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Whole Exome Sequencing in Consanguineous Kashmiri Families Identifies Recurrent Mutations in *TYR* Gene Causing Oculocutaneous Albinism

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SK and KCG carried out experimental work and analyzed data. ZMA and AAA provided resources, supervised the experiments, and analyzed data. RS and SK ascertained the subjects and performed clinical phenotyping. MAK, ZL and MM performed in silico analysis. SK, AAA and ZMA wrote the manuscript. All authors read, edited, and approved the manuscript.

Key words
***TYR* Gene,Whole Exome Sequencing, OCA**

ABSTRACT

Albinism is a complex group of syndromic and non-syndromic disorders that result in either insufficient or no production of the pigment-protein melanin, giving the skin, hair, and eyes a distinctly white or albino coloring. Oculocutaneous albinism (OCA) and ocular albinism (OA) are the two distinctive categories of albinism. OCA is the frequent form of albinism that follows autosomal recessive pattern of inheritance resulting in hypopigmentation in skin, hair and eyes. Genetically it is heterogeneous, resulting in 8 different forms of OCA (gene for OCA5 is not yet reported) by 7 different genes. Genetic investigation in Pakistani families revealed that *TYR* and *OCA2* genes are the common variants that are responsible for OCA. The aim of present study was to investigate the responsible gene for OCA in the two Kashmiri families. Whole exome sequencing along with Sanger validation revealed previously reported c.62C>T: p. Pro21Leu mutation in family A in exon 1 of *TYR* gene. In Family B, the previously reported pathogenic mutation 832C>T; p.Arg278* was reported in the exon 2of *TYR* gene thus enhancing the mutation spectrum of *TYR* gene in Kashmiri population. Additionally, effect of mutations at protein level was studied by using *in silico* analysis. This study provides a base line data for the screening of more families affected with OCA and identification of novel genes involved in hereditary OCA in Kashmiri population.

INTRODUCTION

Ocular albinism (OA) and oculocutaneous albinism (OCA) are the two categories of albinism. In former case, hypopigmentation affects only eyes, whereas, in later case, hypopigmentation occurs in hair, skin and eyes. Globally, OCA is the most common disorder that is distributed between various ancient, linguistic and cultural groups (Hamid *et al*., 2018). It is reported that albinism appears in 1 in 17,000 to 1 in 20,000 people, which suggests that 1 person in 70 people is the carrier of OCA-mutated gene. OCA follows autosomal recessive pattern of inheritance, therefore consanguinity is the major contributing factor (Summers, 2009). Up till now, eight types of OCA have been reported. Clinically, all the reported types show variable clinical features (Garcia Galvão *et al*., 2019).

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OCA1 has two subtypes i.e. OCA1A and OCA1B. OCA1A is the most common type of albinism caused by mutation in *TYR* gene, located on chromosome 11q14.3 (Simeonov *et al*., 2013). Besides OCA1A, the subtype OCA1B is a mild to moderate form of OCA1 (Arshad *et al*., 2018; Norman *et al*., 2017). OCA type 2 is caused due to mutation in *OCA2* gene that is located on chromosome 15 (Preising *et al*., 2007). OCA3 is the third type of OCA in which *TYR*P1 gene is mutated. This gene is located on chromosome 9p23. OCA4 is associated with mutations in *SLC45A2* gene, which is present on 5p13 (Inagaki *et al*., 2006). OCA5 is associated with locus on chromosome 4q24; however the specific defective gene has not been reported yet (Kausar *et al*., 2013). Mutations in the *SLC24A5* gene (solute carrier family-24, member 5) lead to the appearance of OCA6. This gene is located at 15q21.1 (Grønskov *et al*., 2013). *C10orf11* mutations are linked to the OCA7 type of albinism, whose responsible gene is located on chromosome 10q22.2-q22.3 locus. OCA8 is a novel type of OCA that is recently discovered to be associated with mutations in *DCT* on chromosome 13q31-q32 (Garrido *et al*., 2021). Most of the reported genes are involved in melanogenesis. Previous research has reported the presence of *TYR, OCA2, TYRP1, SLC45A2,* and *SLC24A5* genes among the others in Pakistani population (Gul *et al*., 2019; Kausar *et al*., 2013; Morice-Picard *et al*., 2014; Shah *et al*., 2014a; Shahzad *et al*., 2017; Zhang *et al*., 2019).

The present study is designed to explore the genetic basis of OCA in two affected families from Azad Jammu and Kashmir. This region is located between the borders of India and Pakistan. Studies from this area revealed that mutations in *TYR* and *OCA2* genes are the major causative variants in OCA families (Konno *et al*., 2009; Shah *et al*., 2014b; Shahzad *et al*., 2017).

MATERIALS AND METHODS

Two families were recruited from different areas of Azad Jammu and Kashmir. For the extraction of genomic DNA, blood samples were taken from the affected as well as normal members. Interviews were conducted from all the available members of families at the time of enrollment. Pigmentation in the hair, skin and eyes was documented by taking photographs of the affected members.

Whole exome sequencing

Genomic DNA of affected members (IV:6) of family A and V:7 of family B were subjected to whole exome sequencing for the identification of responsible variants of OCA. Whole exome sequencing library enrichment was done with the Roche Nimble-Gen SeqCap EZ Exome v3 kit. Then, Illumina HiSeq 4000 was applied to sequence the selected samples. With the sequencing reads kept at 36x, each read from the target area covered 94% of the average means depth.

After removing adaptors and low-quality reads, the qualifying raw fastq file was processed in accordance with the "Best Practices for Germline SNP and Indel Discovery" published by GATK. The pure sequences were aligned using Burrows-Wheeler Aligner (BWA-MEM v0.7) to the UCSC Genome Browser hg19. 12), and Picard v1.141 was used to remove PCR duplicates. No data containing synonymous, noncoding variations with a minor allele frequency (MAF) of 0:01 is included in the research from the exome aggregation consortium (ExAC), 1000 genomes, or genome aggregation database (gnomAD) (https://gnomad.broadinstitute.org/) databases. Only segments that alter a gene's splice site's coding regions, synonymous splice site variations, nonsense segments, and non-synonymous segments were considered for interpretation. Additionally, a range of *in silico* algorithms, such as SIFT (http://sift.PolyPhen2) (http://genetics.bwh.org), jcvi.org/., mutation taster (http://www.mutationtaster.org/), FATHMM (http://fathmm.biocompute.org.uk/), and Harvard University (http://harvard.edu/pph2/) were utilized to assess potential negative impacts of pathogenic variants on the structure and function of proteins.

Variant validation and segregation testing

Sanger sequencing was used to confirm variant zygosity in all available family members. After filtering, remaining candidate variants were amplified by PCR using primers designed with Primer3 software (Untergasser *et al*., 2012). Bidirectional sequencing of PCR products was performed using standard protocols. Chromas version 2.6.6 and Alamut Visual Plusversion 1.6.2 were used to analyze DNA sequences.

Protein 3D modelling and docking

For designing 3D protein models of normal and mutant TYR protein and its close functional interactor DCT protein, I-TASSER online (Yang *et al*., 2015) tool was used. 3D protein model with highest confidence score (C-Score) was chosen for additional exploration (Muzammal *et al*., 2022). Designed 3D models were visualized by Chimera 1.13.1 (Pettersen *et al*., 2004). After designing and visualizing 3D protein models, protein-protein docking was carried out using online cluspro server tool with its close functional interactors (Kozakov *et al*., 2017). Close functional interactor was predicted via String Data base (Szklarczyk *et al*., 2019).

Multiple sequence alignment

To check the conservation of substituted amino acids

in different species multiple sequence alignment was done using online tool Clustal Omega (Larkin *et al*., 2007; Sievers and Higgins, 2014).

RESULTS

Clinical manifestation

We recruited two consanguineous families affected with OCA from different regions of Azad Jammu and Kashmir. Affected individuals of both families showed clear signs and symptoms of OCA, including hypopigmentation in the eyes, white to pale hair color, reduced pigmentation in skin, impaired vision along with nystagmus, iris trans-illumination and photophobia.

Additionally, in family A, depigmentation was clearly observed in the skin, hair and eyes and impaired vision was reported along with continuous nystagmus. A summary of the clinical characteristics of the affected members of both families is shown in Table I.

Identification of pathogenic variants in OCA-affected families

Analysis of whole exome data of Family A identified previously reported mutation c. 62C>T: p.Pro21Leu in the exon 1 of *TYR* gene. Family B showed previously reported mutation c.832C>T, p.Arg278* in the *TYR* gene. Additionally, the segregation of these variations within their respective families is confirmed by Sanger sequencing.

In Family A, both the affected individual (IV: 5 and IV: 6) were homozygous mutant, while the normal individual (III: 5) was heterozygous carrier for the variant c.62C>T: p.Pro21Leu,while in Family B the normal individuals (IV:2,IV:3 and V:9) were heterozygous carrier for the variant c.832C>T, p.Arg278* and affected members (V: 6) and (V: 7) were homozygous mutant (Fig. 1).

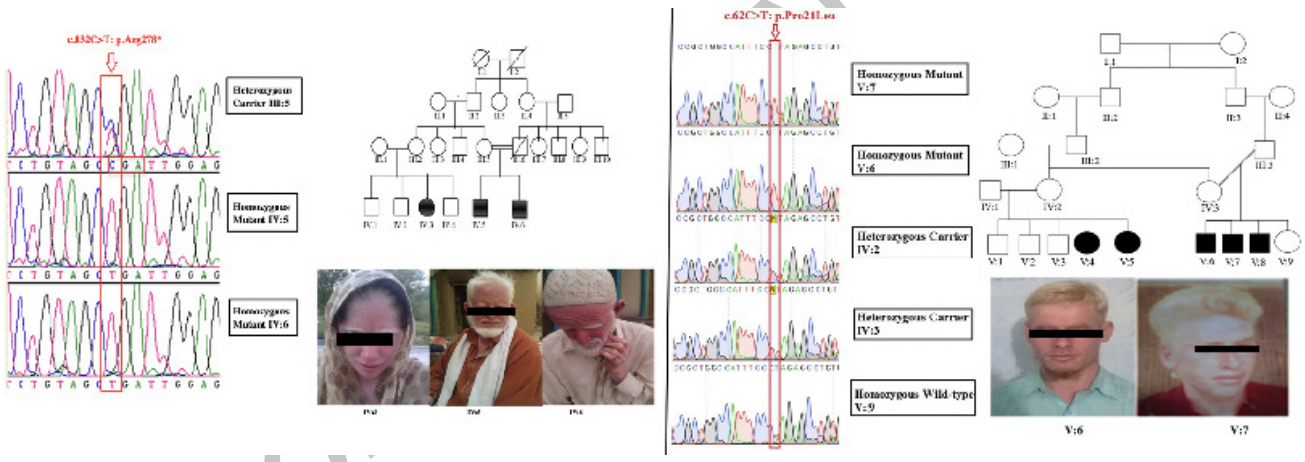

Fig. 1. Sanger sequencing chromatograms, pedigrees and Facial photographs of both the families.

Table I. Clinical findings of two Pakistani families showing mutations in *TYR* gene.

Family	Subject ID	Age (Years)	Sex	Mutation	Ethnolinguistic group	Hair color	Skin tone	Iris color
A	IV:3	54	F	c.62C>T: p.Pro-21Leu	Syed	Black	Black	Black
	III:3	60	M			Black	Black	Black
	V:9	26	F			Black	Black	Black
	V:6	28	M			White	White	Brown
	V:7	30	M			White	White	Brown
B	III:2	47	F	c.1255G>A: p.Gly419Arg	Mughal	Black	Brown	Black
	III:1	50	M			Black	Brown	Black
	IV:3	23	M			White	White	Grey blue
	IV:4	26	M			White	White	Grey blue

Molecular modeling of normal and mutated TYR proteins

3D structure of wild-type and mutant (p. Pro21Leu) TYR proteins were predicted and superimposed to check the similarity (% identity) between two structures (wild-type and mutant (p. Pro21Leu) TYR proteins). The 3D structures of the wild-type and both mutant TYR proteins were overlaid using molecular modeling. The TYR protein wild-type mutant 2 (p. Pro21Leu) had the lowest similarity index of 82.42% (Fig. 2).

Mutant protein showed variable docking sites and docking residues when compared with wild-type TYR protein. These changes in the interacting residues showed the pathogenic effect of these variation in compound heterozygous state, which effect the interaction of TYR protein with its close interactor DCT protein. A study of the protein-protein interactions between the wild-type and both mutant TYR proteins, showed that the mutation caused a significant change in the docking locations, interacting residues, and bonding. Thirteen distinct residues and eighteen hydrogen bonds mediated the contact between the mutant p. Pro21Leu and the DCT protein (Fig. 3).

Multiple sequence alignment

Multiple sequence alignment of substituted amino acid were done and found that the substituted amino acids i.e. i.e. Pro21 is highly conserved throughout several species. Which confirms the importance of these amino acids. Conservation of substituted amino acids are shown in Figure 4.

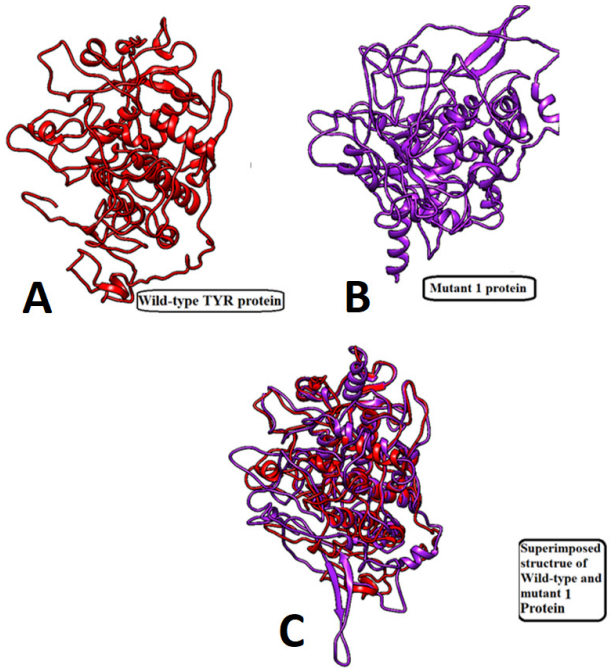

Fig. 2. (A)Tertiary structure of wild-type (B) mutant TYR proteins and (C) their superimposed structure.

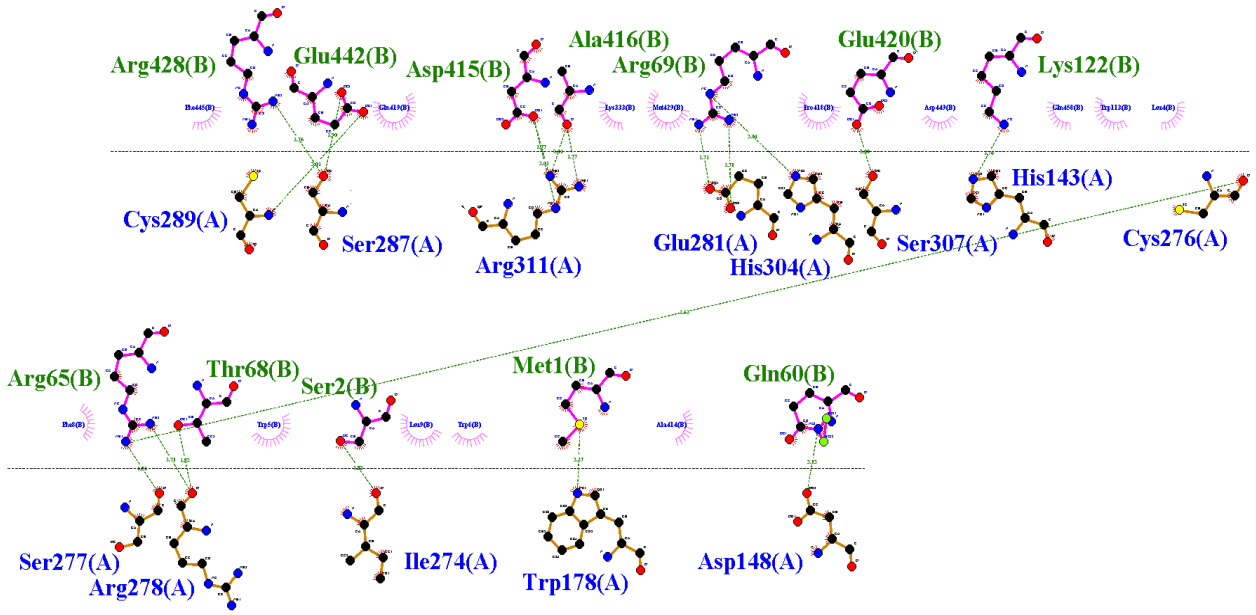

Fig. 3. Protein-Protein docking of mutant p.Pro21Leu and close interactor DCT protein.

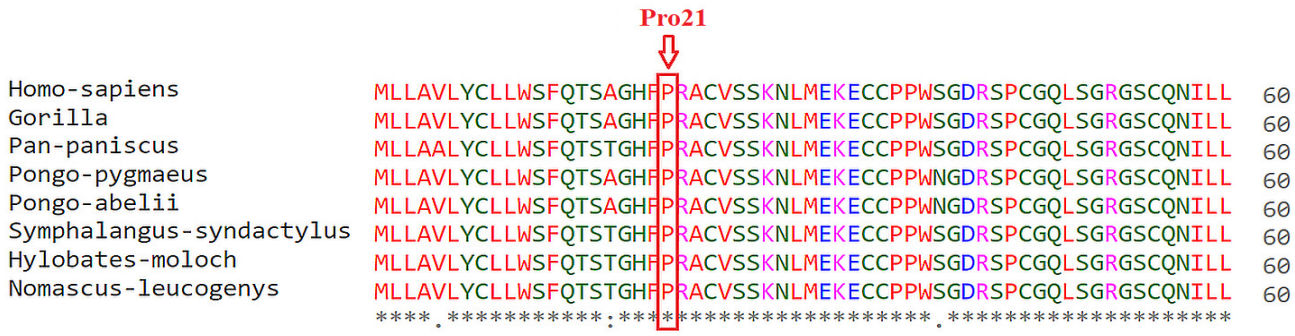

Fig. 4. Multiple sequence alignment of substituted amino acid showing the conservation of substituted amino acids. Red boxes showing position of substituted amino acid.

DISCUSSION

Non-syndromic autosomal recessive OCA is a diverse group of disorders that affects 1 in 20,000 people worldwide. The incidence of the many subtypes of OCA varies significantly based on an individual's ethnic background (Gargiulo *et al*., 2011; Kamaraj and Purohit, 2014). The most common symptom of OCA is a reduction in or absence of melanin synthesis, which leads to a partial or complete loss of skin, hair, and ocular pigmentation. Eight discrete autosomal genetic loci have already been identified to be associated with non-syndromic OCA. It is reported that OCA cases, including those in Pakistan, are primarily caused by mutations in the *TYR* (OCA1) and *OCA2* (OCA2) genes (Gul *et al*., 2017). List of previously reported *TYR* gene mutations in Pakistan population are summarized in Table II.

The overall prevalence of *TYR*-associated pathogenic mutations in Pakistan is approximately 44%, while it is 46% in the European population (Jaworek *et al*., 2012). *TYR* pathogenic variant findings from other Asian ethnicities are inconsistent, but one regional subgroup of the Chinese population has over 70% of these reports, demonstrating considerable variation (Gul *et al*., 2017; Jaworek *et al*., 2012). A study on an Indian ethnic group revealed that individuals with OCA characteristics had a 60% incidence of *TYR* pathogenic mutations (Okamura *et al*., 2019; Sengupta *et al*., 2010). Furthermore, in other populations including Americans, Koreans, Japanese, and Italians, the OCA phenotype is mostly caused by the predominance of TYR pathogenic mutations (Hutton and Spritz, 2008).

With a frequency of about 23%, the pathogenic mutation of the *TYR* gene that is most frequently discovered in Pakistani OCA families is c.1255G>A; p.Gly419Arg, which is mainly displayed by Punjabi. Yet, OCA families from various linguistic groups, such as the Baloch, Saraiki, Sindhi, and Kashmiri language groups, have also been found to have a comparable pathogenic variation (Arshad *et al*., 2018; Gul *et al*., 2019; Hutton and Spritz, 2008; Ghodsinejad Kalahroudi *et al*., 2014; Shahzad *et al*., 2017). Another analysis also identifies the second most prevalent pathogenic variation, which accounts for around 21% of all known pathogenic variants: c.832C>T; p.Arg278*(Gul *et al*., 2019; Shakil *et al*., 2019). Moreover, c.1037-7T>A, a previously identified splice-site pathogenic mutation, has been found in a number of citizens with Pakistani origin (Gul *et al*., 2017, 2019; Shahzad *et al*., 2017).

Numerous research studies have documented novel, well-known, and founder mutations of the *TYR* gene. The most prevalent and common mutations in the population are c.832C>T: p.R278* and c.1255G>A: p.Gly419Arg variants of the *TYR* gene, which have been identified in 21 families (22.6%) and 20 families (23.8%), respectively. On the contrary, the founder mutations, such as c.230G>A (5.9%), are certain mutations that contribute frequently to the ancestry. About 90 variants, including 86.9% missense mutations, 7.1% nonsense mutations and the remainder changes causing splice site errors, deletions, and frame shifts, are connected to *TYR*-linked OCA in consanguineous families in Pakistan (Table II).

In this research, mutational analysis of two families, having non-syndromic OCA, was done by performing Sanger sequencing for the identification of underlying genes that cause disorder to appear. All families were depicting the symptoms of non-syndromic form of OCA. In exon 1 of the *TYR* gene, we discovered already reported pathogenic mutation c. 62C>T: p. Pro21Leu in the exon 1 of the *TYR* gene. Family B showed c.832C>T; p.Arg278* mutation in exon 2 of the *TYR* gene was discovered. As consanguineous marriages account for 60% of all marriages in Pakistan (Elahi *et al*., 1998), consanguinity is the major contributor for causing non-syndromic OCA in both families. The approach used here for the prediction of responsible gene and specific mutations is Sanger sequencing.

Table II. List of previously reported *TYR* gene mutations in Pakistani families associated with OCA.

No of families	Nucleotide change	Amino acid change	Mutation type/ Status	Ethnicity	Reference
1	c.62C>T	p.Pro21Leu	Missense/Homozygous	Punjabi	Kruijt *et al*. (2018)
1	c.1424G>A	p.Trp475*	Nonsense/Homozygous	Saraiki	Jaworek *et al*. (2012)
1	c.1231 T>C	p.Tyr411His	Missense/ Homozygous	Punjab	Shakil *et al*. (2019)
1	c.1217C>T	p. Pro406Leu	Missense/ Homozygous	Punjabi	Shakil *et al*. (2019)
1	c.1184+2 T>C	-	Splicing Error/ Heterozygous	Punjabi	Shakil *et al*. (2019)
1	c.115 Del 340-341	p.R278 *	Frameshift Nonsense	Pakistani	Gul *et al*. (2017)
1	c.1147 G>A	p.Asp383Asn	Missense/ Homozygous	Pakhtoon	Shakil *et al*. (2019)
1	c.1037-18 T>G	-	Splicing Error/ Heterozygous	Urdu speaking	Shakil *et al*. (2019)
4	c.1037-7 T>A	-	Splicing Error/ Heterozygous	Urdu Punjabi	Spritz *et al*. (1995)
1	c.1037G>T	p.Gly346Val	Missense/Homozygous	Punjabi	Shakil *et al*. (2019)
3	c.896A>G	p.Arg299His	Missense/Homozygous	Pakhtoon, Punjabi	Shakil *et al*. (2019)
1	c.593T>C	p.Ile198Thr	Missense/Homozygous	Pakistani	Shah *et al*. (2015)
1	c.585G>A	p.Trp195*	Nonsense/ Homozygous	Punjabi	Shakil *et al*. (2019)
1	c.943-948 deltcagct	p.315-316del	Deletion/Heterozygous	Punjabi	Shakil *et al*. (2019)
1	c.575C>A	p.Ser192Tyr	Homozygous	Kashmiri	Lee *et al*. (2021)
21	c.832C>T	p.Arg278*	Nonsense/ Homozygous	Urdu speaking, Punjabi, Pashtun	Konno *et al*. (2009), Sajid *et al*. (2021), Shahzad *et al*. (2017), Shakil *et al*. (2019), Spritz *et al*. (1995)
2	c.346C>T	p.Arg116*	Nonsense/ Homozygous	Saraiki, Punjabi	Gul *et al*. (2019)
1	c.895C>T	p.Arg299Cys	Missense/Homozygous	Saraiki	Jaworek *et al*. (2012)
1	c.715C>T	p.Arg239Trp	Missense/Homozygous	Kashmiri	Shahzad *et al*. (2017)
1	c.982G>C	p.Glu328Gln	Missense/Homozygous	Pakistani	(Lee *et al*. (2021)
2	c.240G>C	p.Trp80Cys	Missense/Homozygous	Pakhtoon	Arshad *et al*. (2018)
1	c.1037 G>A	p.Gly346Glu	Missense/Homozygous	Punjabi	Shakil *et al*. (2019)
1	c.826C>T	p.Cys276Arg	Missense/Homozygous	Pakhtoon	Konno *et al*. (2009)
1	c.308G>A	p.Cys103Tyr	Missense/Homozygous	Punjabi	Spritz *et al*. (1995)
1	c.272G>A	p.Cys91Tyr	Missense/Homozygous	Punjabi	Spritz *et al*. (1995)
3	c.132T.A	p.Ser44Arg	Missense/Homozygous	Kashmiri, Pakhtoon, Baloch	Arshad *et al*. (2018)
2	Exon 4-5 deletion	-	Deletion/Heterozygous	Punjabi	Shakil *et al*. (2019)
2	c.103T>C	p.Cys35Arg	Missense/Homozygous	Punjabi	Shah *et al*. (2014)
1	c.248T>G	p.Val83Gly	Missense/Homozygous	Pakhtoon	Shahzad *et al*. (2017)
4	c.230G>A	p.Arg77Gln	Missense/Homozygous	Kashmiri	(Shahzad *et al*. (2017)
1	c.1204C>T	p.Arg402*	Nonsense/Homozygous	Punjabi	Shakil *et al*. (2019)
1	c.223G>T	p.Asp75Tyr	Missense/Homozygous	Punjabi	Shakil *et al*. (2019)
1	c.164G>C	p.Cys55Ser	Missense/Homozygous	Punjabi	Shakil *et al*. (2019)
23	c.1255G>A	p.Gly419Arg	Missense/Homozygous	Punjabi, Sindhi, Pakhtoon, Kashmiri, Saraiki	Arshad *et al*. (2018), Shakil *et al*. (2022)

Molecular screening also revealed that all mutations are pathogenic. Besides this, all the families A and B showed homozygosity (Mut/Mut) in affected subjects. However, the carriers were heterozygous (WT/Mut).

CONCLUSION

In this study, we identified previously reported mutation in *TYR* gene in two families affected with non-syndromic OCA, from Azad Jammu and Kashmir, Pakistan. Our current findings and already reported data show that *TYR* gene is the causative gene in families affiliated with OCA in majority of cases in Pakistan. Moreover, research reported that all mutations are showing pathogenic status. This study expands the mutational spectrum of *TYR* gene in Kashmiri families.

DECLARATIONS

Acknowledgement

We are Also thankful to Higher Education Commission Pakistan (HEC) for awarding IRSIP Fellowship to conduct this study.

IRB approval

Informed consent was obtained from the participants prior to blood sampling. The study was also approved by the institutional review board (IRB) of Mirpur University of Science and Technology (MUST) Mirpur, AJK.

Ethical statement

The study was conducted according to the guidelines of the Declaration of Helsinki,and written informed consent was obtained from the participants prior to conduct the study.

Generative AI and AI-assisted technology statement

The authors declare that they have not used generative AI or AI-assisted technologies in the writing or editing of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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