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Exome sequencing as a tool of molecular screening of cataract affected families.

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Abstract
Cataract is the opacity of eye lens which is amongst the major causes of blindness in all age groups around the world. This study involved genetic screening of 24 cataract affected families from Punjab and Balochistan provinces of Pakistan. The screening included linkage analysis for most prevalent cataract genes and two of these families, found linked, were subjected to Sanger sequencing for mutational analysis. Linkage to HSF4 and FYCO1 genes were indicated in two families but no significant mutation could be ascertained upon sequencing HSF4 gene. Three of the unlinked families including a positive control family were submitted to exome sequencing in search for novel mutations. Exome sequencing successfully re-identified an already known mutation (c.233G>A) in the positive control family and revealed potential variants in other families. This mutation was previously identified by using linkage analysis and re-identified in our study by exome sequencing. Indication of linkage in family CCHUS-27 may suggest that FYCO1 is the most prevalent cataract gene in Pakistan. The identification of known mutation in positive control family strengthens the reliability of exome sequencing for identification of mutations causing cataract.

Key Words: Cataract, Linkage Analysis, Sanger Sequencing, Exome Sequencing

1. Introduction

An abnormality or malfunction of eye lens may lead to unfortunate consequences. One of the many malfunctions of lens is cataract which occurs due to loss of lens transparency and it appears with clouded or frosted effect. The cataract may be genetic i-e due to mutation in a gene or it may be due to other reasons such as diabetes, trauma and aging. The inherited form of cataract is now established to be genetically heterogeneous which means that mutations in more than one gene cause cataract (Riazuddin et al. 2009).

Large families with strong interfamily relations and marriage makings are the primary reasons for higher prevalence of genetic disorders in Pakistan (Bhinder et al. 2019). Resultantly, different ethnic groups in Pakistan offer great potential to find novel genes and mutations associated with genetic disorders in affected families. Cataract is widely reported to be the leading cause of blindness in Pakistan. It causes about 66.7% of total cases of blindness among groups distributed in various categories (Hassan et al. 2019). Balochistan is the south western province of Pakistan where least of molecular and genetic studies have been conducted for score of reasons.

Linkage analysis is relatively conventional tool for identification of novel genes associated with segregation of affected trait in a family. It is based on an exception to Mendel's law of independent assortment that two genes (loci) may inherit together if they are located in close proximity on same chromosome. In linkage analysis the two loci are candidate gene and STR marker, both in close proximity.

In this study, twenty-four cataract affected families with two or more than two affected members were enrolled from Balochistan and Punjab provinces. Pedigrees of these families were drawn in consultation with their elders and blood samples were taken after provision of consent. After extraction of DNA, 3-6 STR markers were used to screen the affected members along with their normal parents and siblings for most prevalent cataract genes in Pakistan and Balochistan. In two out of twenty-four families, linkage analysis indicated potential association with already known cataract genes. Family CCUHS-05 indicated linkage to HSF4 gene and family CCUHS-27 was found linked to FYCO1 gene. Two of the rest of unlinked families were subjected to exome sequencing and another family with already known cataract mutation (c.233G>A in LIM2 gene) (Irum, Khan, Ali, Kaul, et al. 2016) was processed as positive control of the study.

2. Methods

The experimental work was carried out in the Departments of Human Genetics & Molecular Biology at University of Health Sciences Lahore, Alama Iqbal Medical College Lahore and the Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore. Genotyping Facility was availed from DNA Core Facility Lab, Centre for Applied Molecular Biology (CAMB). A prior approval was granted by the Ethical Review Committee, University of Health Sciences Lahore. Patients and their guardians were asked at health care units for family history in presence of medical professional on prescribed form. Selected families were visited at their homes for blood sampling, pedigree drawing and associated data collection on prescribed medical history form. A cataract affected family, in which mutation (c.233G>A) was identified by our collaborators, was included as a positive control for comparison of Next Generation Sequencing to linkage analysis.

DNA was quantified by using Nanodrop DNA quantification system. families were investigated for their genetic linkage with most prevalent genes in Pakistan and Balochistan i-e FYCO1 and CRYBB3, and HSF4 respectively through linkage analysis. For every gene 2-3 STR markers were selected in the closest proximate to loci where these genes were situated. STR markers were amplified by conventional PCR (Polymerase Chain Reaction). PCR products were pooled and loaded in a single capillary injection for automated genotyping in ABI PRISM® 3730 Genetic Analyser. Raw data produced was analysed with help of Peak Scanner software v1.0. Analysed results were exported to Excel sheet for haplotype analysis. Excel data spread sheet was prepared to arrange markers along with chromosome in order of cM. Initially five members of family were genotyped for identification of putative linkage to a loci or gene. Any one of a parent, 2 or 3 affected members and a normal kin was executed in initial run. On identification of supposed linkage to a locus, remaining family members were typed for confirmation or exclusion of linkage.

For Sanger Sequencing, PCR products amplified with a primer, either forward or reverse in one reaction, specific for target regions. Sanger sequencing was carried out to confirm a variation which has been already indicated through linkage analysis or exome sequencing as a potential candidate for causing cataract. These sequencing specific amplicons were purified to facilitate the subsequent sequencing reaction in capillary electrophoresis based genetic analyzer. Query data files were exported to software where these were aligned and read against the reference sequence files. Once, a mutation is found in a query sequence file, it is searched in dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) database for the reason whether it is novel mutation or not.

For exome sequencing, libraries were prepared by using Nextera Rapid Capture Expanded Exome kit with instructions provided by the manufacturer (Illumina, San Diego, CA). HiSeq 2000 Genome Analyzer (Illumina) was used to read the samples and generate 59-71 million paired-end raw reads (FASTQ) using Lasergene Genomics Suite (DNASAR, Madison, WI) for genome variants and variants calling. In our exome sequencing operation, an overall >40X coverage was obtained for each sample. These obtained reads were then mapped and aligned to human genome assembly (GRCH38/ hg38) utilizing SeqMan NGen (v12). In order to perform variant analysis including variants calling, DNASTAR-specific format files were obtained by conversion of BAM files. ArrayStar (version 12) was used to carry out the annotations of mapped reads. False positive results were needed to be exclude and a strict sets of filters were applied to narrow down the search to potential variants and exclude the rest. These filters include heterozygosity, variants in intronic regions, and synonymous variations.

3. Results

During the course of this study, about twenty-four families were enrolled among the large number of families identified. These twenty-four families qualified the inclusion-exclusion criteria. Among these twenty-four families, fourteen were recruited from Balochistan and ten of the families belonged to various parts of the Punjab province. Ethnical distribution of enrolled families from Balochistan province included nine families of Pashtun ethnic origin. Four families belonged to Baloch ethnicity and one family was hybrid of Pashtuns and Baloch ethnic groups. Notable castes from families in Punjab were identified as Qureshi, Arrain, Lohar Khokhar, Awan and Kamyar.

Previous studies suggested FYCO1 to be the most prevalent cataract causing gene among Pakistani population and highest number of cataract causing variations were reported in FYCO1 gene among people in Pakistan (Table 1).

Table 1. Homozygous Mutations in Cataract Affected Families from Pakistan

ID	Locus	Gene	DNA Mutation	Protein Mutation	References
1	1p36.13	EPHA2	c.1814C>T	p.T605I	(Chen et al. 2017)
			c.2353G>A	p.A785T	(Kaul et al. 2010)
2	1p32	FOXE3	c.21_24del	p.M7IfsX216	(Iseri et al. 2009)
			c.244A>G	p.M82V	(Iseri et al. 2009)
			c.307G>A	p.E103K	(Chen et al. 2017)
			c.720C>A	p.C240X	(Anjum et al. 2010)
			c.720C>A	p.C240X	(Ali et al. 2010)
3	3p21.31	FYCO1	c.2206C>T	p.Q736X	(Chen et al. 2011)
			c.2206C>T	p.Q736X	(Chen et al. 2017)
			c.2345delA	p.Q782RfsX32	(Chen et al. 2017)
			c.2761C>T	p.R921X	(Chen et al. 2011)
			c.2830C>T	p.R944X	(Chen et al. 2011)
			c.3150+1G>T	splice variant	(Chen et al. 2011)
			c.3151-29_3151-7del	splice variant	(Saleem et al. 2022)
			c.3151-2A>C	p.A1051DfsX27	(Chen et al. 2017)
			c.3755delC	p.A1252DfsX71	(Chen et al. 2011)
			c.3858_3862dupGGAAT	p.L1288WfsX37	(Chen et al. 2011)

			c.4127T>C	p.L1376P	(Chen et al. 2011)
			c.4127T>C	p.L1376P	(Saleem et al. 2022)
4	6p24.2	GCNT2	del (190 kb)		(Irum, Khan, Ali, Daud, et al. 2016)
			del (98kb)		(Happ et al. 2016)
			del (93kb)		(Borck et al. 2012)
			del (93kb)		(Borck et al. 2012)
5	9q22.33	TDRD7	c.1129delG	p.A377PfsX2	(Chen et al. 2017)
6	10q24.2	DNMBP	c.811C>T	p.R271X	(Ansar et al. 2018)
			c.2852_2855delCCAA	p.T951MfsX41	(Ansar et al. 2018)
			c.2947_2948delGA	p.D983X	(Ansar et al. 2018)
7	11q22.1-q23.2	CRYAB	c.31C>T	p.R11C	(Jiaox et al. 2015)
			c.34C>T	p.R12C	(Jiaox et al. 2015)
	12q13	MIP	c.67T>A	p.T23N	(Chen et al. 2017)
8	16q22.1	HSF4	c.433G>C	p.A145P	(Chen et al. 2017)
			c.524G>C	p.R175P	(Forshew et al. 2005)
			c.595-599del5bp	p.G199EfsX15	(Forshew et al. 2005)
			c.1213C>T	p.R405X	(Sajjad et al. 2008)
9	17q25.1	GALK1	c.410delG	p.G137VfsX27	(Yasmeen et al. 2010)
			c.416T>C	p.L139P	(Yasmeen et al. 2010)
			c.766C>T	p.R256W	(Chen et al. 2017)
			c.1067T>C	p.L356P	(Chen et al. 2017)
10	19q13.4	LIM2	c.233 G>A	p.G78D	(Irum, Khan, Ali, Daud, et al. 2016)
11	22q11.23	CRYBB3	c.493G>C	p.G165R	(Riazuddin et al. 2005)
12	22q11.23	CRYBB2	228 kb deletion	Pseudogene conversion	(Irum et al. 2022)
13	22q12.1	CRYBA4	c.440G>T	p.G147V	(Chen et al. 2017)

Crytallins family of genes were identified other potential candidates and amongst the family only CRYBB3 was previously reported from Pakistan. Hence, CRYBB3 was selected as second gene for initial screening. On other hand, HSF4 was the only gene reported from Balochistan to cause cataract, at the time of our experiments (Sajjad et al. 2008). Summarily, FYCO1, CRYBB3 and HSF4 genes were selected to screen the affected families for possible linkage with already known genes. On screening the families using tools of linkage analysis, family CCUHS-05 from Balochistan, was found linked to HSF4 gene (Figure 1A). Another family, CCUHS-27, was found linked to FYCO1 gene and the family was enrolled from Punjab (Figure 1B). Subsequent mutational analysis in family CCUHS-05 could not identify any

mutation in the HSF4 gene segregating in the family (Figure 1C). Mutational analysis in CCUHS-27 is part of the future strategies.

Exome sequencing analysis included three families. Family CCUHS-28 was cataract affected family from Punjab in which mutation in Lim2 gene was already identified by our collaborators (Irum, Khan, Ali, Kaul, et al. 2016). This family was recruited again in our study as a positive control to assess all methods starting from recruitment of families to exome sequencing and identification of cataract causing mutation. Upon Next Generation Sequencing, the family CCUHS-28 provided some potential variants. These variants were filtered to narrow down the search for most eligible variant. These filters included but not limited to quality score, depth reads, homozygosity of variant, clinical significance and expression in tissue relevant. Resultantly, a variation at locus 19q13.41 was found to qualifying all filters set for qualification of a potential variant. It was a homozygous mutation at position c.233G>A of LIM2 gene which translates into p.G78D in its MP19 protein molecule. This was the exact mutation identified in this family previously using linkage analysis (Figure 2).

The rest of two families revealed two-three potential variants each and confirmation by sanger sequencing is part of future strategies. The identification of mutation c.233G>A in our positive control family has greatly assured the integrity and authenticity of not only our nucleic acid sequencing and its analysis but also the rest of our methods.

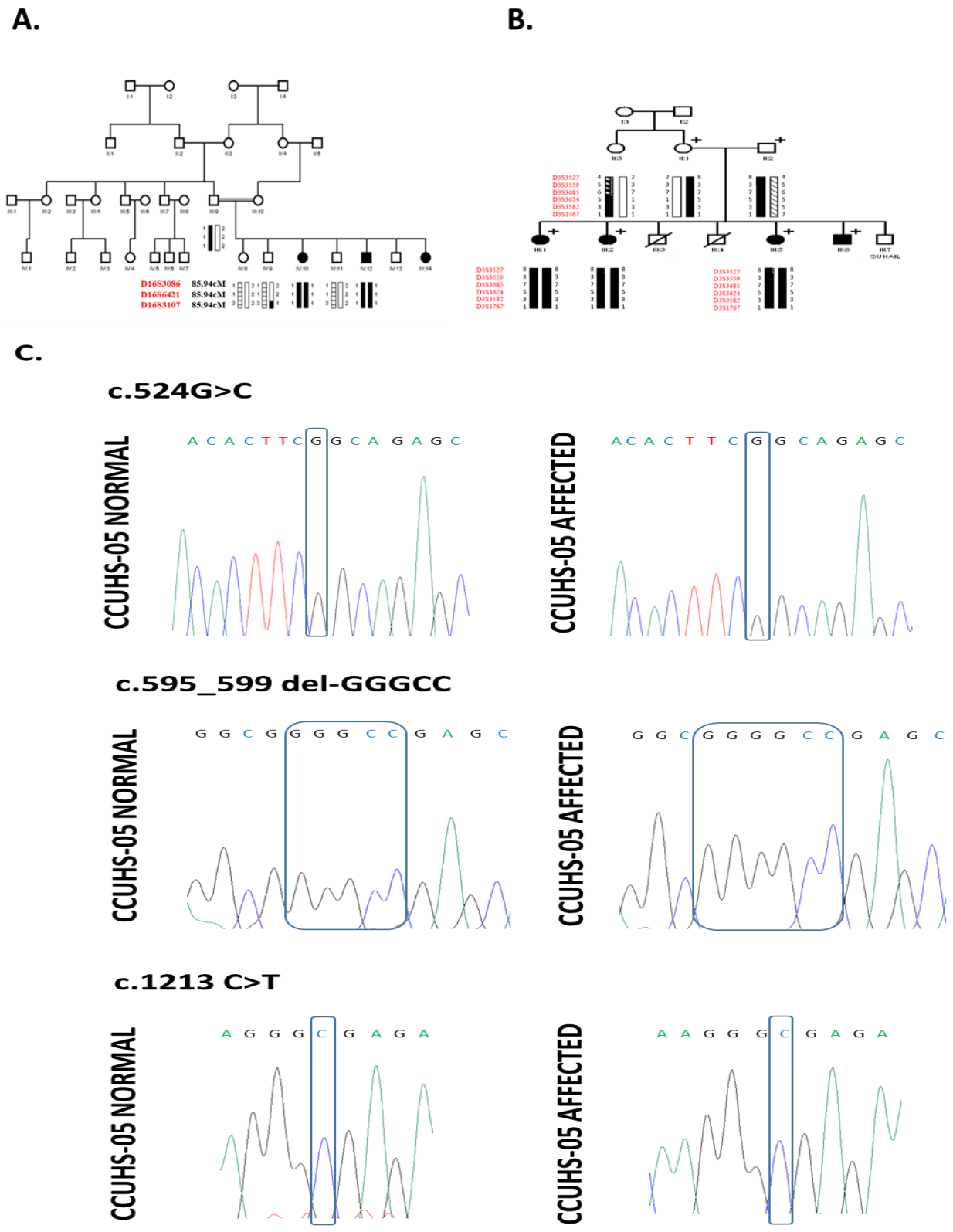
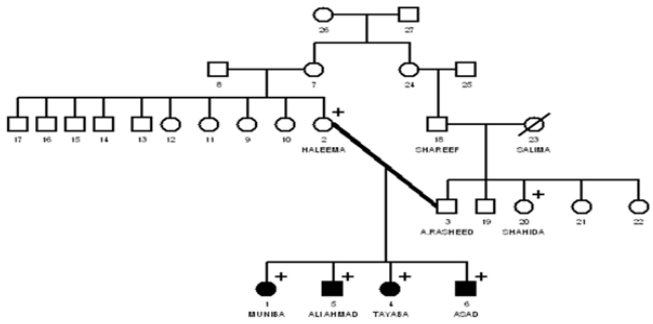


Figure 1. Linkage and Mutational Analysis. A. Pedigree and haplotypes of Family CCUHS-05 linked to HSF4 Gene. **B.** Pedigree and haplotypes of Family CCUHS-27 linked to FYCO1 Gene. **C.** Sequencing of already known HSF4 gene mutations in family CCUHS-05.

A.



B.

S. NO	STUDYN	SEX	AFFECTED	LIM2
1	CCUHS32804	F	AF	AA
2	CCUHS32801	F	AF	AA
3	CCUHS32820	F	UA	GG
4	CCUHS32802	F	UA	GA
5	CCUHS32805	M	AF	AA
6	CCUHS32806	M	AF	AA

C.

CHROM	POS	ID	REF	ALT	QUAL	FILTER	GeneName	Func	Gene	GeneDetail	ExonicFunc	AAChange	Gencode	cytoBand
chr19	51382510	.	C	T	2908.77	PASS	LIM2	exonic	NM_001161748,NM_030657	.	missense SNV	LIM2:NM_001161748:exon3:c.G233A:p.G78D,LIM2:NM_030657:exon3:c.G359A:p.G120D	ENST00000221973.7,ENST00000596399.1	19q13.41

D.

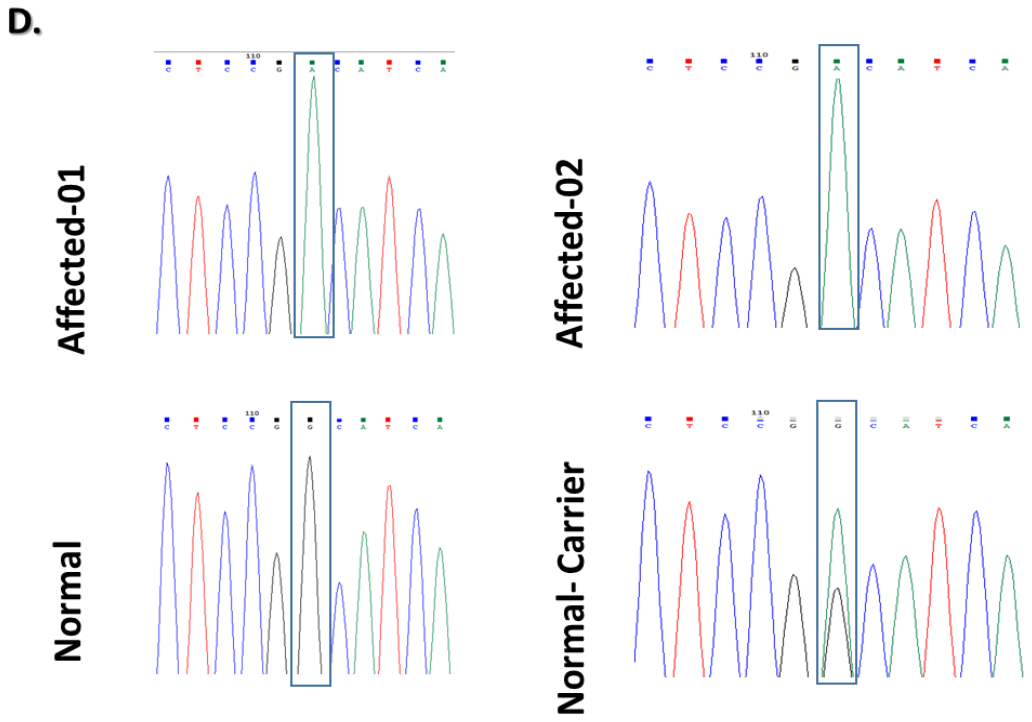


Figure 2. ***Next Generation Sequencing Data of Family CCUHS-28.*** **A.** A pedigree diagram of the family CCUHS-28. **B.** Obtained genotypes of all the family members, both affected and normal, participating in the study. **C.** Results of exome sequencing analysis after application of filters to variants. **D.** Chromatogram of affected, normal and carriers of family CCUHS-28.

4. Discussion

Pakistan is amongst the countries where trends of cousin marriages is the highest [Bhinder et al., 2019; Iqbal et al., 2022]. This has resulted in a society where genetic disorders are not only rampant but also widespread. Cataract is no exception to this; many novel genes and mutations causing cataract are reported in Pakistani population (Table 1). Balochistan is a south western province of Pakistan which comprises 46% area of the country but its population, on the other hand, constitutes only over 6% of the total population. Harsh climate, underdeveloped communication networks and transportation means, and stringent tribal traditions have compelled the people here to live in small towns at vast distances. Resultantly, higher rates of inbreeding in the shape of cousin marriages becomes an understandable phenomenon.

In contrast to other areas of Pakistan, genetic research studies in Balochistan are very rarely conducted, except for few research studies that are carried out in recent years. Therefore, unexplored population in Balochistan offers great potential for discovery of novel genes and mutations associated with genetic disorders. HSF4 was then only cataract causing gene reported from Balochistan and this allows to conclude that HSF4 carries the most potential to be screened in families enrolled from Balochistan.

This study was aimed to screen genes in cataract affected families in search for understanding molecular mechanisms behind cataract genesis in otherwise a normal lens. It also aimed to assess next generation sequencing as an alternative technique for genetic screening in cataract affected families against traditional methods such as linkage analysis. For the purpose, cataract affected families from Pakistan were collected with special focus on Balochistan. Majority of the cataract affected participating in our study were bilateral patients of cataract.

The study advanced to scan all the enrolled families, including positive control, for selected genes using genotyping and haplotype analysis. STR markers for each candidate gene were amplified in two affected, one normal sibling and one of the parents from each family. In family CCUHS-05, a homozygous allelic inheritance was observed in affected members while analysing STR markers of HSF4 gene. However, mutational analysis of all 13 exons of HSF4 gene could not find any significant mutation (Figure 1A & 1C).

Family CCUHS-27 was scanned for genotyping and resulting evidence suggested linkage in this family to FYCO1 gene. On finding initial evidence, then all the members of the family participating were screened. In the family, there was only one normal sibling and he could not participate in the study. Therefore, one of their normal aunt was included. Our future strategy includes to revisit the family CCUHS-27 and look for if there are more affected members born in the family. Including further members of the family may help calculate more indicative LoD score.

Exome sequencing operations analysed three families including CCUHS-28 as a positive control. A range of filters were applied to the refined data obtained after exome sequencing. Majority of the families participating in our study were inferred with autosomal recessive pattern of inheritance hence, heterozygous variants were filtered out. Additionally, variants with less damaging effects were also excluded using data from mutation prediction softwares. Furthermore, variants in non-coding regions were also excluded for their irrelevance to our study. About 2-3 potential variants are identified in the two families other than control family and confirmation of causative mutation is part of future strategies. A missense mutation c.233G>A in LIM2 was re-identified through exome sequencing in positive control family CCUHS-28. LIM2 gene is already reported for causing cataract in Pakistan and other countries (Table 1). However, previous identification of mutation in LIM2 gene was established through linkage analysis and confirmed through Sanger sequencing. Here, the mutation c.233G>A in LIM2 gene is re-identified through exome sequencing and confirmed through sanger sequencing.

This study is a step towards understanding the molecular mechanisms behind complex biological systems. Therapies for most of genetic disorders are not available and therapeutics are largely limited to early intervention. Such studies help the data banks and literature to expand and assist to devise coping strategies. For the purpose, genetic diagnostic panels and pre-natal genetic testing are very important. Studies like this are of great help in maintenance and expansion of databases which in turn are necessary for designing genetic diagnostic tools. Such information become crucial whenever advances in technology may allow development of modern techniques such as gene therapy.

5. Conclusion

Genetic Screening of cataract affected families from Pakistan with special focus towards Balochistan illustrated that cousin marriages has remained the leading cause of genetic disorders in developing countries like Pakistan. Balochistan being the least explored region of our country carries great potential for the identification of novel genes. The re-confirmation of an already known mutation c.233G>A in LIM2 gene causing cataract in positive control family CCUHS-28 not only strengthens the integrity and authenticity of exome sequencing technique but also enhances the reliability of methods carried out in our study. Given the speed and accuracy of exome sequencing technique, it surely outweighs the traditional linkage analysis method when it comes to screen families for genetic disorders.

Future strategies will prioritize the mutational analysis of family CCUHS-27. Furthermore, potential variants identified in families CCUHS-03 and CCUHS-04 will be submitted to Sanger sequencing and subsequent mutational analysis will be carried out for identification of mutations causing cataract.

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