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Mutational Analysis of TYR Gene Causing Oculocutaneous Albinism in Families from District Peshawar

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ABSTRACT Oculocutaneous Albinism is a cluster of genetic diseases specified by full or partial loss of biosynthesis of melanin in melanocytes cells. The aim of current research was to determine the mutational status of this gene in the enrolled OCA diagnosed consanguineous families. DNA was isolated from all collected samples using inorganic method of extraction. The samples were screened for identification of *TYR* mutations in the enrolled families using sequencing of the exons of this gene. A homozygous missense mutation (*c.132T>A*) in the *TYR* gene was recognized in the OCA affected individuals in the consanguineous family-II whereas sequence analysis of normal individuals have shown no sequence change in respective exon. A single missense substitution (*p.Ser44Arg*) was identified in the *TYR* using PolyPhen 2, MutationTaster and I-Mutant software. The current study is important in terms that it confirms important mutation causing Oculocutaneous albinism in this region.

INTRODUCTION

Albus, meaning white, is a Latin word, from which the word albinism is derived. It is an autosomal recessive disease in which formation of melanin in melanocytes cells is partially or totally absent. The earlier used terms for the classification of albinism was "incomplete or complete" and "tyrosinase-positive or tyrosinase- negative", which were replaced by the new classification based on affected genes (Summers et al. 1996; Majeed and Ubaid 2013). Albinism has been classified into two types including non-syndromic and syndromic. Furthermore, there are two forms of non-syndromic albinism, that is, X-linked Ocular Albinism (OA) and OCA (Grønskov et al. 2007). Sir Arcibald Garrod in the beginning indicated that disorder of metabolism in inborn may cause albinism. Currently, it is confirmed that it is a heterogeneous disease and various linked genes are responsible for it (Kamaraj and Purohit 2014). In 1989, the first recognize variant (nonsense variant) was reported in *TYR* gene, causing OCA. A number of different types of mutations have then been identified; large scale and small variations, small indels, splicing, missense and nonsense mutations, missense being the most common (Sundaresan et al. 2004; Lu et al. 2017).

The OCA is clinically recognised by reduction in the pigments of hair, skin and eyes. Ocular abnormalities, involuntary movement of eyes, reduction of pigments in the iris, reduced vision, photophobia are the main symptoms (Khordadpoor-Deilamani et al. 2015). Around the world, 1 out of 20,000 live births are affected by albinism and its frequency alter between dissimilar racial backgrounds (Gargiulo et al. 2011). Around the globe, the highest widespread type is OCA1, indicating more or less 50 percent of all the cases followed by OCA type 2 at 30 percent while prevalence of OCA3 is approximately 3 percent and OCA4 is 17 percent (Rooryck et al. 2008; Rooryck et al. 2009). Various types of OCA prevalence varies commonly in distinct populations (Grønskov et al. 2007). There are seven types of non-syndromic OCA, induced by following genes: OCA1 in *TYR* at 11q14.3, OCA2 in *P gene* at 15q12, OCA3 in *TYRP1* at 9p23, OCA4 in *SLC45A2* at 5p13.3, OCA5 in *ND* at 4q24, OCA6 in *SLC24A5* at 15q21.1

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and OCA7 in *C10ORF11* at 10q22.2- q22.3. The human tyrosinase gene (MIM 606933) at 11q14–q21 is composed of five exons, occupying around 65 kb of the particular region of DNA and which codes for tyrosinase.Activity of tyrosinase is reduced or completely stops by variations in the *TYR* gene, stopping production of melanin in melanocytes throughout life (Shah et al. 2018). The OCA1 has been categorized into two sub forms; OCA1A and OCA1B. In OCA1A a complete loss of the activity of tyrosinase and creates a completely depigmented phenotype having white hair, white skin, and blue irides through entire life span. In OCA1B (yellow albinism), a partial loss of the function of tyrosinase but with the passage of time, some melanin pigments is accumulated. The OCA1B affected patients are born with white hair and with the passage of time, changes to blond or yellow color and exhibit blue eye color (Lu et al. 2017; Norman et al. 2017).

Almost 303, 152, 16 and 77 different pathogenic sequence variations have been accounted in various linked genes including *TYR*, *P*, *TYRP1* and *MATP*, which cause the non-syndromic autosomal recessive OCA(Shah et al. 2018). In Pakistan, recessively genetic disorders are more frequent because of marriages between cousins. (Ullah et al. 2017). As a result of the inimitable socio culture customs in the Pakistan, about sixty percent of the marriages are between cousins, of which greater than eighty percent are among first cousins (Qidwai et al. 2003). These consanguineous marriages are one of the important move in which these mutations are transmitted from generation to generation, further perplexing the disease.

Objectives of the Study

The objectives of the study were to sequenced *TYR* gene for mutational analysis and to determined pathogenic effect of the identified mutation (s) in the selected consanguineous families.

METHODOLOGY

Enrolment of Consanguineous Families

A total of three consanguineous families were enrolled having more than two affected individuals from District Peshawar, Khyber Pakhtunkhwa, Pakistan. For drawing pedigrees of the selected families, the Cyrillic software (Cyrillic 2.1) was used. The selected families showed believable proof for an autosomal recessive way of inheritance (Ali et al. 2011).

Collection and Storage of Blood Samples

Blood (5-10 ml) was taken from the selected families including patients, unaffected brothers, sisters, father and mother in Ethylene Diamine Tetra Acetic Acid tubes.The collected blood tubes were kept at -20°C for not more than a week, before DNA was extracted (Ali et al. 2011).

Extraction and Visualization of Genomic DNA

Inorganic method was utilized to separate DNA from samples of blood. First of all, Red blood cells were lysed with the TE buffer. Then white blood cells were lysed and using 100 µl of 10% SDS, 40 µl of proteinase K and 3 mL TNE Buffer. Finally, DNA was precipitated by adding isopropanol and isolated in the form of pellet with the help of centrifugation.The extracted DNA was visualized using 0.8% agarose gel. 2µl extracted DNA from each sample and 3µl bromophenol blue dye was loaded in the gel wells; bromophenol blue acting as loading and tracking dye. The samples were run on gel in 1X TAE buffer at 80V for 30 minutes. The DNA was seen as fluorescent bands under Ultraviolet (UV) light in gel documentation system. Gel pictures were saved, which was used as reference for further processes (Sambrook et al. 2001).

Optimization of Polymerase Chain Reaction

For all the primers, the conditions were set to achieve more copies of particular portions of DNA. During optimization, various changes were made in the master mixtures ingredients, that is, enzyme, MgCl2, primers, volume of DNA and annealing temperature of primers with template DNA. The DNA of the enrolled consanguineous families was amplified with the help of PCR to achieve more copies of particular segment of DNA. First of all DNA was denatured at 93 °C for 3 min, followed by 30 cycles at 93 °C for 45 sec, 55 °C for 30 sec and 72 °C for 45 sec, and a final extension step at 72 °C for 10 min for all

exons of the gene. The components and its required volume per PCR sample is given in Table 1.

Table 1: Required volumes of the ingredients per PCR sample

S. No.	*Components*	*Volume*
01	Template DNA	2 µl
02	PCR Master Mixture	12.5 µl
03	Forward Primer	0.75 µl
04	Reverse Primer	0.75 µl
05	PCR Water	9 µl

Agarose Gel Electrophoresis

2µl product of PCR was taken and mixed with 3µl of bromophenol blue loading dye. After this, all the mixed PCR samples were loaded in the wells of 1.2 to 2.0% agarose gel based on the PCR product size. The obtained products of PCR was observed as fluorescent band of expected size using UV light and documented by gel documentation system (Kalahroudi et al. 2014).

Designing of Primers

Specific primers for each and every exon of *TYR* gene was made using Primer 3 software and was chemically synthesized by Macrogen (Untergasser et al. 2012). The *TYR* gene is composed of five exons and for these, six primers were designed. The size of exon one was long and therefore two primers were designed to cover the whole exon sequence. Before and after the exon sequence, some region of the intron was also selected. Therefore all the primers were made in the region of the intron so that during sequencing, the sequencer device must easily sequence full region of the exon. The complete detail of all the primers and their sequences are given in Table 2.

Purification and Agarose Gel Electrophoresis of PCR Products

The obtained products of PCR were purified using ethanol purification method. 2µl of ethanol purified PCR product was taken from each purified PCR products and mixed with 3µl of Bromophenol blue. After this, all the samples were loaded in the wells of 1.2 to 2.0 % agarose gel based on the PCR product size. The obtained ethanol purified products of PCR were observed as fluorescent bands of expected size using UV light and documented by gel documentation system (Kalahroudi et al. 2014).

Sequencing of PCR Products

The PCR products purified by ethanol of OCA patients along with unaffected persons of the enrolled families were sequenced with the help of dideoxy chain termination direct Sanger sequencing on ABI genetic analyzer 3130.

Analysis of Results

The obtained data from sequencing were analyzed using BioEdit v7.0.9 and NCBI BLAST. Sequence alignment was performed using NCBI BLAST software to identify mutations in all obtained sequences of the enrolled families. The

Table 2: Sequences of primers of *TYR* exons used in the current study

S. No.	*Name*	*Sequence*	*Exon No*	*Position on chromosome*	*Product size*
1	*TYR 1F*	ACCTTGTGAGGACTAGAGGA	Exon 1	chr11:88911099+88911549	451
	TYR 1R	TGCTTTGCTAAAGTGAGGTA			
2	*TYR 2F*	ACATCTTCGATTTGAGTGC	Exon 1	chr11:88911486+88911980	495
	TYR 2R	TTCCTCCCTACTCTGACATC			
3	*TYR 3F*	ATGATGTTCACGCAATGA	Exon 2	chr11:88924176+88924661	486
	TYR 3R	CCTCCTAGGACTTTGGATAA			
4	*TYR 4F*	TCTCAATACGGAATGAATTTT	Exon 3	chr11:88960850+88961283	434
	TYR 4R	TAAGGTCTGTTTTGGTGACA			
5	*TYR 5F*	CATCTTTCCATGTCTCCAG	Exon 4	chr11:89017828+89018321	494
	TYR 5R	GCAAAATGACCATGACTTT			
6	*TYR 6F*	CAAACCCAGGTGTCTACTCCA	Exon 5	chr11:89028208+89028577	370
	TYR 6R	AGTGAGGTCAGGCTTTTTGG			

pathogenic effect of the identified variation waspredicted using the prediction program Polyphen-2, MutationTaster, I-Mutant software.

RESULTS

The enrolled families were screened for identification of mutations in the selected gene. The ages of the affected individuals ranged from 3 to 50 years. A homozygous missense mutation in the *TYR* gene was recognized in the OCA affected patients in the consanguineous family-II whereas sequence analysis of normal individuals have shown no sequence change in respective exon.

Consanguineous Family-II

This family depicted consanguinity in the third generation and had three affected members represented as 2F3 (IV: 2), 2F4 (IV: 3) and 2F5 (IV: 4). All affected persons showed white hair, white skin, blue irides, involuntary movement of eyes, reduction of pigment in the iris, low vision and photophobia. Picture of the affected individual is given in Figure 1 (A). The pedigree of consanguineous family-II showed three affected persons; IV: 2, IV: 3 and IV: 4, in homozygous condition while their parents were

Fig. 1A. Affected individual having Oculocutaneous Albinism

in heterozygous condition. This family represented a recessive way of inheritance and the pedigree family-II is presented in Figure 1(B).

Quantification, Polymerase Chain Reaction and Agarose Gel Electrophoresis

The quantity of DNA was measured with the help of UV-Visible Spectrophotometry at 260-280nm. The samples were diluted to 1:50 by picking 20μl of extracted DNA in 980μl of autoclaved double distilled water. Optical density of double distilled water was used as a reference and the

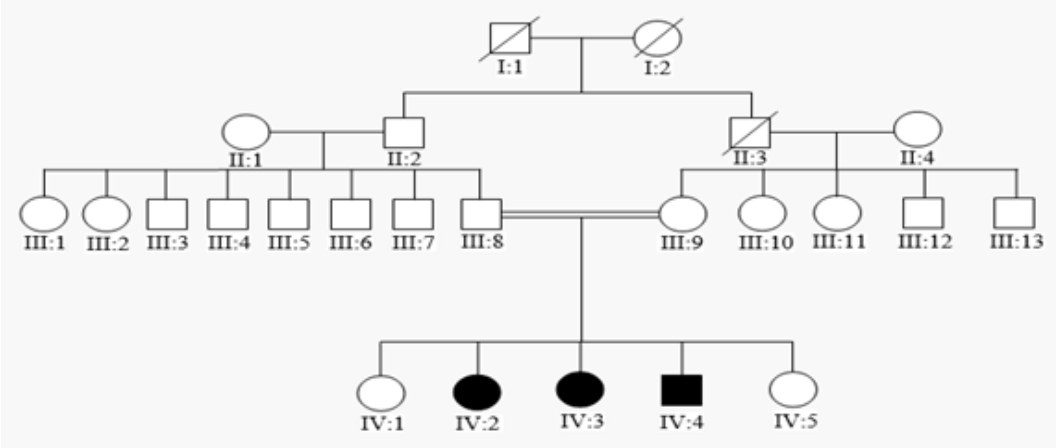

Fig. 1B. Pedigree of consanguineous family-II showing the affected individuals are homozygous while their parents are heterozygous

amount of extracted DNA was determined. The extracted DNA from the whole blood of the collected samples was checked using 0.8% agarose gel. The DNA bands were visualized as fluorescent band under UV light as shown in Figure 2 and documented by gel documentation system. Gel pictures were saved, which was used as reference for further processes.

The DNA of the enrolled families was amplified to get large copies of particular portions of

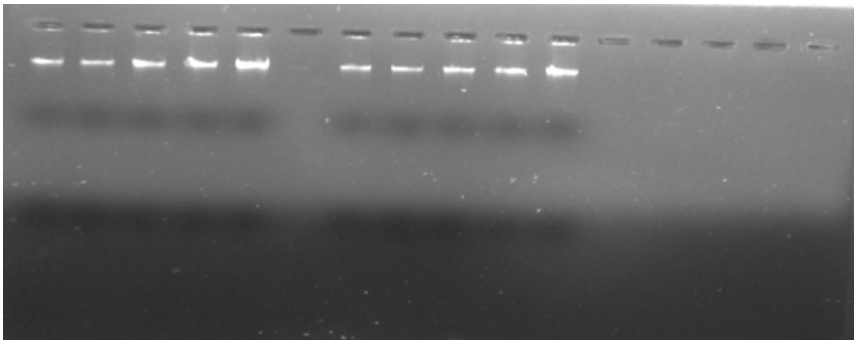

Fig. 2. Gel picture showing Purified extracted DNA from the whole blood samples

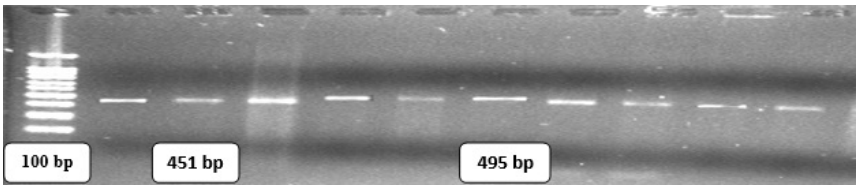

Fig. 3A. Gel picture indicating amplification of the exon 1 of the *TYR* gene

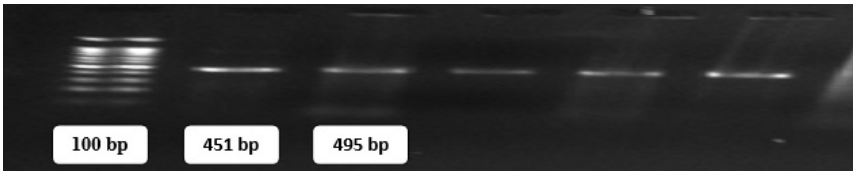

Fig. 3B. Gel picture indicating amplification of the exon 1 of the *TYR* gene

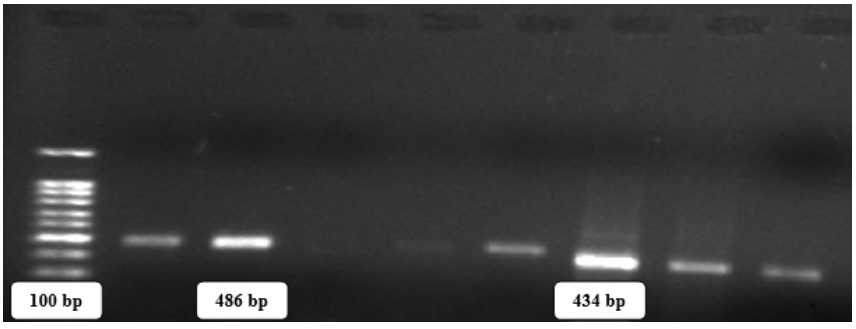

Fig. 3C. Gel picture indicating amplification of the exon 2 and exon 3 of the *TYR* gene

gene and was run 1.2 to 2.0% agarose gel based on the PCR product size. The obtained products of PCR was observed as fluorescent band of expected size using UV light and documented with the help of gel documentation system. The gel pictures showing amplification of the mutated exon as well as other exons of the *TYR* gene in the enrolled consanguineous family-II are given in Figure 3 (A), Figure 3 (B) and Figure 3 (C).

Sequencing and Analysis of PCR Products

The obtained products of PCR by ethanol were sequenced through dideoxy chain termination direct Sanger sequencing on ABI genetic analyser 3130. After sequencing, collected data were analysed using bioinformatics programs. The fasta format of all sequenced exons of the affected individuals as well as normal individual in the enrolled families were taken from BioEdit v7.0.9 and run on NCBI BLAST. After BLAST analysis, a homozygous missense variation (*c.132T>A*) was determined in the affected persons of Family-II.The enrolled consanguineous families data obtained from sequencing were analysed using BioEdit v7.0.9. Mutation was observed in the exon 1 of the *TYR* gene in all affected individuals of consanguineous family-II as shown in Figure 4.

Pathogenic Effect of Identified Mutation Using PolyPhen-2, MutationTaster and I-Mutant Programs

Using PolyPhen-2 program, pathogenic effect of the identified homozygous missense vari-

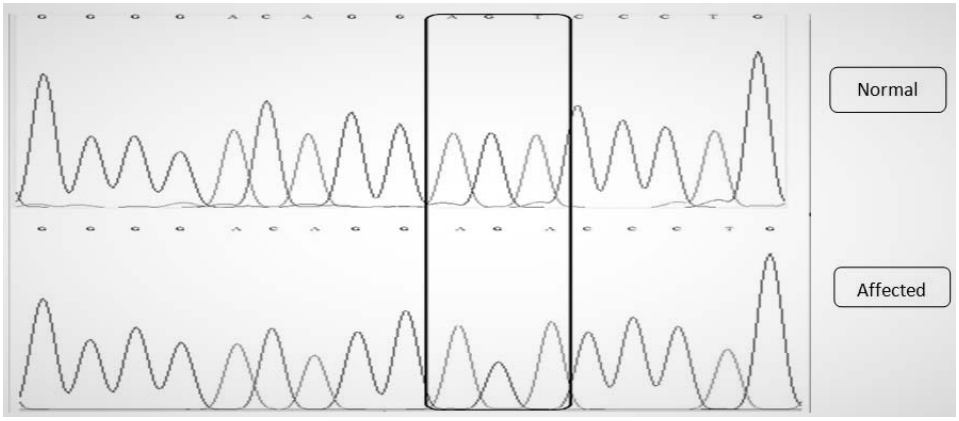

Fig. 4. Electropherogram of Family-II showing a homozygous missense mutation (*c.132T>A*) in exon 1 of the *TYR* gene

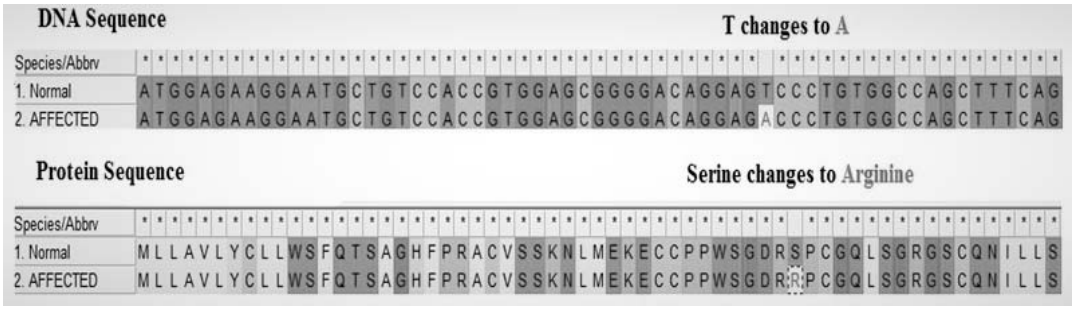

Fig. 5. Analysis showed amino acid substitutions (*p.Ser44Arg*) in the affected individuals

ant in the patients of the family-II was predicted. After Polyphen-2 analysis, an amino acid substitution Serine to Arginine (*p.Ser44Arg)* was identified. This indicated that the identified missense variant was deleterious and can possibly affect the role and features of the translated protein with a score of 0.873. The amino acid substitution (*p.Ser44Arg*) and pathogenic effect of the identified variant in the affected individuals is shown in Figure 5.

Using the MutationTaster online software, pathogenic affect of the homozygous missense mutation was predicted. The result indicated that this mutation probably causing OCA disease and having pathogenic effect on the protein by altering sequence of amino acids, probably affected features of protein as well as changes observed in splice site. Similarly, using the I-Mutant software, pathogenic effect of the homozygous missense mutation was also predicted based on the decrease in the stability of the translated protein. The analysis of homozygous missense mutation using bioinformatics tools is given in Table 3.

DISCUSSION

For the first time, Farabee in 1903 proposed that human albinism can be inherited recessively (Farabee 1903). The first mutation, nonsense, was reported in the *TYR* gene causing OCA in 1989 (Lu et al. 2017). The human tyrosinase gene (*TYR*, 11q14–q21) is composed of 5 exons, occupy around 65kb of the DNA region and codes for tyrosinase. The main role of tyrosinase is biosynthesis of melanin pigments by catalysing the rate-limiting conversions of tyrosine to DOPA and from DOPA to DOPA-quinone. The conversion of 5,6-dihydroxyindole into indole-5,6 quinone is also catalysed by this enzyme (Shah et al. 2014). Till now, almost 548 various pathogenic sequence mutations have been reported, causing non syndromic autosomal recessive OCA (Arshad et al. 2018). There are 303, 152, 16 and 77 different pathogenic sequence variations, which have been reported in various associated genes causing non-syndromic autosomal recessive OCA (Shah et al. 2018). Shah et al. (2015) studied Oculocutaneous albinism in Pakistan and determined various pathogenic variants in the *TYR* gene including; *p. Pro21Leu, p. Cys35Arg, p. Ser44Arg, p. Arg77Gln, p. Ser 192Tyr, p. Ile98Thr, p.Arg239Trp, p.Arg278, c.344 delGA, p. Gln328Glu, p.Glu376, p. Ser315_ a316del, p.Pro43Thr, p.Glu453, p.Gly419Arg, p. Pro431Leu, p.Pro21Leu, p.Tyr411His, p. Cys35 Arg, Arg299His, p.Pro406Leu, (OCA2) p. Met318Ile, p.Asp486Tyr, p.Leu527Arg, p.Pro 743Leu, p.Ala787Thr and (SLC45A2) p.Gln 272Lys.* Similarly Lu et al. (2017) recognised genetic reason of OCA1 in a consanguineous Chinese family. In which, Exome sequencing was performed, followed by confirmation in other individuals of the same family with the help of Sanger sequencing method. A homozygous missense variation, *c.896G>A (p.R299H)*, in the *TYR* gene was recognized. On the other hand Sundaresan et al. (2004) studied a genetic analysis was carried out of OCA1 in Indian families. Bidirectional direct sequencing was performed for mutational study. Two unreported variants *(c.937del8, c.1379del2)* and a previously identified nonsense variant *(R278X)* in the *TYR* gene were recognized. Kalahroudi et al. (2014) studied variations in the tyrosinase coding gene of thirty unrelated Iranian OCA1 affected individuals and hundred normal persons with the help of polymerase chain reaction sequencing. Probable impacts of novel variants were also checked on the features and role of tyrosinase by SIFT, PolyPhen and I-Mutant 2 tools. Two novel pathogenic p.C89S and p.H180R variants were identified in two OCA1 affected individual.

Arshad et al. (2018) reported a novel and reported mutations in *TYR* gene in families affected with OCA from Pakistan. In which an unre-

Table 3: Bioinformatics analysis of homozygous missense mutation

Missense mutation	*Amino acid substitution*	*PolyPhen-2 prediction*	*Mutation taster prediction*	*I-mutant prediction*
c.132T>A	*p.Ser44Arg*	Probably Damaging	Causing Disease & Probably Damaging	Decrease Stability

ported missense mutation in *TYR* (*c.240G>C; p.Trp80Cys*) has been identified. Also, four identified reported mutations in *TYR* gene (*c.649C>T; p.Arg217Trp, c.1255G>A; p.Gly419Arg, c.832C> T; p.Arg278 Term,* and *c.132T>A p.Ser44Arg*) were also recognized in the enrolled OCA affected families. All patients showed the cardinal characters of OCA comprising white hair, pale skin, involuntary movement of the eyes and low vision. In the current research, we studied OCA affected consanguineous families belonging to District Peshawar, Khyber Pakhtunkhwa, Pakistan. The enrolled families were characterized by white hair, dark brown hair, golden hair color, white skin, involuntary movement of the eyes, reduced visual acuity, light sensitivity, color vision impairment, refractive errors, foveal hypoplasia, reduction of pigment in iris and translucency. All of the selected individuals in the enrolled consanguineous families were analysed for detection of the variations in the tyrosinase coding gene. In consanguineous family-II, variation in the *TYR* gene was recognized, in which parents of the affected individuals were heterozygous showing a distinctive phenotype with no symptoms of OCA while the homozygous variant in the *TYR* gene was recognized in the affected individuals, which was absent in the normal individual. A homozygous missense variation (*c.132T>A*) in exon 1of the *TYR* gene was recognized in the OCA affected persons whereas sequence analysis of normal individuals have shown no sequence change in respective exon. Using on computational accesses, a single missense substitution (*p.Ser44Arg*) was identified that might possibly alter the functional behaviour by changing arrangement in the *TYR*, causing OCA type 1.

CONCLUSION

In this study, a homozygous missense variation (*c.132T>A*) was determined in one of the OCA affected family. Using computational accesses, a single missense substitution (*p.Ser44Arg*) was identified that might possibly alter the functional behaviour by altering arrangement in the translated protein, which is responsible for OCA type 1. This study may be helpful by counselling the OCA affected consanguineous families for the purpose to reduce the spreading of this disease in this region.

RECOMMENDATIONS

Genetic counselling of the families affected with OCA should be carried out to stop the spreading of this disease. Also compare a 3D-Model of predicted formed protein with the standard protein using computational programs. Finally, use of proper *TYR* mutant models of animal for the possible development of new treatment strategies for the cure of this problem.

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