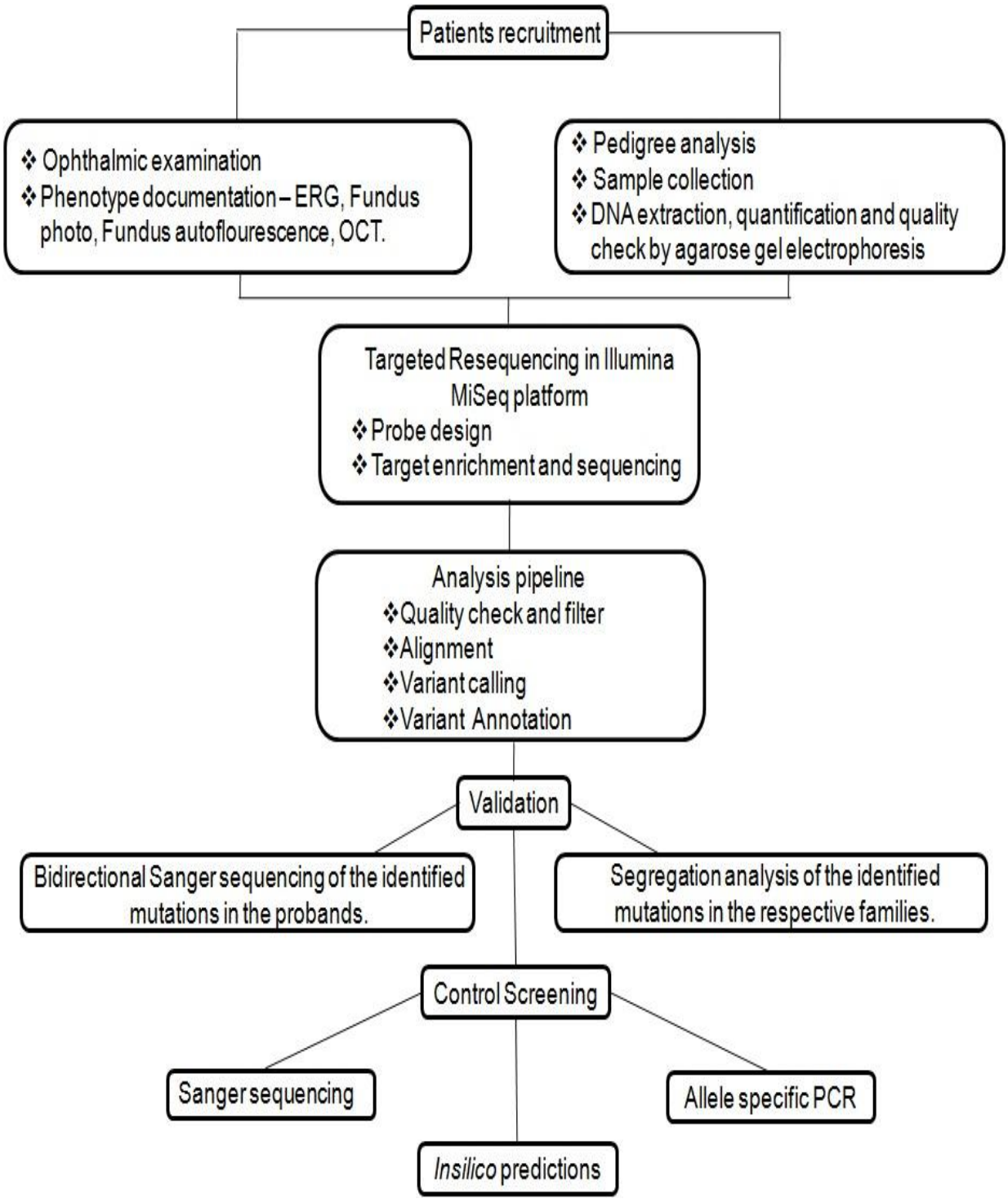


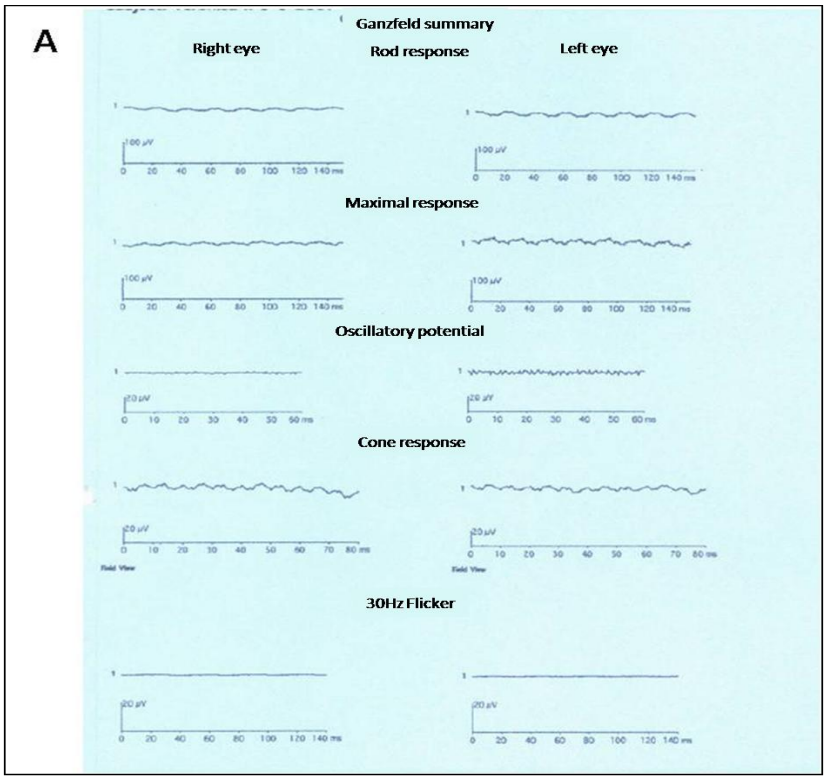
Figure 7: Diagrammatic representation of methodology.

2.1 Patients recruitment

Patients were recruited through the departments of paediatrics ophthalmology, vitreoretina and genetics clinic of Medical Research Foundation, Sankara Nethralaya, Chennai. The work was approved by the Institutional review board and ethical committee and was in accordance with declaration of Helenski. Written Informed consent was obtained from the patients or parents in case the subjects were below 18 years of age.

2.1.1. Clinical documentation:

The patients underwent complete ophthalmic examination and characteristic features of LCA – diagnosed earlier to one year of age, diminished/ absent ERG, nystagmus, hyperopia, photophobia, sluggish pupil and oculo- digital signs were evaluated. They were examined for finer fundus details with posterior pole fundus photograph, fundus autofluorescence (FAF), retinal optical coherence tomography (OCT) measurements if possible (**Figure 8**). In infants or very young children under 5 years of age, fundus photography, FAF and OCT could not be done and ERG alone was documented under general anaesthesia.



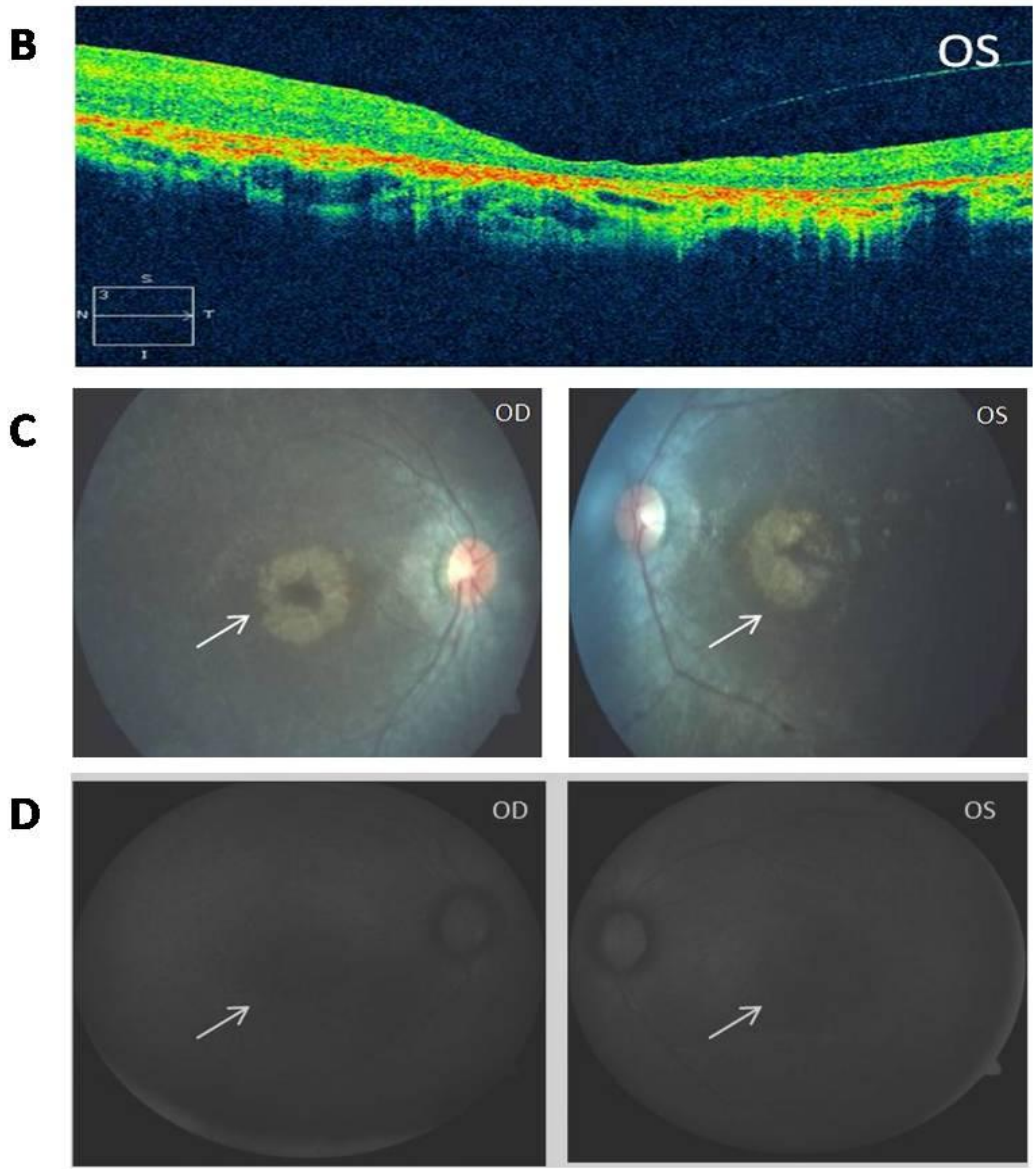


Figure 8: Shows the [A] ERG of LCA patient showing extinguished rod and cone response as a flat wave pattern [B] OCT of patient having mutation in *RDH12* gene showing foveal thinning with RPE atrophy [C] Fundus photo and [D] Fundus autofluorescence of a patient with mutation in *AIPL1* gene showing macular changes (indicated by arrow).

The patients were either simplex cases (78/92) or affected sibs (14/92) from consanguineous or non-consanguineous families (**Figure 9**) representing typically autosomal recessive inheritance. Sixty seven percent of (62/92) families reported consanguinity of which 84% (52/62) were simplex cases.

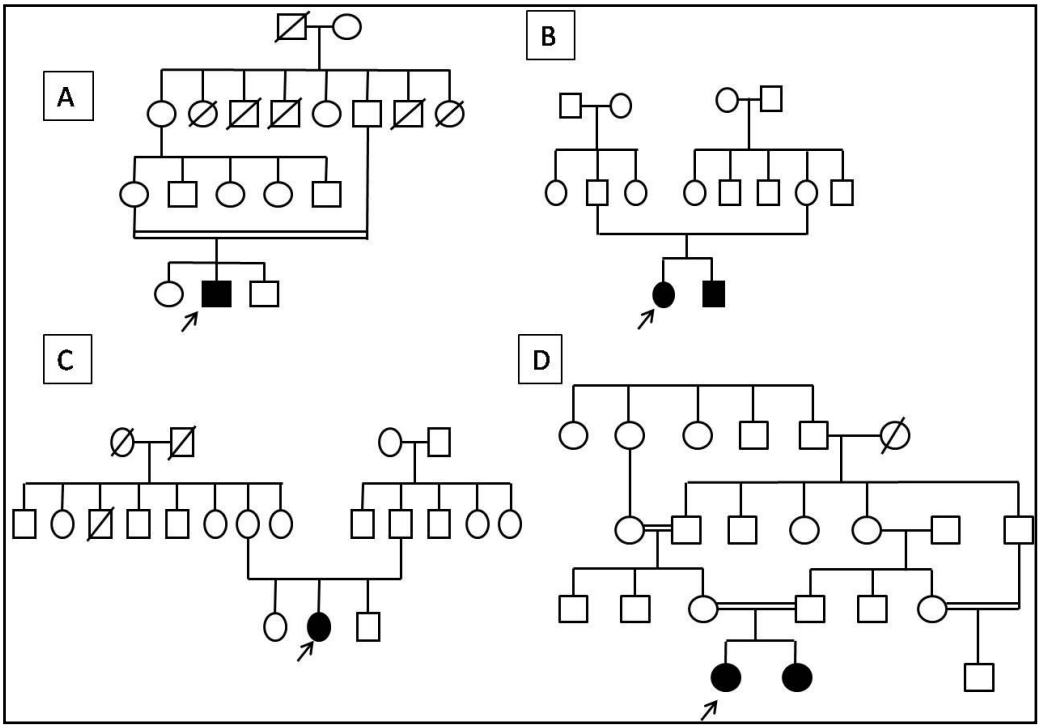


Figure 9: Representative pedigrees of cases recruited. Pedigrees of [A] isolated proband from a consanguineous family [B] affected sibs from non-consanguineous family [C] isolated proband from non-consanguineous family [D] affected sibs from consanguineous family.

2.1.2. Sample collection

Ninety-two LCA patients were recruited in the study from across India. Seventy-three patients are from south India while 19 were from north India. Of these 79% (58/73) and 16% (3/19) are from consanguineous south and north Indian families, respectively **(Figure 10).** Venous blood (8-10 ml) was collected with ACD anticoagulant (BD Vacutainer, BD, New Jersey, USA) from the probands, affected or unaffected sibs (if any) and parents after obtaining their consent.

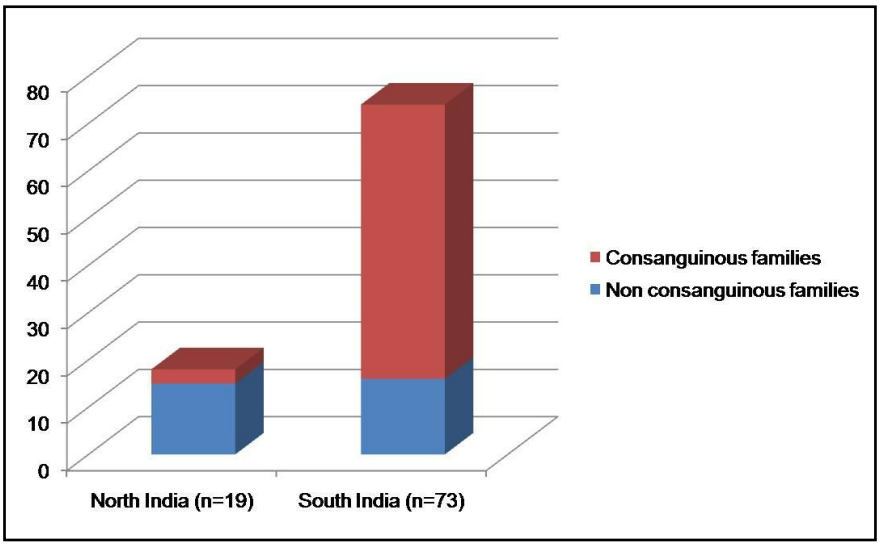


Figure 10: Shows distribution of consanguinity among the north and south Indian cases in the cohort of 92 LCA families.

Control samples were taken from previous epidemiological studies conducted by Medical Research Foundation and they were ruled out for any inherited retinal dystrophies[134, 135]

2.2. DNA extraction

Genomic DNA was extracted from ACD blood samples using NucleoSpin® Blood XL kit (Macherey-Nagel, GmbH, Duren, Germany). [Procedure as in appendix 1]

2.3. DNA Quantification and Quality check

The DNA was quantified using Nano drop Spectrophotometer ND1000 (Thermo Fisher Scientific, Wilmington, USA).The integrity of the DNA was checked by running on 1% agarose gel **(Figure 11)**. [Procedure as in appendix 2]

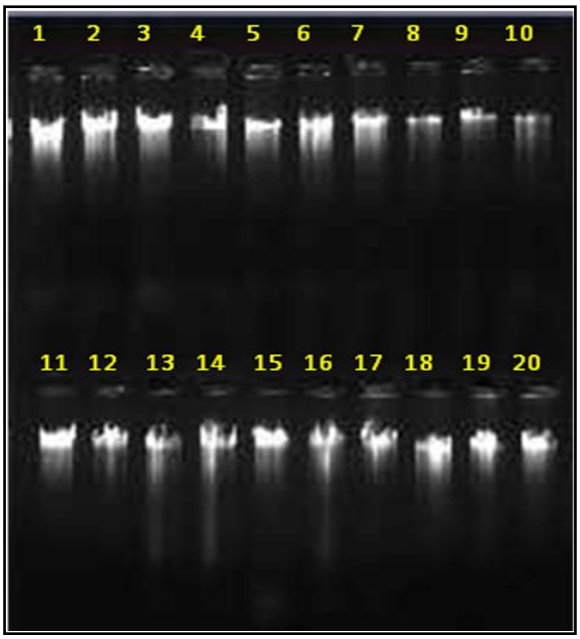
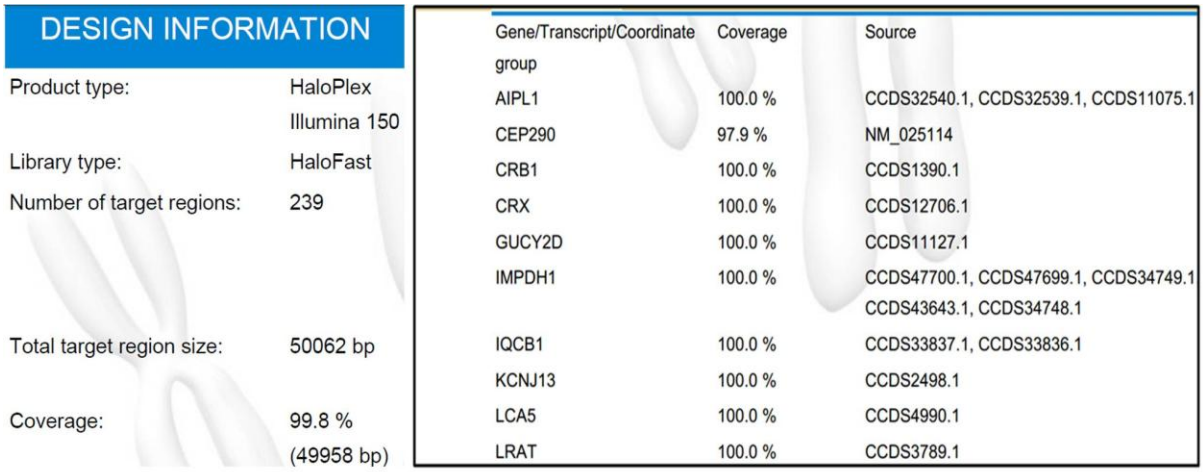


Figure 11: Agarose gel image of DNA samples to check their quality (Lane 1-20).

2.4. Targeted re-sequencing using Illumina MiSeq platform

2.4.1. Designing target enrichment probes:

- Target enrichment probes were designed using Agilent SureDesign HaloPlex standard wizard covering 20 LCA candidate genes *AIPL1, CABP4, CEP290, CRB1, CRX, GUCY2D, IQCB1, IMPDH1, KCNJ13, LCA5, LRAT, MERTK, NMNAT1, OTX2, RD3, RDH12, RPE65, RPGRIP1, SPATA7* and *TULP1* including the exon-intron boundaries, padding each exon with 20 bp sequences upstream and downstream.
- For the probe design the CCDS reference sequence databases were selected for the coding transcripts of all the genes except *CEP290* for which the RefSeq database was used. UTR regions were not included in the designing.
- Two-hundred thirty-nine regions of 50062 bp covering all the coding regions with splice junctions were targeted and probe design was automatically generated by HaloPlex Design Wizard software with 99.8% coverage i.e. 49,958 bp of region of interest **(Figure 12)**.



DESIGN INFORMATION

Product type:	HaloPlex Illumina 150
Library type:	HaloFast
Number of target regions:	239
Total target region size:	50062 bp
Coverage:	99.8 % (49958 bp)

Gene/Transcript/Coordinate group	Coverage	Source
AIPL1	100.0 %	CCDS32540.1, CCDS32539.1, CCDS11075.1
CEP290	97.9 %	NM_025114
CRB1	100.0 %	CCDS1390.1
CRX	100.0 %	CCDS12706.1
GUCY2D	100.0 %	CCDS11127.1
IMPDH1	100.0 %	CCDS47700.1, CCDS47699.1, CCDS34749.1 CCDS43643.1, CCDS34748.1
IQCB1	100.0 %	CCDS33837.1, CCDS33836.1
KCNJ13	100.0 %	CCDS2498.1
LCA5	100.0 %	CCDS4990.1
LRAT	100.0 %	CCDS3789.1

Figure 12: Target enrichment probe design using Agilent SureDesign HaloPlex standard wizard. Snapshot of the output by HaloPlex Design Wizard providing information on the coverage for each gene by the designed custom enrichment probes.

- The region of interest, list of analysable **(Figure 13)** and unanalysable amplicons that were generated by the software was checked manually with the reference sequences to confirm whether all exonic regions of candidate genes were covered or not.

	A	B	C	D	E	F	G	H	I	J	K	L
1	##GFF-version 2											
2	##source-version sedd2gff 0.1											
3	##Type DNA											
4	##date 2012-03-21											
5												
6	track name=Analyzable											
7	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112656173-112656502"			
8	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112686609-112687323"			
9	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112702192-112702336"			
10	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112702384-112702777"			
11	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112704775-112705336"			
12	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112725549-112726007"			
13	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112722629-112723068"			
14	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112732712-112733212"			
15	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112740105-112740715"			
16	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112754648-112754792"			
17	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112754804-112755305"			
18	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112751555-112752312"			
19	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112761190-112761334"			
20	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112760539-112760853"			
21	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112761348-112761795"			
22	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112758545-112759046"			
23	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112767419-112767863"			
24	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112767252-112767415"			
25	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112765687-112766215"			
26	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112776765-112777280"			
27	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112779595-112780217"			
28	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112778685-112779372"			
29	chr2	SEDD	Analyzabl	1.13E+08	1.13E+08	.	.	0	"NC_000002.11:112785609-112786614"			
30	chr2	SEDD	Analyzabl	2.34E+08	2.34E+08	.	.	0	"NC_000002.11:233636253-233636397"			
31	chr2	SEDD	Analyzabl	2.34E+08	2.34E+08	.	.	0	"NC_000002.11:233635289-233636242"			
32	chr2	SEDD	Analyzabl	2.34E+08	2.34E+08	.	.	0	"NC_000002.11:233632814-233633646"			

Figure 13: Above is the snapshot of list of analysable regions for which custom enrichment probes were designed.

❖ Of the 239 targets, 9 were not covered completely. These include few bases in some exons of genes *CEP290, LCA5, RPE65* and *SPATA7* **(Figure 14)**. Bases that were not covered by the custom enrichment probes were genotyped by direct sequencing.

	A	B	C	D	E	F	G	H	I	J	K	L
1	##GFF-version 2											
2	##source-version sedd2gff 0.1											
3	##Type DNA											
4	##date 2012-03-21											
5												
6	track name=Not_Covered											
7	chr1	SEDD	Not_Cove	68896954	68896962	.	.	0	"NC_000001.10:68896954-68896962"			
8	chr6	SEDD	Not_Cove	80197453	80197455	.	.	0	"NC_000006.11:80197453-80197455"			
9	chr6	SEDD	Not_Cove	80197603	80197603	.	.	0	"NC_000006.11:80197603-80197603"			
10	chr12	SEDD	Not_Cove	88454587	88454600	.	.	0	"NC_000012.11:88454587-88454600"			
11	chr12	SEDD	Not_Cove	88471659	88471662	.	.	0	"NC_000012.11:88471659-88471662"			
12	chr12	SEDD	Not_Cove	88510790	88510834	.	.	0	"NC_000012.11:88510790-88510834"			
13	chr14	SEDD	Not_Cove	88862498	88862505	.	.	0	"NC_000014.8:88862498-88862505"			
14	chr14	SEDD	Not_Cove	88893957	88893971	.	.	0	"NC_000014.8:88893957-88893971"			
15	chr14	SEDD	Not_Cove	88904732	88904736	.	.	0	"NC_000014.8:88904732-88904736"			
16												
17												

Figure 14: Above is the snapshot of list of non-analysable regions for which custom enrichment probes could not be designed.

❖ The custom enrichment probes were submitted to the manufacturer for the synthesis of target enrichment kit.

2.4.2. Target enrichment and Sequencing

Target enriched and indexed library was prepared for each sample to be sequenced using **HaloPlex PCR target enrichment and library preparation kit.** This was performed in Scigenom labs Pvt Ltd, Kochi, India

Restriction digestion:

❖ The gDNA was digested with 16 restriction enzymes (RE) in eight different reactions each containing 2 RE. This resulted in fragment library which include both target and non-target gDNA regions.

❖ During each batch of library preparation, an Enrichment Control DNA (ECD) was included to validate the restriction digestion reaction.

❖ The digested ECD was run on Agilent 2100 Bioanalyzer **(Figure 15).**

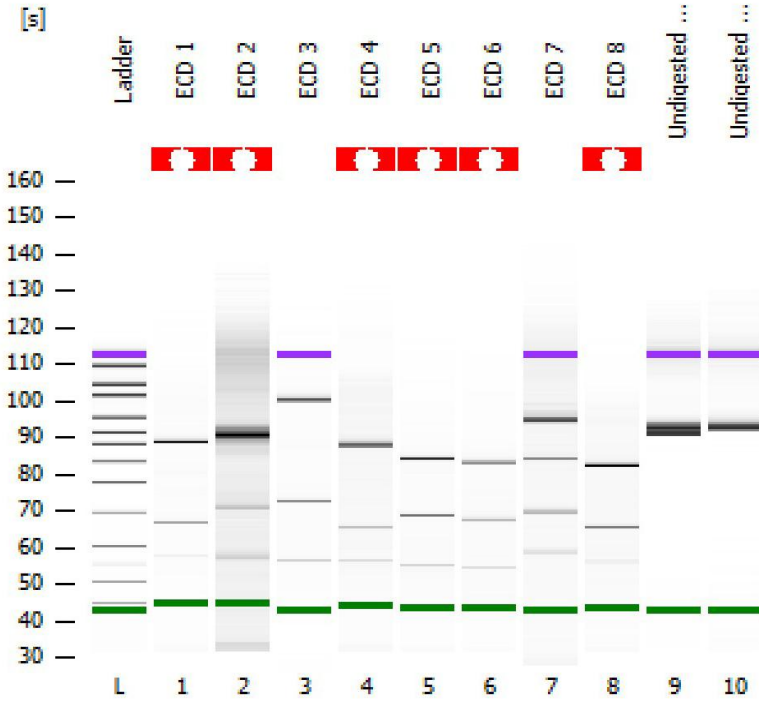


Figure 15: The gel picture of the ECD as run on DNA chip in Agilent 2100 bioanalyzer. 'L' represents 50bp ladder, lane 1-8 represents ECD digested with specific pair of RE and the pattern of digested fragments, lane 9 and 10 represents undigested DNA.

Enrichment:

- The gDNA restricted fragments were then hybridized to HaloPlex probe capture library that were designed to the target regions.
- Each of the eight restriction digested reactions of a sample were pooled and added to the probe capture library.
  - The biotinylated probes are designed along with unique indexes and Illumina paired end sequencing motifs in the centre, thus facilitating indexing of the enriched sequences. The unique indexes help tag the samples during multiplexing for bridge amplification and sequencing on the NGS platform. The presence of target sequence on ends of the probe directs circularization of the target regions during hybridization.
- The circularized target DNA-HaloPlex probe hybrids, containing biotin, were captured on streptavidin beads and the non-target regions were removed.

- This was followed by ligation of the nicks in the circularized target DNA in the HaloPlex probe-target DNA hybrids using DNA ligase.
- The captured, fully circularized targets were eluted from the bead using 50mM NaOH and were PCR amplified.
    - Universal primers complementary to Illumina paired end sequencing motifs were used during PCR amplification.
- Bead based purification of the amplified product was performed which was followed by validating and quantifying the enriched target samples by Agilent 2100 Bioanalyzer prior to sample pooling and sequencing preparation **(Figure 16).**

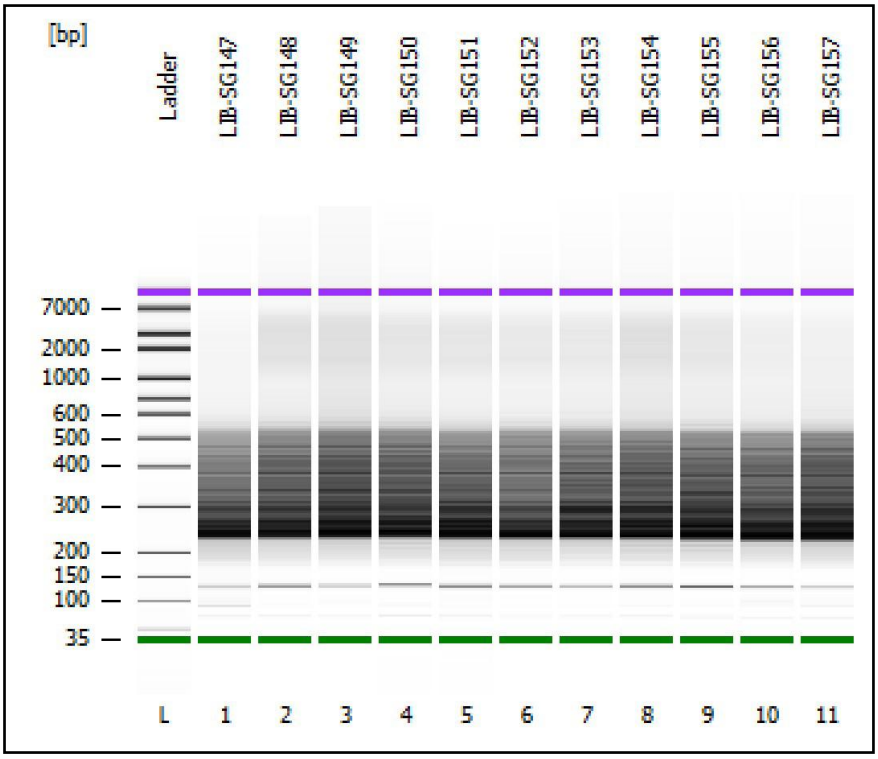


Figure 16: The gel picture of amplified purified samples in the size range between 175-625bp indicating enriched target libraries of target insert sizes 50-500bp. Green line- lower marker; purple line – upper marker.

Sequencing:

- Equimolar amount of each sample was pooled prior to cluster generation by bridge amplification and sequencing on Illumina MiSeq **(Figure 17).** 10nmoles/ 10ul of each sample were pooled and 7 pmoles of this pool was loaded in the

Illumina MiSeq flow cell for cluster generation followed by paired end parallel sequencing.

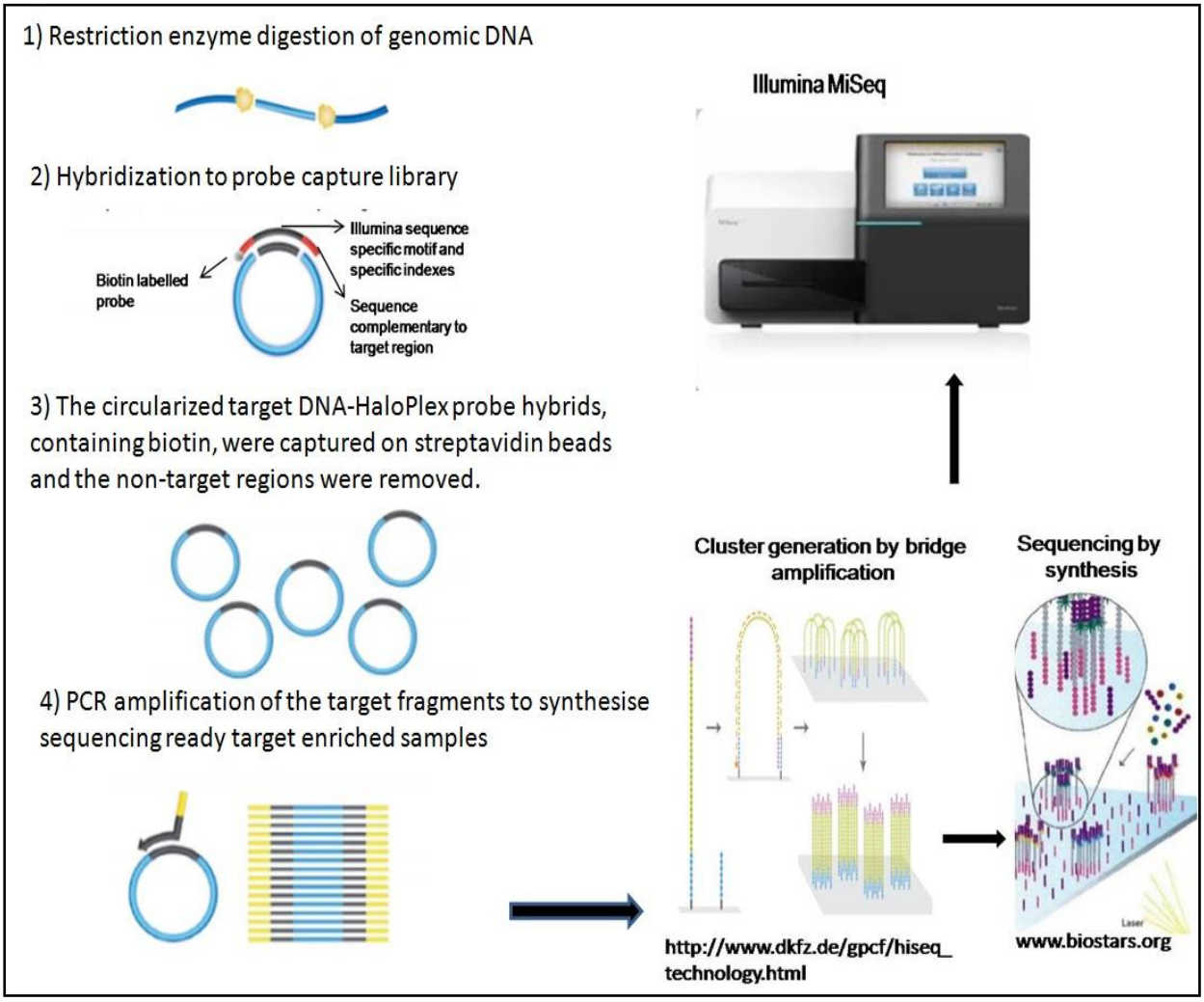


Figure 17: Schematic representation of the steps involved in target enrichment using Agilent HaloPlex target enrichment kit followed by cluster generation by bridge amplification and sequence by synthesis on Illumina MiSeq platform.

2.4.3. Analysis pipeline:

The steps followed to analyse the datasets obtained from next-generation sequencing are briefly described below.

I. **Quality Check and Filter:** First the low quality bases from the reads were removed. Average Q30 score was used as a cut-off to remove low quality bases. Then the adapter sequences were trimmed from both 5' and 3' end of the read sequence. The base quality score distributions, average base content per read and GC distribution in the reads were considered for quality checking.

II. **Alignment:** The filtered reads were aligned to the reference genome (hg19) using BWA program. After alignment only reads with mapping quality ≥20 were used for further analysis. The coding region of each gene was identified for taking into consideration all protein-coding transcripts of the gene in Ensembl 68 database.

III. **Variant Calling:** The variants in the samples were predicted using Samtools toolkit. The variants with quality score ≥ 50 and read depth of at least 5 were taken further for annotation.

IV. **Annotation:** The variants were annotated using GATK and compared with various databases including OMIM, ClinVar, SNPedia, Ensemble (GRCh37) variation table, 1000 genome project data, Exomeserver (ESP5400), ExAC databasev.0.3.1, dbSNP (build 150) and Human genome mutation database for identifying clinically relevant variants.

The initial analysis using the above pipeline was done in Scigenom labs Pvt. Ltd, Kochi, India. The same pipeline was constructed in CLC Cancer Genomics workbench (v.8.0.1) and the samples were re-analysed.

The single nucleotide variations (SNVs) and indels were further analysed manually as defined below

i. As LCA is an autosomal recessive disease, we short listed all the homozygous variants first.
ii. Scrutinized all variants and indels in the coding regions
iii. The canonical splice-sites, regions 10bp upstream and downstream of splice sites (-10 to +10) and variants in the last codon of the exons were identified for further validation and bioinformatics analyses.
iv. The heterozygous variants (MAF< 0.01) were shortlisted to identify possible compound heterozygous mutations.
v. The homozygous and heterozygous SNVs and indels were checked if they are novel or reported in the databases like OMIM [136], ClinVar[137], SNPedia[138], Ensemble variation table [139], Exome server [140], 1000 genome project data [141], ExAC database [142], dbSNP[143] and Human genome mutation database [144].
vi. All other reported and novel, synonymous and intronic variants were noted.

2.5. Validation of the identified pathogenic and likely pathogenic mutations:

The pathogenic or likely pathogenic mutations shortlisted using the above criteria was validated by PCR and bidirectional direct Sanger sequencing of the respective regions. The segregation analysis was performed in parents and available family members. Variants that were validated and segregating within the families were screened in an average of 120 unrelated controls either by custom designed allele specific PCR (ASPCR) or Sanger sequencing.

I. Polymerase Chain Reaction (PCR):

Primers were designed using Primer3 (Version 0.4.0). The primers were designed to amplify the exons in which the pathogenic or likely pathogenic mutations were identified. The primer sequences used for the PCR are provided in appendix 3.

Table 3: Reaction protocol for Polymerase chain reaction.

Reagents	**Stock concentration**	**Volume in µl**
Genomic DNA	50 ng	1
Forward Primer (10pmol/ µl) (Xcelris Genomics, Ahmadabad, India)/ Sigma-Aldrich, Missouri, USA/IDT, Iowa, USA)	5 pmol	0.5
Reverse Primer (10pmol/ µl) (Xcelris Genomics, Ahmadabad, India)/ Sigma-Aldrich, Missouri, USA/ IDT, Iowa, USA)	5 pmol	0.5
Deoxyribo nucleotide phosphates [dNTPs] (Applied Biosystems, Foster City, USA)	250uM	0.125
Taq DNA polymerase (3U/µl) (Thermo Fisher Scientific, Massachusetts, USA)	0.3U	0.1
Taq Buffer (Thermo Fisher Scientific, Massachusetts, USA)	1X	1.25
Betaine (Sigma-Aldrich, Missouri, USA)	5M	1
Sterile water		8.025
Total		**12.5**

The thermal cycler profile is as given below.

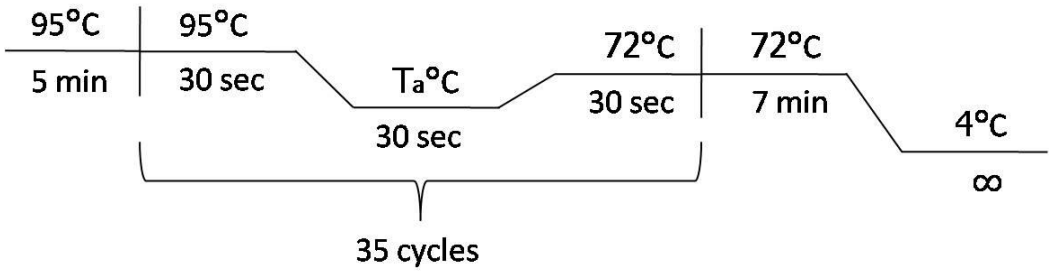


Figure 18: Reaction protocol for PCR amplification in thermal cycler.

The standardized annealing temperature and the product size of the exons that were PCR amplified for validation of identified pathogenic and likely pathogenic mutations from the targeted re-sequencing data and for exons that were incompletely covered by target enrichment probes are given below in the **table 4.** The regions covered by the primers are shown in appendix 6.

Table 4: List of regions analysed by PCR based direct sequencing with annealing temperature and product size

S.No	Gene	Exon	Tm (c°)	Product size (bp)
1	*AIPL1*	6	63	500
2	*AIPL1*	5	60	279
3	*AIPL1*	2	60	316
4	*CRB1*	5	60	317
5	*CRB1*	11	60	283
6	*CRB1*	12A	62	482
7	*CRX*	2	67.8-60.8(-0.5)	314
8	*CRX*	3b	63	294
9	*GUCY2D*	14	69-62(-0.5)	306
10	*GUCY2D*	15	69-62(-0.5)	260
11	*GUCY2D*	17	69-62(-0.5)	168
12	*GUCY2D*	10	69-62(-0.5)	235
13	*GUCY2D*	2b	59	551
14	*GUCY2D*	9	69-62(-0.5)	295
15	*GUCY2D*	3	69-62(-0.5)	381
16	*GUCY2D*	16	69-62(-0.5)	200

17	*GUCY2D*	11	69-62(-0.5)	220
18	*GUCY2D*	2a	64	472
19	*IQCB1*	14	60	351
20	*IQCB1*	11	60	383
21	*IQCB1*	13	60	400
22	*KCNJ13*	3A	56	471
23	*LCA5*	7a	57	340
24	*LCA5*	3	65-58(-0.5)	365
25	*LCA5*	5	56	353
26	*LCA5*	6	56	237
27	*NMNAT1*	2	62	425
28	*RD3*	1	60	480
29	*RDH12*	2	60	359
30	*RDH12*	6	60	492
31	*RPE65*	4	54	501
32	*RPE65*	2	60	360
33	*RPE65*	14	53	453
34	*RPE65*	9	56	292
35	*RPE65*	13	42	199
36	*RPE65*	10	56	226
37	*RPGRIP1*	21	58	287
38	*RPGRIP1*	14	52	554
39	*RPGRIP1*	6	52	282
40	*RPGRIP1*	24	56	205
41	*SLC19A2*	2	63-56(-0.5)	700
42	*SPATA7*	10	60	243
43	*SPATA7*	1	60	144
44	*SPATA7*	7	60	477
45	*TULP1*	11	59	247
Unanalysable regions				
46	*CEP290*	Intronic	63	294
47	*CEP290*	40	58	592
48	*CEP290*	18	62	495
49	*RPE65*	12	60	214
50	*SPATA7*	3	65	312
51	*SPATA7*	6	60	250
52	*SPATA7*	11c	63	396

II. ExoSap digestion of PCR product.

The amplified products were digested by ExoSap prior to cycle sequencing. This is an enzymatic procedure for purifying the PCR product where the unused primers and dNTPs are hydrolysed.

Table 5: ExoSAP protocol

Reagents	Stock concentration	Volume (µl)
Exonuclease I (Thermoscientific, Massachusetts, USA)	20U/µl	1
Exonuclease buffer (Thermoscientific, Massachusetts, USA)	10X	4
FastAP thermosensitive alkaline phosphatase (Thermoscientific, Massachusetts, USA)	1U/µl	2
FastAP buffer (Thermoscientific, Massachusetts, USA)	10X	3

Procedure

- 1.5 µl of the above cocktail and 5 µl of amplified PCR product were mixed well, microfuged and reaction proceeded.

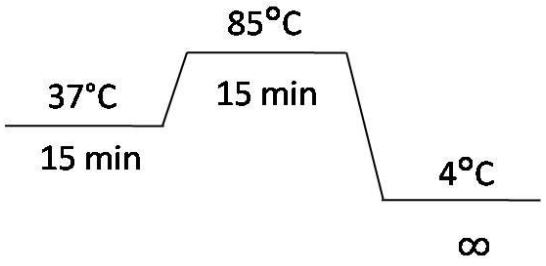


Figure 19: Reaction protocol for ExoSap digestion in thermal cycler.

III. Cycle Sequencing:

Cycle sequencing was done using big dye terminator v3.1 kit (Applied Biosystems, Foster City, USA). Sequencing of all amplified products was carried out using the either forward primer/ reverse primer**.** A 10 µl reaction was prepared as given in the protocol below, mixed and microfuged.

Table 6: Reaction protocol for cycle sequencing.

Reagents	Volume in µl
5X sequencing Buffer (Applied Biosystems, Foster City, USA)	1.5
Primer (100pmol/ µl)	1
RR mix (Applied Biosystems, Foster City, USA)	0.5
Exo Sap product	1
Sterile water	6
Total	**10**

- The reaction was set in a thermal cycler as given in the conditions below.

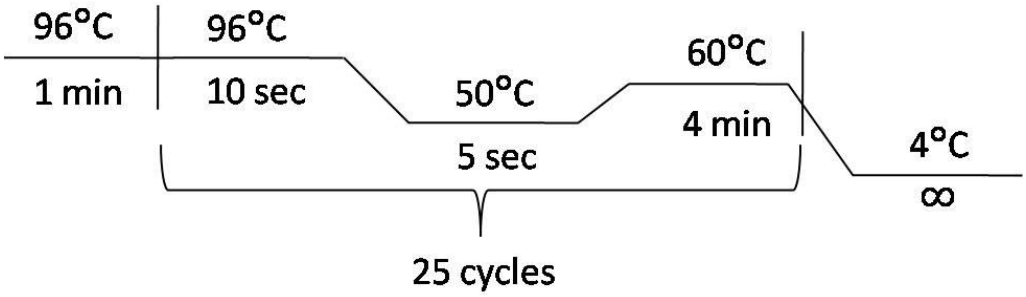


Figure 20: Thermal cycle reaction protocol for cycle sequencing

The samples were purified by ethanol/ EDTA/ Sodium Acetate precipitation method. The precipitated product was resuspended in Hi-Di™ Formamide (Applied Biosystems, Foster City, USA) and was loaded on to ABI 3100 *Avant* or 3730 Genetic analyzer (Applied Biosystems, Foster City, USA). [Procedure as in appendices 4 & 5]

IV. Reverse transcriptase PCR

1. RNA extraction

RNA extraction was performed from whole blood (two families) of the probands, parents and affected/ unaffected sib using Trizol method.

Procedure

- 2 ml Trizol (TRI Reagent® (Sigma Aldrich, Missouri, USA)) was added to 1ml whole ACD/ EDTA blood, mixed, incubated for 10 minutes at room temperature

- It was centrifuged at 12000 rpm for 15 minutes at 4°C.
- To the supernatant 2ml Trizol and 400µl chloroform (Merck, New Jersey, USA) was added, mixed, incubated for 10 minutes at room temperature and centrifuged at 12000 rpm for 20 minutes at 4°C.
- The aqueous phase was separated and equal volume of isopropanol (Merck, New Jersey, USA) was added, mixed well, incubated for 10 minutes at room temperature and centrifuged at 12000 rpm for 10 minutes at 4°C.
- To the pellet, 2ml of 75% ethanol (Hayman Limited, Witham, England) was added and washed twice by centrifuging at 8400 rpm for 5 minutes at 4°C.
- The pellet was air dried and dissolved in RNAse free water.
- The RNA was quantitated using Nano drop Spectrophotometer ND1000 and electrophoresed in 1% agarose gel to check the quality.

2. cDNA conversion:

cDNA conversion was performed using Verso cDNA Synthesis Kit, (Thermo Scientific, Massachusetts, USA).

Procedure

- A 20 µl reaction was prepared as given in the protocol below, mixed and microfuged.

Table 7: Reaction protocol for cDNA Synthesis.

Reagents	Volume in µl
Template RNA (~1µg)	1
Verso enzyme mix	1
RT enhancer	1
RNA primer mix (Random hexamer: oligodt = 3:1)	1
dNTP mix	2
5X cDNA synthesis buffer	4
Nuclease free Water	10
Total	**20**

- The reaction was set in a thermal cycler as given in the condition below.

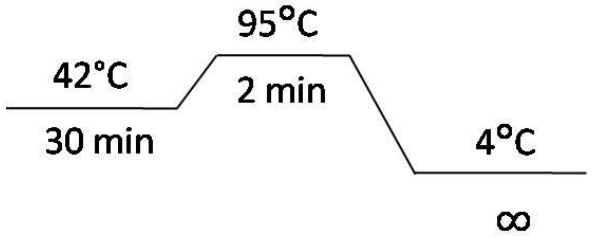


Figure 21: Thermal cycler reaction protocol for cDNA synthesis.

3. Reverse transcriptase PCR:

Reverse transcriptase PCR was performed on cDNA using primers encompassing exons 7-10 and 9-11 covering the mutations c.913-2A>G and c.1215+5C>A of the *SPATA7* gene to identify the altered splicing. PCR was performed using the reaction protocol as in **table 3** and thermal profile as in **figure 18.** The standardized annealing temperature and product size is provided in **table 8**. The regions covered by the primers are shown in appendix 6.

Table 8: cDNA region analysed by PCR based direct sequencing with annealing temperature and product size

S.No	Region	Target exon	Tm (c°)	Product size (bp)
1	SPATA7RT1	7-10	63-56(-0.5)	575
2	SPATA7RT2	9-11	61	418

V. Gel extraction

As there were two different transcripts of *SPATA7* gene expressed in blood, gel extraction followed by sequencing was performed to identify them. Gel extraction was done using QIAquick Gel Extraction Kit (Qiagen, Hilden**,** Germany)

Procedure

- The specific amplified band was excised from agarose gel with a clean scalpel and weighed.
- Buffer QG (provided in kit) was added to the gel slice such that for 100mg gel slice 100µl buffer QG was added.

- The tube was incubated at 50°C until the gel slice dissolved completely. It was also vortexed intermittently.
- Equal volume of isopropanol was added to the sample and mixed.
- The sample was added to QIAquick column (provided in kit) and centrifuged at 13000 rpm for 1 min.
- The flow through was discarded and 500 µl of buffer QG was added to the column and centrifuged at 13000 rpm for 1 min.
- To the column, 750 µl of wash buffer PE (provided in kit) was added, incubated for 5 minutes and centrifuged at 13000 rpm for 1 minute. This step was repeated twice.
- The flow through was discarded and the column was centrifuged at 13000 rpm for 1 minute to remove residual buffer PE.
- The column was transferred to 1.5 ml microfuge tube and 15 µl elution buffer EB (provided in kit) was added, incubated for 4 minutes and centrifuged at 13000 rpm for 1 minute.
- The eluted products (2.0 ul each) were directly used for bidirectional Sanger sequencing.

2.6. Insilico Predictions:

Online bioinformatics prediction tools like SIFT[145], PolyPhen[146], PMut[147], Mutationaccessor (http://mutationassessor.org/v1), Mutpred[148] and Variant effect predictor[149] were used to predict the effect of the missense mutations. Mutation taster[150], Netgene2[151], NNsplice[152], Fsplice (http://www.softberry.com/berry.phtml?topic=fsplice&group=programs&subgroup=gfind) and Splice finder[153] predicted the effect of splice-site mutations. CADD analysis was performed for all identified variants.

2.7. Screening of *SLC19A2* in a patient diagnosed with Thiamine Responsive Megaloblastic Anaemia (TRMA) with LCA as ocular feature.

A patient, reported to our hospital at 5 months of age and showed typical characteristics of LCA, later developed megaloblastic anaemia, non-type 2 diabetes mellitus and sensory neural deafness. The proband **(Figure 22)** underwent complete clinical, hematological, ophthalmic and auditory examination and presented the triad features - anaemia, diabetes and

deafness which are typical characteristics of syndrome called 'Thiamine responsive megaloblastic anaemia, an autosomal recessive disorder[154]. The only known causative gene for the syndrome *SLC19A2,* encodes for thiamine transporter protein, THTR1[155]. Blood samples were collected from the patient and her family members after an informed consent.

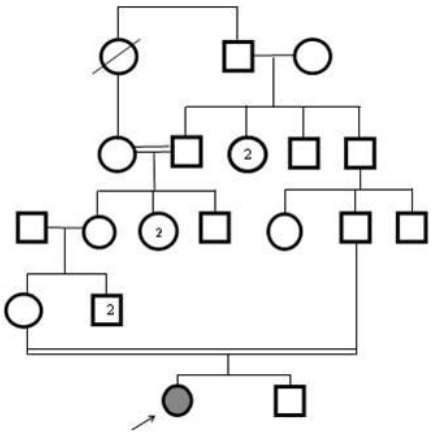


Figure 22: Pedigree of the proband showing typical autosomal recessive pattern of inheritance

The PCR primers for all 6 exons of the *SLC19A2* gene were designed using Primer3 software (Version 0.4.0). PCR was standardized as in **table 3**. Standardization of PCR for all six exons of *SLC19A2* gene was done (**Table 9**). The regions covered by the primers are shown in appendix 12.

The Exo-SAP treated PCR products were genotyped by direct sequencing using 3100-Avant genetic analyzer. SIFT and PolyPhen analyses were performed to predict the possible impact of amino acid substitution on the structure and function of protein.

Table 9: List of regions in *SLC19A2* analysed by PCR based direct sequencing with annealing temperature and product size. ASPCR- Allele specific PCR for the identified mutation in exon 2.

S.No	Region	Tm (c°)	Product size (bp)
1	*SLC19A2*- Exon 1	61	589
2	*SLC19A2*- Exon 2	63-56 (-1ºC)	700
3	*SLC19A2*- Exon 3	61	391
4	*SLC19A2*- Exon 4	61	429
5	*SLC19A2*- Exon 5	61	300
6	*SLC19A2*- Exon 6	61	295
7	*SLC19A2*-Exon 2-ASPCR	56	186

CHAPTER 3 – RESULTS

3.1. Targeted resequencing

3.1.1. Data analysis

I. **Quality Check and Filter:**

a. Raw read summary

The reads/ data obtained after sequencing was analysed using specific analysis pipeline. This involves trimming of reads depending on their quality, removal of the adapter sequence, and then aligning to the reference sequences before the variants are called and annotated. Below (**Table 10**) is the summary of raw fastq files obtained from NGS for the processed 92 samples.

Table 10: Raw read summary

	All samples
# of paired-end reads	75,006,077
# of bases (Gb)	9.74
% of data >= Q30	83.2
Raw read length (bp)	151 x 2

II. **Alignment:**

a. Coverage:

The reads covering the coding region of all genes is represented as coverage. For all 20 genes that were studied we obtained an average coverage of 99.03%. The coverage of each gene is provided in **figure 23** and **table 11**

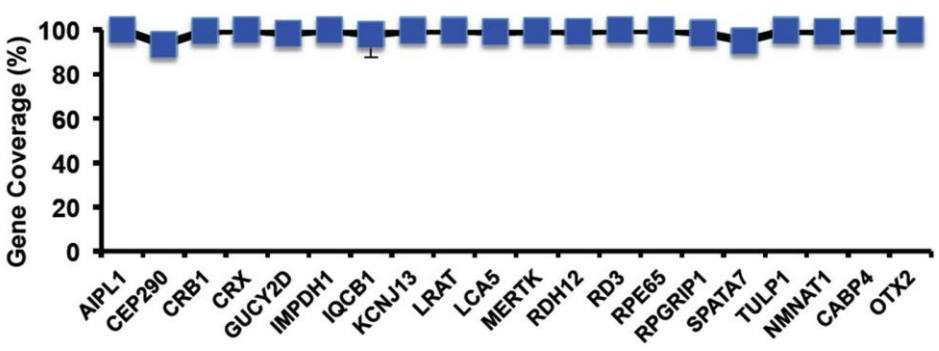


Figure 23: Average read coverage graph for all 20 genes

Table 11: Average coverage and depth for each 20 genes studied.

Gene	Average coverage (%)	Average depth (X)
AIPL1	100	224.783653
CEP290	93.45964192	37.08527504
CRB1	99.69204983	93.72696533
CRX	100	103.4220974
GUCY2D	98.51097797	147.0135295
IMPDH1	99.97926786	269.9190111
IQCB1	98.09025253	69.26971055
KCNJ13	99.87620773	80.8880666
LRAT	99.85664776	98.76046058
LCA5	99.11889464	39.42222961
MERTK	99.60507028	173.1452721
RDH12	99.43473675	208.0075136
RD3	99.93682646	173.8373646
RPE65	99.99320227	156.7695652
RPGRIP1	98.63304982	100.09021
SPATA7	95.10451291	78.28896321
TULP1	99.91919031	233.9596462
NMNAT1	99.43671388	96.9768017
CABP4	100	167.9272444
OTX2	99.97560142	139.8586469

b. Depth:

The average number of reads covering the coding region is represented as depth. An average depth of 134X was obtained for all 20 genes. The depth for each gene is provided in **table 11** and **figure 24**.

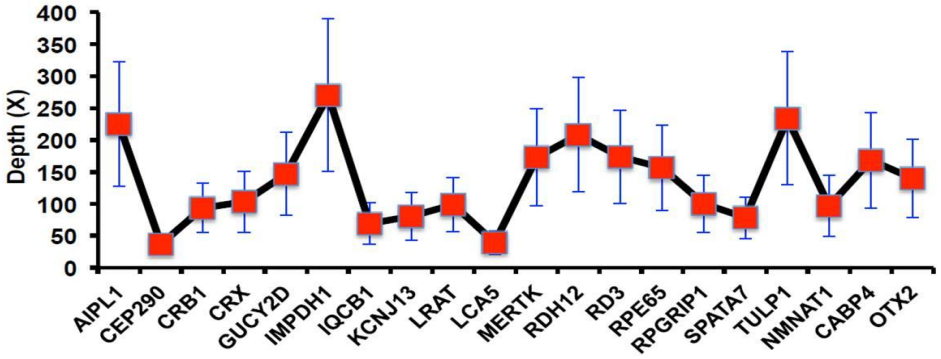


Figure 24: Average read depth graph for all 20 genes

III. **Variant calling**

The variants were predicted using Samtools program. An average of 78variants was identified in each sample for the 20 selected genes. The average number of total variants identified for each gene over all samples is shown in **figure 25**. Highest number of variants was observed for *MERTK* gene. Overall, 508 (378 SNPs, 130 Indels) different variants were identified for 20 genes in 92 samples.

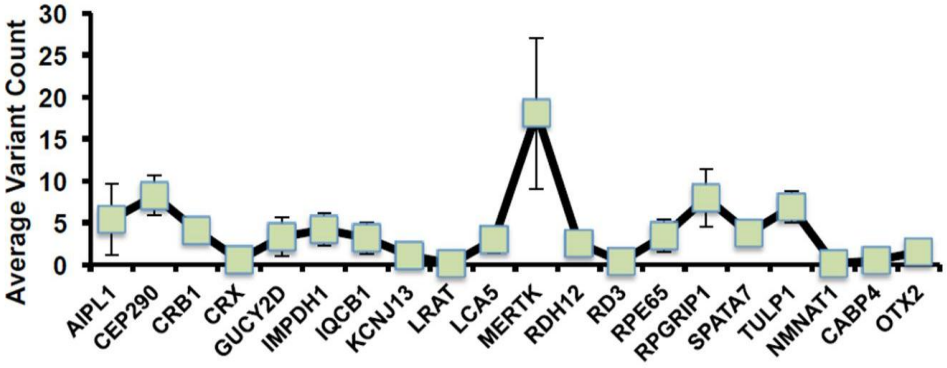


Figure 25: Average number of variants detected for all 20 genes

IV. **Variant Annotation:**

The variants were annotated and compared with various databases including OMIM, ClinVar and SNPedia, Ensemble variation table, Exome server, 1000 genome project data and Human genome mutation database for identifying clinically relevant variants. In total, we identified 126 SNVs and 13 Indels present in coding region of the selected 20 genes in the 92 samples screened. Four out of all 92 samples were positive controls with known mutations (**Table 12**).

Table 12: Shows the list of positive control sample and variants

Sample ID	Gene	Variants
Sample 2	*RPE65*	c.1409C>T (p.Pro470Leu)
Sample 12	*RPE65*	c.1109T>A (p.Leu370His)
Sample 58	*RPE65*	c.362insT (p.Ser121Phefs1290)
Sample 60	*LCA5*	c.955G>A (activates cryptic splice-site)

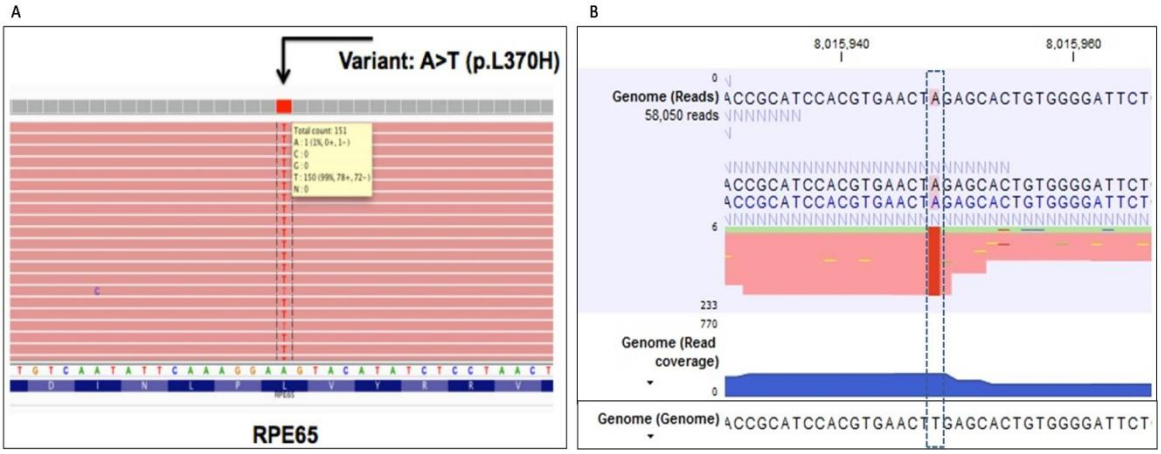


Figure 26: [A] IGV view of c.1109T>A (p.L370H) variation identified in *RPE65* gene of LRS 12 in IGV viewer[156] [B] IGV view of c.3065T>A (p.L1022X) variation identified in *GUCY2D* gene of LRS 1 from CLC genomics workbench (v.8.0.1).

3.1.2. Validation of the identified pathogenic and likely pathogenic mutations:

The single nucleotide variations (SNVs) and indels were further analysed manually as defined in materials and methods (C.IV). The analysis by the mentioned criteria identified fifty-four cases with homozygous pathogenic / likely pathogenic variants including double mutations (2 homozygous mutations in same gene) in two cases. Compound heterozygous pathogenic variants in one case and digenic likely pathogenic variants in another were also identified. (**Table 13**).

Table 13: Lists of the pathogenic variants identified in fifty-six cases. The details of sample ID, gene and its ensemble transcript ID, genomic, cDNA and protein positions of the variants, variant type and reported or novel, CADD scoring, dbSNP ID and LOVD ID are given

S.NO	LRS ID	GENE	TRANSCRIPT ID	MUTATIONS IDENTIFIED		PREDICTED CHANGE IN PROTEIN	MUTATION TYPE	REPORTED/ NOVEL	CADD PHRED LIKE SCORE$^{\phi}$	rs ID (dbSNP147)	LOVD DB-ID
				GENOMIC	cDNA						
1	LRS 37	*GUCY2D*	NM_000180.3/ NP_000171.1	g.7906889 T>G	c.524T>G	p.(Leu175Arg)	Missense	Novel	23.8	rs867412298	*GUCY2D*_ 000013
2	LRS 31	*GUCY2D*	NM_000180.3/ NP_000171.1	g.7907287 C>G	c.839C>G	p.(Thr280Arg)	Missense	Novel	29	rs868557040	*GUCY2D*_ 000016
3	LRS 43	*GUCY2D*	NM_000180.3/ NP_000171.1	g.7915502 G>A	c.1790 G>A	p.(Gly597Glu)	Missense	Novel	27.7	rs867266072	*GUCY2D*_ 000014
4	LRS 32	*GUCY2D*	NM_000180.3/ NP_000171.1	g.7915789 C>T	c.1978 C>T	p.(Arg660*)	Nonsense	Reported	42	rs61750161	***
5	LRS 83	*GUCY2D*	NM_000180.3/ NP_000171.1	g.7915789 C>T	c.1978 C>T	p.(Arg660*)	Nonsense	Reported	42	rs61750161	***
6	LRS 55	*GUCY2D*	NM_000180.3/ NP_000171.1	g.7915873 G>A	c.2062 G>A	p.(Gly688Arg)	Missense	Reported	18.42	rs144291605	***
				g.7915474 C>T	c.1762 C>T	p.(Arg588Trp)	Missense	Reported	35	CM078223 (HGMD)	***
7	LRS 74	*GUCY2D*	NM_000180.3/ NP_000171.1	g.7916489 G>A	c.2182 G>A	p.(Asp728Asn)	Missense	Reported	29.8	rs766646217	*GUCY2D*_ 000017
8	LRS 54	*GUCY2D*	NM_000180.3/ NP_000171.1	g.7918263 delG	c.2663 delG	p.(Gly888Alafs*8)	Frameshift	Novel	27.2	rs868328481	*GUCY2D*_ 000020
9	LRS 39	*GUCY2D*	NM_000180.3/ NP_000171.1	g.7918761 delC	c.2885 delC	p.(Thr962Ilefs*16)	Frameshift	Novel	32	rs867821640	*GUCY2D*_ 000011
10	LRS 51	*GUCY2D*	NM_000180.3/ NP_000171.1	g.7919153 G>A	c.3037 G>A	p.(Gly1013Arg)	Missense	Reported	34	rs868612148	*GUCY2D*_ 000015

11	LRS 1	*GUCY2D*	NM_000180.3/ NP_000171.1	g.7919266 T>A	c.3065 T>A	p.(Leu1022*)	Nonsense	Novel	36	rs866511152	*GUCY2D*_ 000012
12	LRS 14[#]	*GUCY2D*	NM_000180.3/ NP_000171.1	g.7919319 C>G	c.3118 C>G	p.(Arg1040Gly)	Missense	Reported	29.9	rs61750194	***
				g.7906524 delG	c.159delG	p.(Phe54Ser fs*31)	Frameshift	Novel	26.1	rs868091683	*GUCY2D*_ 000018
13	LRS 48	*AIPL1*	NM_014336.4/ NP_055151.3	g.6337268 G>A	c.247G>A	p.(Glu83Lys)	Missense	Reported	31	rs267605009	*AIPL1*_ 000014
14	LRS 4	*AIPL1*	NM_014336.4/ NP_055151.3	g.6330030 A>G	c.689A>G	p.(Asn230Ser)	Missense	Novel	12.96	rs866980715	*AIPL1*_ 000008
15	LRS 9	*AIPL1*	NM_014336.4/ NP_055151.3	g.6329101 G>A	c.834G>A	p.(Trp278*)	Nonsense	Reported	38	rs62637014	*AIPL1*_ 000010
16	LRS 71	*AIPL1*	NM_014336.4/ NP_055151.3	g.6329101 G>A	c.834G>A	p.(Trp278*)	Nonsense	Reported	38	rs62637014	*AIPL1*_ 000010
17	LRS 13	*AIPL1*	NM_014336.4/ NP_055151.3	g.6329091 G>T	c.844G>T	p.(Glu282*)	Nonsense	Novel	39	rs779454542	*AIPL1*_ 000009
18	LRS 22	*AIPL1*	NM_014336.4/ NP_055151.3	g.6329091 G>T	c.844G>T	p.(Glu282*)	Nonsense	Novel	39	rs779454542	*AIPL1*_ 000009
19	LRS 81	*AIPL1*	NM_014336.4/ NP_055151.3	g.6329025 G>T	c.910G>T	p.(Glu304*)	Nonsense	Reported	42	rs746116735	*AIPL1*_ 000006
20	LRS 25	*RPE65*	NM_000329.2/ NP_000320.1	g.68914352_ 68914355 delTTTG	c.46_49 delTTTG	p.(Phe16Lys fs*14)	Frameshift	Novel	35	rs866809120	*RPE65*_ 000009
21	LRS 28	*RPE65*	NM_000329.2/ NP_000320.1	g.68910458 G>T	c.353+1 G>T	*r.spl?*	Splice-site	Reported	26.4	rs61752876	***
22	LRS 23	*RPE65*	NM_000329.2/ NP_000320.1	g.68910347 insA	c.361dupT	p.(Ser121Phe fs*10)	Frameshift	Reported	35	rs121918844	*RPE65*_ 000073
23	LRS 58	*RPE65*	NM_000329.2/ NP_000320.1	g.68910347 insA	c.361dupT	p.(Ser121Phe fs*10)	Frameshift	Reported	35	rs121918844	*RPE65*_ 000073

24	LRS 12	*RPE65*	NM_000329.2/ NP_000320.1	g.68903889 T>A	c.1109 T>A	p.(Leu370His)	Missense	Reported	29.6	rs776250699	***
25	LRS 2	*RPE65*	NM_000329.2/ NP_000320.1	g.68896789 C>T	c.1409 C>T	p.(Pro470Leu)	Missense	Reported	26.2	***	*RPE65_* 000128
26	LRS 86	*RPE65*	NM_000329.2/ NP_000320.1	g.68895547 T>G	c.1514 T>G	p.(Leu505Arg)	Missense	Novel	29.8	rs868473289	*RPE65_* 000012
				g.68904660 T>G	c.963T>G	p.(Asn321Lys)	Missense	Reported	4.096	rs149916178	*RPE65_* 000013
27	LRS 47	*RPGRIP1*	NM_020366.3/ NP_065099.3	g.21775984 G>T	c.895G>T	p.(Glu299*)	Nonsense	Novel	39	***	***
28	LRS 65	*RPGRIP1*	NM_020366.3/ NP_065099.3	g.21793055 C>T	c.2041 C>T	p.(Gln681*)	Nonsense	Novel	37	rs868548381	*RPGRIP1_* 000010
29	LRS 45	*RPGRIP1*	NM_020366.3/ NP_065099.3	g.21793055 C>T	c.2041 C>T	p.(Gln681*)	Nonsense	Novel	37	rs868548381	*RPGRIP1_* 000010
30	LRS 15	*RPGRIP1*	NM_020366.3/ NP_065099.3	g.21811289 delA	c.3434 delA	p.(Glu1145Glyfs*18)	Frameshift	Novel	34	rs866944524	*RPGRIP1_* 000009
31	LRS 26	*RPGRIP1*	NM_020366.3/ NP_065099.3	g.21819302 T>C	c.3788 T>C	p.(Leu1263Pro)	Missense	Novel	25.7	rs866154508	*RPGRIP1_* 000011
32	LRS 87	*LCA5*	NM_181714.3/ NP_859065.2	g.80203350 C>T	c.838C>T	p.(Arg280*)	Nonsense	Reported	35	rs866395428	*LCA5_* 000041
33	LRS 60	*LCA5*	NM_181714.3/ NP_859065.2	g.80202268 G>A	c.955G>A	p.(Ala319Thr)	Missense	Reported	23.3	CS081931	*LCA5_* 000008
34	LRS 20	*LCA5*	NM_181714.3/ NP_859065.2	g.80201335_80201341 delGTTTTCG	c.1062_1068 delCGAAAAC	p.(Tyr354*)	Frameshift	Novel	35	***	***

35	LRS 30	*LCA5*	NM_181714.3/ NP_859065.2	g.80201335_ 80201341 delGTTTTCG	c.1062_ 1068 delCGAA AAC	p.(Tyr354*)	Frameshift	Novel	35	***	***
36	LRS 21	*LCA5*	NM_181714.3/ NP_859065.2	g.80197393 delT	c.1422 delT	p.(Ile474Met fs*11)	Frameshift	Novel	23.2	rs866833585	*LCA5_* 000040
37	LRS 10	*IQCB1*	NM_00102357 0.3/ NP_00101886 4.2	g.121509055 G>A	c.994C>T	p.(Arg332*)	Nonsense	Reported	36	CM050645 (HGMD)	***
38	LRS 92	*IQCB1*	NM_00102357 0.3/ NP_00101886 4.2	g.121500667 G>A	c.1333 C>T	p.(Arg445*)	Nonsense	Novel	38	rs867772426	*IQCB1_* 000004
39	LRS 77	*IQCB1*	NM_00102357 0.3/ NP_00101886 4.2	g.121500637 G>A	c.1363 C>T	p.(Arg455*)	Nonsense	Reported	37	rs866982675	***
40	LRS 79	*IQCB1*	NM_00102357 0.3/ NP_00101886 4.2	g.121491413 G>A	c.1558 C>T	p.(Gln520*)	Nonsense	Reported	40	rs779858591	*IQCB1_* 000006
41	LRS 88	*CRB1*	NM_201253.2/ NP_957705.1	g.197326045 _1973260 insTGAG	c.1073ins TGAG	p.(Ser359Glu fs*20)	Frameshift	Novel	26.6	rs867681420	*CRB1_* 000040
42	LRS 56	*CRB1*	NM_201253.2/ NP_957705.1	g.197411423 G>A	c.4005+1 G>A	*r.spl?*	Splice site	Reported	28.6	***	*CRB1_* 000092
43	LRS 7	*CRB1*	NM_201253.2/ NP_957705.1	g.197446956 C>T	c.4168 C>T	p.(Arg1390*)	Nonsense	Reported	42	rs763324776	*CRB1_* 000045
44	LRS 78	*CRB1*	NM_201253.2/ NP_957705.1	g.197446956 C>T	c.4168 C>T	p.(Arg1390*)	Nonsense	Reported	42	rs763324776	*CRB1_* 000045

45	LRS 17	*RDH12*	NM_152443.2/ NP_689656.2	g.68191267 C>T	c.146C>T	p.(Thr49Met)	Missense	Reported	34	rs28940314	*RDH12_* 000003
46	LRS 49	*RDH12*	NM_152443.2/ NP_689656.2	g.68191305 C>T	c.184C>T	p.(Arg62*)	Nonsense	Reported	36	rs104894471	IKBKG_ 000009
47	LRS 93	*RDH12*	NM_152443.2/ NP_689656.2	g.68195995 G>T	c.746G>T	p.(Arg249Leu)	Missense	Novel	19.61	rs868385141	*RDH12_* 000010
48	LRS 11	*SPATA7*	NM_018418.4/ NP_060888.2	g.88852180 A>G	c.18A>G	r.(spl?)/p.(Arg6=)	Splice-site/ Silent	Novel	18.54	***	***
49	LRS 67	*SPATA7*	NM_018418.4/ NP_060888.2	g.88895690 A>G	c.913-2 A>G	r.913_919del insac/ p.(Ala305Thrfs*6)	Splice-site	Reported	23.2	***	*SPATA7_* 000003
50	LRS 29	*SPATA7*	NM_018418.4/ NP_060888.2	g.88903946 G>A	c.1215+5 C>A	r.1161_1215del/ p.(Phe388Lysfs*10)	Splice-site	Novel	13.55	***	***
51	LRS 8	*NMNAT1*	NM_022787.3/ NP_073624.2	g.10032184 A>G	c.53A>G	p.(Asn18Ser)	Missense	Reported	15.16	rs748902766	*NMNAT1_* 000003
52	LRS 62	*NMNAT1*	NM_022787.3/ NP_073624.2	g.10032240 G>A	c.109G>A	p.(Gly37Arg)	Missense	Novel	23.5	rs865992941	*NMNAT1_* 000012
53	LRS 68	*CRX*	NM_000554.5/ NP_000545.1	g.48339521 G>A	c.122G>A	p.(Arg41Gln)	Missense	Reported	29.9	rs61748436	***
54	LRS 80	*RD3*	NM_001164688.1/ NP_001158160.1	g.211654461 G>A	c.296+1 G>A	*r.spl?*	Splice site	Reported	26.4	rs386834260	***
55	LRS 40	*TULP1*	NM_003322.5/ NP_003313.3	g.35473583 T>G	c.1047 T>G	p.(Asn349Lys)	Missense	Reported	26	rs763272975	*TULP1_* 000043
56	LRS 90†	*AIPL1*	NM_014336.4 / NP_055151.3	g.6329101 G>A	c.834G>A	p.(Trp278*)	Nonsense	Reported	38	rs62637014	*AIPL1_* 000010
		KCNJ13	NM_002242.4/ NP_002233.2	g.233633499 C>T	c.485G>A	p.(Arg162Gln)	Missense	Reported	34	rs757304681	*KCNJ13_* 000006

†Indicates **digenic inheritance involving *AIPL1* and *KCNJ13 gene*** #Indicates **compound heterozygous mutation in *GUCY2D* gene.** All variations were observed in homozygous state except *AIPL1* c.834G>A, *KCNJ13* c.485G>A in LRS 90, *GUCY2D* c.3118C>G and *GUCY2D* c.159delG in LRS 14.

***Indicates ID not available.

ϕ Indicates Phred like CADD scoring – Score ≥ 10 is considered deleterious. The higher the CADD score, the more likely that the variant has deleterious effects.

The 53 pathogenic/ likely pathogenic variants include 9 frameshift, 14 nonsense, 24 missense and 6 splice-site mutations (**Figure 27b**). Further 21 of these were novel (8 frameshift, 4 nonsense, 7 missense and 2 splice-site mutations) (**Figure 27c**). These disease-causing variants were observed in fourteen of the twenty candidate genes screened (**Figure 28**).

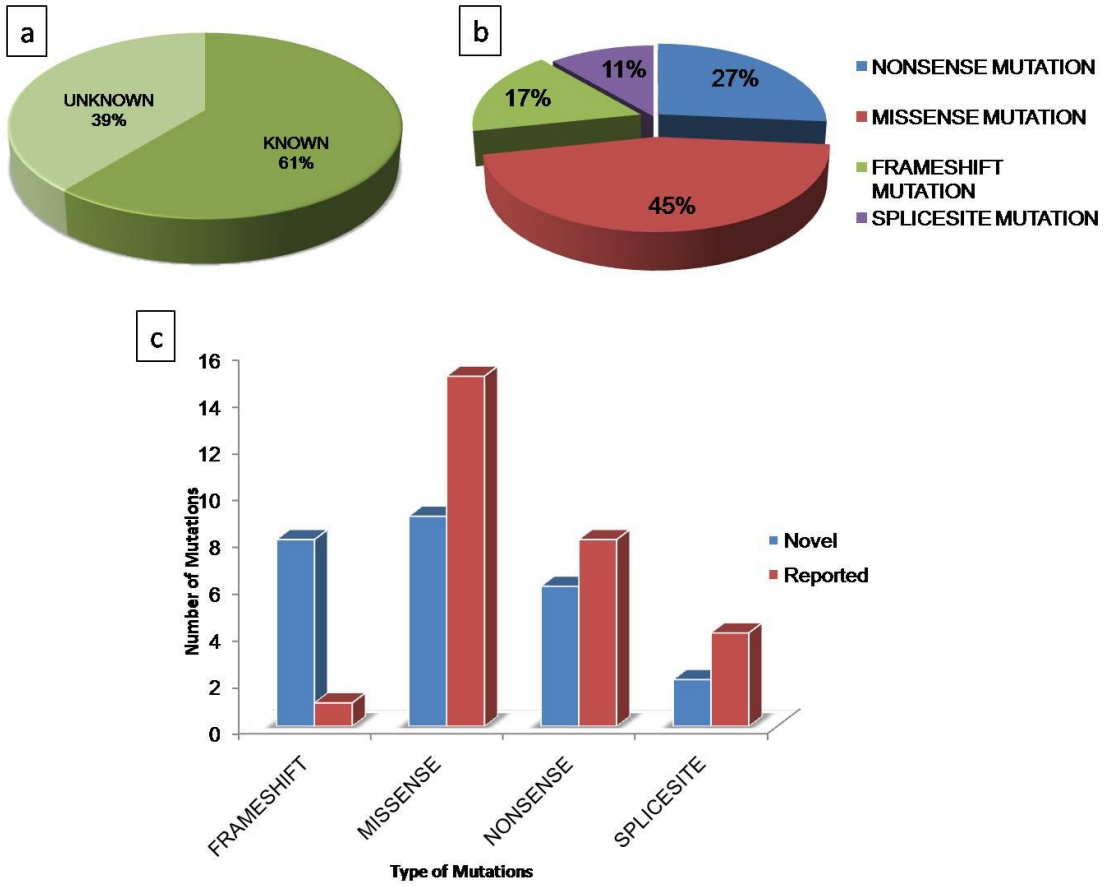


Figure 27: A: Pie chart showing the percentage of the cohort where mutations were identified (including the positive controls) and not identified in the twenty known LCA genes screened. B: Pie chart showing the percentage distribution of different types of mutations among the mutation positive cases. C: Bar diagram showing the distribution of reported and novel mutations among the different types of identified mutations.

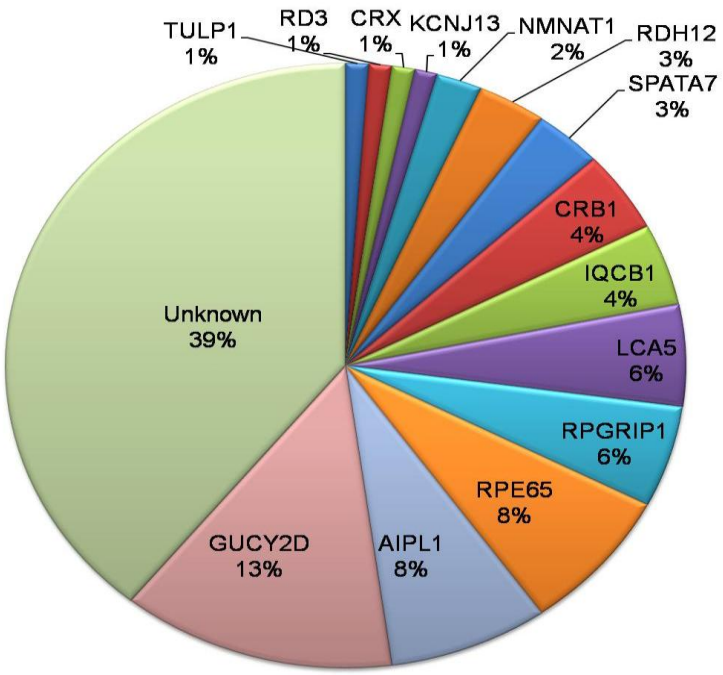
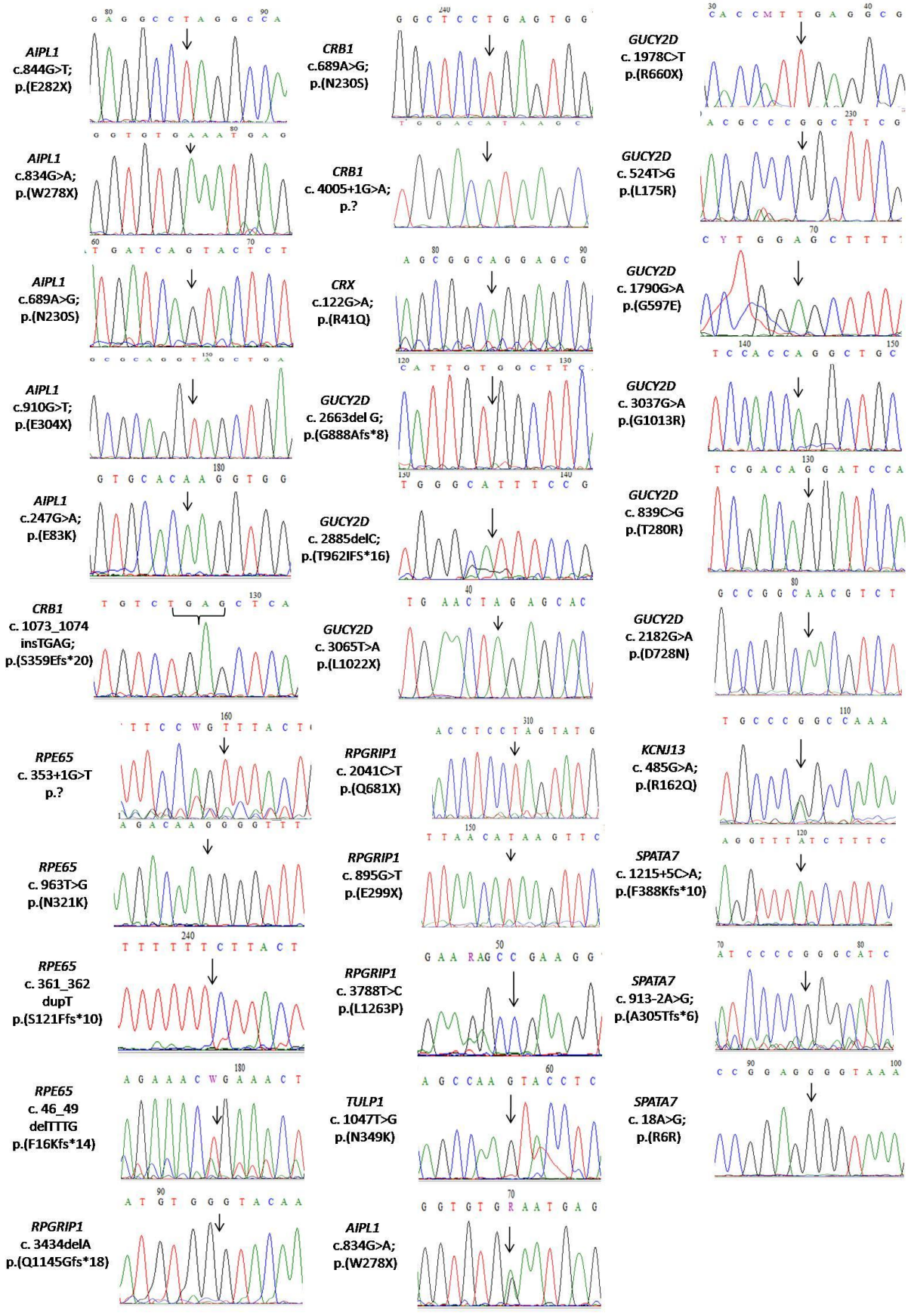


Figure 28: Pie chart showing frequency of mutations in the twenty LCA candidate genes in the Indian cohort studied.

Screening of the unanalysable regions (regions not covered by probes in target enrichment) and *CEP290* intronic variant, c.2991+1655A>G by PCR direct sequencing in the negative cases revealed no disease-causing variants in these regions.

- **Validation of the identified mutations:**

All identified pathogenic and likely pathogenic variants were validated (**Figure 29**) and segregated in the families.


AIPL1
c.844G>T;
p.(E282X)
AIPL1
c.834G>A;
p.(W278X)
AIPL1
c.689A>G;
p.(N230S)
AIPL1
c.910G>T;
p.(E304X)
AIPL1
c.247G>A;
p.(E83K)
CRB1
c. 1073_1074
insTGAG;
p.(S359Efs*20)
RPE65
c. 353+1G>T
p.?
RPE65
c. 963T>G
p.(N321K)
RPE65
c. 361_362
dupT
p.(S121Ffs*10)
RPE65
c. 46_49
delTTTG
p.(F16Kfs*14)
RPGRIP1
c. 3434delA
p.(Q1145Gfs*18)
CRB1
c.689A>G;
p.(N230S)
CRB1
c. 4005+1G>A;
p.?
CRX
c.122G>A;
p.(R41Q)
GUCY2D
c. 2663del G;
p.(G888Afs*8)
GUCY2D
c. 2885delC;
p.(T962IFS*16)
GUCY2D
c. 3065T>A
p.(L1022X)
RPGRIP1
c. 2041C>T
p.(Q681X)
RPGRIP1
c. 895G>T
p.(E299X)
RPGRIP1
c. 3788T>C
p.(L1263P)
TULP1
c. 1047T>G
p.(N349K)
AIPL1
c.834G>A;
p.(W278X)
GUCY2D
c. 1978C>T
p.(R660X)
GUCY2D
c. 524T>G
p.(L175R)
GUCY2D
c. 1790G>A
p.(G597E)
GUCY2D
c. 3037G>A
p.(G1013R)
GUCY2D
c. 839C>G
p.(T280R)
GUCY2D
c. 2182G>A
p.(D728N)
KCNJ13
c. 485G>A;
p.(R162Q)
SPATA7
c. 1215+5C>A;
p.(F388Kfs*10)
SPATA7
c. 913-2A>G;
p.(A305Tfs*6)
SPATA7
c. 18A>G;
p.(R6R)

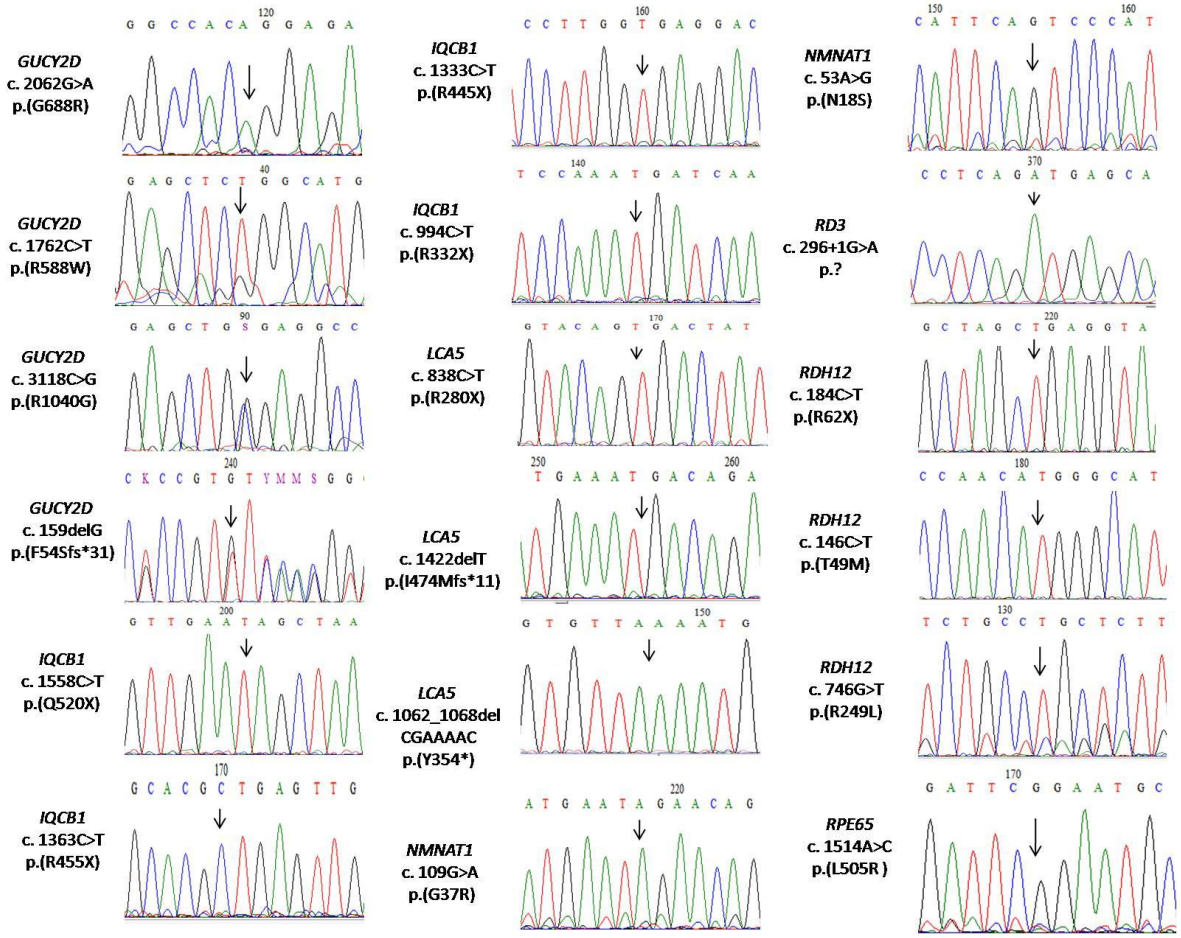


Figure 29: Electrophoretogram of the identified mutations (marked by arrows) in the LCA probands.

The parents were heterozygous carriers and the unaffected sibs were either heterozygous carriers or homozygous wild type **(Figure 30).** Of the 92 samples, ten probands; LRS 7, LRS 12, LRS 20, LRS 28, LRS 29, LRS 30, LRS 31, LRS 32, LRS 40 and LRS 90 had affected sibs segregating the same homozygous pathogenic variant. Control screening of 240 chromosomes by either direct sequencing or ASPCR for the identified pathogenic and likely pathogenicvariantsrevealed wild type sequence for all. The identified novel variants were submitted to LOVD (http://www.lovd.nl/3.0/home) and dbSNP147.

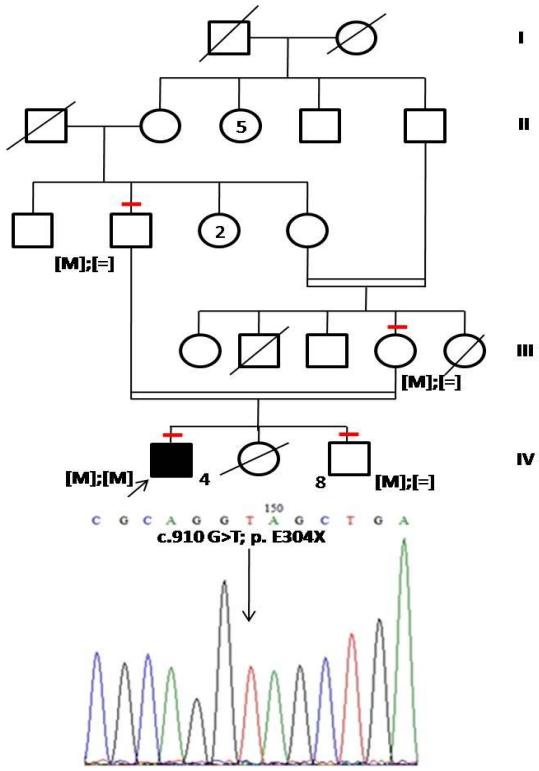


Figure 30: Representative pedigree of the family LCARS-81, segregating a homozygous nonsense mutation, c.910G>T p. (E340X) in *AIPL1* gene.

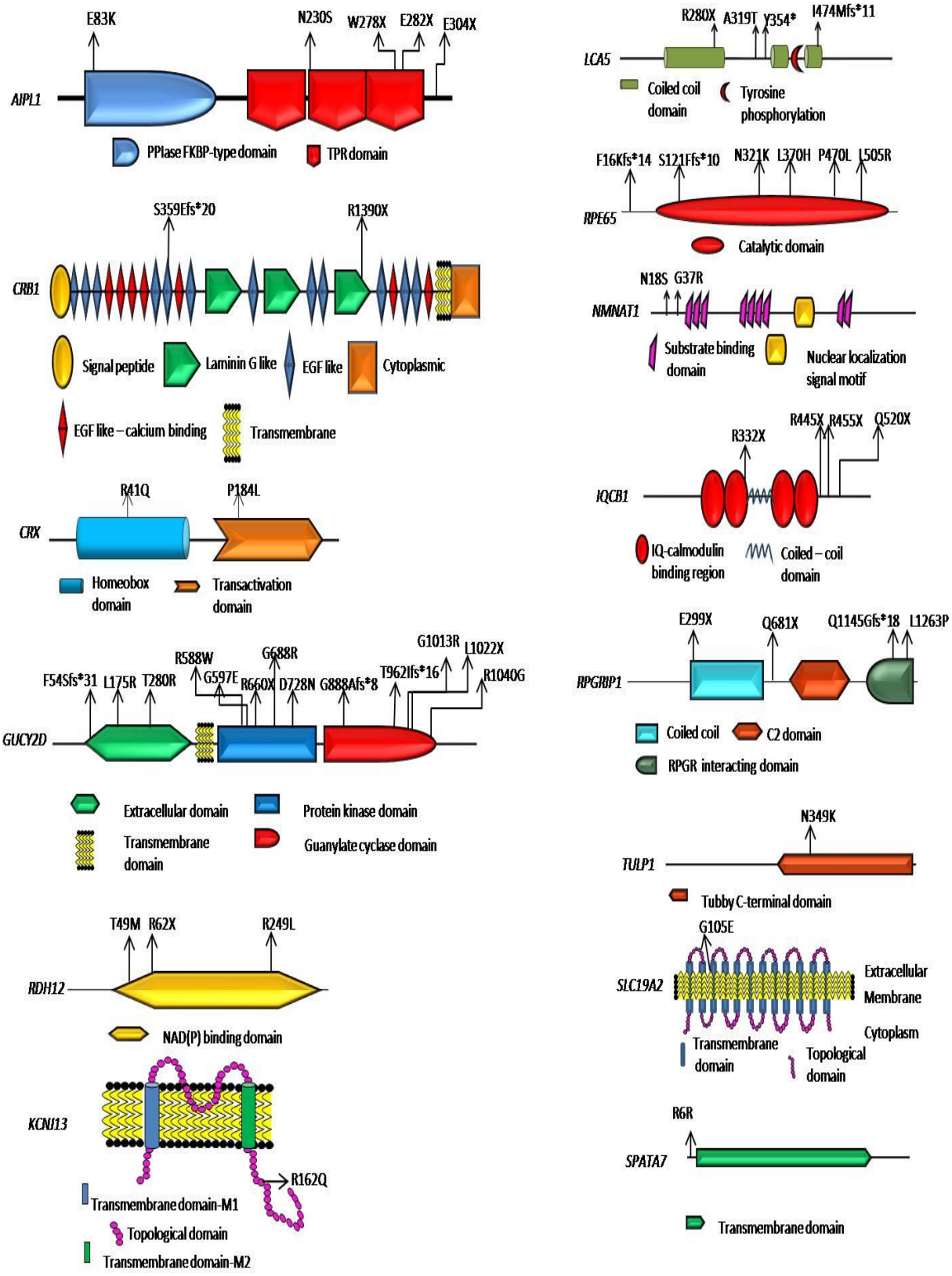


Figure 31: Putative protein structure showing domains and mutations (marked by arrows) in proteins of LCA candidate genes identified by targeted resequencing.

- **Possible Digenic Inheritance**

We observed heterozygous mutations in two different genes in one of our case, LRS 90. The proband had heterozygous variant in *AIPL1,* c.834G>A; p.(Trp278*) and *KCNJ13,* c.485G>A; p.(Arg162Gln). The affected sib also inherited the same variants. On segregation, the mother was found to be heterozygous for *AIPL1* c.834G>A; p. (Trp278*) and the father for *KCNJ13* c.485G>A; p.(Arg162Gln). The parents were found to be wild type for the other alternate allele thus confirming the digenic status **(Figure 32).** Both variants were absent in the controls screened. The *AIPL1* c.834G>A; p.(Trp278*) (rs62637014) variant has been shown to be pathogenic in many study population[157].

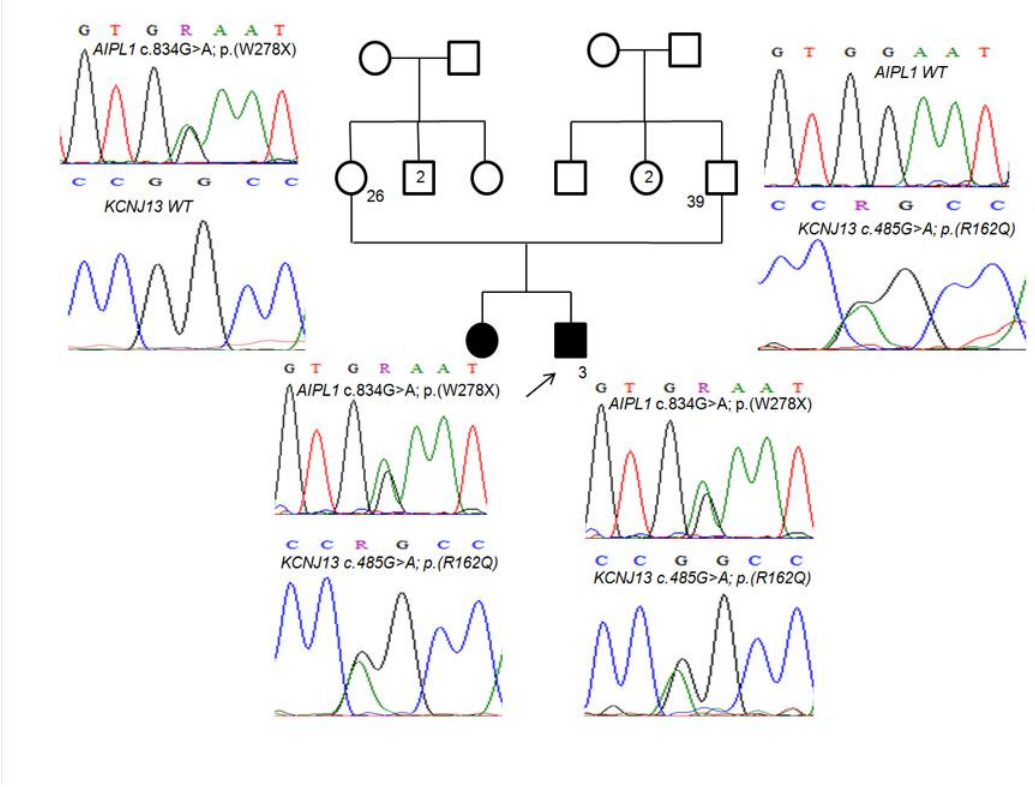


Figure 32: Pedigree of LRS 90 showing digenic inheritance segregating in the family. Proband and affected sib are heterozygous for both *AIPL1* c.834G>A; p.(Trp278*) and *KCNJ13* c.485G>A; p.(Arg162Gln) mutations. The father and the mother are heterozygous for *KCNJ13* and *AIPL1* mutations, respectively.

3.1.3. Other reported or novel variants and SNPs in coding and non–coding regions

- **Triallelic variants**

Among mutation positive cases, 12 cases with identified pathogenic mutations in the coding regions also had a reported heterozygous variant in another gene with MAF ≤ 0.005. Seven different variants were observed and three of these heterozygous variants are reported as mutations either in homozygous or compound heterozygous state. Segregation of above variants showed heterozygosity in either or both parents. These could contribute to triallelism, modifying the disease severity (**Table 14**).

- **Novel heterozygous variants:**

Novel heterozygous variants with no other mutation/ variant were observed in three cases in this study. The variants are *MERTK*- c.1627T>C, p. (Ser543Pro) in LRS51; *RPE65*- c.814C>T, p. (Gly272Arg) in LRS24; *RPGRIP1*- c.1480delA, p. (Asn495Thrfs*11) in LRS89. The possibility of these novel variants being pathogenic and hence representing a carrier state in respective individuals or being a novel SNP can be defined only when the population frequency of these variants are known. The frequency of these variants in Indian cohort is unavailable. However, the regulatory regions of the genes have not been screened and possibility of any variant in these regions cannot be ruled out.

We have also observed one novel homozygous and thirteen novel heterozygous synonymous variants; thirty-five novel homozygous and ninety novel heterozygous intronic variants in this cohort.

Table 14: Lists the heterozygous variants observed in mutation positive cases possibly contributing to triallelism

S.NO	LRS ID	IDENTIFIED PATHOGENIC VARIANTS		OBSERVED HETEROZYGOUS VARIANTS				
		GENE	PATHOGENIC VARIANTS	GENE	VARIANTS	NOVEL / REPORTED	CADD	MAF (ExAc)
1	LRS 21	*LCA5*	g.80197392delT c.1422delA p.(Ile474Metfs*11) (rs866833585)	*AIPL1*	g.6331702 A>T c.401A>T p.(Tyr134Phe)	Reported (rs16955851)	24.4	0.004
2	LRS 58	*RPE65*	g.68910347insT c.362dupT p.(Ser121Phefs*10) (rs121918844)	*CABP4*	g.67223001 C>T c.107C>T p.(Pro36Leu)	Reported (rs766418692)	8.996	0.00003808
3	LRS 28	*RPE65*	g.68910458G>T c.353+1G>T *r.(spl?)* (rs61752876)	*MERTK*	g.112786031 G>T c.2590G>T p.(Val864Phe)	Reported (rs557004700)	10.59	0.0004366
4	LRS 15	*RPGRIP1*	g.21811289delA c.3434delA p.(Glu1145Glyfs*18) (rs866944524)	*MERTK*	g.112786031 G>T c.2590G>T p.(Val864Phe)	Reported (rs557004700)	10.59	0.0004366

5	LRS 68	*CRX*	g.48339521G>A c.122G>A p.(Arg41Gln) (rs61748436)	*MERTK*	g.112786031 G>T c.2590G>T p.(Val864Phe)	Reported (rs557004700)	10.59	0.0004366
6	LRS 40	*TULP1*	g.35473583T>G c.1047T>G p.(Asn349Lys) (rs763272975)	*MERTK*	g.112777002 C>A c.2092C>A p.(Gln698Lys)	Reported (rs761295704)	28.1	0.00006589
7	LRS 78	*CRB1*	g.197446956C>T c.4168C>T p.(Arg1390*) (rs763324776)	*RPE65*	g.68904660 T>G c.963T>G p.(Asn321Lys)	Reported (rs149916178)	4.096	0.005362
8	LRS 80	*RD3*	g.211654461G>A c.296+1G>A *r.(spl?)* (rs386834260)	*RPE65*	g.68904660 T>G c.963T>G p.(Asn321Lys)	Reported (rs149916178)	4.096	0.005362
9	LRS 88	*CRB1*	g.197326045insTGAG c.1073_1074insTGAG p.(Ser359Glufs*20) (rs867681420)	*RPE65*	g.68904660 T>G c.963T>G p.(Asn321Lys)	Reported (rs149916178)	4.096	0.005362

10	LRS 53	*AIPL1*	g.6337268G>A c.247C>T p.(Glu83Lys) (rs267605009)	*MERTK*	g.112732891 A>G c.986A>G p.(Asn329Ser)	Reported (rs34943572)	22.2	0.0002553
11	LRS 8	*NMNAT1*	g.10032184A>G c.53A>G p.(Asn18Ser) (rs748902766)	*RPE65*	g.68904660 T>G c.963T>G p.(Asn321Lys)	Reported (rs149916178)	4.096	0.005362
12	LRS 14	*GUCY2D*	g.7919319C>G c.3118C>G p.(Arg1040Gly) (rs61750194) g.7906524delG c.159delG p.(Phe54Serfs*31) (rs868091683)	*CRB1*	g.197326047 T>G c.1075T>G p.(Ser359Ala)	Reported (rs533227950)	3.021	0.000832

3.2. cDNA analysis

RT-PCR using cDNA was standardized using ARPE-19 cell lines as positive control for *SPATA7* gene expression. The same RT-PCR reactions were performed using cDNA obtained from peripheral blood of probands and their families, LRS67 (**Figure 33**) and LRS29 (**Figure 34**) to check the effect on splicing for the mutations, c.1215+5C>A and c.913-2A>G, respectively. cDNA primers targeting *GAPDH* was used as an internal control. Two transcripts of *SPATA7* gene were observed on 3% agarose gel electrophoresis. Gel extraction was performed followed by sequencing of the extracted product.

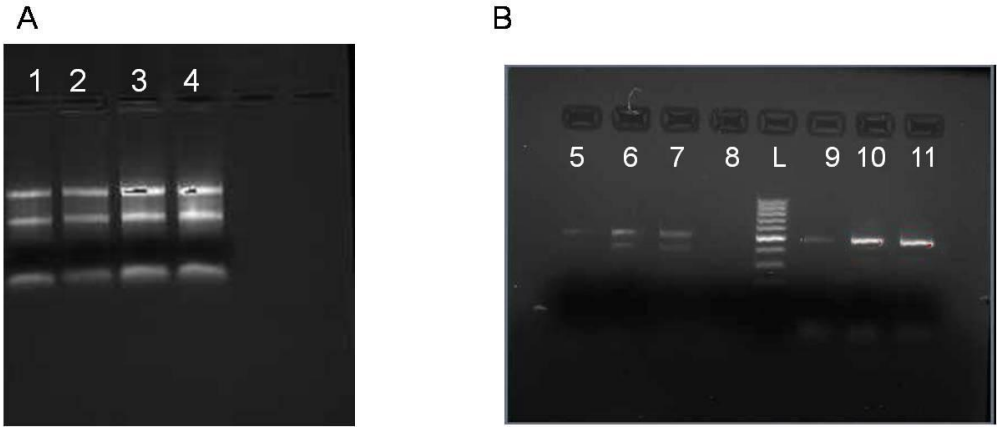


Figure 33: In family LRS 67 [A] 0.7% agarose gel picture showing RNA in Lane 1-4: 1. Proband 2.Sib. 3.Father 4.Mother [B] 2% agarose gel picture showing cDNA amplified products of 525bp size targeting *SPATA7*- c.913-2A>G optimized at 63-56(-0.5°C) touchdown protocol Lane 5-8: 5.Proband 6.Father 7.Sib 8. NC. Lane 9-11: GAPDH amplification of the corresponding cDNA 9. Proband 10. Father 11. Sib.

The bidirectional Sanger sequencing in family LRS 67 revealed an indel, where the first 7 bases of exon 8 were deleted and 2 bases inserted resulting in predicted truncated protein p.(Ala305Thrfs*6) (**Figure 35**). The parents were heterozygous for the deletions. This confirms the c.913-2A>G mutation in the canonical splice-site in intron 7 affects splicing. In family LRS 29, the presence of variant c.1215+5C>A in the intron 10 resulted in the deletion of exon 10, leading

to predicted truncated protein p. (Phe388Lysfs*10) (**Figure 36**). The parents were heterozygous for the deletion.

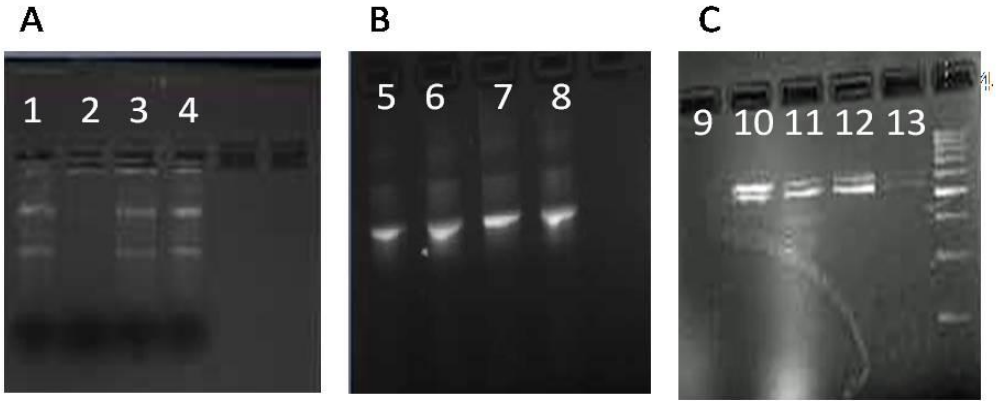


Figure 34: In family LRS 29 [A] 0.7% agarose gel picture showing RNA in Lane 1-4: 1. Proband 2.Father 3.Mother 4.Sib [B] 2% agarose gel picture showing *GAPDH* amplification of the corresponding cDNA Lane 5-8: 5. Proband 6.Father 7.Mother 8.Sib [C] cDNA amplified products of 596bp size targeting *SPATA7*-c.1215+5C>A optimized at 65-58(-0.5°C) touchdown protocol. Lane 9-13: 9.NC 10.Proband 11.Father 12.Mother 13.Sib

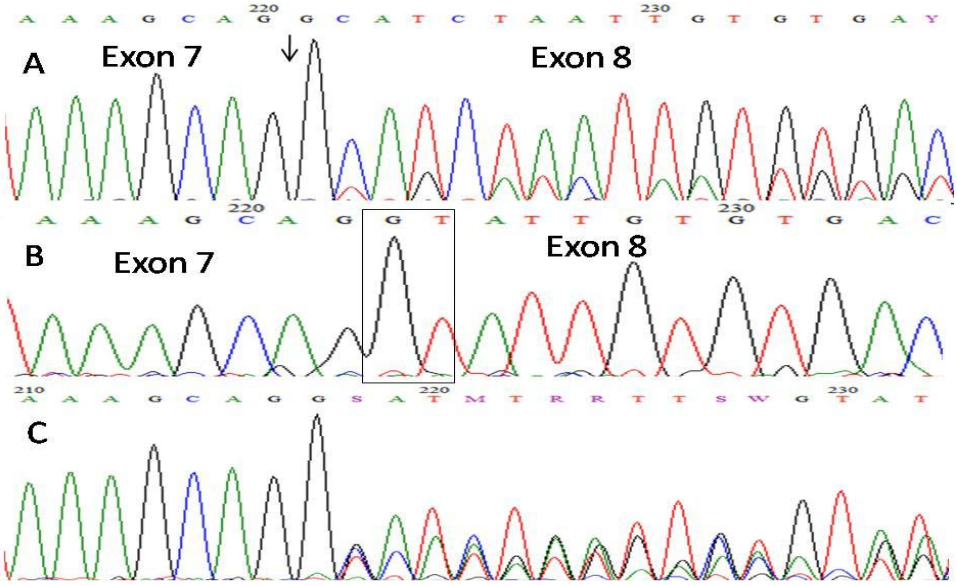


Figure 35: Electrophoretogram of cDNA analysis for splice variants in *SPATA7* gene. [A] Wild type cDNA sequence (forward) in control showing exon 7 and exon 8 [B] homozygous indel (insertion of GT and first 7 bases deletion in exon 8) in LRS 67 due to splice mutation c. 913-2A>G [C] heterozygous indel in parent of LRS 67 .

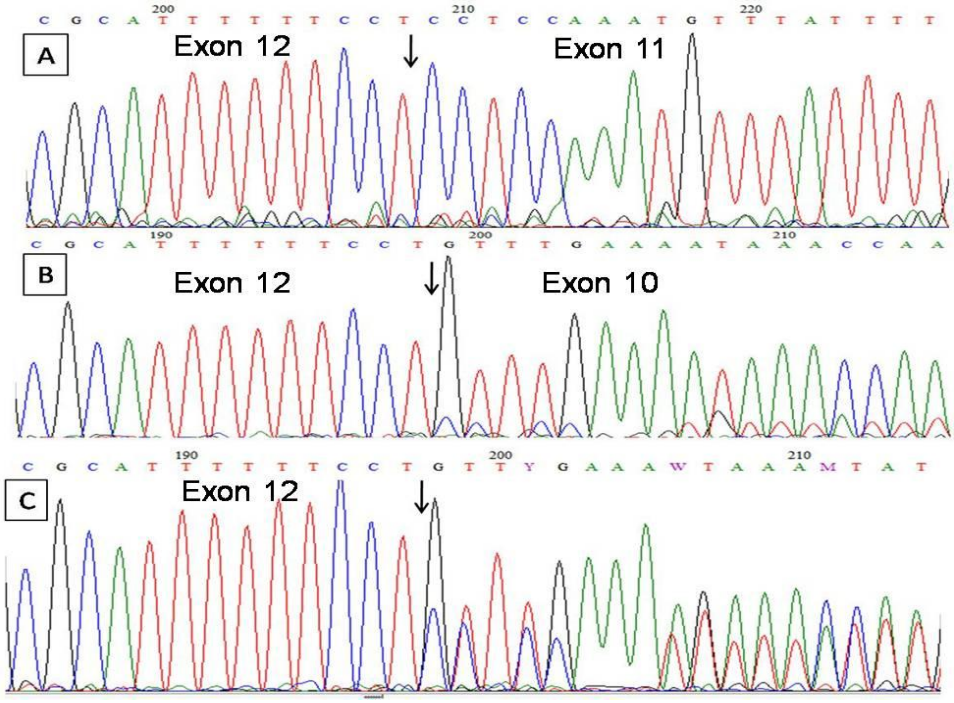


Figure 36: *SPATA7*- c.1215+5C>A electrophoretogram showing representative reverse primer sequence of [A] Wild type control cDNA [B] Homozygous deletion of exon 10 in proband [C] Heterozygous deletion of exon 10 in parent.

The cDNA analyses for the synonymous variantc.18A>G; p.(Arg6Arg), in the penultimate base of exon 1 in *SPATA7,* predicted to alter splicing, identified in family LRS 11*,* couldn't be performed as the family was unavailable for recollection and analysis.

3.3. Bioinformatics Analyses

Apart from CADD scoring, six online bioinformatics tools were used for predicting the effect of the identified missense variants. Of the 24 missense variants, 12 were predicted to be damaging by 5/6 tools and 9 by more than 3/6 tools **(Table 15)**. **Table 13** and **table 14** include Phred-like CADD scores for all the identified variants.

Table 15: Bioinformatic analyses of missense variants

S.NO	SAMPLE ID	GENE	cDNA	PREDICTED CHANGE IN PROTEIN	SIFT	POLYPHEN	Mutation taster	Pmut	Variant effect predictor	Mutation accessor
1	Sample 4	*AIPL1*	c.689A>G	Asn230Ser	Damaging	Benign	Disease causing	Neutral	moderate	low
2	Sample 53	*AIPL1*	c.247G>A	Glu83Lys	Damaging	Probably damaging	Disease causing	Pathological	moderate	medium
3	Sample 29	*CEP290*	c.5237G>A	Arg1746Gln	Tolerated	Benign	Disease causing	Neutral	moderate	neutral
4	Sample 68	*CRX*	c.122G>A	Arg41Gln	Damaging	Probably damaging	Disease causing	Pathological	moderate	medium
5	Sample 11	*CRX*	c.551C>T	Pro184Leu	Tolerated	Benign	Polymorphism	Pathological	moderate	neutral
6	Sample 37	*GUCY2D*	c.524T>G	Leu175Arg	Damaging	Possibly damaging	Polymorphism	Neutral	moderate	Low
7	Sample 43	*GUCY2D*	c.1790G>A	Gly597Glu	Damaging	Probably damaging	Disease causing	Neutral	moderate	medium
8	Sample 46	*GUCY2D*	c.3037G>A	Gly1013Arg	Damaging	Probably damaging	Disease causing	Pathological	moderate	high
9	Sample 31	*GUCY2D*	c.839C>G	Thr280Arg	Damaging	Probably damaging	Disease causing	Pathological	moderate	medium
10	Sample 74	*GUCY2D*	c.2182G>A	Asp728Asn	Damaging	Probably damaging	Disease causing	Neutral	moderate	high
11	Sample 55	*GUCY2D*	c.2062G>A	Gly688Arg	Tolerated	Benign	Disease causing	Pathological	moderate	neutral
12	Sample 55	*GUCY2D*	c.1762C>T	Arg588Trp	Damaging	Probably damaging	Disease causing	Pathological	moderate	high
13	Sample 14	*GUCY2D*	c.3118C>G	Arg1040Gly	Damaging	Probably damaging	Disease causing	Pathological	moderate	high
14	Sample 60	*LCA5*	c.955G>A	Ala319Thr	Damaging	Benign	Polymorphism	Neutral	moderate	Low
15	Sample 62	*NMNAT1*	c.109G>A	Gly37Arg	Tolerated	Possibly damaging	Polymorphism	Pathological	moderate	neutral
16	Sample 8	*NMNAT1*	c.53A>G	Asn18Ser	Tolerated	Probably damaging	Disease causing	Neutral	moderate	Low

S.NO	SAMPLE ID	GENE	CDNA	MUTATION	SIFT	POLYPHEN	Mutation taster	Pmut	Variant effect predictor	Mutation accessor
17	Sample 17	*RDH12*	c.146C>T	Thr49Met	Damaging	Probably damaging	Disease causing	Neutral	moderate	medium
18	Sample 93	*RDH12*	c.746G>T	Arg249Leu	Tolerated	Benign	Polymorphism	Neutral	moderate	neutral
19	Sample 86	*RPE65*	c.1514T>G	Leu505Arg	Damaging	Probably damaging	Disease causing	Pathological	moderate	medium
20	Sample 86	*RPE65*	c.963T>G	Asn321Lys	Tolerated	Benign	Polymorphism	Neutral	moderate	low
21	Sample 2	*RPE65*	c.1409C>T	Pro470Leu	Damaging	Probably damaging	Disease causing	Pathological	moderate	medium
22	Sample 12	*RPE65*	c.1109T>A	Leu370His	Damaging	Probably damaging	Disease causing	Neutral	moderate	medium
23	Sample 26	*RPGRIP1*	c.3788T>C	Leu1263Pro	Damaging	Probably damaging	Disease causing	Pathological	moderate	medium
24	Sample 40	*TULP1*	c.1047T>G	Asn349Lys	Damaging	Probably damaging	Disease causing	Neutral	moderate	medium

All the identified splice-site variants, canonical and in region within 10bp of exon-intron junction were predicted to alter donor or acceptor sites probably affecting splicing. In LRS 11, a variant in the penultimate base of exon 1 of *SPATA7* gene translates to a synonymous change, p.(Arg6Arg). However, the splice prediction tools predicted this variant to cause alteration of the donor splice-site affecting splicing.

3.4. Genotype- phenotype Correlation

Genotype and clinical phenotype correlation (**Figure 37**) was performed for subjects with identified pathogenic and likely pathogenic variants. All these cases had profound loss of vision with only 3/56 patients having a best corrected visual acuity better than 6/60. Five patients (LRS 12, LRS 15, LRS 17, LRS 39, and LRS 40) were myopic and the rest were hyperopic. Extinguished rod and cone response in ERG within first year of life and pendular nystagmus were observed in all cases. Among the mutation positive cases, keratoconus in 7% (4/56), delayed milestone in ~4% (2/56), astigmatism in 16% (9/56), cleft palate, mental retardation, autism, hearing deficit and cataract in ~2% each (1/56) were observed. The clinical data of the mutations positive cases are detailed (**Table 16**)

AIPL1:

Patients with *AIPL1* mutations had optic disc pallor with attenuated vessels. Macular atrophy and yellowish spots in the macular region which corresponded to dark spots in FAF were noted. RPE granularity, metallic sheen and coarse roundish pigments were seen in the periphery.

CRB1:

Patients with *CRB1* mutations presented with mild optic disc pallor, attenuated vessels with para arteriolar sparing of the retinal pigment epithelium (PPRPE) typical of *CRB1* mutation. Macula shows atrophy with surrounding pigmentation. Nummular (roundish coin shaped) pigmentation was observed more in posterior pole than in the periphery.

CRX:

Posterior pole of the patient with *CRX* mutation had pale disc with attenuated vessels. Macular atrophy and pinhead size round shaped pigments at the posterior pole were observed.

GUCY2D:

Phenotype of patients with *GUCY2D* mutations showed mild to nil disc pallor and arterial attenuation. The predominant feature observed was a greyish tapetal reflex and minimal peripheral RPE granularity.

IQCB1:

Patients with *IQCB1* mutations presented with minimal to nil disc pallor and arteriolar attenuation. Mild peripheral granularity and metallic sheen in the fundus was observed.

LCA5:

All patients with *LCA5* mutation showed mild optic disc pallor and mild arteriolar attenuation. The typical changes observed in phenotype were macular atrophy, pinhead sized round pigments and peripheral white spots beyond the arcade which were seen as dark spots on FAF.

NMNAT1:

Patients with *NMNAT1* mutation showed mild optic disc pallor, arteriolar attenuation, macular atrophy with coarse pigment more in the posterior pole and few bony spicules in the periphery.

RD3:

Patient had mild optic disc pallor, arteriolar attenuation and macular atrophy with yellowish pigment. Pinhead sized round pigments more evident as dark spots in FAF were observed with widespread RPE damage.

RDH12:

The fundus phenotype of *RDH12* patients showed optic disc pallor and arteriolar attenuation, macular coloboma and bony spicules like pigmentation seen in both posterior pole and periphery.

RPE65:

These patients showed disc pallor and arterial attenuation. Varied presentations were seen among the patients, with white spots in posterior pole in three patients, metallic sheen in three and macular atrophy in two. LRS 12 (13 yrs) showed yellow macular scaring and coarse pigmentation.

RPGRIP1:

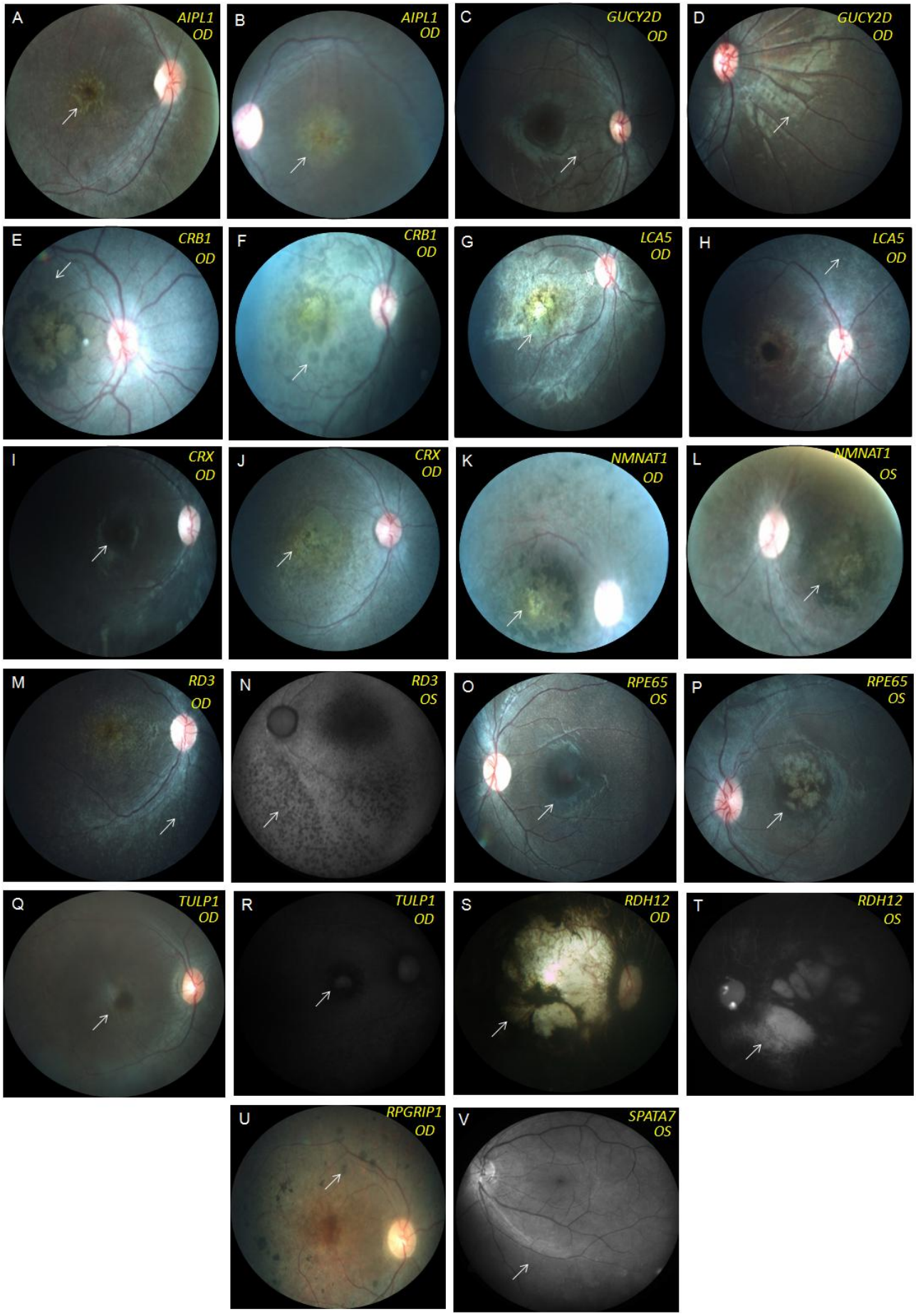
All patients showed mild disc pallor with attenuated vessels. Macular atrophy with coarse coin shaped early bony spicules and RPE mottling was observed in all the patients.

SPATA7:

Patients with mutation in *SPATA7* showed minimal to nil disc pallor and attenuated vessels. Tapetal reflex was observed wide spread on peripheral fundus. One patient also showed presence of peripheral pigment.

TULP1:

Fundus showed optic disc pallor with marked attenuated arterioles. Perifoveal yellow annular ring with macular atrophy and mild peripheral bone spicules pigments were observed.


A
AIPL1
OD
B
AIPL1
OD
C
GUCY2D
OD
D
GUCY2D
OD
E
CRB1
OD
F
CRB1
OD
G
LCA5
OD
H
LCA5
OD
I
CRX
OD
J
CRX
OD
K
NMNAT1
OD
L
NMNAT1
OS
M
RD3
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N
RD3
OS
O
RPE65
OS
P
RPE65
OS
Q
TULP1
OD
R
TULP1
OD
S
RDH12
OD
T
RDH12
OS
U
RPGRIP1
OD
V
SPATA7
OS

Figure 37: Fundus photograph showing Genotype- phenotype correlation in LCA patients with mutation in genes [A,B] *AIPL1* having macular atrophy and yellow pigment in the macula [C,D] *GUCY2D* showing greyish tapetal reflex and arteriolar attenuation [E,F] *CRB1* presenting macular atrophy and nummular pigmentation [G,H] *LCA5* with pinhead sized round pigments and peripheral white spots [I,J] *CRX* showing macular atrophy and pinhead size round pigments [K,L] *NMNAT1* projecting central island of pigmented atrophy [M,N] *RD3* having pinhead sized round pigments seen as dark spots in autofluorescence [O,P] *RPE65* showing metallic sheen and macular atrophy [Q,R] *TULP1* with perifoveal yellow annular ring[S, T] *RDH12* showing macular coloboma like phenotype [U] *RPGRIP1* with coarse coin shaped early bony spicules [V] *SPATA7* presenting tapetal reflex.

Table 16: Shows the clinical features of mutation positive index cases affected by LCA.

S.No	Patient ID	Sex	Age (year)	Affected Gene	Nystagmus	Visual Acuity		Refractive Error						ERG	Fundus	OCT	Others
						OD	OS	OD			OS						
								Sphl	Cyl	axis	Sphl	Cyl	axis				
1	LRS 13	F	5	*AIPL1* c.844G>T	Jerk	PLPR	PLPR	+3.50	-1.75	180	+3.50	-1.50	180	Extinguished	Pale disc, attenuated vessels, Yellowish spots in the macula. Metallic sheen and RPE granules visible	No OCT	**
2	LRS 22	M	5	*AIPL1* c.844G>T	Jerk	follows and fixates light	follows and fixates light	+8.50	+2.00	40	+8.50	+2.50	130	Extinguished	Arteriolar attenuation with disc pallor present, few yellow spots in macula and RPE granules visible	No OCT	**
3	LRS 9	F	11	*AIPL1* c.834G>A	Wandering	HM+1; less than N36	HM+; less than N36	+3.50	**	**	+3.50	**	**	Extinguished	Pale disc, attenuated vessels, yellowish spots in macula, RPE granules and coarse roundish pigments observed	RPE atrophic changes seen in macula	**

4	LRS 71	M	3	*AIPL1* c.834G>A	Pendular	follows and fixates light	follows and fixates light	+5.50	**	**	+5.00	**	**	Extinguished	Disc pallor, mild arteriolar attenuation, Yellowish spots in the macular region and metallic sheen in macula seen	OD-Foveal thinning with thinning of IS-OS junction noted at macula	**
5	LRS 4	M	5	*AIPL1* c.689A>G	Jerk	PLPR; less than N36	PLPR; less than N36	+9.00	-0.50	180	+9.00	-0.50	180	Extinguished	Pale disc, attenuated vessels, yellow spots, and coarse roundish pigments observed in periphery	No OCT	Delayed milestone
6	LRS 81	M	17	*AIPL1* c.910G>T	Jerk	PLPR; less than N36	PLPR; less than N36	+0.75	-2.75	40	+1.00	-2.00	160	Extinguished	Pale disc, attenuated vessels, atrophic yellowish disc shaped macula with coarse pigment granules and metallic sheen	No OCT	OS-keratoconus

7	LRS 48	F	5	*AIPL1* c.247G>A	Wandering	follows and fixates light	follows and fixates light	+5.50	-1.00	180	+6.00	-1.25	180	Extinguished	Mild arteriolar attenuation, disc pallor, yellowish spots, RPE granules and metallic sheen observed.	No OCT	Astigmatism, cleft palate
8	LRS 88	M	3	*CRB1* c.1073_1074 insTGAG	Jerk	Eccentric fixation, follows light	Eccentric fixation, follows light	+6.00	**	**	+2.00	-0.75	180	Extinguished	Pale disc, minimal attenuation of vessels, greyish reflex in the macula, para arteriolar sparing and RPE loss in the periphery	No OCT	Astigmatism
9	LRS 78	F	10	*CRB1* c.4168 C>T	Jerk	PLPR; less than N36	PLPR; less than N36	+12.50	-1.00	180	+13.00	-1.00	180	Extinguished	Pale disc, minimal attenuation of vessels, greyish reflex in the macula and roundish coin shaped pigmentary clumps are seen	Foveal thinning (OD>OS) with RPE atrophy noted. Thin ERM seen	Astigmatism

10	LRS 56	M	13	*CRB1* c.4005+1 G>A	Jerk	PLPR; less than N36	PLPR; less than N36	+8.00	-1.5	180	+8.50	-1.50	180	Extinguished	Pale disc, attenuated vessels, minimal para arteriolar sparing, coarser pigment and atrophic patches in the macula and calcareous yellowish deposits observed	No OCT	**
11	LRS 7	F	2	*CRB1* c.4168 C>T	Jerk	follows and fixates light	follows and fixates light	+6.50	-2.25	155	+7.00	-3.00	180	Extinguished	Pale disc, minimal attenuation of vessels, para arteriolar sparing greyish reflex in the macula and roundish coin shaped pigmentary clumps are seen	Altered foveal contour seen Thinning of IS-OS junction noted, RPE alterations seen	Keratoconus

12	LRS 68	M	9	*CRX* c.122G>A	Jerk	PLPR accurate	PLPR accurate	+2.25	-1.00	180	+2.25	-1.00	180	Extinguished	Pale disc, minimal attenuation of vessels, macular atrophy and pinhead sized pigmentary clumps are seen	Foveal thinning noted with loss of photoreceptor layer and RPE atrophy	Mental retardation, Astigmatism
13	LRS 54	M	5	*GUCY2D* c.2663 delG	Wandering	Does not follow light	Does not follow light	+5.00	-1.00	180	+5.50	-1.25	180	Extinguished	Minimal attenuated vessels with tapetal reflex in macula.	Normal foveal dip seen with RPE alterations	**
14	LRS 39	F	7	*GUCY2D* c.2885 delC	Jerk	CFCF/ less than N36	CFCF/ Less than N36	-1.00	-1.00	10	-1.25	-1.00	170	Extinguished	Mild disc pallor with tapetal reflex and peripheral RPE granules seen	No OCT	Keratoconus
15	LRS 1	F	5	*GUCY2D* c.3065 T>A	Jerk	follows and fixates light	follows and fixates light	+7.00	-2.00	10	+7.00	-2.00	170	Extinguished	Normal disc and vessels, tapetal reflex and pigmentary granules observed in the background	Foveal contour seen with thinned IS-OS junction at the fovea	**

16	LRS 83	F	6	*GUCY2D* c.1978 C>T	Rotatory	PLPR	PLPR	+6.50	**	**	+7.00	**	**	Extinguished	Minimal attenuation of vessels, tapetal reflex in the macula.	No OCT	**
17	LRS 32	M	14	*GUCY2D* c.1978 C>T	Jerk	2/60	3/60	+6.50	**	**	+6.00	**	**	Reduced	Tapetal reflex observed with peripheral RPE granules.	No OCT	**
18	LRS 37	F	11	*GUCY2D* c.524T>G	Wandering	CFCF/ Less than N36	CFCF/ Less than N36	+3.50	**	**	+3.75	**	**	Reduced	Tapetal reflex and peripheral RPE granules observed	No OCT	**
19	LRS 43	M	3	*GUCY2D* c.1790 G>A	Wandering	follows light	follows light	+5.50	**	**	+5.50	**	**	Extinguished	peripheral granularity observed	No OCT	Autism
20	LRS 51	F	7	*GUCY2D* c.3037 G>A	Jerk	6/60; N36	6/60;N36	+3.50	-1.25	180	+3.50	-1.25	180	Extinguished	Tapetal reflex observed in both eyes	No OCT	**
21	LRS 31	F	13	*GUCY2D* c.839C>G	Pendular	1/60 less than N36	1/60 close to N36	+5.00	-0.75	180	+5.50	-1.00	180	Extinguished	Yellowish spots in macula and coarse RPE granules in periphery observed	No OCT	**

22	LRS 74	F	3	*GUCY2D* c.2182 G>A	Wandering	follows and fixates light	follows and fixates light	+3.50	-1.00	180	+3.50	-1.00	180	Extinguished	Minimal attenuation of vessels, tapetal reflex in the macula.	No OCT	**
23	LRS 55	M	6	*GUCY2D* c.2062 G>A c.1762 C>T	Rotatory	follows and fixates light	follows and fixates light	+5.00	-1.5	90	+5.00	-1.50	90	Extinguished	Tapetal reflex observed in both eyes	No OCT	**
24	LRS 14	M	10	*GUCY2D* c.3118 C>G c.159delG	Wandering	occasionally follows light	occasionally follows light	+0.00	**	**	+3.50	**	**	Extinguished	Mild disc pallor and arteriolar attenuation present, tapetal reflex and RPE granules visible	No OCT	**
25	LRS 79	F	9	*IQCB1* c.1558 C>T	Jerk	HM+1; less than N36	HM+1; less than N36	+7.50	**	**	+8.50	-1.00	100	Extinguished	Minimal attenuation of vessels and metallic sheet in the fundus seen	Normal foveal dip seen. Thinning of IS-OS junction noted	**
26	LRS 77	M	4	*IQCB1* c.1363 C>T	Jerk	PLPR inaccurate; less than N36	PLPR inaccurate; less than N36	+6.50	**	**	+6.50	-0.50	180	Extinguished	Minimal attenuation of vessels and metallic sheet in the fundus seen	No OCT	**

27	LRS 92	M	5	*IQCB1* c.1333 C>T	Wandering	Does not follow light	Does not follow light	+0.00	**	**	+1.00	**	**	Extinguished	Metallic sheet in the fundus and RPE granules visible.	No OCT	Enlarged kidney on scan at 5 months
28	LRS 10	F	11	*IQCB1* c.994C>T	Pendular	HM+1; less than N36	HM+1; less than N36	+7.50	-1.50	180	+7.50	-1.50	180	Extinguished	Mild disc pallor, Minimal attenuation of vessels and metallic sheet in the fundus seen	No OCT	Myopic, Astigmatism
29	LRS 87	M	3	*LCA5* c.838C>T	Wandering	follows and fixates light	follows and fixates light	+6.50	-1.50	180	+6.00	-1.50	180	Extinguished	Mild disc pallor, Arteriolar attenuation present, shiny reflex and pinhead sized round shaped pigment observed.	No OCT	Hearing problem, delayed milestone
30	LRS 21	F	8	*LCA5* c.1422 delT	Jerk	follows and fixates light	follows and fixates light	+4.00	-1.50	180	+4.00	-1.50	180	Extinguished	Pale disc, mild attenuated vessels, macular atrophy observed	No OCT	**

31	LRS 60	F	6	*LCA5* c.955G>A	Wandering	Does not fix light	Does not fix light	+6.50	**	**	+5.50	**	**	Extinguished	Pale disc, mild attenuated vessels, macular atrophy and white spots observed in fundus	No OCT	Astigmatism
32	LRS 20	F	11	*LCA5* c.1062_1068 delCGAAAAC	Jerk	follows and fixates light	follows and fixates light	+4.50	-1.25	150	+5.00	-1.25	150	Extinguished	Pale disc, mild attenuated vessels, macular atrophy and peripheral white spots observed	No OCT	**
	Affected sib of LRS 20	F	3	*LCA5* c.1062_1068 delCGAAAAC	Jerk	follows and fixates light	follows and fixates light	+3.00	-1.00	180	+3.00	-1.00	180	Extinguished	Pale disc, mild attenuated vessels, macular atrophy seen	No OCT	**
33	LRS 30	M	14	*LCA5* c.1062_1068 delCGAAAAC	Wandering	follows and fixates light	follows and fixates light	+1.00	-1.75	10	+1.00	-1.00	10	Extinguished	Pale disc, mild attenuated vessels, macular atrophy, shiny reflex seen and white dots in RPE observed.	No OCT	**

34	LRS 62	F	9 months	*NMNAT1* c.109G>A	Pendular	follows and fixates light	follows and fixates light	+6.00	-0.75	180	+5.50	-0.75	180	Extinguished	Pale disc, mild attenuated vessels, coarse pigment with macular atrophy observed	No OCT	**
35	LRS 8	M	5	*NMNAT1* c.53A>G	Jerk	PLPR accurate	PLPR accurate	+6.00	**	**	+6.50	-1.00	70	Extinguished	Pale disc, mild attenuated vessels, coarse pigment with macular atrophy observed, typical bony spicules in the periphery	Foveal thinning with RPE atrophy noted at fovea. Pigmentation causing shadowing noted.	**
36	LRS 80	M	8	*RD3* c.296+1 G>A	Jerk	HM+;1 less than N36	CFCF/ Less than N36	+3.50	-2.50	20	+3.00	-1.75	160	Extinguished	Pale disc, mild attenuated vessels and early macular scarring seen. Pinhead sized round pigment observed	No OCT	**

37	LRS 49	F	3	*RDH12* c.184C>T	Jerk	6/24	6/24	+8.00	-0.75	180	+8.00	-0.75	180	Extinguished	Pale disc, attenuated vessels and macular coloboma with bony spicules and pigmentary clumps observed	No OCT	**
38	LRS 17	M	29	*RDH12* c.146C>T	Nil	CF at 50;N36	CF at 50;N36	-6.00	**	**	-5.00	**	**	Extinguished	Pale disc, marked attenuated vessels and large area of macular coloboma with bony spicules observed	No OCT	**
39	LRS 93	F	17	*RDH12* c.746G>T	Jerk	3/60	1/60	+3.00	**	**	+3.50	-1.00	180	Reduced	Pale disc, marked attenuated vessels and macular coloboma with bony spicules observed	Foveal thinning with RPE atrophy and pigmentation causing shadowing noted.ERM noted with IPVD.OS: IPVD	**

															noted causing slight traction just temporal to fovea	
40	LRS 86	F	7	*RPE65* c.1514 T>G c.963T>G	Jerk	6/36;N6	CFCF/ Less than N36	+2.50	-0.50	180	+3.50	**	**	Extinguished	Minimal optic disc pallor with attenuated vessels, metallic sheen and macular atrophies observed	Foveal contour seen with IS-OS junction thinning at the fovea. RPE atrophy noted.	Astigmatism
41	LRS 28	M	5	*RPE65* c.353+1 G>T	Jerk	6/36;N12	6/36;N12	+2.50	-0.75	180	+2.25	-0.75	180	Extinguished	Mild optic disc pallor with attenuated vessels and metallic sheen observed	No OCT	**
42	LRS 23	F	11	*RPE65* c.362 dupT	Jerk	6/24;N18	2/60;N36	+0.75	-1.50	170	-2.25	-1.50	10	Extinguished	Pale tilted disc with attenuated vessels, white spots in posterior pole observed	No OCT	Myopic

43	LRS 25	F	7	*RPE65* c.46_49 del TTTG	Jerk	6/18;N6	6/18;N6	+1.75	**	**	+1.75	**	**	Extinguished	Optic disc pallor with attenuated vessels, white spots in posterior pole and metallic sheen observed	Foveal thinning noted. RPE alteration noted	**
44	LRS 2	F	9	*RPE65* c.1409 C>T	Jerk	6/18;N6	6/60;N18	+2.50	-1.00	20	+4.00	-1.50	160	Extinguished	Optic disc pallor with attenuated vessels, with white spots observed	Minimal foveal thinning noted with thinning of IS-OS junction.	**
45	LRS 12	M	12	*RPE65* c.1109 T>A	Pendular	HM+;1less than N36	CF at 50;less than N36	-4.00	-2.00	180	-16.00	-4.00	170	Extinguished	Pale disc, attenuated vessels, yellowish scarring seen in macula. coarse pigmentary clumps more in posterior pole observed	No OCT	Myopic, astigmatism, keratoconus
46	LRS 58	M	36	*RPE65* c.362 dupT	Jerk	CF at 50;less than N36	CF at 1m;less than N36	+3.50	**	**	+4.00	**	**	Extinguished	Optic disc pallor with attenuated vessels and peripheral spicule pigmentation observed	No OCT	**

47	LRS 15	M	34	*RPGRIP1* c.3434 delA	Jerk	PLPR accurate; less than N36	PLPR inaccu rate; less than N36	-3.00	**	**	-5.50	**	**	Extingui shed	Pale disc, attenuated vessels, macular atrophy with coarse coin shaped early bony spicules.	No OCT	Myopic
48	LRS 65	M	4	*RPGRIP1* c.2041 C>T	Rotato ry	Does not follow light	Does not follow light	+10.50	-2.00	180	+9.50	-1.00	180	Extingui shed	Minimal attenuation of vessels with RPE mottling seen.	No OCT	Astigma tism
49	LRS 45	M	6	*RPGRIP1* c.2041 C>T	Wande ring	CFCF	HM+	**	**	**	**	**	**	Extingui shed	Mild optic disc pallor, attenuation of vessels with RPE mottling seen.	OU: Foveal contour seen with thinning of IS-OS junction noted. FT- OD- 170 micron, OS- 180 micron	**
50	LRS 26	M	22	*RPGRIP1* c.3788 T>C	Jerk	PLPR accurate	PLPR accura te	+6.50	-3.00	180	+6.50	-3.00	180	Extingui shed	Mild optic disc pallor, attenuation of vessels, extensive	No OCT	**

															RPE mottling with early bony spicule pigmentation seen. Calcified deposits observed		
51	LRS 47	F	27	*RPGRIP1* c.895G>T	Wandering	PLPR accurate	PLPR accurate	+5.50	-1.00	30	+5.00	-1.00	130	Extinguished	Mild optic disc pallor, attenuation of vessels, with bony spicule pigmentation seen.	Foveal thinning noted with RPE atrophy at the fovea, thinning of IS-OS layer seen.FT-OD-45 microns, OS-63 microns	**
52	LRS 40	M	22	*TULP1* c.1047 T>G	Jerk	1/60	1/60	-5.00	-0.75	90	-3.50	-0.75	60	Extinguished	Pale disc, marked attenuated vessels, Peri foveal yellow annular ring with macular atrophy and mild peripheral bone spicules like pigmentation seen	Foveal thinning noted with RPE atrophy seen sparring fovea. Thin ERM noted with ILM wrinkling seen.	Myopic, cataract

53	LRS 29	F	22	*SPATA7* c.1215+5 C>A	wandering	PLPR accurate; less than N36	PLPR accurate; less than N36	+7.50	**	**	+8.00	**	**	Extinguished	Pale disc, attenuated vessels, tapetal reflex seen in periphery	NO OCT	**
	Affected sib of LRS 29	M	14	*SPATA7* c.1215+5 C>A	wandering	PLPR accurate; less than N36	PLPR accurate; less thanN36	+6.00	-0.5	180	+6.00	**	**	Extinguished	Pale disc, attenuated vessels, RPE atrophy	NO OCT	**
54	LRS 67	M	15	*SPATA7* c.913-2A>G	Jerk	PLPR accurate	PLPR accurate	+1.00	-1.00	170	+1.00	-2.25	170	Extinguished	Pale disc, attenuated vessels with RPE atrophy seen.	Normal foveal dip seen with thinning of IS-OS junction noted. RPE alterations seen	**
55	LRS 11	F	11	*SPATA7* c.18A>G	Jerk	3/60	HM+	+2.00	-1.50	20	+2.00	-3.00	120	Extinguished	Pale disc, attenuated vessels with tapetal reflex and peripheral pigmentary changes seen.	NO OCT	**

Table 11: Shows the clinical features of mutation positive index cases affected by LCA. IS-OS = Inner segment-outer segment; ERM = Epiretinal membrane; RPE = Retinal pigment epithelium; ILM = Internal limiting membrane; PLPR= Perception and projection of light; HM = Hand movements; CFCF = Counting finger close to face.

3.5. Screening of *SLC19A2* in a patient diagnosed with Thiamine Responsive Megaloblastic Anaemia (TRMA) with LCA as ocular phenotype.

Figure 38: 2% Agarose gel picture of the amplified products of the six exons of *SLC19A2* gene. Lane 1: Exon 1- 589 bp, Lane 2: Exon 2- 700 bp, Lane 3: Exon 3- 391 bp, Lane 4: Exon 4- 429 bp, Lane 5: Exon 5- 300 bp, Lane 6: Exon 6 - 295 bp.

Screening identified a novel point mutation in exon 2, a c.314G>A transition resulting in a missense mutation p.(Gly105Glu) (**Figure 39**). SIFT and PolyPhen analysis predicted the mutation to be “damaging” (score-0.02) and “probably damaging” (score-0.994), with glycine residue conserved across vertebrates and human SLC19 family. Parents were also screened and were found to be heterozygous for the mutation and the unaffected sib homozygous for wild type.

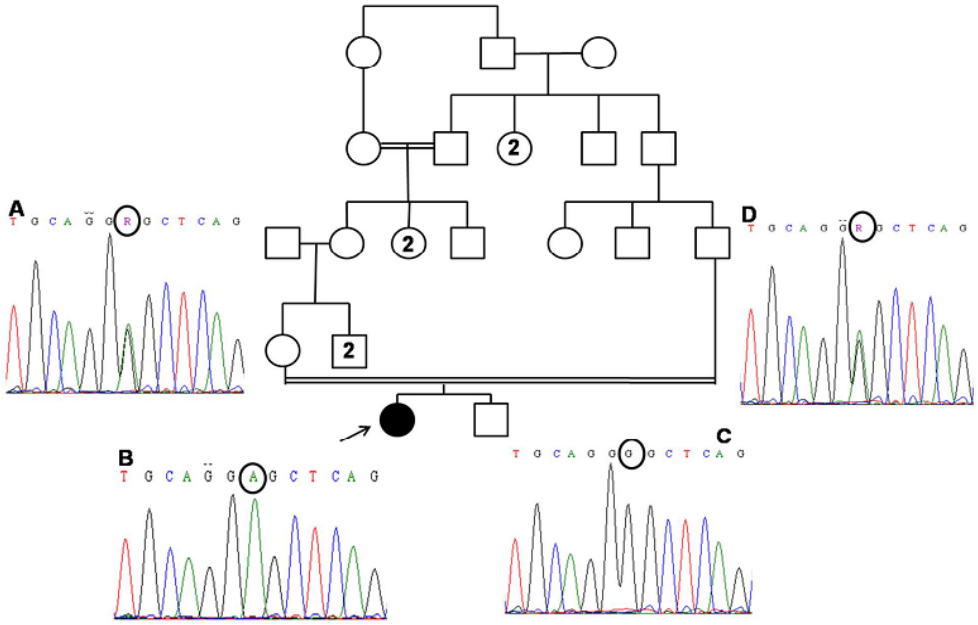

Figure 39: Sequence showing a novel point mutation in exon 2, a c.314G>A transition resulting in a missense mutation p.(Gly105Glu). [A] Heterozygous mutation in mother [B] Homozygous mutation in proband [C] Homozygous wild type in sib [D] Heterozygous mutation in father.

CHAPTER-4

DISCUSSION

Next generation sequencing has transformed genomic research from a technically laborious, time consuming and an expensive experimentation to a fast, cost effective and efficient tool in investigating genetic basis of many diseases. Clinical implementations of NGS are still evolving; however, it has increased the understanding of genetics in everyday medicine. The massively parallel sequencing on NGS platforms has made possible the simultaneous sequencing of multiple genes in a single reaction thus increasing the knowledge on genetic basis of various Mendelian diseases especially those that are genetically heterogeneous.

Leber congenital amaurosis is characterised by both clinical variability and genetic heterogeneity. The autosomal dominant and recessive LCA are shown to be caused by 29 different genes. Therefore, next generation sequencing technologies is currently the most promising approach to identify mutations in candidate genes of LCA.

4.1. Targeted resequencing in Indian LCA cohort:

In this study, after complete ophthalmic examination, phenotype documentation and pre-test genetic counselling, genotyping by NGS based targeted re-sequencing of twenty LCA candidate genes was performed to identify the disease-causing variant in ninety-two LCA families from an Indian cohort. Except the four positive controls[122, 127] (LRS 2, LRS 12, LRS 58, LRS 60) the index cases were not screened for mutations by any other methodologies.

The frequency of mutations in LCA genes varies in different populations. In a Chinese cohort of 145 patients screening 163 genes by retinal gene panel, 76.6% were mutation positive with *GUCY2D* mutations being more common [27] while in an Italian cohort of 95 patients, screening 8 LCA genes identified mutations in 28% with *RPE65* being most frequent[158]. Screening 13 genes in 37 Saudi Arabian families showed 24% mutation positivity with *TULP1* being common[159]. In a Belgium cohort of 91 cases, 69% were positive with *CEP290* mutations being frequent [160]. In a Spanish cohort eight genes screened in 84 patients revealed 23.8% mutation identification with highest mutation rate in *CRB1*[161]. Screening of nine genes in 64

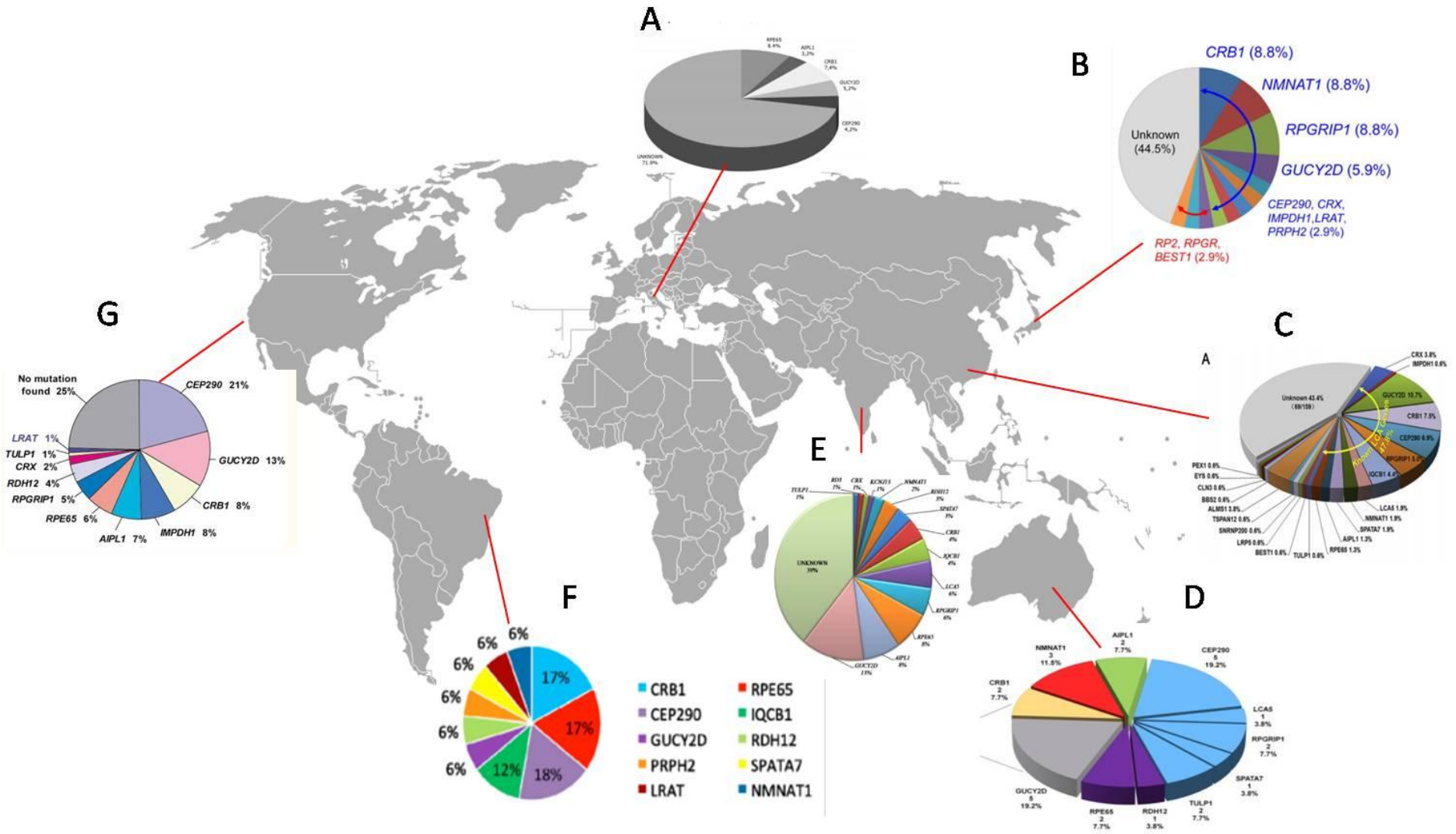

Figure 40: Pie charts of cohort studies showing frequency of LCA candidate gene mutation in the population. A. Italy[158] B. Japan[162] C. China[27] D. Australia[163] E. India (Current study) F. Brazil[164] G. Western cohort[124]

Danish patients identified 49% mutation positivity with high contribution of *RPE65* gene mutations[165].

In the current study, after the analysis, validation and segregation of the annotated data, we found 61% (56/92) of recruited LCA cases to be mutation positive in the twenty known candidate genes. The mutation spectrum was found to be as follows - *GUCY2D* gene contributing the highest (13%) followed by *AIPL1* and *RPE65* (8%), *RPGRIP1* and *LCA5* (6%), *IQCB1* and *CRB1* (4%), *SPATA7* and *RDH12* (3%), *NMNAT1* (2%), *KCNJ13, CRX*, *RD3* and *TULP1* contributing to 1%. We also observed same mutation in two or more unrelated individuals in our cohort.

No mutations were observed in *CABP4, CEP290*, *LRAT, MERTK, OTX2* and *IMPDH1* in this cohort. According to world-wide reports, *CEP290* is the most frequently mutated gene[11] followed by *GUCY2D*. However, we did not observe any mutations in *CEP290* gene in the cohort studied.

In LRS 11 (with consanguineous parentage), we observed a homozygous silent variant, c.18A>G; p.(Arg6Arg) in the penultimate base of exon 1 in *SPATA7*. The mother was heterozygous for the variant but the father was unavailable for analysis. This variant was not seen in controls screened. Although a synonymous change, computational predictions showed that it affects the wild type donor splice-site probably affecting splicing. This could either lead to a truncated protein or result in non-sense mediated decay[166]. The patient and the family were unavailable for cDNA analysis to confirm the exact splicing defect. However, reports on silent variants in the last codon of exon leading to disease phenotype suggest this variant to be likely pathogenic[167]. The same patient had a heterozygous missense variant c.551C>T in *CRX.* Bioinformatics tools predicted the variant to be damaging. The mother was wild type and the father was unavailable for gene testing. Hence, this *CRX* variant is defined as variant of unknown significance.

We observed specific bilateral changes in fundus features of our patients. Distinct phenotypes as found in earlier reports were observed in our study too; macular atrophy and yellowish spots in the macular region in *AIPL1* mutation [168], paraarteriolar sparing of RPE and nummular pigmentation in *CRB1*[169]. Greyish tapetal reflex was significantly observed in many patients with *GUCY2D* mutation in our cohort. *LCA5* mutation positive patients had hypopigmented whitish spots [127],

while a typical macular colobomatous atrophy and bony spicules were evident in *RDH12* [170] and *RPGRIP1* mutation, respectively. Typical fundus features of these various mutations are almost like the footprints of the disease that can be easily evaluated on retinal examination. These allow clinicians to consider few possible underlying mutated gene(s) and to some extent prognosticate the progression of the disease without subjecting all patients to a detailed genetic evaluation.

Among the 8 *AIPL1* mutation positive individuals, we observed the most frequent mutation p.(Trp278*) in three patients however the characteristic occurrence of keratoconus associated with *AIPL1* mutation was observed in only one. In *RPE65* affected individuals, we observed deterioration of vision with increase in age which correlates with the absence of macular involvement in early years and development of atrophy in later ages.

As reported, the visual acuities in our subjects varied from no light perception to 20/100 in many[171] and cycloplegic refractions revealed predominantly hyperopic and few myopic LCA patients[172]. Other ocular phenotypes like astigmatism, keratoconus and cataract that were reported in other studies were also observed in this cohort, astigmatism in a *SPATA7* mutated patient[173], keratoconus in *AIPL1, CRB1, RPGRIP1, LCA5, RPE65* and *TULP1* mutated LCA patients[172, 174]. Cataract was seen in some patients with *AIPL1, TULP1, RPGRIP1, LCA5* and *CRB1* mutated patients[174]. Other anomalies that are reported to be associated with LCA, like cleft palate[171], mental retardation, autism, delayed milestone[175] and hearing deficit were observed in few of our patients which signifies the importance of a thorough systemic evaluation in these subjects.

Double mutations i.e. two homozygous pathogenic variants in same gene were observed in two of our patients. LRS 86, with homozygous *RPE65* mutations, (p.(Asn321Lys) and p.(Leu505Arg)), had a severe macular phenotype with multiple small atrophic patches which is different from other *RPE65* cases. While LRS 55 with *GUCY2D* mutations (p.(Gly688Arg) and p.(Arg588Trp)), showed no significant difference compared to other *GUCY2D* mutation positive cases.

In LRS 90 family, the proband (3 yrs) and the affected sib (6 yrs) with digenic variants in *KCNJ13* and *AIPL1* showed diffused RPE mottling. The characteristic fundus phenotypes may be observed at a later age.

Among the mutation positive cases, there were twelve possible triallelic cases, where a heterozygous variant was observed along with disease causing pathogenic homozygous variant. The role of the third heterozygous allele remains unclear as we did not observe any significant difference in the fundus phenotype due to this allele. Although all the third heterozygous allele segregated in the families, the low CADD scores of *CABP4* and *CRB1* variants excludes the possibility of triallelism in LRS 58 and LRS 14 respectively. The pathogenicity of heterozygous *RPE65*, p.(Asn321Lys) variant in LRS 8, LRS 78, LRS 80 and LRS 88 as a disease allele remains unclear because of relatively high MAF and low CADD scoring, though the variant has been defined as disease causing in many studies[176, 177]. Further molecular characterization of the variants might clarify the functional consequences, if any.

4.1.2. Possible functional impact of mutations on their protein:

Mutations in specific domains of protein are shown to have a major impact on their structure and functions. In our study, about 80% of mutations are in the functional domain and 44% of the mutations are either nonsense or frameshift which are present on or before the functional domain and predicted to result in truncated protein (**Figure 31**).

AIPL1:

The PPIase domain binds to farnesylated PDE6 protein[178] and mutation in this domain disrupts its binding. Glu83Lys mutation identified in this study is present in this domain and was predicted to alter the structure as the amino acid change is from acidic to a basic residue. The tetratricopeptide repeat (TPR) domain in *AIPL1 is* essential for the protein to function as chaperone heterocomplex for assisting in retinal protein maturation. Mutations in TPR domain or deletion of the domain are shown to disrupt this interaction between *AIPL1* and its co-chaperone *Hsp70*[179]*.* Three of the identified mutations p.(Asn230Ser), p.(Trp278*) and p.(Glu282*) are present in this domain. The p.(Trp278*) mutation is shown to result incomplete loss of protein[180].

CRB1:

The CRB1 protein has a signal peptide, EGF-like domains, laminin AG-like domains and cytoplasmic domains. We identified frameshift mutation p.(Ser359Glufs*20) in

the 5th epidermal growth factor-like (EGF) domain and p.(R1390X) in the 3rd laminin aglobular like domain leading to premature truncation. Mutations in EGF domain are said to affect interdomain packing[181]. The laminin G-like domains are conserved and are predicted to affect calcium binding, protein folding and interactions[182]. The functional impact of the identified canonical splice variant c.4005+1G>A is unknown but is predicted to be damaging by *insilico* analysis.

CRX

The CRX protein contains an otd/ Otx-like paired homeodomain (HD) in the N-terminus that are required for DNA-binding and nuclear localization and a transcription activation domain at the C-terminal[183]. The mutation identified in this study p.(Arg41Gln) is present in HD domain while p.(Pro184Leu) is present in transcription activation domain. p.(Arg41Gln) has already been characterized by co-transfection assays and was shown to significantly reduce DNA-binding activity[184].

GUCY2D

GUCY2D protein has N-terminal extracellular domain (ECD), a single membrane-spanning domain, an intracellular kinase homology domain (KHD), and a catalytic domain (CD). In our study, p.(Phe54Serfs*31) in ECD, p.(Arg660*) in KHD, p.(Gly888Alafs*8), p.(Thr962Ilefs*16), p.(Leu1022*) in CD all lead to premature protein truncation causing the disruption in protein activity. Studies have shown that the protein activity is compromised even with point mutations in KHD and CD domains [185]. All other missense variants in our study are distributed in all three domains and are predicted to be disease causing by bioinformatics tools.

RDH12

RDH12 has a NAD(P) binding domain that is essential for its catalytic activity possessing cofactor or substrate binding sites[186]. The nonsense variant p.(Arg62*) leads to protein truncation while one of the missense mutations identified, p.(Thr49Met), was well characterized and had shown to be rapidly degraded leading to decreased cellular availability, thus causing the disease[187]

LCA5

LCA5 has 4 coiled coil domains that aid in the interaction with its other partners like *OFD1* [188], *Nlp*^{isoB} etc. All mutations identified in *LCA5* in this study are frameshift p.(Ile474Metfs*11), and nonsense p.(Tyr354*), p.(Arg280*) leading to truncation of

the protein. The c.955G>A lead to change in the last base of exon 6 leads to amino acid change from alanine to threonine. This mutation has been characterized by cDNA analysis leading to a 5 bp insertion of intronic sequence, causing a frameshift and premature truncation of protein[189].

RPE65

RPE65 has a catalytic domain that is responsible for its isomerohydrolase activities[190]. The 2 frameshift mutants observed in the study, p.(Phe16Lysfs*14) and p.(Ser121Phefs*10) are before and in the beginning of the catalytic domain, respectively. Hence there would be disruption of whole domain resulting possibly in a non-functional protein and causing disease. Although studies on the functional consequence of the other mutants p.(Leu505Arg), p.(Asn321Lys), p.(Pro470Leu) and p.(Leu370His) are unavailable, we find that all these mutants lie on the catalytic domain and are predicted to have change in amino acid charge and polarity except for p.(Asn321Lys).

NMNAT1

NMNAT1 has substrate binding domain, isoform-specific targeting and interaction domains that are essential for neuroprotective function of the protein[191]. We identified 2 missense variants that are not characterized before. By *Insilico* analysis[192], the p.(Asn18Ser) mutant is predicted to affect correct protein folding as the hydrophobicity of the wild-type and mutant residue differs. In p.(Gly37Arg) mutant, the glycine to arginine change introduces a charge (neutral to positive) in this position which might result in a repulsion between the altered residue and the adjacent amino acid residues. Also, glycine with its neutral charge is flexible for any change in protein conformation thereby helping in protein function. Its alteration to arginine might decrease the flexibility thus affecting protein function.

IQCB1

IQCB1 possess IQ calmodulin-binding and coiled coil domains [193, 194].The functional domain is essential for its interaction with RPGR and calmodulin during ciliogenesis[29]. All *IQCB1* mutants identified in the study are nonsense mutants (Gln520*, Arg455*, Arg445*, Arg332*), and might have a functional impact on protein.

RPGRIP1

RPGRIP1 contains a C-terminal *RPGR* interacting domain (RID) and a coiled-coil (CC) domain. These domains are homologous among the proteins that are involved in vesicular trafficking[195]. The mutants identified in our study- p.(Glu1145Glyfs*18), p.(Gln681*) and p.(Glu299*) result in proteins that lack *RPGR* interacting domain causing disease.

TULP1

TULP1 has carboxy-terminal tubby domain which acts as DNA binding domain and the N-terminal regions helps in transcription activation[196]. The p.(Asn349Lys) mutation is found to be in the tubby domain. The mutation segregated with the disease phenotype in the family with the affected sib harbouring the same mutation and is predicted to be damaging by insilico tools. The functional impact of this possibly pathogenic variant is yet to be studied.

SPATA7

The N-terminal domain of SPATA7 functions as binding region for other ciliary proteins to perform ciliary trafficking [76]. The mutants from this study c.913-2A>G, c.1215+5C>A and c.18A>G are splice mutants. The two splice variants c.913-2A>G and c.1215+5C>A were characterized by RT-PCR and cDNA sequencing that confirm the altered splicing which would lead to premature truncation of protein. The cDNA mutant is predicted by bioinformatic analysis to alter splicing as well.

4.2. Observed syndromic LCA

4.2.1. Senior-Loken syndrome (SLSN)

SLSN is an autosomal recessive syndrome. It is a nephronophthisis-associated disorder, where the patient presents cystic kidney disease and retinal dystrophy. Retinal abnormalities like retinitis pigmentosa or Leber congenital amaurosis is reported so far. Prognosis with respect to vision is usually poor and treatment is still unavailable. But for the kidney, renal transplantation is recommended for the end stage renal failure[197]. Mutations in *IQCB1* have been shown to cause Senior-Loken syndrome (SLSN). In the study by Estrada Cuzcano et*. al*, LCA patients with *IQCB1* mutations were recalled and assessed for renal abnormalities and those positive were subsequently re-diagnosed as SLSN[198]. Patients with *IQCB1* mutations are

shown to be at a high risk of renal failure during or after second decade of life.In our study, we observed pathogenic variants in *IQCB1* gene in four patients – LRS 10, LRS 77, LRS 79 and LRS 92. Following the results obtained from our targeted re-sequencing assay, we re-called the *IQCB1* mutation positive patients, re-counselled and advised for comprehensive renal evaluation at regular intervals. The case LRS 92 first reported to us at seven months of age and was diagnosed with LCA. Following gene testing and re-counselling at 5 years of age, the patient's family revealed history of bilateral marginal nephromegaly diagnosed at 9 months. At 7yrs, he developed chronic kidney disease stage II and presented with behavioural issues both characteristic of *IQCB1* mutation and thus re-diagnosed as Senior-Loken syndrome. The age ranges of other *IQCB1* mutation positive patients are between 4 and 11 years and no renal abnormality has been observed yet.

4.2.2. Thiamine responsive megaloblastic anaemia:

TRMA Syndrome was first reported in 1969 by Rogers et al. [199]. It is autosomal recessive and the onset of the syndrome is in infancy or in early childhood with main features being megaloblastic anemia, diabetes mellitus (non-type 1) and sensorineural deafness, responding in varying degrees to thiamine treatment[200, 201]. Other associated findings described are congenital cardiac malformations, cardiomyopathy andretinal abnormalities.

In a case of cone-rod dystrophy with TRMA syndrome, deafness was the first feature to be noticed at 1 year of age followed by diabetes mellitus. At 7 years of age, the patient developed pancytopenia, cardiomyopathy and vision loss, where the ERG was consistent with cone-rod dystrophy. By 15 years of age, vision in both eyes deteriorated and fundi showed bull's eye maculopathy with peripheral pigmentary changes[202]. Contrarily, in the current study, the patient complained severe vision loss with photophobia, nystagmus and hyperopia consistent with LCA manifesting within one year of age. ERG was extinguished. This was followed by sensorineural deafness at one year and diabetes mellitus later at three and half years. The manifestation of all features of TRMA was much early in our patient. Homozygosity mapping and subsequent gene screening identified on chromosome 1 (1q23.2-23.3), *SLC19A2,* a member of solute carrier family 19, encoding a thiamine transporter, THTR1 as the candidate gene, for TRMA[155, 203]. Many mutations in *SLC19A2* gene in TRMA patients have been reported[204-206]. Gene

screening aids in the differential diagnosis of neonatal diabetes as *SLC19A2* is not mutated in patients with permanent neonatal diabetes mellitus (PNDM) in which diabetes is presented in the first 6 months of life[207].

A higher expression of hTHTR-1 and moderate expression of hTHTR-2 were found to be present in ARPE-19 cells as well as in native human retinal tissue. By confocal imaging, ARPE-19 cells expressing TRMA associated hTHTR-1 mutants (Gly172Asp, Ser143Phe and Asp93His) have shown impaired cell surface expression or function of the transporter in this cell. However, it is still not known as to why only some TRMA patients present with retinal degeneration and why there is a difference in the clinical presentation of the same[208].

In our patient, we report a novel missense mutation in exon 2, a c.314G>A transition resulting in p.(Gly105Glu), converting nonpolar hydrophobic glycine to negatively charged hydrophilic glutamic acid. The retinal degenerative (RD) phenotypes so far described as part of TRMA are cone rod dystrophy[202], retinitis pigmentosa[209] and optic atrophy[210]. Here for first time, we report Leber's congenital amaurosis (LCA) as a component of RD in our patient.

In the last decade, tremendous advances in genotyping and genomic research have improved molecular diagnosis aiding in genetic counselling and development of therapeutic strategies for the treatment of various diseases like cancer, genetic defects, infectious diseases or autoimmune disorders. Even when significant progress has been made in recent years in gene therapy, most genetic diseases do not have therapies, and it will be important to continue developing treatment strategies for individual genetic diseases even if they occur only rarely[211]. Advancements in understanding the genetic and molecular basis of a variety of retinal diseases has led to the development of gene therapy for these and the original hope that gene therapy could cure otherwise untreatable, inherited genetic diseases has been rekindled by gene therapy clinical trial for LCA. The difficulties in designing gene therapy are due to the choice of suitable gene transfer vehicle, the efficiency of gene transfer and problems related to immune reactions of the treated patient against the vector carrying the therapeutic gene. AAV2-mediated gene transfer to the human retina does not elicit the cytotoxic T-lymphocyte responses to AAV capsids unlike that were observed in muscle and central nervous system, thus supporting the use of AAV-mediated gene augmentation therapy for treatment of

few inherited retinal diseases. Molecular testing aids in the diagnosis of LCA and knowledge of the disease gene in LCA patients are important in counselling patient's family and in determining their suitability for gene-specific therapies for very few now and for many subjects in future.

In conclusion, this study provides an insight on the genetic profile and mutation spectrum of candidate genes of LCA from a larger Indian cohort. The molecular findings have also helped in re-diagnosis, medical management, carrier testing, prenatal testing and genetic counseling.

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CONCLUSION

In conclusion, this is the first comprehensive study on the genetic profile and mutation spectrum of candidate genes of LCA from a larger Indian cohort. Also, this study for the first time reported association of TRMA with LCA as the retinal disease component. In this cohort, mutation was found in 62% (57/92 - 56 by targeted resequencing and 1 TRMA) of patients recruited. Among the mutations found 39% (21/53) were novel. The mutation spectrum and the presence of large number of novel mutations suggests that using custom made chips designed for targeting specific mutations for genetic testing would not be an efficient method of genetic diagnosis, especially in genetically heterogeneous diseases.

The current study also reports a possible digenic inheritance which proves the advantage of using NGS over other screening methodologies, as a large set of genes are screened and analysed for possible disease pathogenicity. The triallelic inheritance reported in the current study provides an insight on the modifier alleles that could probably make a difference in the disease severity among. However, we did not observe any alteration in the disease severity in our cohort with triallelic variants. Mutation was not identified in 35/92 cases (38%) where screening of mutations in unscreened deep intronic and regulatory regions might contribute to a small percentage of disease etiology in these patients. These negative cases also represent cohort where other retinal genes or novel genes could be involved. Also, large structural rearrangements such as duplications are not identified by targeted re-sequencing. Whole exome or genome sequencing would identify all these including novel genes.

SPECIFIC CONTRIBUTIONS

- First report on mutation spectrum in LCA from a large Indian cohort
- First report on association of TRMA with LCA as an ocular disease phenotype.
- Distinct phenotype to genotype correlation in a large number of LCA patients.
- 21 novel pathogenic variants submitted to public databases.
- Genetic testing data of 57 patients positive for mutation in candidate genes screened aided in

❖ Genetic counselling

Genetic diagnostics has strengthened genetic counselling and management of genetic diseases. Although gene therapies for ocular genetic diseases are still underway, gene testing confirms the clinical diagnosis and has helped the patient and their families in rehabilitation and disease management.

❖ Carrier testing

Inthe current study, after identifying the mutation in the probands we extended carrier testing to other members of the families. As a part of segregation analysis and carrier testing the siblings were checked for carrier status. This had aided in genetic counselling for the carrier sibling by providing the risk of disease for the next generation, especially if they decide on consanguineous marriages.

❖ Prenatal testing

Molecular genetic test, prenatally, are usually carried out in the fetal DNA from chorionic villi sample. In two of our patients, LRS 43 with p.(Gly597Glu) and LRS 74 with p.(Asp728Asn) mutations in *GUCY2D,* following the genetic test in proband, the results were discussed with the family and the proband's parents opted for prenatal testing for the successive pregnancy. Both the foetuses were found to be heterozygous carriers and had normal vision after birth.

❖ Management of genetic disease

The patients with *IQCB1* mutations are shown to be at a high risk of renal failure during or after second decade of life. We identified *IQCB1* pathogenic variants in four patients and all were re-called, re-counselled and advised for comprehensive renal evaluation at regular intervals. LRS92 with *IQCB1* mutation, at 7yrs, developed chronic kidney disease stage II and presented with behavioural issues both characteristic of *IQCB1* mutation. Monitoring the renal function of patients with *IQCB1* mutation periodically help in avoiding end stage complication and proper management of disease.

Screening of *SLC19A2* in the TRMA patient helped in confirming the diagnosis and thereby management. The megaloblastic anaemia was corrected using a pharmacological dose of thiamine (25-75 mg /day) and for diabetes; the patient is under 18U and 10U of insulin at morning and night, respectively.

LIMITATIONS

Large structural rearrangements such as duplications were not analyzed in this study. Deep intronic and regulatory regions of the twenty candidate genes screened were not sequenced in the current study. This might contribute to a small percentage of mutations in the negative cases. Three cases, LRS 46, LRS 24 and LRS 89 with novel heterozygous variants *MERTK,* c.1627T>C, p.(Ser543Pro); *RPE65,* c.814C>T, p.(Gly272Arg) and *RPGRIP1,* c.1480delA, p.(Asn495Thrfs*11) could harbour deep intronic variants. Recently, more candidate genes; *ALMS1, CCT2, CNGA3, CLUAP1, DTHD1, GDF6, IFT140, MYO7A* and *PRPH2* have been reported which were not screened in the current study. Although variants in each of these genes were reported in only one case, it might also contribute to the mutation spectrum in our cohort.

FUTURE SCOPE OF THE STUDY

- Whole exome or genome sequencing might help in identifying the causative variant involved in the mutation negative cases, either variants in deep intronic and regulatory regions of known candidate genes or novel genes.
- The phenotype and genotype correlation understood by the current study can be extrapolated to design phenotype based diagnostic testing to reduce the cost of gene testing for the patients.
- The available data on the mutation spectrum of the patients would help in the application of gene replacement therapy in the future.
- Identifying novel genes and understanding their function and disease mechanism may pave way for new therapeutic strategies.

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Appendix

1. DNA extraction:

Requirements:

- NucleoSpin® Blood XL kit (Macherey-Nagel, GmbH, Duren, Germany)
- 50 ml Collection tubes
- 50 ml Collection tubes with Nucleospin® Blood XL column*
- REMI R-23 Centrifuge
- Water Bath with Shaker
- Pasteur pipettes

Reagents:

- Proteinase K [Lyophilized] *– 126 mg of proteinase K [lyophilized form] was dissolved in 5.75 ml of proteinase buffer. It was stored at 4ºC
- Proteinase Buffer*
- Sterile Dulbecco's Phosphate Buffered Saline - pH - 7.4 (HiMedia, Mumbai, India)
- Lysis Buffer BQ1*
- Absolute Alcohol (Hayman Limited,Witham, England)
- Wash Buffer concentrate BQ2*
- Elution Buffer BE*

* Provided in the kit.

Procedure:

- Five hundred µl of proteinase K was added to 50ml collection tube.
- The blood sample was added along the sides of the labeled tubes. If the volume of blood was ≤ 10 ml, it is made up to 10 ml with phosphate buffer saline.
- Ten ml of lysis buffer was added to the blood sample and the tube was shaken vigorously for 5 minutes.
- The tube was then placed in 56ºC shaker water bath for 15 minutes.
- The lysate was cooled at room temperature.
- Ten ml of chilled absolute alcohol was added to the lysate and shaken vigorously for 5 minutes.

- The lysate was transferred to Nucleospin® Blood XL column and centrifuged at 4500 rpm for 2 minutes.
- The flow through was discarded and the sides of the tubewas wiped clean.
- The column was washed with 7.5 ml of wash buffer, centrifuged at 4500 rpm for 2 minutes.
- The above step was repeated, with the centrifugation time being increased to 20 minutes, to ensure complete drying of the column.
- The column was transferred to a fresh 50 ml collection tube.

Elution of DNA

- Seven-hundred fifty µl of elution buffer pre-heated to 70ºC was added to the column and left at room temperature, overnight.
- The DNA was eluted completely by centrifuging at 5000 rpm for 6 minutes. This was transferred into a 1.5 ml vial, labeled as 1st elute, and stored at 4ºC for immediate use or at – 20ºC for long term storage.
- Thousand µl of elution buffer pre-heated to 70ºC was added to the column and left at room temperature overnight. It is centrifuged at 5000 rpm for 6 minutes. The eluted DNA was again added to the same column. The same was repeated for 4 more days.
- The elute was collected on the fifth day into a 1.5 ml vial and stored at -80ºC. This was labeled as the 2nd elute.

2. Agarose Gel Electrophoresis

Requirements:

- Gel electrophoresis unit (Bio-Rad laboratories, California, USA)
- BIO-RAD, Gel Doc^{TM}XR Gel Documentation system (Bio-Rad laboratories, California, USA)
- Agarose (SeaKem® LE Agarose, Lonza, Maine, USA)
- 10X TBE buffer
    - Tris (Merck, New Jersey, USA) - 54 g
    - Boric acid (Merck, New Jersey, USA) - 27.5 g
    - EDTA (GeNei^{TM}, Bangalore, India) - 3.72 g
    - Distilled water - 500ml

- Ethidium bromide (Sigma-Aldrich, Missouri, USA)- 2mg/ml – dissolved in distilled water
- Tracking dye- Bromophenol blue (BPB)
  - ❖ Bromophenol blue (HiMedia, Mumbai, India) - 250 mg dissolved in 50ml 1X TBE buffer.
  - ❖ Sucrose (Merck, New Jersey, USA) - 40 g dissolved in 50ml Milli Q water.

The above contents are mixed and stored in brown bottle.

Procedure

- ➢ The gel trough was cleaned with ethanol and the ends were sealed with cellophane tape. Combs were placed to form wells.
- ➢ Agarose was weighed and dissolved in 100 ml of 1X TBE buffer (1% for DNA, 2% for PCR products, 3% for ASPCR products and gel extraction)
- ➢ The agarose was melted in microwave oven and 20 µl of ethidium bromide was added to the molten agarose.
- ➢ This was mixed and poured on to the sealed trough and allowed to set.
- ➢ After the gel solidified, the cellophane tapes and combs were removed and the trough was placed in electrophoresis tank containing 1X TBE buffer.
- ➢ 4ul of sample and 4ul of tracking dye were mixed and loaded on to the wells.
- ➢ 1ul of molecular weight maker (100bp ladder) and 2ul of tracking dye were mixed and loaded on to the well.
- ➢ The electrophoresis was run at 120V for 20 to 30 minutes.
- ➢ The gel was docked in BioRad Gel Documentation system for visualization using Quantity one (v.4.6.2) software.

3. Polymerase Chain Reaction (PCR):

3.1. Primer Design:

For Direct sequencing:

The primers were designed using Primer 3 input (v.0.4.0) software. The primers were designed in such a way that they flank the complete exon sequence including 60-100bp intronic sequences. The properties like GC content, self-annealing, dimer formation, hairpin loop formation was checked for both forward and reverse primers using the Oligocalc software.

The table below lists the forward and reverse primer sequences for amplification of specific exons of the candidate genes.

S. No	Gene	Exon	Primer (5' - 3')	
			Forward Primer	**Reverse Primer**
1	*AIPL1*	2	GGGCCTTGAACAGTGTGTCT	GCTGCTGTGTTTCGGGAAA
2	*AIPL1*	5	GCAGCTGCCTGAGGTCATG	GTGGGGTGGAAAGAAAAG
3	*AIPL1*	6	CTGGGAAGGGAGCTGTAG	AAAAGTGACACCACGATCC
4	*CEP290*	Intronic	ACAGGCACCCACCATCAT	TTCTTGTGGCAGTAAGGAGGA
5	*CEP290*	40	GCCAAAATGAAACGTGCATA	TGAAAGTCAGTTTTAACAAATACCAT
6	*CEP290*	18	TGGAGGGATTTTGGAAACAA	CCGGCTAAAACGGTGAAAC
7	*CRB1*	5	CCTCCTTTTAGGCAAATGCTC	AAAGCCATGGTCTGCCATAA
8	*CRB1*	11	TTCCCATTTCACAACCAATGT	GCTCGTCATTCATACGCAAA
9	*CRB1*	12A	GCTTGCTCTGGTTGGTCTTC	GCGGAACCACTGTGAAAGTT
10	*CRX*	2	GGATGGAATTCTTGGCATCCCAC	CTCTTTGTTCCGGGCAGGCCTC
11	*CRX*	3b	CCTCCACAGATGTGTGTCCAGAC	TGGGAGAAAGGTAGGGGTCTAGG
12	*GUCY2D*	3	GGACGGCGCCGCGAGCCAAGC	TCCCCTCTCCCTTGCCTTCTT
13	*GUCY2D*	9	CCCACATTGCCCTGGGCAGAA	CCTGCCCCCAGGACGTCACCC
14	*GUCY2D*	10	AGCAGGCTGAGGCTGCCTCTT	TCCCGGTGGATCCTCGTCTGC
15	*GUCY2D*	11	GCGGCGCCTCAGCCCCTTCCC	CCCTGTGGGCCGAACTCCACG
16	*GUCY2D*	14	CAGCTGGAGCCCAGCCAGGTA	CTGGTGAAGCTGAATTGAAGG
17	*GUCY2D*	15	GTGGAGACAATAGGGGACGCC	CTAAAGAGGGAGATGGGCTGG
18	*GUCY2D*	16	CCCCGAGGCCCTACCTAGGTG	ACCTCCCCGTCTTGTCCCCGT
19	*GUCY2D*	17	GGTGAGTCCCGAGCTCACGGC	CGGGGCCTCCGGGAGGGGTTGG
20	*GUCY2D*	2a	GGGTTACTCGGGCTTGGA	GTTCACCGGACCCACGAG
21	*GUCY2D*	2b	GTCCCCGCTTCGAGGTAGCGC	CCGAGTGCATCACCATGATCAC
22	*IQCB1*	11	CACAACAGCAGCAGATGACA	TCATCACGTAGCTAGAAAAGTTGG
23	*IQCB1*	13	TCCCCTCCTTATACACACTCAGA	CAATGCATTACCTTATACCAGCA
24	*IQCB1*	14	CCTCTGCTAAGTGGTTGGGTA	TTTCCTGAGGTTAGGGGATGA
25	*KCNJ13*	3a	TGGCACCAAGAAATGTGTAAA	CCTGCATTGCTGAAAGGAA
26	*LCA5*	3	CACTGCCATGTGTTATGTTTCA	CCAACAAACCTTTTCTAAGTGC
27	*LCA5*	5	CATGCCAGCTGAATTCCATA	CCCTTCAAAAAGGCTAGTCG
28	*LCA5*	6	CCCCCTTTTCTATAATTTGTGA	TTCTTTCTCAAGGGATGCTGA
29	*LCA5*	7a	AAATATGGTGGTTTTATGAAAGTT	AGGTAACAATGGCAAAACAGG
30	*NMNAT1*	2	GGTGGCAGAGCAAGACCTTA	ACAGGCACAGTGAATTTTCG
31	*RD3*	1	CTCTCTCCCCCTTGGGTTC	CTCTAGTCCTGGTGGCCTCA
32	*RDH12*	2	ACCCTTCTTTGAGGCTGGAT	TTGAATCCCAGGTTCCTTGA
33	*RDH12*	6	TTGCTGCAGGAGATAAGCTG	GAAAAGCGGCACACGAGTAT

34	*RPE65*	2	CCCACCCAGCTGAGACTAGA	TAAAAAGCCCAAACCACCTG
35	*RPE65*	4	CTGTACGGATTGCTCCTGTC	TTAGAATCATACATTCGCAGCATG
36	*RPE65*	9	GTACACTTTTTTCCTTTTTAAATG CATC	GTTTTAGATGTGATTCAGATTGAGTG
37	*RPE65*	10	TTGTCATTGCCTGTGCTCATG	TGAGAGAGATGAAACATTCTGG
38	*RPE65*	13	GCATATTGACTGATTGCTTG	GCAGTAAGAAGAGTATTCAG
39	*RPE65*	14	AGTCAGAAAAAGAAGTCAGGTC	ATTGCTTGCTCAACTCAGTGC
40	*RPE65*	12	CACACGGGAGTGAACAAATG	GCATATACTCAAAGCACTGTTCAAA
41	*RPGRIP1*	6	AGGGCATAGTCAAGGAGAA	CTGAATTGTGGCTTCTCATA
42	*RPGRIP1*	14	GAAAGAGCTCCCTACCCTT	GGAAATTCTGCATTGGTGC
43	*RPGRIP1*	21	CTTGGAGCCTCACTAACC	TTCATCAGACTTCCTCACC
44	*RPGRIP1*	24	AGTGTTCAACTGAGTGATGC	ACTATGGTTCCCTCAGAGAC
45	*SLC19A2*	2	CCAGGTCCTTTCATCACTAATGT	TGTAATTGCTACTATGGGGGC
46	*SPATA7*	1	GTCGGCTCCTCTTTTCCAG	CCCTGACAGCTGCCCTTTAC
47	*SPATA7*	3	ACAGCTGCAAGGTCTGGAAC	ACCTCCAGAACTCTCTTTATACTGC
48	*SPATA7*	6	TTCTAGCCAGTAAACCTTGTTACC	CCACCAACAGATTATTCTTC
49	*SPATA7*	7	AAAAAGTGCTGGATGGATAGAA	CATTTCAACTTTTACTAAGCACTTCA
50	*SPATA7*	10	CAACCTTTGTAGTTTCAGTGTTACG	GCACTTGCTTTTAATGTATTGTTTG
51	*SPATA7*	11c	AGAACGAGATATTCCCTTCACCA	TGAGTTACTGGCCATTTGAGG
52	*SPATA7*RT1	cDNA exon 7-10	CCAGTGGGGATCTTTTGGAT	GTCGCTCGAACAGTCGTTCT
53	*SPATA7*RT2	cDNA exon 9-11	CCAAGGGCAATGTGTCAGTA	CATCCTTTGGTGCCGACAAT
54	*TULP1*	11	TTCCCATCCTCTCACCTGTC	ACTGTGGTGGGTGCTCTACC
55	*SLC19A2*	1	CAATGGAAGAGCAGGCAAGT	CGCTTTTCTCGGTCCTCTCT
56	*SLC19A2*	2	CCAGGTCCTTTCATCACTAATGT	GCCCCCATAGTAGCAATTACA
57	*SLC19A2*	3	TGGGCCTGTAAATTGCTTTC	CAAATTTGGGAGGGGTGAAT
58	*SLC19A2*	4	GCAACAGCATTTGTGTAGCA	TGGGAATAAACAAAACTTGCCTA
59	*SLC19A2*	5	GTTGGAAAGGCAATTGACAG	TTCGATGCCAAAGGAGAGAT
60	*SLC19A2*	6	GGCACGTGGTGTAAGTATGC	TGCTGTGCAGAGTTCTTGCT

For ASPCR:

Primer designing for ASPCR is similar to that as above where either the forward or reverse primer is specific to the wild and mutant type alleles at 3' end while the other primer in the pair is designed complementary to the wild type. Two PCR reactions, one with wild type and other with mutant primer were set for

each mutation to be screened. ASPCR was performed to screen control samples for certain identified mutations.

S.No	PRIMER	SEQUENCE 5'-3'
1	*AIPL1*-X5-N230S-FP-WT	AAGCTGGAGAAGATGTTCAA
	AIPL1-X5-N230S-FP-MT	AAGCTGGAGAAGATGTTCAG
2	*AIPL1*-X2-E83K-RP-WT	ACCAGAACTCGGCCTCCTC
	AIPL1-X2-E83K-RP-MT	ACCAGAACTCGGCCTCCTT
3	*IQCB1*-R332X-RP-WT	TCTCCAGCAACATCTTTCATCG
	IQCB1-R332X-RP-MT	TCTCCAGCAACATCTTTCATCA
4	*RPGRIP1*-L1263P-FP-WT	CTACCCCAATAGGATGGCT
	RPGRIP1-L1263P-FP-MT	CTACCCCAATAGGATGGCC
5	*TULP1*-N349K-FP-WT	AACGGAGCAAGACAGGCAAT
	TULP1-N349K-FP-MT	AACGGAGCAAGACAGGCAAG
6	*IQCB1*-Q520X-FP-WT	GATCAGCACCAACGTGGAAC
	IQCB1-Q520X-FP-MT	GATCAGCACCAACGTGGAAT
7	*RPE65*-N321K-FP-WT	ACATCAACACCTATGAAGTCAAT
	RPE65-N321K-FP-MT	ACATCAACACCTATGAAGTCAAG
8	*RPE65*-L505R-FP-WT	AGCCTGCTTATCTCCTGTTTCT
	RPE65-L505R-FP-MT	AGCCTGCTTATCTCCTGTTTCG
9	*CRB1*-R1390X-RP-WT	ATCAAGTTCCACATTTCCTCTCG
	CRB1-R1390X-RP-MT	ATCAAGTTCCACATTTCCTCTCA
10	*GUCY2D*-G1013R-FP-WT	GGATGCACTTAACAAGGCT
	GUCY2D-G1013R-RP-WT	GTCACACTCACGCAGCAC
	GUCY2D-G1013R-RP-MT	GTCACACTCACGCAGCAT
11	*LCA5*-FP-R280X-WT	TTCTTCAAAAGGAGGTTCAGC
	LCA5-FP-R280X-MT	TTCTTCAAAAGGAGGTTCAGT
12	*RDH12*-R249L-RP-WT	GACAAAGGGGGAGAATAGCC
	RDH12-R249L-RP-MT	GACAAAGGGGGAGAATAGCA
13	*RPGRIP1*-Q40X-FP-WT	CCTCTATGACTTCACGTCCC
	RPGRIP1-Q40X-FP-MT	CCTCTATGACTTCACGTCCT
14	*GUCY2D*-L175R-FP-WT	GGATGCCCTCTACCCCCT
	GUCY2D-L175R-FP-MT	GGATGCCCTCTACCCCCG

15	*SLC19A2*-Exon 2-FP	CCAGGTCCTTTCATCACTAATGT
	SLC19A2-Exon 2 –WT-RP	CCATGTAACAATAAGGCTGAGAC
	SLC19A2-Exon 2 –MT-RP	CCATGTAACAATAAGGCTGAGAT

4. Purification of cycle sequenced extension products

Requirements:

- Microfuge
- Cycle sequenced product.
- 0.5M EthyleneDiamineTetraacetic Acid (EDTA) (GeNei^{TM}, Bangalore, India) [pH 8.0] - 1 in 4 dilution of above stock to obtain 0.125M EDTA- Freshly prepared
- 3M Sodium Acetate (GeNei^{TM}, Bangalore, India) [pH 4.6]
- 100% absolute alcohol (Hayman Limited, Witham, England)
- 70% absolute alcohol- 70ml of 100% absolute alcohol made up to 100ml with distilled water. Freshly prepared.
- Sterile Water

Procedure

- Ten µl of sterile water, 2 µl of 0.125M EDTA, 2 µl of 3M sodium acetate (pH 4.6) and 50µl of chilled absolute ethanol were taken in a 0.5ml vial.
- To this mixture 10 µl of the cycle sequenced product was added, vortexed and incubated at room temperature for 15 minutes.
- It was microfuged at 13000 rpm for 20 min.
- The supernatant was discarded without disturbing the pellet.
- Two-hundred µl of chilled 70% ethanol added, vortexed and microfuged for 10 minutes at 13000 rpm.
- The supernatant was discarded and the precipitate was once again washed with chilled 70% ethanol, centrifuged at 13000rpm for 10 minutes.
- The pellet was air dried completely before loading to the sequencer.

5. Sequencing in genetic analyzer

Reagent:

- Hi-Di^{TM} Formamide (Applied Biosystems, Foster City, USA)

Procedure

- The cycle sequenced, purified sample was re-suspended in 12µl of Hi-Di™ formamide.
- The sample was mixed well and microfuged. It was then loaded onto 96 well optical plate.
- The sample was then denatured in the thermal cycler at 95°C for 3 minutes.
- After denaturation, the plate was loaded and linked to the sequencer ABI 3100 *Avant* or 3730 Genetic analyzer (Applied Biosystems, Foster City, USA) for sequencing by capillary electrophoresis.
- The data obtained was viewed by ABI PRISM DNA Sequencing Analysis Software v. 5.1.1 and also analyzed by BioEdit sequence alignment editor v.7.2.5.

6. Target sequences

- ***AIPL1*– Exon 2**

GGGCCTTGAACAGTGTGTCTAGAGCAGAGTGCACCGTCTCGGTGACTAGG
TGATCTTTCATTTCCGCACCATGAAATGTGATGAGGAGCGGACAGTCATTG
ACGACAGTCGGCAGGTGGGCCAGCCCATGCACATCATCATCGGAAACATG
TTCAAGCTCGAGGTCTGGGAGATCCTGCTTACCTCCATGCGGGTGCACGA
GGTGGCCGAGTTCTGGTGCGACACCATCGTAAGTAGGCCCTGCGCGCCTG
TCTCCTGGGACTAGTCTTTTCTGGGCTCACCCACCCGCTTTGCGGG**GCTGC**
TGTGTTTCGGGAAA

- ***AIPL1– Exon 5***

GCAGCTGCCTGAGGTCATGGCTGACCTTCTCCCTGGGCAGGAGAAGCCAT
GGGAGGTGCAGTGGCTGAAGCTGGAGAAGATGATCAATACTCTGATCCTCA
ACTACTGCCAGTGCCTGCTGAAGAAGGAGGAGTACTATGAGGTGCTGGAG
CACACCAGTGATATTCTCCGGCACCACCCAGGTGCGCGGGGCTGCAGGGG
CGGACAGTGAGGGGGCGCCCAGCCCAGGGCCACGGAGACACCTGCCATA
GCCTTCCTGGA**CTTTTCTTTCCACCCCAC**

- ***AIPL1 – Exon 6***

CTGGGAAGGGAGCTGTAGCTGGATGCTCCCTGCTCCCCACAGGCATCGTG
AAGGCCTACTACGTGCGTGCCCGGGCTCACGCAGAGGTGTGGAATGAGGC
CGAGGCCAAGGCGGACCTCCAGAAAGTGCTGGAGCTGGAGCCGTCCATG

CAGAAGGCGGTGCGCAGGGAGCTGAGGCTGCTGGAGAACCGCATGGCGG
AGAAGCAGGAGGAGGAGCGGCTGCGCTGCCGGAACATGCTGAGCCAGGG
TGCCACGCAGCCTCCCGCAGAGCCACCCACAGAGCCACCCGCACAGTCAT
CCACAGAGCCACCTGCAGAGCCACCCACAGCACCATCTGCAGAGCTGTCC
GCAGGGCCCCCTGCAGAGCCAGCCACAGAGCCACCCCCGTCCCCAGGGC
ACTCGCTGCAGCACTGAGCCCCCTGAGGCCCACAGCCACCCAGGCAGGG
AGCAAGTGGCCTGGTCACTTCTGGTTCGATTGACCA**GGATCGTGGTGTCAC**
TTTT

- ***CEP290 - Intronic- c.2991_1655A/G***

ACAGGCACCCACCATCATGCCCGGCTAATTTTTTGTATTTTTAGTAGAGATG
GGGTTTCACCTTGTTAGCCAGGATGGTGTCGATCTCCTGAACTCGTGATCC
ACCCGCCTCGGCCTCCTAAAGTGCTGGGATTACAGATGTGAGCCACCGCA
CCTGGCCCCAGTTGTAATTGTGAATATCTCATACCTATCCCTATTGGCAGTG
TCTTAGTTTTATTTTTTATTATCTTTATTGTGGCAGCCATTATTCCTGTCTCTA
TCTCCAGTCTTACA**TCCTCCTTACTGCCACAAGAA**

- ***CEP290 –Exon 40***

GCCAAAATGAAACGTGCATATTTGATAAAATTTTGTTTGTGGGTGTTTGTTG
AAGATGAATTGCTTTACACTAGTTTTTTTTTTTTTTCTCAAAGTCGACTTTTTT
CCTCAAGGTAGACTTGACATGAATATGGAAAAATATATGTAGTTTGTGGTTA
TTTTTTTTCTCTTGTGTACTTAAAAATTCAGACTGAATTTTTCTTATAATGGTA
TATTTTCTGTTTTATGTTCCTTTTATCATTGATACTTCTTGAAGAGTCATGAAT
AATACCTTTCTTTTTCTCTTATTAGACACAAGTTGAAGATTTAAATGAAAATCT
TTTAAAATTGAAAGAAGCACTTAAAACAAGTAAAAACAGAGAAAACTCACTA
ACTGATAATTTGAATGACTTAAATAATGAACTGCAAAAGAAACAAAAAGCCT
ATAATAAAATACTTAGAGAGAAAGAGGAAATTGATCAAGAGAATGATGAACT
GAAAAGGCAAATTAAAAGACTAACCAGTGGATTACAGGTAATTTTATATTTAA
CTCTGATAATGTCTGATTTACAATATAGAGGTAGTAGTTTATTTCTACTTTAT
CATTTTATCT**ATGGTATTTGTTAAAACTGACTTTCA**

- ***CEP290 –Exon 18***

TGGAGGGATTTTGGAAACAATTATTCTACCTTTGCATTAAAACTTGATTGTA
GGTTTTAAGAATTAAAGTGTTGGAATAGTAGGAGGGTTATTTTAATGTTTTTA
GTTTGTTAATTCTCTTATATATAGGATTAACCACTGAGGACCTGAACCTAACT

GAAAACATTTCTCAAGGAGATAGAATAAGTGAAAGAAAATTGGATTTATTGA
GCCTCAAAAATATGAGTGAAGCACAATCAAAGGTAATAGTAAAGTATTGCAA
AGAGAGTAAAGGAAAATATTTTTTTTTTTTTTTTTTTTGAGACGGAGTCTCG
CTCTGTCTCCCAGGCTGGAGTGCAGTGGCGCGATCTCGGCTCACTGCAAG
CTCCGCCTCCCGGGTTCATGCCATTCTCCTGCCTCAGCCTCCCAAGTAGCT
GGGACTACAGGCGCCCGCCACCACGCCCGGCTAATTTTTTGTATTTTTAGT
AGAGACGGG**GTTTCACCGTTTTAGCCGG**

- ***CRB1* – Exon 5**

CCTCCTTTTAGGCAAATGCTCTATAATTCAACACCTTTGACTTAGCAGCTTC
TCTGAATTTTCATCATGCAGGATACACAGGTGCCCAGTGTGAGATCGACCT
CAATGAATGCAATAGTAACCCCTGCCAGTCCAATGGGGAATGTGTGGAGCT
GTCCTCAGAGAAACAATATGGACGCATCACTGGACTGCCTTCTTCTTTCAG
CTACCATGAAGCCTCAGGTTATGTCTGTATCTGTCAGCCTGGATTCACAGGT
GAGGCCAAGGAGATGGGATATGACTTGACTTTCTGGTATT**TTATGGCAGAC**
CATGGCTTT

- ***CRB1* – Exon 11**

TTCCCATTTCACAACCAATGTATTCAACAGGGACCTGGGTTTCTGCTGTTCT
GTTTATTTTGAAGGTGTGAAAAGGACATTGATGAGTGTGCCTCTGATCCGTG
TGTCAATGGAGGTCTGTGCCAGGACTTACTCAACAAATTCCAGTGCCTCTG
TGATGTTGCCTTTGCTGGCGAGCGCTGCGAGGTGGACGTAAGCAGCCTCT
CCTTTTATGTCTCTCTCTTATTCTGGCAGAATCTTTTTCAGCTTCTTTCTTAC
CTCAT**TTTGCGTATGAATGACGAGC**

- ***CRB1* – Exon 12a**

GCTTGCTCTGGTTGGTCTTCATTCCTGAGTAGTTCCATTGTCCTGAATATTT
ATTTGCCTTTGCTATAGAATTCGCATCCCAATGATTTCAATCTTTCCAGTTGG
CAGATGACTTGATCTCCGACATTTTCACCACTATTGGCTCAGTGACTGTCGC
CTTGTTACTGATCCTCTTGCTGGCCATTGTTGCTTCTGTTGTCACCTCCAAC
AAAAGGGCAACTCAGGGAACCTACAGCCCCAGCCGTCAGGAGAAGGAGGG
CTCCCGAGTGGAAATGTGGAACTTGATGCCACCCCCTGCAATGGAGAGACT
GATTTAGGAGCATTGTGTCCCTTCGAGATGGGGATCCACACACTGTGAATG
TGATGACTGTACTTCAGGTATCTCTGACATACCTGACAATGTTAATCTGCAA

CTGGGATTACACTGGAACTACAGGAATGATTCCTTTGACCACCTTAAA**AACT**
TTCACAGTGGTTCCGC

- ***CRX* – Exon 2**

GGATGGAATTCTTGGCATCCCACCCAGCCTCAGGGCCTCACACCAGCCCA
TGTGGATGACCTGAGGGTCCTGTTTCCCATCCCACCCCAGGCGCCCCCAG
GAAGCAGCGGCGGGAGCGCACCACCTTCACCCGGAGCCAACTGGAGGAG
CTGGAGGCACTGTTTGCCAAGACCCAGTACCCAGACGTCTATGCCCGTGA
GGAGGTGGCTCTGAAGATCAATCTGCCTGAGTCCAGGGTTCAGGTGGGGT
GGTGGGTCCCTGGACCCCTCCCGACACTTCCTGTGATCTCAG**GAGGCCTG**
CCCGGAACAAAGAG

- ***CRX* – Exon 3b**

CCTCCACAGATGTGTGTCCAGACCCTCTGGGCATCTCAGATTCCTACAGTC
CCCCTCTGCCCGGCCCCTCAGGCTCCCCAACCACGGCAGTGGCCACTGTG
TCCATCTGGAGCCCAGCCTCAGAGTCCCCTTTGCCTGAGGCGCAGCGGGC
TGGGCTGGTGGCCTCAGGGCCGTCTCTGACCTCCGCCCCCTATGCCATGA
CCTACGCCCCGGCCTCCGCTTTCTGCTCTTCCCCCTCCGCCTATGGGTCTC
CGAGCTCCTATTTCAGCGG**CCTAGACCCCTACCTTTCTCCCA**

- ***GUCY2D* – Exon 2a**

GGGTTACTCGGGCTTGGAGAAACTCGGGGTTACGGGGAGAACCCTAGGG
GAGGCCGGGGTCTCAGTCGCTCAGCCTGCTCCGTCTGTGTTCGCAGAAGC
CGGCAATGACCGCCTGCGCCCGCCGAGCGGGTGGGCTTCCGGACCCCGG
GCTCTGCGGTCCCGCGTGGTGGGCTCCGTCCCTGCCCCGCCTCCCCCGG
GCCCTGCCCCGGCTCCCGCTCCTGCTGCTCCTGCTTCTGCTGCAGCCCCC
CGCCCTCTCCGCCGTGTTCACGGTGGGGGTCCTGGGCCCCTGGGCTTGC
GACCCCATCTTCTCTCGGGCTCGCCCGGACCTGGCCGCCCGCCTGGCCGC
CGCCCGCCTGAACCGCGACCCCGGCCTGGCAGGCGGTCCCCGCTTCGAG
GTAGCGCTGCTGCCCGAGCCTTGCCGGACGCCGGGCTCGCTGGGGGCCG
TGTCCTCCGCGCTGGCCCGCGTGTCGGGC**CTCGTGGGTCCGGTGAAC**

- ***GUCY2D* – Exon 2b**

GTCCCCGCTTCGAGGTAGCGCTGCTGCCCGAGCCTTGCCGGACGCCGGG
CTCGCTGGGGGCCGTGTCCTCCGCGCTGGCCCGCGTGTCGGGCCTCGTG
GGTCCGGTGAACCCTGCGGCCTGCCGGCCAGCCGAGCTGCTCGCCGAAG

AAGCCGGGATCGCGCTGGTGCCCTGGGGCTGCCCCTGGACGCAGGCGGA
GGGCACCACGGCCCCTGCCGTGACCCCCGCCGCGGATGCCCTCTACGCC
CTGCTTCGCGCATTCGGCTGGGCGCGCGTGGCCCTGGTCACCGCCCCCCA
GGACCTGTGGGTGGAGGCGGGACGCTCACTGTCCACGGCACTCAGGGCC
CGGGGCCTGCCTGTCGCCTCCGTGACTTCCATGGAGCCCTTGGACCTGTC
TGGAGCCCGGGAGGCCCTGAGGAAGGTTCGGGACGGGCCCAGGGTCACA
GGTAGGCTCCCTTGCAGGGTGCGAGGAGGTCGGCTGGTCCTGCCGGCAG
CCGGACGGCGCCGCGAGCCAAGCCTCTGTCCGCAGCA**GTGATCATGGTG**
ATGCACTCGG

- ***GUCY2D* – Exon 3**

GGACGGCGCCGCGAGCCAAGCCTCTGTCCGCAGCAGTGATCATGGTGAT
GCACTCGGTGCTGCTGGGTGGCGAGGAGCAGCGCTACCTCCTGGAGGCC
GCAGAGGAGCTGGGCCTGACCGATGGCTCCCTGGTCTTCCTGCCCTTCGA
CACGATCCACTACGCCTTGTCCCCAGGCCCGGAGGCCTTGGCCGCACTCG
CCAACAGCTCCCAGCTTCGCAGGGCCCACGATGCCGTGCTCACCCTCACG
CGCCACTGTCCCTCTGAAGGCAGCGTGCTGGACAGCCTGCGCAGGGCTCA
AGAGCGCCGCGAGCTGCCCTCTGACCTCAATCTGCAGCAGGTAGACGGTC
CCGGGAGGAGGG**AAGAAGGCAAGGGAGAGGGGA**

- ***GUCY2D* – Exon 9**

CCCACATTGCCCTGGGCAGAAAATGCAAGTCAACTCTCCCCCTCTCAGCTC
CAGGAGCTCCGGCATGAGAACGTGGCCCTCTACCTGGGGCTTTTCCTGGC
TCGGGGAGCAGAAGGCCCTGCGGCCCTCTGGGAGGGCAACCTGGCTGTG
GTCTCAGAGCACTGCACGCGGGGCTCTCTTCAGGACCTCCTCGCTCAGAG
AGAAATAAAGCTGGACTGGATGTTCAAGTCCTCCCTCCTGCTGGACCTTAT
CAAGGTGTGTGTCTGGGGGTGGTG**GGGTGACGTCCTGGGGGCAGG**

- ***GUCY2D* – Exon 10**

AGCAGGCTGAGGCTGCCTCTTACCCTACCCATTCCAAGGGAATAAGGTATC
TGCACCATCGAGGCGTGGCTCATGGGCGGCTGAAGTCACGGAACTGCATA
GTGGATGGCAGATTCGTACTCAAGATCACTGACCACGGCCACGGGAGACT
GCTGGAAGCACAGAAGGTGCTACCGGAGCCTCCCAGAGCGGAGGGTAAG
AG TCCCCTGT**GCAGACGAGGATCCACCGGGA**

- ***GUCY2D* – Exon 11**

GCGGCGCCTCAGCCCCTTCCCCATCCCCAGACCAGCTGTGGACAGCCCCG
GAGCTGCTTAGGGACCCAGCCCTGGAGCGCCGGGGAACGCTGGCCGGCG
ACGTCTTTAGCTTGGCCATCATCATGCAAGAAGTAGTGTGCCGCAGTGCCC
CTTATGCCATGCTGGAGCTCACTCCCGAGGGTAAGGCTGCCCTGTG**CGTG
GAGTTCGGCCCACAGGG**

- ***GUCY2D* – Exon 14**

CAGCTGGAGCCCAGCCAGGTAGAGTGGCCCCCAGGTGACCTCACTGCCT
GCCATCCCTAGGTCTGTGGCTGAGGCCTTGAAGACGGGGACACCAGTGGA
GCCCGAGTACTTTGAGCAAGTGACACTGTACTTTAGTGACATTGTGGGCTT
CACCACCATCTCTGCCATGAGTGAGCCCATTGAGGTTGTGGACCTGCTCAA
CGATCTCTACACACTCTTTGATGCCATCATTGGTTCCCACGATGTCTACAAG
GTGCAGTGTGTAGGGGACAAGCCCTCCTGA**CCTTCAATTCAGCTTCACCA
G**

- ***GUCY2D* – Exon 15**

GTGGAGACAATAGGGGACGCCTATATGGTGGCCTCGGGGCTGCCCCAGC
GGAATGGGCAGCGACACGCGGCAGAGATCGCCAACATGTCACTGGACATC
CTCAGTGCCGTGGGCACTTTCCGCATGCGCCATATGCCTGAGGTTCCCGT
GCGCATCCGCATAGGCCTGCACTCGGGTAACTCCCGGGTCTTCCCAGGCT
CCAGCCCATCTCCCTCTTTAG

- ***GUCY2D* – Exon 16**

CCCCGAGGCCCTACCTAGGTGCAGCCCAGGGCCGGCCCTGCTAGCCCCG
CCGACCCCCAGCATCTCCACAGGTCCATGCGTGGCAGGCGTGGTGGGCCT
CACCATGCCGCGGTACTGCCTGTTTGGGGACACGGTCAACACCGCCTCGC
GCATGGAGTCCACCGGGCTGCGTGAGTGTG**ACGGGGACAAGACGGGGAG
GT**

- ***GUCY2D* – Exon 17**

GGTGAGTCCCGAGCTCACGGCGTCCCCCACCGCCACAGCTTACCGCATCC
ACGTGAACTTGAGCACTGTGGGGATTCTCCGTGCTCTGGACTCGGGCTACC
AGGTGGAGCTGCGAGGCCGCACGGAGCTGAAGGTGAGGCAGGGCC**CCAA
CCCCTCCCGGAGGCCCCG**

- ***IQCB1* – Exon 11**

CACAACAGCAGCAGATGACAAGCTATTTTATCATACCTTATTAACACTAGAA
ATTATCTTAAAATTTTTTCTGTTTACATTTATTTGATTACTAGTGAGGTTATGC
ATTTTTTCATATGATTTATGAAAATTATGTATAACACACTGTTTCCATCACAGA
TCCAAACGATCAAAGATGTTGCTGGAGATAAATAGGCAGAAGGAAGAAGAG
GACCTCAAATTACAATTGCAACTTCAAAGACAGAGAGCCATGAGACTTTCCC
GAGAATTGCAGCTGAGTATGCTCGAAATAGTTCATCCAGGTGAGTGGCAGT
ATCAACTTTATCAATATAAGAATATTTAGGTTATCTTTTAACAAA**CCAACTTTT**
CTAGCTACGTGATGA

- ***IQCB1* – Exon 13**

TCCCCTCCTTATACACACTCAGAGTTAATCATTATTGCAATGTGATCACTAC
CATATGTTGTAGTGAGAAAATAGTTCTATTAGTTAGGGGAGAAAAATCACAA
ACCTTCAATTTTTTTTCTTCCAGGCGCTTAAATTCCTAGCGAAGTGCCGTAA
GAAAAAGAAACTATTTGCTCCTTGGCGAGGACTCCAAGAACTCACTGATGC
ACGCCGAGTTGAACTGAAGAAACGAGTGGATGACTATGTCAGAAGACATTT
GGTATGAAGCTTCTGTACCATATCAGTATTACATTATTCCAATGCATGATATT
GATAACTGTACACACACACACATATATTGTGTGTGTGTGTGTGTGTGTGTGT
GTGTGTACATTTAT**TGCTGGTATAAGGTAATGCATTG**

- ***IQCB1* – Exon 14**

CCTCTGCTAAGTGGTTGGGTACTTTGATACCTATGTCCTCTCTGTGACAGG
TTTTTCTGTCCTGTGTTTCCAGGGCTCTCCAATGTCAGATGTGGTCAGTAGG
GAGCTCCATGCCCAAGCTCAAGAACGACTGCAACACTACTTTATGGGCAGG
GCCCTAGAAGAGCGAGCCCAGCAGCACAGAGAAGCTCTGATAGCACAGAT
CAGCACCAACGTTGAACAGCTAATGAGTATGTGCTCATTTTACTTTCGTTCT
CTAAAAGGAACAACCTATGCGAGGTTTGTGACATCCAAAACCTTTAGAAGG
AAACCATTACTAAGCATCTTTAGC**TCATCCCCTAACCTCAGGAAA**

- ***KCNJ13*- Exon 3a**

TGGCACCAAGAAATGTGTAAATATTTGATATACACTTGTCATTCAGAAAAAG
TATTGATATTTAAAGTCTTCATTCTAAAAAAAAATCAATAATTTCTTATTTAGG
TGCTTTTGTGGCGAAGATTGCCCGGCCAAAAAATCGAGCTTTTTCAATTCGC
TTTACTGACACAGCAGTAGTAGCTCACATGGATGGCAAACCTAATCTTATCT
TCCAAGTGGCCAACACCCGACCTAGCCCTCTAACCAGTGTCCGGGTCTCA

GCTGTACTCTATCAGGAAAGAGAAAATGGCAAACTCTACCAGACCAGTGTG
GATTTCCACCTTGATGGCATCAGTTCTGACGAATGTCCATTCTTCATCTTTC
CACTAACGTACTATCACTCCATTACACCATCAAGTCCTCTGGCTACTCTGCT
CCAGCATGAAAATCCTTCTCACTTTGAATTAGTTGTA**TTCCTTTCAGCAATG**
CAGG

- ***LCA5* – Exon 3**

CACTGCCATGTGTTATGTTTCATTGTTGGAATTTTTTTAAAGGACTGCTTTTT
ATGTATTTATACATATAGGAGCTATCGAAAAACCTTGAACTGAGTACTAACA
GTTTCCAACGACAGTTGCTTGCTGAAAGGAAAAGGGCATATGAGGCTCATG
ATGAAAATAAAGTTCTTCAAAAGGAGGTACAGCGACTATATCACAAATTAAA
GGTAAGTATTTTAAATTGCATTGTTTTCCATAATGTGTATTAGACGAAAATTA
AATATTTTAAAACACTAAATGTTACTATTCATATTTGTAAGTTGGTACAATATA
ATTTACAAAATTCTGATATTATCTTTA**GCACTTAGAAAAGGTTTGTTGG**

- ***LCA5* – Exon 5**

CATGCCAGCTGAATTCCATATATTTTAGCATTATTCATTGTAATAATTTTTTA
AAAATAATTTTCACTGTAGCTGCATGCCAGAGTGATTTTGCAGACCTGTGTA
CAAAAGGAGTACAAACCATGGAAGACTTCAAGCCAGAAGAATATCCTTTAAC
TCCAGAAACAATTATGTGTTACGAAAACAAATGGGAAGAACCAGGACATCTT
ACTTTGGTGAGTTTAGCAGTATTGTTATGTACCAAACTCGGTCTGAAGTTAT
TCCTAAAAACACAATATCAGACATTTTAAAATTTCAGTAATGAAGCTAGTACT
ATATCTATATGAATATG**CGACTAGCCTTTTTGAAGGG**

- ***LCA5* – Exon 6**

CCCCCTTTTCTATAATTTGTGAACTTGCATTTTTTTTAACGTATAATAGGACT
TGCAATCTCAAAAGCAAGACAGGCATGGAGAAGCAGGGATTCTAAACCCAA
TTATGGAAAGAGAAGAAAAATTTGTTACAGATGAAGAACTCCATGTCGTAAA
ACAGGAGGTTGAAAAGCTGGAGGATGGTAAGAAAAAGAGTTTGTTTAAGCA
TGTGACAAG**TCAGCATCCCTTGAGAAAGAA**

- ***LCA5* – Exon 7a**

AAATATGGTGGTTTTATGAAAGTTGATAGTTTTCCTATTTAAAAATATTGACT
GTTACAAAATCCCATTTAAAAAATTTGATTTCATACAGAATGGGAAAGAGAA
GAACTTGATAAAAAGCAAAAAGAAAAGGCATCTTTACTGGAAAGAGAAGAAA

AGCCAGAGTGGGAAACTGGAAGGTACCAACTAGGAATGTATCCAATTCAGA
ATATGGATAAATTGCAAGGAGAGGAAGAAGAAAGACTGAAGAGAGAAATGC
TACTTGCTAAACTGAATGAAATTGACAGAGAACTCCAAGATTCTCGAAATCT
AAAATAC**CCTGTTTTGCCATTGTTACCT**

- ***NMNAT1* – Exon 2**

GGTGGCAGAGCAAGACCTTATCTTAGGGAAAAAAAAATGCATAACTGAATT
TATTTTCTTTTTCCTTTGTAGACAACAAGGGAGGTGTCACAGTTTTCCATTTA
GATCAACAACTTCAAGTTCTTACCATGGAAAATTCCGAGAAGACTGAAGTGG
TTCTCCTTGTGGTTCATTCAATCCCATCACCAACATGCACCTCAGGTTGTTT
GAGCTGGCCAAGGACTACATGAATGGAACAGGTAGGAGCAGTAACCAAAA
GTGGCTTAAGACTAGAGAACCAGCCGGGTGCAGTGGCTCACACCTGCAAT
CCCAGCATTTTGGGAGGCTGAGACGGGCAGATCACAAGGTCAAGAGATCG
AGACCATCCTAGCCAACATGGTAAAACCCTGTCTCTACTAAAAAATA**CGAAA**
ATTCACTGTGCCTGT

- ***RD3* – Exon 1**

CTCTCTCCCCCTTGGGTTCCCAGGTTCCCCACTCTGACTAACAGAGGGGCC
CAGGAAAGATGCACATCTGTCCTGTCTCAGCAGGGCCAGGGGCTATGTCT
CTCATCTCATGGCTTCGGTGGAACGAGGCCCCATCCCGGCTGTCCACCAG
GAGCCCTGCTGAGATGGTGCTGGAGACGCTTATGATGGAGCTGACGGGGC
AGATGCGAGAGGCTGAGAGGCAGCAGCGGGAGCGCAGCAATGCGGTCAG
AAAGGTCTGCACCGGTGTGGACTACAGCTGGCTGGCCAGCACACCCCGGT
CCACCTATGACCTCAGCCCCATTGAGCGGTTGCAGCTGGAAGATGTCTGC
GTTAAGATCCACCCATCCTATTGTGGGCCTGCTATCCTCAGGTGAGCACTG
GGATGGGGACCCTTGCTGGGCTGCAGGCTCCAGGAAAGGTGGCTGCAGT
GGCAGGGGCC**TGAGGCCACCAGGACTAGAG**

- ***RDH12* – Exon 2**

ACCCTTCTTTGAGGCTGGATAGAGTTTTTTTTTTTTTTTTTAACGTATCTTAG
TGTGAGCTCGTGAAGGATGGTACGTGATGCTCTTGTTTCCCTTGCCGATAG
GAAGTTCTTTGCTGGTGGAGTGTGTAGAACAAATGTGCAGCTTCCTGGCAA
GGTAGTGGTGATCACTGGCGCCAACACGGGCATTGGCAAGGAGACGGCCA
GAGAGCTCGCTAGCCGAGGTAAGTGTTTCCCCTTTAGTCTCCAAAGGGCCA
TGCCTCCCACCCTTCTTCCCACTGGGGCCTCTGTCCATATTGCTTTGTGTTT

CCTCCTAGGCTTGGGGGCTCTGACTAGAAAT**TCAAGGAACCTGGGATTCA**
A

- ***RDH12* – Exon 6**

TTGCTGCAGGAGATAAGCTGTTTTCCTGGGCTCAGAGTGTGTCCCTGATCT
AATTGTGCCCTCTTTGTCCCAGGCACCGGGGTCACCACCTACGCAGTGCAC
CCAGGCGTCGTCCGCTCTGAGCTGGTCCGGCACTCCTCCCTGCTCTGCCT
GCTCTGGCGGCTCTTCTCCCCCTTTGTCAAGACGGCACGGGAGGGGGCGC
AGACCAGCCTGCACTGCGCCCTGGCTGAGGGCCTGGAGCCCCTGAGTGG
CAAGTACTTCAGGTGTGTGAAGGCAATGCGGTTCTCTCCACCACCTGTGTG
CATGGGAGGTGCCGGACTCGCTGGGCTGTTCATCCTGAGAAGCTGAGTTT
GTGCCTGATGATGCAATCCAGGTTTGGGTTGGGCCTGCAAACAGAATGCC
GTTGCTTTGTTAAGGAAACTTACAGTACAAACTTATGTGTTGGGAAGAGTTG
CTTTTCTGGCTTTATTTT**ATACTCGTGTGCCGCTTTTC**

- ***RPE65* – Exon 2**

CCCACCCAGCTGAGACTAGACCGGCAGGAGTGAACAGGCTTTGAGCCAG
CCCTAGAGTGCCTTCTCTCCTGCAGCTCTGCCTCTATCTCTGCGGACTTTG
AGCATCAACATGGGCTTCTTCCTTATTCTTCCACCATTTCAGGGTTGAGCAT
CCTGCTGGTGGTTACAAGAAACTGTTTGAAACTGTGGAGGAACTGTCCTCG
CCGCTCACAGCTCATGTAACAGGTTGGTCTCGCCCATCTTGAAGCCATCCT
CTTTTATGTCAGTCTCTCTTCTCTGGCTTCCTATTCCTTGGCGTCTCTCAGT
GCAGACTCCCCCTGTGGGTCACAGGGAGAGGGAT**CAGGTGGTTTGGGCTT**
TTTA

- ***RPE65* – Exon 4**

CTGTACGGATTGCTCCTGTCTATACTCTTCCCTATGTTTCAATGTCCTTCAG
GTTCATCCGCACTGATGCTTACGTACGGGCAATGACTGAGAAAAGGATCGT
CATAACAGAATTTGGCACCTGTGCTTTCCCAGATCCCTGCAAGAATATATTT
TCCAGGTTACTGAACCCAAACTGAATGTTACTCAAGACATTTTATATTAGCC
CTTTTTCTCTCATGGCTTGAAAATTACTGGACTGAAAAATTCATTTGTTTCTA
CAGGTTTTTTTTCTTACTTTCGAGGAGTAGAGGTTACTGACAATGCCCTTGT
TAATGTCTACCCAGTGGGGGAAGATTACTACGCTTGCACAGAGACCAACTT
TATTACAAAGATTAATCCAGAGACCTTGGAGACAATTAAGCAGGTGGGACA

CAGTGCTAGGTGATGTTCAGGAATTTAGAATTTGGAACTTAAAATTAATTCA
ACATAAATTATT**CATGCTGCGAATGTATGATTCTAA**

- ***RPE65* – Exon 9**

GTACACTTTTTTCCTTTTTAAATGCATCAAAATATTTTCCTCATTTTTCAAGG
TTTGGCTTCATATTGCTGACAAAAAAAGGAAAAAGTACCTCAATAATAAATAC
AGAACTTCTCCTTTCAACCTCTTCCATCACATCAACACCTATGAAGACAATG
GGTTTCTGATTGTGGATCTCTGCTGCTGGAAAGGGTAAGAAAGGACACTGG
ACAAATGGGACACCTCCCATTGTTCCTGGAAATTACGGGGTTTTTACAGAG
CTGCTG**CACTCAATCTGAATCACATCTAAAAC**

- ***RPE65* – Exon 10**

TTGTCATTGCCTGTGCTCATGTTTGACTTTTTATTTTTGCAGATTTGAGTTTG
TTTATAATTACTTATATTTAGCCAATTTACGTGAGAACTGGGAAGAGGTGAA
AAAAAATGCCAGAAAGGCTCCCCAACCTGAAGTTAGGAGATATGTACTTCC
TTTGAATATTGACAAGGTAACCTGCTTCTCTGTAGATTTCAGATTTAA**CCAG
AATGTTTCATCTCTCTCA**

- ***RPE65*- EXON 12**

CACACGGGAGTGAACAAATGTTTCTTTCAAAGAGATTAAGAGTTTTCCTAA
GCATGTGCTCTATTTCGTAGCATTTGAGTTTCCTCAAATCAATTACCAGAAG
TATTGTGGGAAACCTTACACATATGCGTATGGACTTGGCTTGAATCACTTTG
TTCCAGATAGGGTAATTAATCCTTCTTACTAATA**TTTGAACAGTGCTTTGAGT
ATATGC**

- ***RPE65* – Exon 13**

GCATATTGACTGATTGCTTGATTGATTTTTCTTTCTCACAAACAGCTCTGTAA
GCTGAATGTCAAAACTAAAGAAACTTGGGTTTGGCAAGAGCCTGATTCATAC
CCATCAGAACCCATCTTTGTTTCTCACCCAGATGCCTTGGAAGAAGATGATG
GTAATGAAAGCAATTGTTGTGT**CTGAATACTCTTCTTACTGC**

- ***RPE65* – Exon 14**

AGTCAGAAAAAGAAGTCAGGTCATATGGTTTTCTATATTTGTCAATGTAATA
CCTCCTATATTATTTCAATGACATTCAATCTATAGCTTGGGCTTTTAAAAACT
CAATATTGCCTAATTTACTTCTGATAAACAGGTGTAGTTCTGAGTGTGGTGG
TGAGCCCAGGAGCAGGACAAAAGCCTGCTTATCTCCTGATTCTGAATGCCA
AGGACTTAAGTGAAGTTGCCCGGGCTGAAGTGGAGATTAACATCCCTGTCA

CCTTTCATGGACTGTTCAAAAAATCTTGAGCATACTCCAGCAAGATATGTTT
TTGGTAGCAAAACTGAGAAAATCAGCTTCAGGTCTGCAATCAAATTCTGTTC
AATTTTAGCCTGCTATATGTCATGGTTTTAACTTGCAGATGCGCACAATTTTG
CAATGTTTTACAGAAA**GCACTGAGTTGAGCAAGCAAT**

- ***RPGRIP1*** **– Exon 6**

AGGGCATAGTCAAGGAGAAAATGTCTTTAATTCTATCCATGTTCAGACAGA
ATAATTTAGCGCCTTTCTCTGCAGAGCTTCCATTAAAGAGAAGGTAGAGCTG
ATTCGACTTAAGAAGCTCTTACATGAAAGAAATGCTTCATTGGTTATGACAA
AAGCACAATTAACAGAAGTTCAAGAGGTGAGTTGCCATCATCAGCTGTGCT
TTCTTGGTGGGGGGAAACCCCAATTAAGAGATTCATATTATTTTCTCCATTTT
AAA**TATGAGAAGCCACAATTCAG**

- ***RPGRIP1*** **– Exon 14**

GAAAGAGCTCCCTACCCTTTAACGGATAGGCAGCTTTCTTTCCCCTCTAGA
ACAGCTCAAAGATGTTGCTTATGGCACCCGACCGTTGTCGTTATGTTTGGAA
ACACTGCCAGCCCATGGAGATGAGGATAAAGTGGATATTTCTCTGCTGCAT
CAGGGTGAGAATCTTTTTGAACTGCACATCCACCAGGCCTTCCTGACATCT
GCCGCCCTAGCTCAGGCTGGAGATACCCAACCTACCACTTTCTGCACCTAT
TCCTTCTATGACTTTGAAACCCACTGTACCCCATTATCTGTGGGGCCACAGC
CCCTCTATGACTTCACCTCCCAGTATGTGATGGAGACAGATTCGCTTTTCTT
ACACTACCTTCAAGAGGCTTCAGCCCGGCTTGACATACACCAGGCCATGGC
CAGTGAACACAGCACTCTTGCTGCAGGATGGATTTGCTTTGACAGGGTGCT
AGAGACTGTGGAGAAAGTCCATGGCTTGGCCACACTGATTGGTAAGTGCC
GTTGGCTTCCTGCGGCTCCTAA**GCACCAATGCAGAATTTCC**

- ***RPGRIP1*** **– Exon 21**

CTTGGAGCCTCACTAACCTTTAGGAACTAAATAAACATTTTCCTTATCAGGA
TTCAGAGAAGATGTGCATTGAAATTGTCTCCCTGGCCTTCTACCCAGAGGC
AGAAGTGATGTCTGATGAGAACATAAAACAGGTGTATGTGGAGTACAAATTC
TACGACCTACCCTTGTCGGAGACAGAGACTCCAGTGTCCCTAAGGAAGCCT
AGGGCAGGAGAAGAAATCCACTTTCACTTTAGCAAGGGTGAGGCATCCTGT
GTGGTTACTGG**GGTGAGGAAGTCTGATGAA**

- ***RPGRIP1* – Exon 24**

AGTGTTCAACTGAGTGATGCTGTTTTTTTCCCTTTCCCAACAGTTGTTAGCC
CTGAAGATCTGGCTACCCCAATAGGAAGGCTGAAGGTTTCCCTTCAAGCAG
CTGCTGTCCTCCATGCTATTTACAAGGAGATGACTGAAGATTTGTTTTCATG
AAGGAACAAGTGCTATTCCAATCTAAAA**GTCTCTGAGGGAACCATAGT**

- ***SLC19A2* – Exon 2**

CCAGGTCCTTTCATCACTAATGTTTTTTGTTTTTTTTTTTTCTCTTTCTACCAG
GTCTTCAATGAAATTTATCCAGTATGGACTTACTCTTACCTGGTGCTACTGTT
TCCTGTGTTCCTTGCCACAGACTACCTCCGTTATAAACCTGTTGTTCTACTG
CAGGGGCTCAGCCTTATTGTTACATGGTTTATGCTGCTCTATGCCCAGGGA
CTGCTGGCCATTCAATTTCTAGAATTTTTTTATGGCATCGCCACAGCCACTG
AAATTGCCTATTACTCTTATATCTACAGTGTGGTGGACCTGGGCATGTACCA
GAAAGTCACAAGTTACTGTCGAAGTGCCACTTTGGTGGGCTTTACAGTGGG
CTCTGTCCTAGGGCAAATCCTTGTCTCAGTGGCAGGCTGGTCGCTGTTCAG
CCTGAATGTCATCTCTCTTACCTGTGTTTCAGTGGCTTTTGCTGTGGCCTGG
TTTTTACCTATGCCACAGAAGAGCCTCTTCTTTCACCACATTCCTTCTACCTG
CCAGAGAGTGAATGGCATCAAGGTACAAAATGGTGGCATTGTTACTGACAC
CCCAGCTTCTAACCACCTTCCTGGCTGGGAGGACATTGAGTCAAAAATCCC
TCTAAATATGGAGGAGCCTCCCGTGGAGGAACCGGTAAGCTCAGCCTTAAA
TATCT**TGTAATTGCTACTATGGGGGC**

- ***SPATA7*– Exon 1**

GTCGGCTCCTCTTTTCCAGTCCTCCACTGCCGGGGCTGGGCCCGGCCGCG
GGAAGGACCGAAGGGGATACAGCGTGTCCCTGCGGCGGCTGCAAGAGGA
CTAAGCATGGATGGCAGCCGGAGAG**GTAAAGGGCAGCTGTCAGGG**

- ***SPATA7 – Exon 3***

ACAGCTGCAAGGTCTGGAACATTTTGTGATTTCCACATTTGGTATTTGTCAT
TTATAAATACTGACAATATTATTAAACTATTACCTTTCTCTTTCAGCTGCAGTA
GACTGCTCGGTTCCAGTAAGCGTGAGTACCAGCATAAAGTGTAAGTAATTTT
TGGACATTATTACCTTTTTAAAAAAAAATTAAGGTAAATATACATAACATAAAA
TTTACTGTCTTAACCATTTTTAAGTGTCCAGTTCAATATTAAGTACATTAATAT
TGTGGTACAATGATAACTACT**ACCTCCAGAACTCTCTTTATACTGC**

- ***SPATA7*– Exon 6**

TTCTAGCCAGTAAACCTTGTTACCACAGTGCTTATATTTTGAAGGATTAACA
ATTATTTATTTTAAATTATTACAGCTTTAAATCTGAGTTGGGGACAGCTGAGA
CTAAAAACATGACAGATTCAGAAATGAACATAAAGCAGGTAATAAGTATGAA
ATCTTTTGGTATTGCTACATTTGAATTACAGATGTTTTTCAGTAAATAGAATAT
GTACAGCTATTTAGACTGT**GAAGAATAATCTGTTGGTGG**

- ***SPATA7*– Exon 7**

AAAAAGTGCTGGATGGATAGAAAAATTATTCTAAAATATTCTTTGTCGTACT
GTATAATTTTTATCTACTGGATATCTCTGTTCAATCACTTAAAATTTGCTGTGT
TATATTCTGCTTTCGTAATGTATTTTTAAGCAAAAATAAATATTTTTTTTATTG
CATCCCCAGGCATCTAATTGTGTGACATATGATGCCAAAGAAAAAATAGCTC
CTTTACCTTTAGAAGGGCATGACTCAACATGGGATGAGATTAAGGATGATG
CTCTTCAGCATTCCTCACCAAGGTAAACAGTTCACAGGAGAAATAATTTCAA
CTGTCTTTAATTGCCTTCTTGTATAACAGACATATAGTATTTGCCGCATAAGT
ACTATTTAATTGCATGCTCCAATCTATTTTGGTGTTATGTAATTCAATAACAG
TTTTAGTTTTACCAAGGAAATTTTACAAAT**TGAAGTGCTTAGTAAAAGTTGA
AATG**

- ***SPATA7*– Exon 10**

CAACCTTTGTAGTTTCAGTGTTACGTAGCTAGTTTATATTTAGATGATTTTCT
GATTTTGAGACATTAACATTTTTGTTTATCATTTGTAGGTTTTTAGAACGACT
GTTCGAGCGACATATAAAACAAAATAAACATTTGGAGGAGGTTTGTCTTTCC
TTATAACTTCATTAGAAAAATTATAATGTAAAAATAATTGTATGGTTTTTTTCA
TACCTT**CAAACAATACATTAAAAGCAAGTGC**

- ***SPATA7*– Exon 11c**

AGAACGAGATATTCCCTTCACCAACTGAATTTTTCATGCCTATTTATAAATC
AAAGCATTCAGAAGGGGTTATAATTCAACAGGTGAATGATGAAACAAATCTT
GAAACTTCAACTTTGGATGAAAATCATCCAAGTATTTCAGACAGTTTAACAG
ATCGGGAAACTTCTGTGAATGTCATTGAAGGTGATAGTGACCCTGAAAAGG
TTGAGATTTCAAATGGATTATGTGGTCTTAACACATCACCCTCCCAATCTGTT
CAGTTCTCCAGTGTCAAAGGCGACAATAATCATGACATGGAGTTATCAACTC
TTAAAATCATGGAAATGAGCATTGAGGACTGCCCTTTGGATGTTTAATCTTC
ATTAATAAATA**CCTCAAATGGCCAGTAACTCA**

- ***TULP1*– Exon 11**

TTCCCATCCTCTCACCTGTCTCCCCTTTTCCCCAGGTGTTCCTCTTGGCTGG
CAGGAAACGAAAACGGAGCAAGACAGCCAATTACCTCATCTCCATCGACCC
TACCAATCTGTCCCGAGGAGGGGAGAATTTCATCGGGAAGCTGAGGTGGG
GCTGGGCTTCCTGGGGCTGGGGGGACTTGGGTCAGCAAAAGGCCATAGC
CCGTCATCTCTGTCCTGCTGTGCCT**GGTAGAGCACCCACCACAGT**

- **SPATA7RT1 – Exon 7-10**

CCAGTGGGGATCTTTTGGATAAACATTCTGAACTCTTTTCTAACAAACAATT
GCCATTCACTCCTCGCACTTTAAAAACAGAAGCAAAATCTTTCCTGTCACAG
TATCGCTATTATACACCTGCCAAAAGAAAAAAGGATTTTACAGATCAACGGA
TAGAAGCTGAAACCCAGACTGAATTAAGCTTTAAATCTGAGTTGGGGACAG
CTGAGACTAAAAACATGACAGATTCAGAAATGAACATAAAGCAGGCATCTAA
TTGTGTGACATATGATGCCAAAGAAAAAATAGCTCCTTTACCTTTAGAAGGG
CATGACTCAACATGGGATGAGATTAAGGATGATGCTCTTCAGCATTCCTCAC
CAAGGGCAATGTGTCAGTATTCCCTGAAGCCCCCTTCAACTCGTAAAATCTA
CTCTGATGAAGAAGAACTGTTGTATCTGAGTTTCATTGAAGATGTAACAGAT
GAAATTTTGAAACTTGGTTTATTTTCAAACAGGTTTTT**AGAACGACTGTTCG**
AGCGAC

- **SPATA7RT2 – Exon 9-11**

CCAAGGGCAATGTGTCAGTATTCCCTGAAGCCCCCTTCAACTCGTAAAATC
TACTCTGATGAAGAAGAACTGTTGTATCTGAGTTTCATTGAAGATGTAACAG
ATGAAATTTTGAAACTTGGTTTATTTTCAAACAGGTTTTTAGAACGACTGTTC
GAGCGACATATAAAACAAAATAAACATTTGGAGGAGGAAAAAATGCGCCAC
CTGCTGCATGTCCTGAAAGTAGACTTAGGCTGCACATCGGAGGAAAACTCG
GTAAAGCAAAATGATGTTGATATGTTGAATGTATTTGATTTTGAAAAGGCTG
GGAATTCAGAACCAAATGAATTAAAAAATGAAAGTGAAGTAACAATTCAGCA
GGAACGTCAACAATACCAAAAGGCTTTGGATATGTT**ATTGTCGGCACCAAA**
GGATG

- ***SLC19A2*–EXON1**

CAATGGAAGAGCAGGCAAGTATTCCCGGCGTCCGCTGTGATTGGTTCCCG
GAGTGGAGGCGGTGGCAGAGGGTGGGCCTTAGGACGGGTCTCCCTTAAA
CTGGGCGATCAGGCAGCGACCCTAGAGGCGTCTGTAGGGTAAAGCTGGG

GGTTCTGTAGCCGGAGGCGGCGGCGAGTCCAGAACGTCCTGGCCTTACAG
GGAGAAGGCGTCACTCGCGGTTACAAGTGCCTGACCCTCACTCCAGTTGG
CGGAGGAGGAGAAGGAAGGGGCCGGGCCGGGTCCCCTCCCCTCGCGCC
CCGGATGGATGTGCCCGGCCCGGTGTCTCGGCGGGCGGCGGCGGCGGC
GGCCACTGTGCTCCTGCGGACCGCTCGGGTCCGTCGCGAATGCTGGTTCT
TGCCGACCGCGCTGCTCTGCGCCTACGGCtTTCTTCGCCAGCCTCAGGCCG
TCCGAGCCCTTCCTGACCCCGTACCTGCTGGGGCCGGACAAGAACCTGAC
CGAGAGGGAGGTACGCGCGGCGGACGCGGGGCGCGGGAAGGGCGGGCG
ACCGGCCTGCGGGTGGGCAGCGGAGGA**AGAGAGGACCGAGAAAAGCG**

- ***SLC19A2*- EXON 2**

CCAGGTCCTTTCATCACTAATGTTTTTTGTTTTTTTTTTTTCTCTTTCTACCAG
GTCTTCAATGAAATTTATCCAGTATGGACTTACTCTTACCTGGTGCTACTGTT
TCCTGTGTTCCTTGCCACAGACTACCTCCGTTATAAACCTGTTGTTCTACTG
CAGGGGCTCAGCCTTATTGTTACATGGTTTATGCTGCTCTATGCCCAGGGA
CTGCTGGCCATTCAATTTCTAGAATTTTTTTATGGCATCGCCACAGCCACTG
AAATTGCCTATTACTCTTATATCTACAGTGTGGTGGACCTGGGCATGTACCA
GAAAGTCACAAGTTACTGTCGAAGTGCCACTTTGGTGGGCTTTACAGTGGG
CTCTGTCCTAGGGCAAATCCTTGTCTCAGTGGCAGGCTGGTCGCTGTTCAG
CCTGAATGTCATCTCTCTTACCTGTGTTTCAGTGGCTTTTGCTGTGGCCTGG
TTTTTACCTATGCCACAGAAGAGCCTCTTCTTTCACCACATTCCTTCTACCTG
CCAGAGAGTGAATGGCATCAAGGTACAAAATGGTGGCATTGTTACTGACAC
CCCAGCTTCTAACCACCTTCCTGGCTGGGAGGACATTGAGTCAAAAATCCC
TCTAAATATGGAGGAGCCTCCCGTGGAGGAACCGGTAAGCTCAGCCTTAAA
TATCT**TGTAATTGCTACTATGGGGGC**

- ***SLC19A2* - EXON 3**

TGGGCCTGTAAATTGCTTTCTATATAATTGCCTTCATCAGAGTTTGAGCATT
GTTCCCTTTTGCTATGTAGGAACCCAAGCCAGACCGTCTCCTGTATTGAAAG
TACTATGGAATGATTTCCTGATGTGCTACTCCTCTCGCCCTCTTCTCTGCTG
GTCTGTGTGGTGGGCCCTCTCTACCTGTGGCTATTTTCAAGTTGTGAACTAC
ACACAGGGCCTGTGGGAGAAAGTGATGCCTTCTCGCTATGCTGCTATCTAT
AATGGTGGCGTGGAGGCCGTTTCAACCTTACTGGGTAAGCAATGCAGGATA
ATAGAGGGTCGTGGTGGGGGAGGGATTCCTCTGGT**AGTTAAGCCAGCCAT**
AATCA

- ***SLC19A2* - EXON 4**

GCAACAGCATTTGTGTAGCAAAATAATTGGATTTTTTTTGTTTGTTCTGTAG
GTGCTGTTGCTGTGTTTGCAGTTGGTTATATAAAAATATCCTGGTCAACTTG
GGGAGAAATGACATTATCTCTCTTTTCTCTCCTGATTGCTGCTGCAGTGTAT
ATCATGGACACTGTGGGTAACATTTGGGTGTGCTATGCATCCTATGTTGTCT
TCAGAATCATCTACATGTTACTCATCACGATAGCAACGTATGTATTTTGGCT
CATAGAAGCCTTAGGAAAAATTGTAACAGGATAAATTTTTTGGTTTCTTTACA
AACCAAAATATTCAAGTTTCTAGTTAAATGAGGCAAATGGGAGGAAGCATTG
TATTATATGTGCATTATCAGTTTTTCTCCTGAGGAAGCATT**TAGGCAAGTTTT**
GTTTATTCCCA

- ***SLC19A2* - EXON 5**

GTTGGAAAGGCAATTGACAGTAGAAAGAAGGACTCTTCATGTTTAAAGATA
AATGTTTACTTTTATTTAGAATAAATATTATAAGTACTCCAGAAGTAATTCTTA
TTTATTCACTCTTTTATTTTTTTTTTAAGTTTTCAAATTGCTGCAAACCTCAGCA
TGGAACGCTATGCCCTAGTATTTGGTGTAAATACCTTCATTGCCCTGGCACT
GCAGACGCTGCTCACTCTAATTGTGGTAGATGCCAGTGGCCTTGGATTAGA
AATTACCACTCAGGTAAG**ATCTCTCCTTTGGCATCGAA**

- ***SLC19A2* - EXON 6**

GGCACGTGGTGTAAGTATGCAATATTTTTTGAGGCTTTATGGACAAATTCTGA
ATATACTTTATTATAGAGTAATCCTCTTGTCATTGATAATTCAATAAACTGCTTT
TCCCCCTTCTAGTTTTTGATCTATGCCAGTTATTTTGCACTCATCGCTGTGGT
TTTCCTGGCCAGTGGTGCAGTCAGTGTTATGAAGAAATGTAGAAAGCTGGAA
GATCCACAATCAAGTTCTCAAGTAACCACTTCATAATATACTGCTGAAGGGCT
TCTTCTTAT**AGCAAGAACTCTGCACAGCA**

The sequence in bold indicate the forward and reverse primer sequences.

LIST OF PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

- **Natarajan N. Srikrupa**, Sundaramurthy Srilekha, Parveen Sen, Tharigopala Arokiasamy, Swaminathan Meenakshi, Muna Bhende, Suman Kapur and Nagasamy Soumittra. Genetic Profile and Mutation Spectrum of Leber Congenital Amaurosis in a Larger Indian Cohort using High Throughput Targeted Re-sequencing. *Clin Genet*. 2017 Oct 25. doi: 10.1111/cge.13159. [Epub ahead of print].

- **Srikrupa NN**, Meenakshi S, Arokiasamy T, Murali K, Soumittra N. Leber's congenital amaurosis as the retinal degenerative phenotype in thiamine responsive megaloblastic anaemia: a case report. *Ophthalmic Genet*. 2014 Jun; 35(2):119-24. doi: 10.3109/13816810.2013.793363. PMID: 23638917.

- Srilekha S, Arokiasamy T, **Srikrupa NN**, Umashankar V, Meenakshi S, Sen P, Kapur S, Soumittra N. Homozygosity Mapping in Leber Congenital Amaurosis and Autosomal Recessive Retinitis Pigmentosa in South Indian Families. *PLoS One*. 2015; 10(7):e0131679. doi: 10.1371/journal.pone.0131679. PMID: 26147992.

- Bende P, **Natarajan K**, Marudhamuthu T, Madhavan J. Severity of familial isolated retinitis pigmentosa across different inheritance patterns among an Asian Indian cohort. *J Pediatr Ophthalmol Strabismus*, 2013; 50(1):34-6. PMID: 23463886.

- Dhandayuthapani Sudha, Srividya Neriyanuri, **Srikrupa Natarajan**, Mamatha Gandra, Arokiasamy Tharigopala, SathyaBharathi Ravichandran, Al Ameen Muhammed, Umashankar Vetrivel, Lingam Gopal, VikasKetan, Rajiv Raman, Subbulakshmi Chidambaram and Jayamurugapandian Arunachalam. Variable disease severity among XLRS patients with similar mutant RS1 secretion profile: An insight on the localization of mutant RS1. *Experimental Eye Research*. (Under review)

PRESENTATIONS

- **Natarajan N. Srikrupa**, Srilekha Sundar, Swaminathan Meenakshi, Parveen Sen, Muna Bende, Tharigopala Arokiasamy, Suman Kapur, Nagasamy Soumittra. High throughput gene screening of patients with Leber Congenital Amaurosis in an Indian Cohort. NextGen Genomics, Biology, Bioinformatics and Technologies (NGBT) Conference 2016, Cochin. **Awarded Travel fellowship.**

- **Natarajan N. Srikrupa,** Srilekha Sundar, Swaminathan Meenakshi, Parveen Sen, Muna Bende, Tharigopala Arokiasamy, Suman Kapur, Nagasamy Soumittra. Swarnalatha Punshi Award presentation 2015. Vision research foundation, Chennai. **Awarded Swarnalatha Punshi Award for Best Research in Basic Science.**

- **Natarajan Srikrupa N**, Swaminathan Meenakshi, Bhende Muna, Tharigopala Arokiasamy, Nagasamy Soumittra.Screening of LCA9 locus gene, *NMNAT1*, in LCA cases of Indian cohort. ASIA ARVO 2013, New Delhi. **Awarded Second Best poster.**

- **Srikrupa Natarajan,** Meenakshi S, Kaushik Murali, Nagasamy Soumittra. A novel mutation in *SLC19A2* gene in a south Indian patient with thiamine-responsive megaloblastic anaemia (TRMA). Indian Society of Human Genetics 2012, Chandigarh.

- **Srikrupa Natarajan,** Manoharan Aarthi, Ronnie George, Manmath Kumar Das, VL Ramprasad, G Kumaramanickavel, Lingam Vijaya, Sripriya S, Parveen Sen. Association between the indel variant in the *LOC387715/ ARMS2* gene and Age-related Macular Degeneration in South Indian population. Indian Eye Research Group 2010, Hyderabad. **Awarded Travel fellowship.**

- D Sudha, **Srikrupa Natarajan**, T Karthiyayini, S Muthukumaran, V Umashankar, L. Gopal, VikasKhetan, A Jayamuruga Pandian. Molecular characterization of *RS1* gene mutants implicated in retinoschisis. Indian Society of Human Genetics 2012, Varanasi.

- Sudha. D, Srividya. N, **Srikrupa. N. N**, Dharanija. M, Mamatha. G, Karthiyayini. T, Arokiasamy. T, SathyaBharathi. R, Muthukumaran. S, Al Ameen. M, Umashankar. V, Lingam. G, Vikas. K, Rajiv. R, Subbulakshmi. J, JayamurugaPandian. A. Nonsecreted mutant retinoschisin localizes to the plasma membrane. Indian Society of Human Genetics 2016, Chennai.

BRIEF BIOGRAPHY OF THE CANDIDATE

Ms. Srikrupa N N completed her bachelor's degree in Biochemistry at Dr. MGR Janaki College of arts and science for women affiliated to University of Madras, Chennai in the year 2006. She joined Medical Research Foundation, Sankara Nethralaya for her post-graduation degree in MS(MLT) (2006-2009) affiliated to BITS Pilani, Following her post-graduation, she joined the SNONGC department of Genetics and Molecular biology as a research fellow in a DBT funded project on age related macular degeneration (AMD) for a year (2009-2010). She was awarded a travel fellowship to present this work in IERG (2010) conference. She was then deputed in a 3 year DBT funded project on "Molecular diagnostics of LCA - a chip based resequencing approach". During this fellowship tenure she registered for PhD in BITS Pilani, under the guidance of Dr. N. Soumittra from Vision Research Foundation, Chennai and Prof. Suman Kapur from BITS Pilani, Hyderabad campus as co-supervisor. She had attended national and international conferences. She was awarded travel fellowship to attend and present her work in Indian Eye Research Group (IERG) 2010 and NextGen Genomics, Biology, Bioinformatics and Technologies (NGBT) Conference 2016. She is a recipient of the Swarnalatha Punshi Award for Best Research in Basic Science (2015) by Vision Research Foundation and awarded best poster in ASIA ARVO, the association for research in vision and ophthalmology (2013). She has 3 published research papers, and two manuscripts under revision. She has hands on experience in performing molecular biology techniques like PCR and its variants, Sanger sequencing, analysis and interpretation of next generation sequencing (NGS) data, cloning, transformation and tissue culture techniques. Her area of research is on genetics of retinal degenerative disease specifically Leber congenital amaurosis, mutation screening by NGS, phenotype genotype correlation and assisting in genetic counselling.

BRIEF BIOGRAPHY OF THE SUPERVISOR

Dr. N.Soumittra, Associate Professor and Principal Scientist, joined as Senior Scientist in the department of Genetics and Molecular Biology, Vision Research Foundation, Chennai in 2006. She completed her PhD from University of Madras and her doctoral work was on genetics of hereditary cancers. She is a recipient of Best outgoing student in biochemistry and overall Best outgoing student in the post graduate programme, MSMLT. She is UGC-CSIR lectureship qualified and was awarded "Best Paper award**"** by the Indian Society of Oncology Conference, 2004, "Young Scientist award" for the year 2004by the Indian Society of Human Genetics and "Best free paper" in Genetics at Asia ARVO 2013 conference. Her current research interests are genetics of retinal diseases and corneal dystrophies. Her technical areas of expertise include medium and high through put genotyping and expression analyses studies like linkage analyses, homozygosity mapping, next generation sequencing analyses (targeted re-sequencing and whole exome sequencing) and whole transcriptome expression using microarray. She has received grants from DBT and ICMR, Govt. of India, as principal and co-investigator and has two current and four completed grants to her credit including an ICMR-INSERM collaborative project. She has published twenty-two research articles in international journals with two publications in Nature Genetics and two book chapters. She is involved in teaching genetics and molecular biology courses to post graduate students and is also a recognized PhD guide by SASTRA University, Thanjavore and University of Madras. Currently, she has two PhD students under her supervision and one candidate who has completed her PhD. Dr. N. Soumittra is a genetic counselor and is involved in molecular diagnostics of inherited ocular diseases.

BRIEF BIOGRAPHY OF THE CO-SUPERVISOR

Dr. Suman Kapur joined BITS Pilani on 17th July 2004 as Professor in the centre for Biotechnology, department of Biological Sciences. She is presently working as the Dean, International Programs and Collaboration Division since June 2012. She has earlier served in several senior administrative positions like Dean, Research & Consultancy at the Hyderabad Campus and Chief of Community Welfare and International Relations Unit at BITS-Pilani.

Dr. Kapur is a popular teacher at BITS, Pilani and has been instrumental in introducing several new courses, namely MPH G513, MPH G522, MPH G692, MPH G539, MPH G521, MPH G681,BIO G515 and has developed the curriculum for a new degree program “Master of Public Health”, incorporating learning through field visits and interdisciplinary teaching. Several of her students have gone to make excellent careers for themselves as CEO’s of start up companies and faculty at some of the best institutions in USA. She has published more than 100 research articles in International and national journals.

Dr. Kapur’s research interests lie in identifying biomarkers for unravelling the genetic basis of human diseases such as psychiatric disorders like depression, schizophrenia, addiction and Alzheimer’s disease and metabolic disorders such as diabetes (T2DM), obesity, cataract and metabolic syndrome. Early and specific diagnosis is the backbone of effective treatment and reduction of both disease associated morbidity and mortality. Ours is the first group to show that in the Indian population a mutation in the mu opiate receptor is linked to risk for addiction to opiates, a mutation in the Ob (leptin) gene may be linked to hypertension in depressed individuals and similarly mutations in *CRYGA& B*, *SPHK* and *SPAG 16* genes are linked to Cataract.

Modern day integration of electronics and biological possibilities on an integrated chip can be successfully used to develop POC devices, especially suited for low-cost settings and our group has already developed two such devices for bacterial antibiotic susceptibility and blood glucose monitoring. She has founded a biotechnology start up company for commercialization of the same.

Revival of research on Traditional Medicine/Herbal Remedies with a locally relevant evidence-based, disease-oriented approach is particularly relevant for India. Her group is also involved in developing clonal variants of Indian Medicinal plants and screening natural products for anti-diabetic, anti-inflammatory and anti-obesity activities in specific animal models for these diseases. Several industry sponsored projects are also in progress for bio-conversion, remediation & effluent treatment using consortia of microbial populations.