Genetic Analysis of Children with Congenital Ocular Anomalies in Three Ecological Regions of Nepal: A Phase II of Nepal Pediatric Ocular Diseases Study

Srijana Adhikari (srij_a@yahoo.com)
Tilganga Institute of Ophthalmology https://orcid.org/0000-0003-4894-9533
Neelam Thakur
National Academic of Medical Sciences
Ujjowala Shrestha
Sudrishti Eye Centre
Mohan Shrestha
Tilganga Institute of Ophthalmology
Murari Man Shrestha
TUTH
Bijaya Thapa
Patan Academy of Health Sciences
Manish Poudel
Tilganga Institute of Ophthalmology
Ajay Kunwar
Kathmandu Centre for Genomics and research laboratory

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Abstract

Background: Genetic eye diseases constitute a large and heterogeneous group of childhood ocular morbidity. Individual diseases may cause multiple structural anomalies and developmental features. The Nepal Pediatric Ocular Disease Study (NPODS) was a population-based epidemiological study conducted across three ecological regions of Nepal to determine the prevalence and etiology of childhood ocular morbidity and blindness. In Phase II of this study, genetic analysis was performed for children who were found to have congenital ocular anomalies.

Methodology: It was a cross sectional descriptive study. A total of 10,270 children across three different ecological regions in Nepal (terai, hills, and mountains) underwent ocular examinations. 374(3.6%) of these children were found to have ocular abnormalities, 30 of whom were thought to be congenital in nature. Targeted genetic analysis, including genotyping for specific genes specific to the presenting phenotype, was performed for 25 children using serum samples.

Results: Out of 25 children, 18 children had meaningful genetic results. Analysis revealed one missense alteration G12411T of ZFHX4 gene in one participant among 10 with congenital ptosis and another missense variation T>C P.Y374Cof STRA6 gene in one participant among 3 with microphthalmos.

Conclusion: The study is first of its kind from Nepal and mutant genes were unique to Nepalese Population. Further analysis of genetic factors is crucial to better understand genetic association with ocular diseases and conditions . This helps further in genetic counseling and probably gene therapy to prevent blindness from these conditions.

Background

Visual impairment is one of the most common disabilities affecting children. There is an estimated1.4 million children worldwide who are blind, two-thirds of whom live in developing countries such as Nepal (1). Controlling childhood blindness has been a top priority of the World Health Organization since its launch of the VISION 2020: The Right to Sight global initiative in 1999(2). Congenital ocular anomalies are one of the important causes of childhood ocular morbidity and blindness.) The genetic and hereditary eye diseases account for 11–39% of childhood blindness with more common in developed than in the developing world (3) Treatment is unfortunately often difficult or nonexistent for many of these congenital anomalies. Genetic testing in these situations can help to confirm diagnoses and thus provide prognostic information, guide interventions for the child, and assist in counseling regarding risk of disease occurrence in subsequent children. The Genetic Eye Disease Task Force of the American Academy of Ophthalmology has recommended various genetic testing of different congenital ocular anomalies (4)

Phase I of the Nepal Pediatric Ocular Disease Study(NPODS) determined the prevalence of childhood ocular morbidity and blindness across three ecological regions (lowlands, hills, mountains) of Nepal (5). Phase II of NPODS was the nested case control study to find out the risk factors of ocular morbidities

identified in the first study (6). In this study, we conducted genetic analysis in children with congenital anomalies to understand the genetic implications of these diseases in Nepalese children.

Method

It was a cross sectional descriptive study. Children identified as having congenital ocular anomalies in Phase 1 of NPODS were called for further genetic testing. Children aged 0 to 16 years were included. After getting pre informed written consent at the time of examination, the children's parents were instructed to report to primary health center for blood sample collection. The genetic test was carried out in Kathmandu Centre for Genomics and Research Laboratory (KCGRL) in Kathmandu Nepal A single 5 ml venous blood sample was collected from each child while awake. DNA was extracted from the blood samples using a commercially-available DNA Extraction Kit (Qiagen; city, state) per the manufacturer's protocol and evaluated for quality. Genotyping was done for a selected number of specific genes which corresponded to the presenting phenotype of each child. (Fig. 1) Locations of mutations were identified by conventional methods as well as reference Single Nucleotide Polymorphism (SNP) ID available. Genomic reference sequences spanning the mutation locus were downloaded from db SNP as well as Genbank database. Polymerase chain reaction primers to amplify these loci were designed using Primer design version 3 online tool. Primers were chosen such that they amplify 300–500 bp size amplicons and location of mutation is central to the amplicon where Sanger sequencing reads are having high QV bars. Furthermore, Primers were designed using Chromosomal DNA reference sequence, db SNP reference sequences and primer 3 design software available at bioinfo.ut.ee/primer3-0.4.0. to amplify each locus. (Table 1) The Polymerase Chain Reaction (PCR) was carried out in a 25 µl volume with the final mix containing 10 × PCR buffer, 1.25 mM dNTPs, 25 mM $MgCl_2$, 10 pmole of each primer, 2.5 U of *Taq* polymerase and 2.5 µl of DNA template. The sample was heated to 95 °C for 5 min, followed by 35cycles of 95 °C for 30 sec, 55 °C for 30sec, 72 °C for 30 seconds and a final extension at 72 °C for 10 min. (Fig. 2)

Table 1
Primer name with sequence in different diseases

Disease Name	Primer Name	Primer Sequence	PCR Product
Congenital Ptosis	ATPL_ZF2	5'TGTAGTGCAGCAACAGTTTGG 3'	445
	ATPL_ZF3	5' CATGGCAAGCAAAACAAAAAT 3'	
Congenital Cataract	ATPL_GJ1	5' CTGCTGAGGACCTACATCTGC 3'	405
	ATPL_GJ2	5' CAAGGGGAAATAGTGGGAAAC 3'	
Crouzen Syndrome	ATPL_FG1	5' CAGCCAATAACCTGGGATGTA 3'	458
	ATPL_FG2	5' TCCTCACCTTGAGAACCTTGA 3	
Crouzen Syndrome	ATPL_FG3	5' GATCAAGCACGTGGAAAAGAA 3'	448
	ATPL_FG4	5' AGCTCTTTCCATCCACTCCAT 3'	
Micropthalmus	ATPL_ST1	5' AGGTGGGCTCCATGACAG 3'	380
	ATPL_ST2	5' CATTGGCTTACACTGCATCTG 3'	
Micropthalmus	ATPL_ST3	5' TATTAGCCCCATTTTGCAATG 3'	405
	ATPL_ST4	5' GAGGGTAGTAGAGGGCAGCAT 3'	
Micropthalmus	ATPL_CR1	5 'GCTCCTGGAGGAATCTGAGTT 3'	317
	ATPL_CR2	5 'AAAAGTCCTAGCACCCGTCTC 3'	
Micropthalmus	ATPL_OT1	5' AGCCTCCCCAACTTTCTTACA 3'	404
	ATPL_OT2	5' GAAGCTGGGCTCCAGATAGAC 3'	
Micropthalmus	ATPL_OT3	5' TGAGACCTGCCAAAAAGAAGA 3'	387
	ATPL_OT4	5' AAGCTGGGGACTGATTGAGAT 3'	
Colobama	ATPL_AB1	5' CCTGAGTCCCTGCTTCTTCTT 3'	366
	ATPL_AB2	5' AACTTGATCCAGATGCCCATT 3'	

Through the confirmation from correct size of amplification, samples were further subjected to DNA sequencing. (Table 2). Cycle sequencing reaction for each sample was performed in 0.2 mL PCR tube. The reaction included Terminator Ready Reaction Mix (BigDye®) Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems) (0.5 µL), BigDye® Sequencing Buffer (1.8 µL), One sequencing primer (3.2 pmol), and template DNA (2 µL) with Standard Milli Q (SMQ) water (4.7 µL) to make up the volume of 10.0 µL. For each sample two sequencing reactions were performed one using forward primer and other with reverse primer. The cycle sequencing protocol was as follows: Initial denaturation at 96 ºC for 1 min, followed by 25 cycles of 96ºC for 10 secs, annealing at 50 ºC for 5 sec, and elongation at 60 ºC for 4 minutes.

Table 2
Samples with further DNA sequencing

Lane Number	Sample Number	Gene	Mutation covered
M	100–1000 bp ladder	-	-
1	A	ZFHX4	G12411T L4137F
2	B	GJA8_Cx50	c.649G > A (Val196Met)
3	F	FGFR2	S267P (T-C)
3	F	FGFR2	C278F (G-T)
3	F	FGFR2	Q289P (A-C)
4	F	FGFR2	C342S (G-C)
4	F	FGFR2	C342Y (G-A)
4	F	FGFR2	C342W (C-G)
4	F	FGFR2	A344A (G-A)
4	F	FGFR2	S347C (C-G)
5	D	STRA6	T > C P.Y374C
6	D	STRA6	A > T P.L152M
7	D	CRYBA4	C > T P.R25W
8	D	OTX2	p. Gln104 X
8	D	OTX2	p. Gln106 His
9	D	OTX2	p. Thr186 Fs < frame shift
10	C	ABCB6	p. Ala 57 Thr G◊A
A: Congenital Ptosis			
B: Congenital Cataract			
C: Colobama			
D: Micropthalmus			
F: Crouzen Syndrome			

Data management and Analysis

Data were entered into the electronic database The codes, recodes, consistency, outlines etc were assessed through the use of Microsoft Excel. Data analysis was done in Statistical Package for Social

Science (SPSS) version16.

Results

In Phase 1 of NPODS, a total of 10,270 children, aged 0 to 16 years across three different ecological regions of Nepal underwent vision screening Within this cohort, 5208 children (50.7%) were from terai, 3136 (30.5%) were from the hill, and 1926 (18.8%) were from the mountain region. Ocular abnormalities were present in 374 (3.6%) of all the children examined. The ocular abnormalities in 30 of these children were thought to be congenital in nature. Within this group, 17 children were from terai, 4 were from the hill region, and 4 were from the mountain region.

Among the 30 children thought to have congenital ocular anomalies, 19 (63.3%) were male and 11 (36.7%) were female. Fourteen (46.7%) of the children had congenital ptosis, 6 (20.0%) of the children had congenital cataract, 4 (13.3%) of the children had iris and chorioretinal coloboma, 3 (10.0%) of the children had microphthalmos, 1 (3.3%) had Nevus of Ota, 1 (3.3%) had proptosis secondary to Crouzon syndrome, and 1 (3.3%) had Retinal dystrophy. Table 3 shows the pattern of congenital ocular diseases across the three ecological regions. Most of the children with congenital anomalies were from the Terai region. The total morbidity was also high in terai region. Genetic analysis was carried out in 25 children. Five (16.7%) children were non responders. The summary of different gene mutations has been shown in Table 4

Table 3
Pattern of congenital ocular diseases in three ecological regions

Disease pattern	Terai N(%)	Hills N(%)	Mountains N (%)	Total N (%)
Coloboma	4 (13.3)	0 (0)	0 (0)	4 (13.3)
Congenital Ptosis	10 (33.3)	2 (6.6)	2 (6.6)	14 (46.7)
Congenital cataract	3 (10)	3 (10)	1 (3.3)	6 (20)
Microphthalmous	3 (10)	0	0	3 (10)
Nevus of Ota	0	0	1(3.3)	1 (3.3)
Proptosis (Crouzen syndrome)	0	1(3.3)	0	1(3.3)
Retinal dystrophy	1(3.3)	0	0	1(3.3)
Total				

Table 4
The result summary of genetic analysis

RN	SN	Gene	Mutation	BASE Call	Result
1	A	*ZFHX4*	G12411T L4137F	T	G12411T detected
2	B	*GJA8_Cx50*	c.649G > A (Val196Met)	G	c.649G > A Not detected
3	F	*FGFR2*	S267P (T-C)	T	Not detected
3	F	*FGFR2*	C278F (G-T)	G	Not detected
3	F	*FGFR2*	Q289P (A-C)	A	Not detected
4	F	*FGFR2*	C342S (G-C)	-	Not detected
4	F	*FGFR2*	C342Y (G-A)	-	Not detected
4	F	*FGFR2*	C342W (C-G)	-	Not detected
4	F	*FGFR2*	A344A (G-A)	-	Not detected
4	F	*FGFR2*	S347C (C-G)	-	Not detected
5	D	*STRA6*	T > C P.Y374C	C	T > C P.Y374C detected
6	D	*STRA6*	A > T P.L152M	T	L152L present, mutation not detected.
7	D	*CRYBA4*	C > T P.R25W	C	Mutation not detected
8	D	*OTX2*	p. Gln104 X	C	p. Gln104 X Not detected
8	D	*OTX2*	p. Gln106 His	C	p. Gln106 His Not detected
9	D	*OTX2*	p. Thr186 Fs < frame shift	G	p. Thr186 Fs < frame shift Not detected
10	C	*ABCB6*	p. Ala 57 Thr G > A	G	p. Ala 57 Thr G > A Not detected

SN = Sample number, RN = reaction number Where, A: Congenital Ptosis, B: Congenital Cataract, C: Colobama, D: Microphthalmus, F: Crouzen Syndrome.

Ten SNP among seven genes for five congenital eye disorders listed above were selected. One missense alteration G12411T of *ZFHX4* gene was identified in patient with congenital ptosis. (Fig. 3). Another missense variation T > C P. Y374C of STRA6 gene was identified in patient with microphthalmos (Fig. 4).

Discussion

In the current era of genetic advances, the diagnosis of genetic eye diseases is increasing and facilitated by collaborations between ophthalmologists and geneticists. Genetic testing can not only help confirm

suspected diagnoses, but also provide important prognostic information and guide management for the individual affected but also help provide appropriate genetic counseling for parents. A comprehensive genetic evaluation requires a thorough clinical examination, a detailed family history, and access to advanced technology for molecular investigation.

We carried out the genetic analysis to find out molecular pattern and any significant mutations of inherited eye diseases in Nepalese children. In our study, congenital anomalies were most commonly present in terai region with 18 cases followed by 4 in mountains and 5 in hills. Five types of congenital anomalies were studied ie congenital ptosis, microphthalmos, congenital cataract, coloboma and Crouzon syndrome. Among these only crouzen syndrome was only found to be familial where mother was also affected.

Hereditary cataracts are clinically and genetically heterogeneous, often presenting as congenital or developmental cataracts that arises at birth or during the first few decades of life (7. Approximately 25% of non-syndromic cataracts are inherited (8. They can also be grouped into three major classes, based on the functions of known underlying genes, those that code for crystallins, membrane/cytoskeleton proteins, and transcription factors. At least 35 independent loci, including more than 20 known genes, have been identified for non-syndromic cataract (Cat- Map) (9,10). Mutations in crystallin genes account for the majority of missense mutations (nearly 50%) followed by mutations in the genes for cytoskeletal or membrane proteins (nearly 35%) (9). The Gap Junction Protein Alpha 8 (*GJA8) and* Gap Junction Protein Alpha 3 (*GJA3)* mutations together account for 20% of the reported total non-syndromic familial cataracts worldwide (9). We selected a known SNP c.649G > A (Val196Met) from the same gene *GJA8* and tested for the children with congenital cataract. This variation was not found in our patient with congenital cataract.

We selected one alteration in the ZFHX4 gene, G12411T L4137F in the children with congenital ptosis. This alteration was also previously reported in a Japanese family (11). This change was also detected in one child with congenital ptosis.

Another group of children were with iris and chorioretinal coloboma and microphthalmia. Microphthalmia and iris and chorioretinal coloboma are related structural, congenital eye malformations which display a spectrum of severity and can occur in isolation or as part of a syndrome. Microphthalmia refers to a small eye, defined by axial length. Iris coloboma is a segmental ocular defect resembling a key hole deficiency in iris. Chorioretinal coloboma is associated with iris choloboma and visually significant if posterior pole is involved. (12). The STRA6 gene plays a key role in normal ocular development, encoding a transmembrane receptor for the retinol-binding protein (RBP) and is responsible for mediating vitamin A uptake from circulation to target organs including the eye (13). We selected two SNP in STRA6 (T > C P. Y374C and A > T P. L152M) gene which are already found in database. We could identify one of these mutations in as well.

The second gene we have selected was Crystalline Beta A 4 (CRYBA4). It is known that complex microphthalmia in association with genetic cataracts has been attributed to mutations in the *CRYBA4*

gene (14). We selected one SNP C > T P.R25W from CRYBA4 gene which could not be identified in our patient.

The next gene is Orthodenticle Homeobox 2 (OTX2). The *OTX2* gene encodes a transcription factor critical for forebrain and eye development (15). The OTX2 protein contains a homeodomain, responsible for DNA binding, SGQFTP and SIWSPA motifs involved in protein–protein interactions, and two C-terminal tandem OTX-tail motifs responsible for transactivation (16). We have selected three SNP p. Gln104 X, p. Gln106 His, p. Thr186 Fs < frame shift from OTX2 gene which could not be identified in our patient.

We have also selected one SNP p. Ala 57 Thr G◊A from ATP Binding Cassette Sub Family B Member 6 (ABCB6) gene, which is involved in the active transport of various compounds vital for CNS development, but could not identify this mutation in our patient. Crouzen syndrome is an autosomal dominant craniosynostosis disorder that can be caused by mutation in the Fibroblast Growth Factor Receptor 2 (FGFR2) genes. (18) We also selected few SNP from FGFR2 gene, which codes for fibroblast growth factor receptor, which are mentioned in table for Crouzon syndrome but we were not able to find any mutation. (19). The limitation of our study was small sample size. Apart from the diseases included in the study, we could widen our research in other types of congenital ocular anomalies.

Conclusion

Our study was the first of its kind in Nepal to identify genetic mutations associated with congenital ocular anomalies. Through our analysis, two new mutations were identified in children with congenital ptosis and the microphthalmos unlike other studies on genetic analysis of congenital ptosis and microphthalmos.

List Of Abbreviation

NPODS (Nepal Pediatric Ocular Disease Study)

STRA6: Signaling Receptor and Transporter Retinol 6

ZFHX4: Zinc Finger Homeobox 4

KCGRL: Kathmandu Centre for Genomics and Research Laboratory

SNP: Single Neucleotide Polymorphism

PCR: Polymerase Chain Reaction

dNTPs: Deoxy Nucleotide Triphosphates

SMQ: Standard Milli Q

SPSS: Statistical Package for Social Science

GJA8: Gap Junction Protein Alfa 8

RBP: Retinol Binding Protein

CRYBA4: Crystallin Beta A 4

OTX2: Orthodenticle Homeobox 2

ABCB6 Gene: ATP binding Cassette Subfamily B Member 6

FGFR 2: Fibroblast Growth Factor Receptor 2

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Review Committee of the Tilganga Institute of Ophthalmology. The study adheres to the tenets of the Declaration of Helsinki. The written informed consent was obtained from a parent or guardian for participants under 16 years old.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interest

The authors declare that they have no competing interests.

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Author's contribution

All authors have read and approved the manuscript. Each author's contribution in carrying out this research and writing the manuscript has been listed as follows

SA: Principal investigator of this research and wrote most parts of the manuscript

NT: Clinical genetic specialist helped in manuscript preparation and genetic analysis

US: Helped in manuscript preparation

MKS: Helped in manuscript preparation and study design

MMS: Helped in study design

MP: Helped in data analysis

BT: Helped in study design

AK: Helped in research design and genetic analysis

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Figures

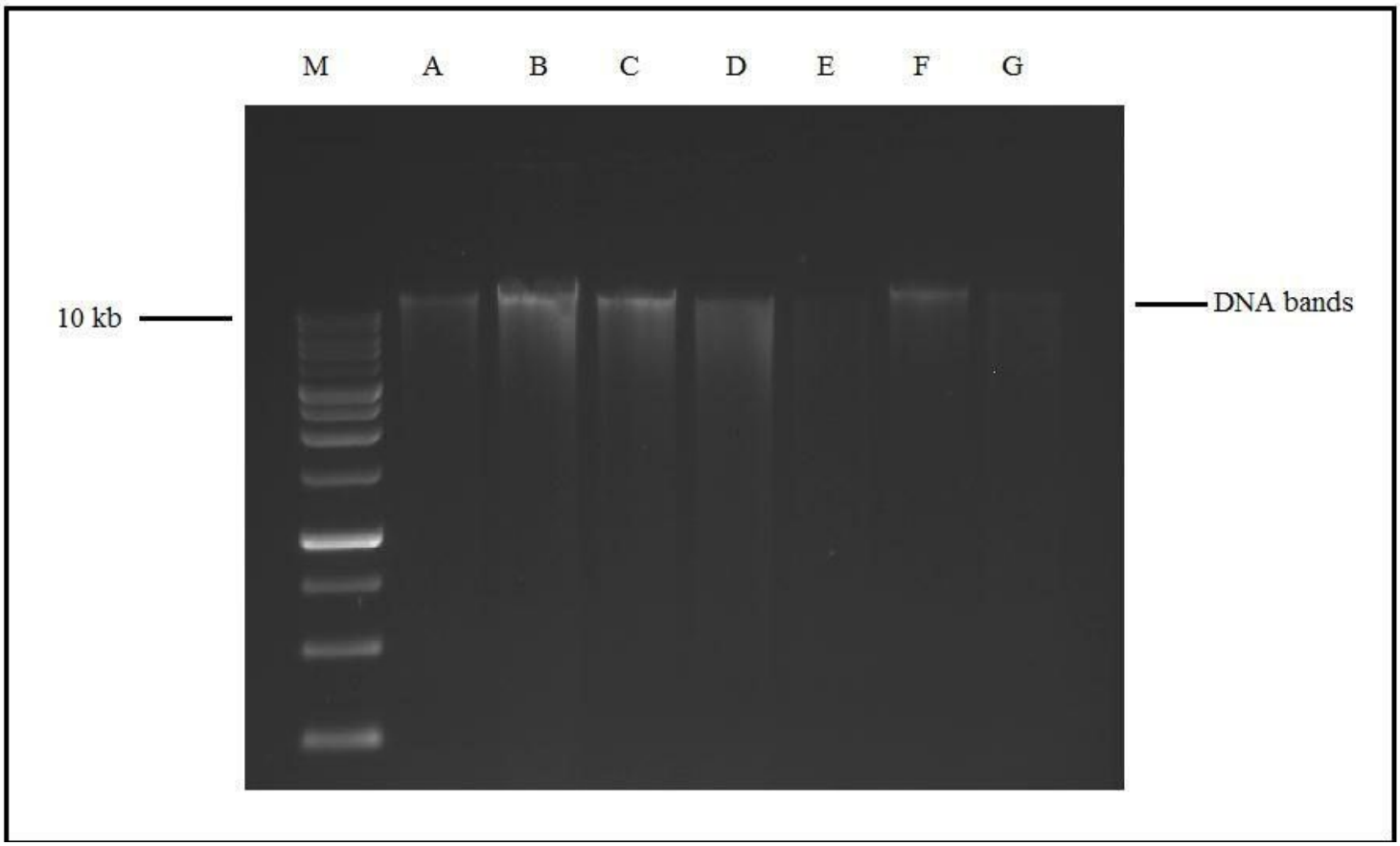

Figure 1

Agarose Gel electrophoresis A: Congenital Ptosis B: Congenital Cataract C: Colobama D: Microphthalmus, F: Crouzen Syndrome

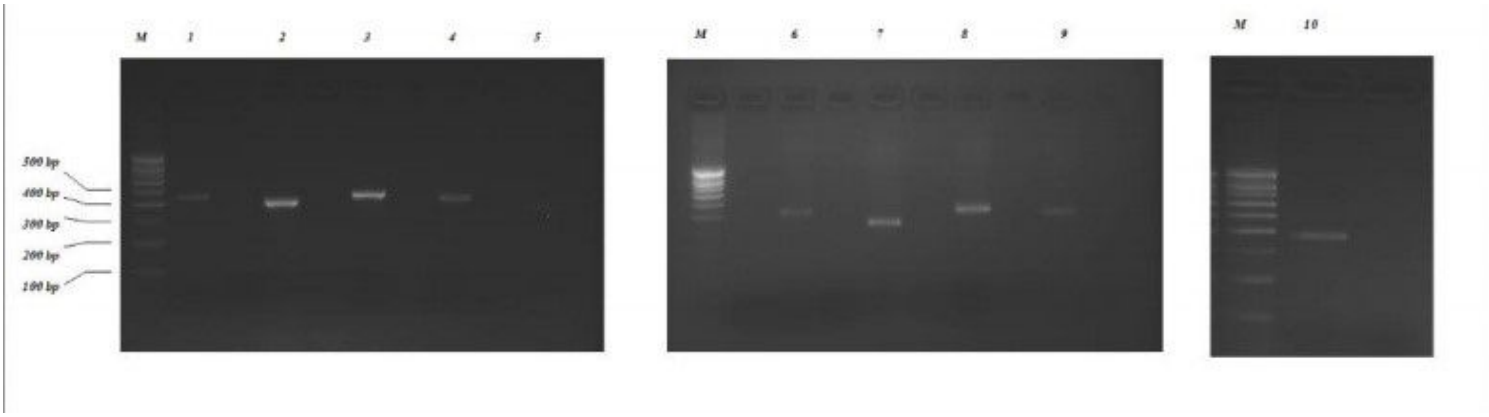

Figure 2

Agarose Gel electrophoresis of PCR products on 2% (w/V) agarose gel

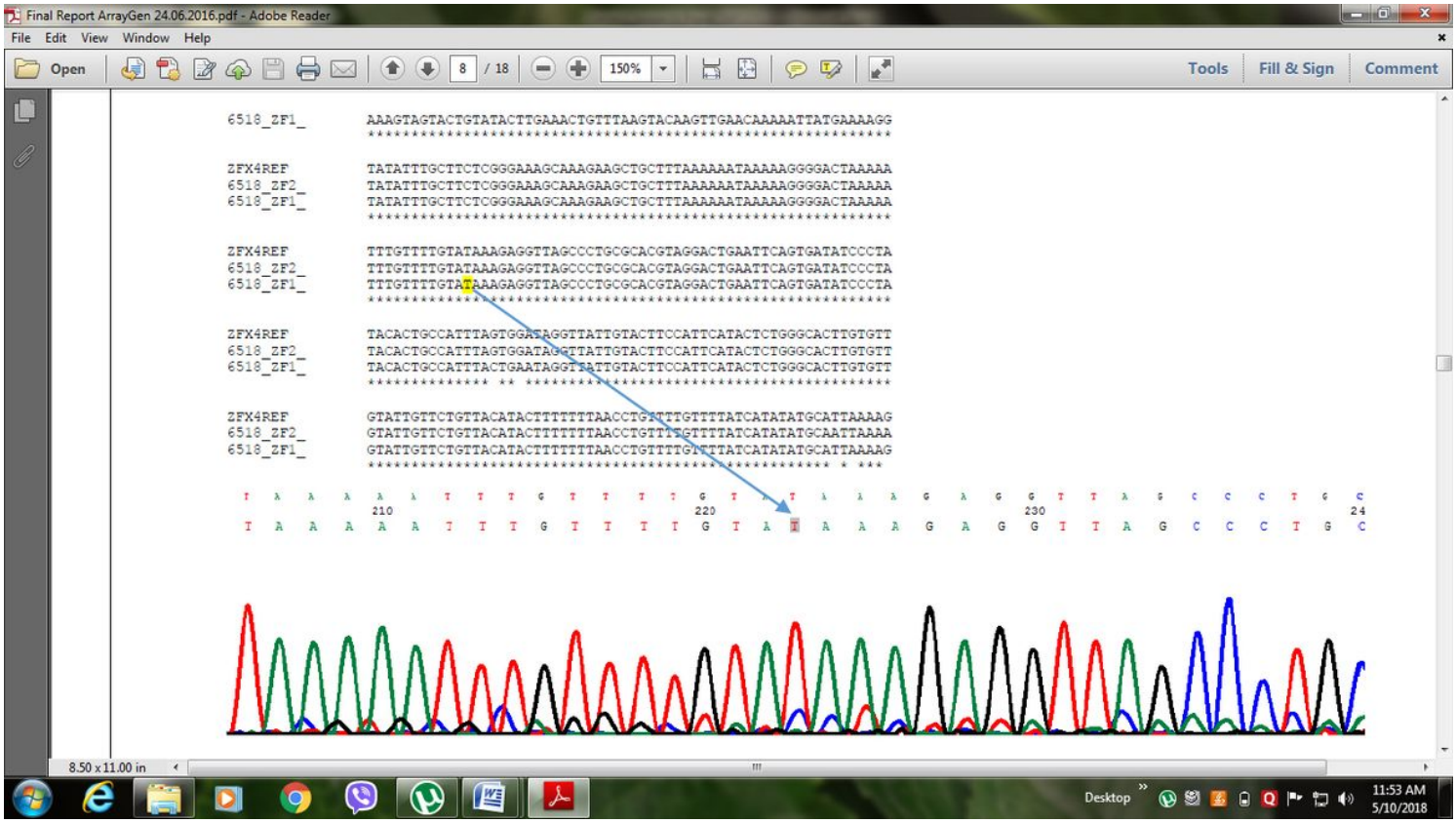

Figure 3

Shows sequence result of G12411TL4137F mutation in ZFHX4 gene in a child with congenital Ptosis

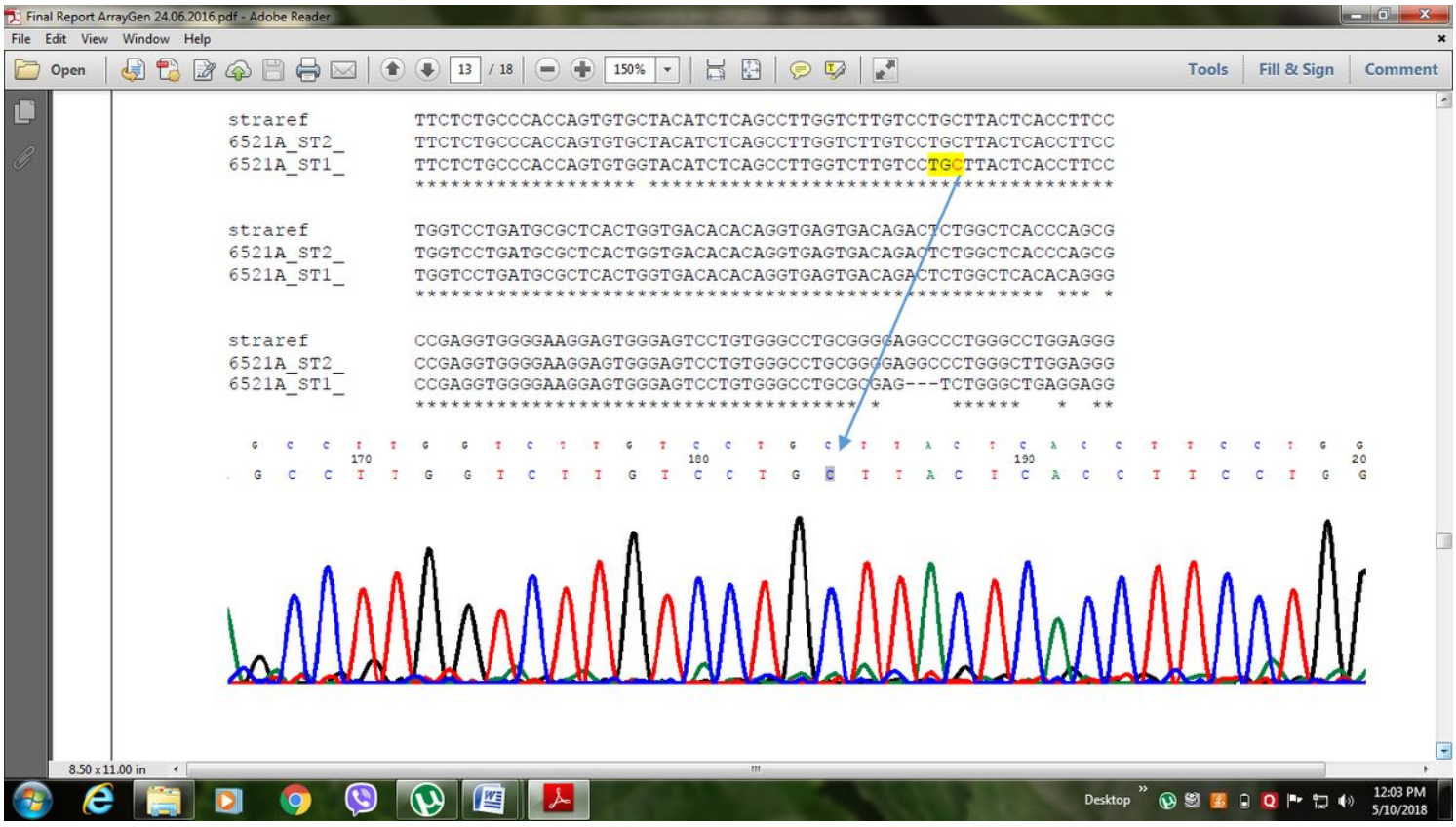

Figure 4

Showing T>C P. Y374C mutation in STRA6 gene in a child with micropthalmous

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- STROBEchecklistv4combined1.doc
- Thestrobestatementchecklistforgeneticsmanuscript.docx