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Molecular Genetic Testing for Carrier - Prenatal Diagnosis and Computational Analysis of Oculocutaneous Albinism Type 1

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Abstract

Purpose: Specific genetic markers have been used to detect pathological association for oculocutaneous albinism through carrier detection and prenatal diagnosis. To understand the impact and improper functioning of tyrosinase enzyme with c.715 C>T (Arg239Trp) mutation in TYR gene for albinism, molecular and bioinformatic tools are being explored. The present study aims to focus on molecular testing for oculocutaneous albinism and to develop structural model of human tyrosinase by computational approach to analyze the structural properties of Arg239Trp residue.

Methods: Blood samples were collected from the proband and available family members. A Chorionic Villus Sample (CVS) was collected from the proband's mother during the third month of her third pregnancy for prenatal diagnosis. The human tyrosinase structural model was generated for the wild type by homology modeling using tyrosinase of *Streptomyces castaneoglobisporus* as a template protein and validated. The mutant model was generated based on the structure of wild type by mutating the native residue arginine 239 to tryptophan. The predicted structures were analyzed and compared for the differences in their physico-chemical and structural properties to correlate the dysfunction of tyrosinase enzyme in mutant type.

Results: Molecular testing of a fetal CVS sample of the proband's mother revealed a normal sequencing pattern which is not containing R239W mutation. As expected, the mother delivered a normal baby without any ocular or cutaneous abnormalities in a familial case. With the same mutation in an affected father and normal mother from sporadic family participated in carrier detection, were delivered phenotypically normal baby. The hydrophobicity has increased slightly in the mutant model. It is observed that there is a conformational change in the overall structure of tyrosinase and the orientation of three histidine residues that interact with two copper ions as well.

Conclusions: This is the first successful result revealing the prenatal genetic profile of Arg239Trp mutant in a fetus from a familial oculocutaneous albinism family and also in a sporadic case by carrier detection. This analysis contributed a wide range of knowledge to correlate, pathogenicity of the predicted mutant model with wild type of human tyrosinase.

Keywords: Oculocutaneous albinism I; Molecular testing; Carrier detection; Prenatal diagnosis; Human tyrosinase; Homology modeling; Ramachandran plot; Hydrophobicity

Introduction

In India epidemiological-communicable diseases are on the decline due to better living conditions and healthcare delivery in the society. On the other hand, the relative increase in the prevalence of genetic diseases threatens to be a public health problem. One such group of metabolic disorder is Albinism. General population based oculocutaneous albinism (OCA) carrier screening is controversial in all the races. Because of the occurrence of this disease in prior generations, it is necessary to create the knowledge, so that even uneducated affected family members will be willing to diagnose the disease status. As a result, the carrier detection in general population has become necessary in Indian population. OCA, a recessive genetic disorder, diagnosed by performing molecular diagnostic analysis during the early pregnancy period. Prenatal diagnosis is a newly emerging technique, especially in genetically-inherited diseases, as a preventive measure and to provide a profile of genetic information about the fetus.

In humans, albinism generally leads to medical, social, and psychological problems. Due to this genetic inheritance, some parents choose to have the disease status of the fetus determined early in pregnancy in order to have options to continue if the fetus is disease-free or, for parents who decide to continue the pregnancy with an abnormal fetus, better preparation to care for the infant by being informed about the disease in advance. For example, genetic diseases that have a diet intolerance component may be treated with specialized diets for the mother and newborn baby. Likewise, at least few genetic diseases can be manageable by introducing prenatal diagnosis.

In general, congenital hypopigmentation of skin, hair, and eyes is known as oculocutaneous albinism (OCA); when primarily in the eye alone, it is called ocular albinism (OA). This occurs mainly due to dysfunction of the melanin pigment-producing cells (melanocytes) resulting in an abnormal disease-causing tyrosinase (EC 1.14.18.1) gene. There are four types of oculocutaneous albinism such as OCA1, OCA2, OCA3 and OCA4 based on the level of melanin pigmentation. Approximately 1 in 17,000 people have one of the types of albinism [1]. The frequency of OCA1 is approximately 1/40,000 and the carrier rate is 1/100 in most populations throughout the world [2]. There are two subtypes of OCA1; completely inactive enzyme and no melanin production is OCA1A, whereas minimally active and a small amount of melanin is produced is called OCA1B.

Since 1989, a large number of mutations of the tyrosinase gene, which results in OCA, have been reported (http://albinismdb.med.umn.edu/oca1mut.html- edited by William Oetting, Last update September 9, 2009). In previous study, we have observed *TYR* mutation c.715C>T in the proband recruited from the South Indian

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familial family which was diagnosed as oculocutaneous albinism type I-A (negative form) [3]. The same mutation has also been previously reported in different populations. In the same family, this mutation was not observed in the chorionic villus sample of proband's mother that was examined during the third month of pregnancy and the baby was normal. In second family, the same mutation (R239W) was observed in the albinotic father. Based on this observation, we hypothesized that R239W substitution is the primary cause for the OCA type 1-A which leads to improper functioning of tyrosinase and further affects the melanin synthesis.

In humans, tyrosinase encodes 529 amino acids and five exons with 50 kb genomic DNA [4,5], and is located on 11q14–q21 and encoded by the TYR gene [6]. Tyrosinase is classified under the type 3 copper-containing protein family, with a catalytic center formed by di-nuclear copper molecules [7]. The presence of two $Cu2^{+}$ ions in the active site of tyrosinase is observed across numerous organisms [8]. Each $Cu2^{+}$ ion in the active site of tyrosinase are coordinated by three histidine residues (180, 202, 211, 363, 367, 390) and are essential for the enzyme's catalytic activity [9]. The mutation c.715 C>T was originally reported [10] in a family with tyrosinase-negative OCA proband.

Generally, abnormal/inactive tyrosinase affects the entire mechanism of melanin production and thus leads to an albinotic condition. Because of the mutation in the amino acid sequence, it is hypothesized that there would be a change in the structure of mutant tyrosinase compare to the wild type. Therefore, the present study aims to emphasis the importance of carrier detection and prenatal diagnosis of specific OCA mutation that was previously diagnosed in the proband and parent's samples to reveal the fetal condition during next pregnancy before birth. Also, this study is carried out to analyze the structural impact of the above mentioned point mutation in WT and mutant tyrosinase. However, there is no crystallographic structure of human tyrosinase available in Protein Data Bank (PDB) so far [11]. It is important to develop molecular model to understand the variations in structural properties of wild with mutant and to correlate its phenotypic features with disease causing mutations. As the mutational analysis has proved that this variation is causative for the albinotic condition in this proband, there would be some differences in the overall structure of mutant tyrosinase which affects the functional properties of melanin bio-synthesis. Therefore, this report explores the variation in the structural and physico chemical properties of wild and mutant models and further evaluate the structure function relationship to provide structural insights in the field of 'Ocular Genetics'.

Materials and Methods

Sample recruitment and clinical evaluation

The study protocol had the approval of the Institutional Review Board of Aravind Eye Hospital, and all studies were performed according to the tenets of the Declaration of Helsinki. The families for carrier detections were recruited from different institutions; four families from Mediscan systems, Chennai, Tamil Nadu; one from Amrita Institute of Medical Sciences and Research Center, Cochin, Kerala and molecular diagnosis were performed in Aravind Medical Research Foundation, Madurai, Tamil Nadu. Mediscan Systems is registered for prenatal diagnostic techniques under the Regulation & Prevention of Misuse Act 1994. A certificate was issued by the Government of Tamil Nadu (Reg. No: PNA/364/99 DT. 15.09.1999). The Chorionic Villus Sample (CVS) collection and genomic DNA isolation were performed by the specialist group of Mediscan systems for prenatal diagnosis; the proband, his affected maternal grandmother, and other members of the family were clinically well diagnosed by the investigator from the Paediatric Ophthalmology Clinic, Aravind Eye Hospital, Madurai. After the nature of the study was explained, an informed consent was obtained from the proband's parents to collect peripheral blood samples for molecular diagnosis.

Preparation of genomic DNA

Peripheral blood samples were collected from the individuals and family members and genomic DNA was extracted by the salting out method [12]. Prenatal molecular diagnosis was performed on the extracted amniotic fluid by CVS collection from the proband's mother during her third month of pregnancy. The minimum amount of 30 to 40 mg of processed chorionic villus sample was extracted, and genomic DNA was isolated by Mediscan group and sent us for molecular diagnosis. The genomic DNA of participants was isolated from white blood cells and the concentration was quantified using a Nano-spectrophotometer (NanoDrop Technologies, Inc.; Wilmington, DE) and saved as a template for further mutation screening analysis. The results were blast analyzed by Finch TV (http://www.geospiza.com/Products/finchtv.shtml) and Chromas (http://www.technelysium.com.au/chromas.html) softwares. The observed mutations were reconfirmed by sequencing with the new set of PCR products. Heterozygous patients were systematically resequenced to ensure that the screening had not overlooking mutations.

Evolutionary conservation of human variant amino acid residues was evaluated using Expasy (http://www.expasy.ch/) tools by alignment to related mammalian species.

Bi-Direct sequencing of PCR amplified genomic DNA

The DNA fragments were amplified by PCR; the mutations were detected by using the cyclic PCR reactions with reported primers. The direct sequencing reactions were performed with genomic DNA as the template by using 3130 ABI Genetic Analyzer (Applied Biosystems; Foster City, CA). For the CVS sample the DNA corresponding to the Arg239Trp mutation in the first exon were PCR amplified with 50 ng of genomic DNA (extracted from blood lymphocytes from all participants and the amniotic fluid sample collected from the three months pregnant mother): 0.2 mM concentration of each primer [13], 200 mM dNTPS (Medox, Biotech PVT Ltd.; Chennai, India), 10 mM Tris-HCL (pH 8.3), 50 mM KCL, 1.5 mM Mgcl2, and 0.2 U of Taq DNA polymerase (Sigma Aldrich; St. Louis, MO) in 20 µl volume of reaction mix by using MJ Research-PTC-200 (Peltier Thermal Cycles; Taunton, MA). For this PCR amplicon, the genomic DNA was initially denatured at 94°C for 5 min followed by 28 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 1 min. For the carrier detection two different primer source were selected, for first exon to third [13] and for fourth to fifth exons [14]. The PCR reactions were performed by initial denaturation at 94°C for 5 min followed by 28 cycles of denaturation at 94°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 1 min with different annealing temperatures. The PCR amplified fragments were purified (Bio Basic, Inc.; Toronto, Canada) and then amplified with single primers by big dye termination chemistry. Bi-directional DNA sequencing for the mutant Arg239Trp was performed for all available family members. The sequencing results were compared to the gene sequence of *TYR* (NT_008984), using Chromas software. Heterozygous patients were systematically resequenced to ensure that the screening had

not overlooked mutations. Evolutionary conservation of human TYR amino acid residue R239 was compared to other related mammalian species: pig (*Susscrofa*: Q4R1H3), mouse (*Musmusculus*: P11344), Japanese rice fish (*Oryziaslatipes* (ORYLA): P55025), Canfa (*Canisfamiliaris*: P54834), and Bovine (Bostaurus: Q8MIU0), using the T-Coffee Multiple Sequence Alignment tool (http://www.ebi.ac.uk/Tools/services/web/toolform.ebi?tool=tcoffee) [15]. Then the sequence was uploaded to WebLogo http://weblogo.berkeley.edu/logo.cgi [16,17] for better representation of a particular position.

SIFT analysis of missense mutation R239W

The evolutionary model was used to predict the functional consequence of genetic variations due to the missense mutation R239W. The probability that a given coding variant will cause a deleterious functional change is estimated by the substitution position-specific evolutionary conservation (sub-PSEC) score. The Sorting Intolerant From Tolerant tool (SIFT: http://sift.bii.astar.edu.sg) [18] was used to predict the functional impact of missense changes identified in this study.

Structure prediction of human Tyrosinase

Human tyrosinase protein sequence was retrieved from Uniprot database (Accession number: P14679). The protein was modeled using Prime module -Schrodinger 2011, LLC. (Prime version 3.0, Schrödinger, LLC, New York, NY, 2011). The sequence homologs were searched and alignment was made with suitable template using BLAST [19] search tool with default parameters. The crystal structure of tyrosinase protein of *Streptomyces castaneoglobisporus* (PDB-ID: 1WX2) [20] with 1.80 Å analyzed by X-ray diffraction was selected as template based on percentage of identity and E-value. A position specific substitution matrix (PSSM) for the query sequence, derived from PSI BLAST, is used to match the template sequence. Finally, the build process was carried out in four steps such as copying the backbone atom coordinates for aligned regions and side chains of conserved residues, optimization of side chains, minimization of non-template residues and building of insertions and closing of deletions in the alignment. Further, the structure was refined by side chain modeling, loop modeling and energy minimization using OPLS 2005 force-field. Loop modeling was performed with serial loop sampling method to refine each loop independently. The mutant tyrosinase was also modeled using the wild type model as a template, by mutating arginine to tryptophan amino acid at 239th position. Conjugate gradient energy minimization was done for both the structures and the energy values are noted.

Structural validation of the predicted model

The protein model was validated by Ramachandran plot obtained from PROCHECK based on the sterochemical properties in residue by residue geometry and overall structure geometry (http://www.ebi.ac.uk/thornton-srv/software/PROCHECK/) [21,22]. The extensive checking of many sterochemical parameters of the residues in the model was performed with this program. The model was superimposed with the template to calculate the root mean square deviation (RMSD).

Physico-chemical and structural analysis of wild and mutant tyrosinase

The physico-chemical properties of the amino acids generally determine the structure and biological function of proteins. The predicted models of wild and mutant tyrosinase (R239W) were analyzed for the physico-chemical properties such as molecular weight, theoretical pI, instability index, aliphatic index, Grand average of hydropathicity (GRAVY). The hydropathic effect was monitored by analyzing the hydropathy plot obtained from TopPred(http://mobyle.pasteur.fr/cgi-bin/portal) [23,24]. As the hydrogen bond is a key factor for the stability and specificity of protein, the number of intramolecular hydrogen bonds of the 239thresidue was measured and counted for both wild and mutant tyrosinase. Di-copper centre plays a major role in biological dioxygen activation. Hence, the distance between the two copper ions with the 239th residue was measured. As each of the two copper ions surrounded by three conserved histidine residues which are crucial for its function, the distance between di-copper atoms and the histidine residues were also examined.

Results

Family Ascertainment

Family 1: In proband from familial family I, the ophthalmologic examinations of hypo-pigmented iris, nystagmus, photophobia, refractive error with combined myopic or hypermetropic astigmatism, and albinotic fundus with foveal hypoplasia were well documented and a reported *TYR* mutation R239W was observed [3]. In addition, the proband was examined by physicians to check the presence of other systemic abnormalities.

In family I, the parents belongs to non-consanguineous marriage, and the disease was previously observed in a maternal relation (maternal grandmother, Figure 1A-III:6; proband's mother was also a carrier, Figure 1A-IV:9), but there was no affected individual in the paternal relations, to the best of their knowledge. The father (Figure 1A-IV:10) was, unfortunately, also carrier of the same mutation, R239W, which was inherited from his carrier mother (Figure 1AIII:11). The first pregnancy was a stillbirth (Figure 1A-V:6) for unknown reasons, and the second baby (Figure 1A-V:7) was affected by the R239W mutant, inherited from both carrier parents. The maximum number of family members from the pedigree was recruited for genetic analysis (Figure 1), and the genotypic status of R239W is tabulated in Table 1.

Among these, 20 members were screened; 10 evidenced a wild

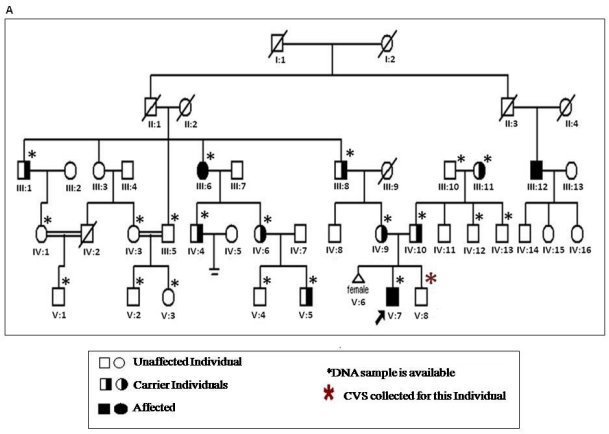

Figure 1A: One South Indian pedigree participated in prenatal diagnosis. The individual (V:8) with coloured asterisk indicates -CVS sample collected from the mother during her 3rd month of pregnancy.

Table 1: Pedigree Information showing the genotypic status of Missense Mutation c.715 C>T (p.Arg239Trp) in this family members.

Individuals	Relationships	Disease status	Genotypic status
55-1	Proband	Affected	Affected Homozygous
55-2	Father	Normal	Carrier Heterozygous
55-3	Mother	Normal	Carrier Heterozygous
55-4	Grand Father (M)	Normal	Carrier Heterozygous
55-5	Grand Mother (M)	Affected	Affected Homozygous
55-6	Aunty (M)	Normal	Normal Homozygous
55-7	Grand Father (M)	Normal	Carrier Heterozygous
55-8	Uncle (M)	Normal	Carrier Heterozygous
55-9	Aunty (M)	Normal	Carrier Heterozygous
55-10	Cousin(M)	Normal	Normal Homozygous
55-11	Cousin (M)	Normal	Carrier Heterozygous
55-12	Brother (M)	Normal	Normal Homozygous
55-13	Grand Father (M)	Normal	Normal Homozygous
55-14	Grand Mother (M)	Normal	Normal Homozygous
55-15	Uncle (M)	Normal	Normal Homozygous
55-16	Aunty (M)	Normal	Normal Homozygous
55-17	Grand Father (P)	Normal	Normal Homozygous
55-18	Grand Mother (P)	Normal	Carrier Heterozygous
55-19	Uncle (P)	Normal	Normal Homozygous
55-20	Uncle (P)	Normal	Normal Homozygous

Note: 55-Family Number; 55-1 to 55-20 – Individuals from 1 to 20 in family 55; (P) –Paternal; (M) –Maternal

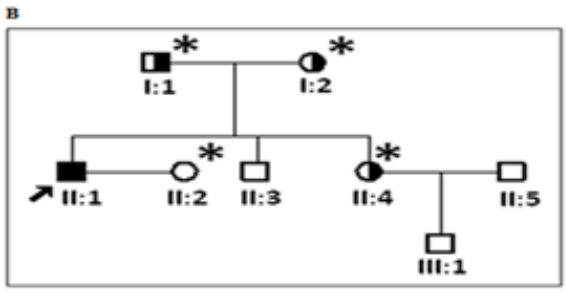

Figure 1B: One south Indian pedigree participated in carrier detection.

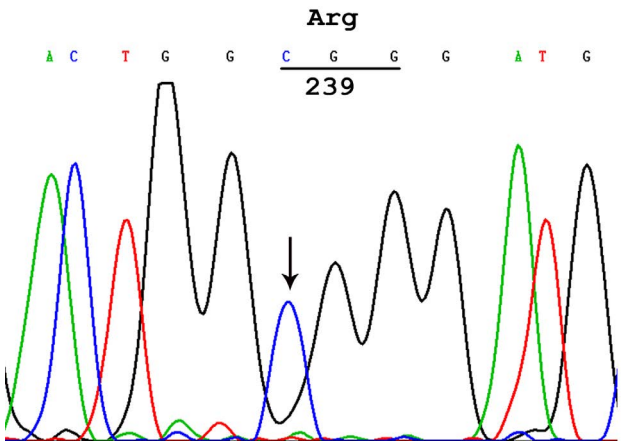

Figure 2A: Chromatogram showing the sequence pattern of p.R239W. Normal genotype from control sample.

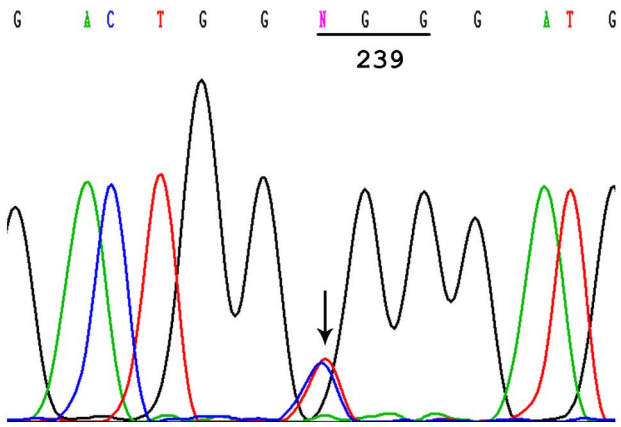

Figure 2B: Carrier (Heterozygous) genotype from unaffected parents and family members.

type sequence pattern and eight were heterozygous for the R239W mutation, including both parents. The remaining two were affected with OCA1A (Figure 1A-V:7–proband; Figure 1A-III:6–maternal grandmother), and showed homozygous mutant sequence pattern C to T transition, resulting in an amino acid substitution of Arg239Trp (p.R239W/c.715 C>T) in exon 1 of the TYR gene.

This family-I was further concerned about the prenatal diagnosis of the third baby due to their involvement in this gene-based diagnostic studies. Our DNA-based analysis of the CVS sample showed a wild type sequence pattern for the R239W mutant (Figure 2) and the family was given genetic counseling; the mother delivered a genotypically normal baby without any phenotypic features of albinism.

Family 2: In a sporadic case (family-II) the proband and his spouse were undergone diagnosis for OCA type and its inheritance before pregnancy as they belongs to the same caste, but not relatives. A reported missense mutation c.715C>T (p.Arg239Trp) was identified in the proband and diagnosed as OCA type 1-A-negative form (Figure 1B). This mutant c.715C>T (p.Arg239Trp) was observed as heterozygous form in proband's parents (non consanguineous marriage) and also in sibling, whereas wild type sequence pattern was observed in proband's spouse.

The sequence profile of the wild type, heterozygous and normal conditions were illustrated in Figure 2 for these two cases involved in molecular testing procedures. Figure 2: Profile of chromatogram; Figure 2A: Control; Figure 2B: Carrier; Figure 2C: Mutant; and Figure 2D: CVS DNA sample. This change in arginine 239 tryptophan is proximal to the copper-binding site A (CuA), and this position is very well conserved among other related mammalian species Figure 2E.

This mutation may alter the structure of this position, and thus prevent the normal binding of copper; this may lead to changes in the conformational properties and function of the catalytic tyrosinase enzyme. Amino acid conservation at a specific position within related mammalian species (i.e., an ensemble sequence of proteins sharing a related ancestor) is considered to be an indication of strong evolutionary pressure at that site (Figure 2E). Thus, the R239W residue was highly conserved and thus, probably crucial for maintaining the function of tyrosinase. Figure 2E represents the conserved position of Arg239 among the different species was illustrated by CLUSTALW and Weblogo software.

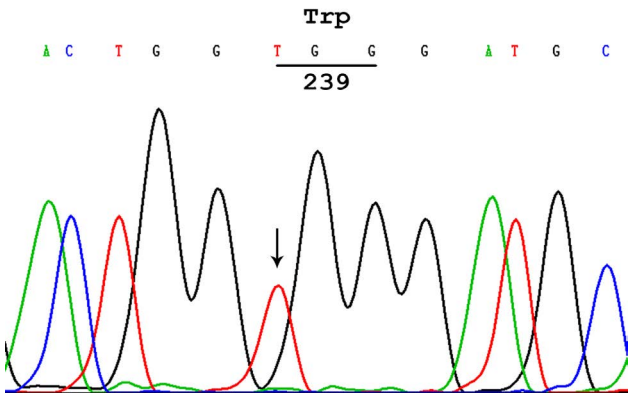

Figure 2C: Mutant genotype from Proband and maternal Grandmother.

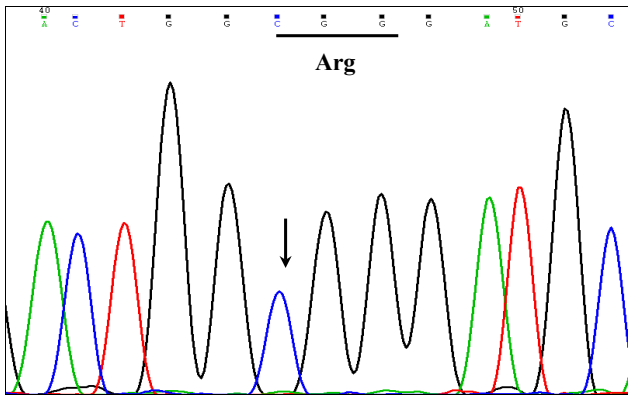

Figure 2D: Chorionic Villus Sample showing normal sequence pattern.

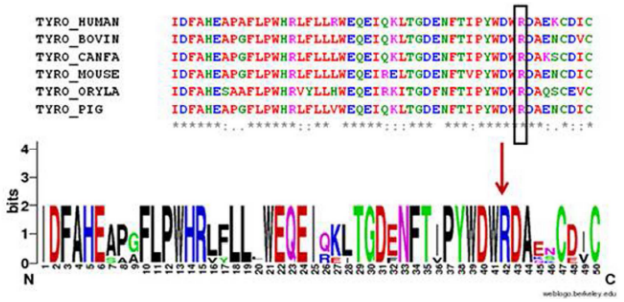

Figure 2E: Evolutionary Conservation of R239th position among the other related mammalian species (Human, Bovin, Canfa, Mouse, Oryla, Pig). The amino acid residue Arginine in the 239th position shown in the box is highly conserved.

Implications of the R239W mutant in the molecular surface of tyrosinase

The absence of arginine at 239 and the replacement of tryptophan may affect the catalytic properties of the enzyme and thus result from the absence of tyrosinase activity in the melanocyte. The change of arginine (having polar-side chain polarity and with positive charge) to tryptophan (having non-polar-side chain polarity and with neutral charge) may affect the regular structure and functional interaction properties of codon 239 and thus, produced OCA type I-A (negative) pattern. The presence of arginine residues, essential for catalytic activity, has been reported in more than a hundred enzymes [25,26], and some enzymes were shown to lose or change their catalytic activity through the replacement of arginine with neutral residues such as glutamine [27-29] charged residues are also known to be important for folding stability, and play an significant role in enzyme stability in some proteins [30]. As a result, the change of positively charged arginine to neutral group tryptophan affects the folding properties of tyrosinase, and that ultimately leads to changes in the functional character of the tyrosinase enzyme.

In-silico analysis

SIFT online tools were used for potential functional prediction of mutant proteins. The SIFT scores were 0.99 and 0.00, after input the amino acid sequences of the wild-type TYR protein and their mutant R239W respectively, which meant that the variant (TYR:p.R239W) was predicted as "pathogenic" with high confidence (SIFT Score value: 0.00; threshold for intolerance: less than 0.05). This replacement in the conserved region (239) may be sufficient to change the secondary structure of the protein and resulting pathogenic effects.

Computational analysis of R239W to compare the effects

3D Structure prediction of wild and mutant human tyrosinase: We attempt to model the 3D structure of human tyrosinase by homology modeling and further refined by energy minimization. The protein sequence of human tyrosinase is 529 amino acids in length; contain signal peptide sequence from 1-18aa, one transmembrane region (477- 497 aa) and two topological domains such as extra cellular region (19-476 aa) and cytoplasmic region (498-529 aa). Tyrosinase from *Streptomyces castaneoglobisporus*- sT (PDB ID: 1WX2) [18] was selected as template, as it has 27% identity and 41% positives. The template structure of *Streptomyces castaneoglobisporus* contains 415 residues and the template has shown alignment from 117 to 429 with the wild type human tyrosinase (Figure 3A) which covers extracellular region of tyrosinase. The central region of human tyrosinase overlaps with the prokaryotic tyrosinase-*Streptomyces castaneoglobisporus* (sT) from 117aa to 429 aa (Figure 3A). Therefore, human tyrosinase was modeled only from 117 to 429 residues. The region that modeled includes the active di-copper centers with six histidine residues such as 180, 202, 211, 363, 367 and 390 and also the mutated residue which is essential for structural analysis. The secondary structural elements are predicted by Psipred and Sspro where represented as H for helix and E for β sheets. Side chain modeling was done wherever required. There were five gaps in the alignment that included Ile145 to leI170, Trp195 to Phe200, Gly295 to Arg311, Ala348 to Lue351 and Ala355 to Ser360 which was modeled by loop modeling (Figure 3A). The protein structure was furtherrefined by energy minimization using OPLS 2005 force field. The modeled structure was validated by PROCHECK and Ramachandran plot. Seventy percentage of the residues are in most favored regions and 22% in additional allowed region. Only 3% are in disallowed region (Supplementary Figure 1A). The energy of the model is -10784.5 kcal/mol. The RMSD is 1.8Å between the template and the predicted model. The final model is given in figure 4A. The mutant tyrosinase model (R239W) was constructed based on the wild type model of human tyrosinase as template (Figure 3B) which was also energy minimized using OPLS 2005 force field. The Ramachandran plot of the mutant model was obtained for validation (Supplementary Figure 1B). The predicted mutant structure is illustrated in Figure 4B.

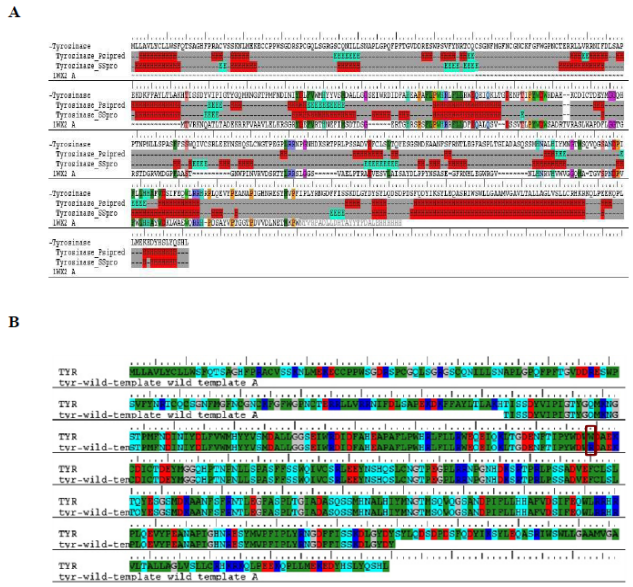

Figure 3: Sequence alignment of human tyrosinase.
A: Sequence alignment of human tyrosinase (hT) wild type with *Streptomyces castaneoglobisporus* (1WX2_A) tyrosinase (sT) showing alignment from 117-429 residues. Secondary structural elements predicted using Psipred and SSPro.
B: Alignment of human tyrosinase wild type with mutant sequence by changing R to W at 239th position shown in brown box.

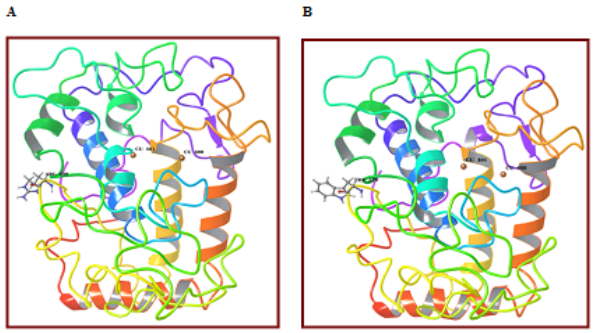

Figure 4: Predicted 3D model of human tyrosinase (hT).
A: 3D structure of human tyrosinase-wild type showing Arginine residue at 239th position
B: 3D structure of human tyrosinase-mutant type showing Tryptophan residue at 239th position. In both structures the CuA and CuB Domains were shown in brown.

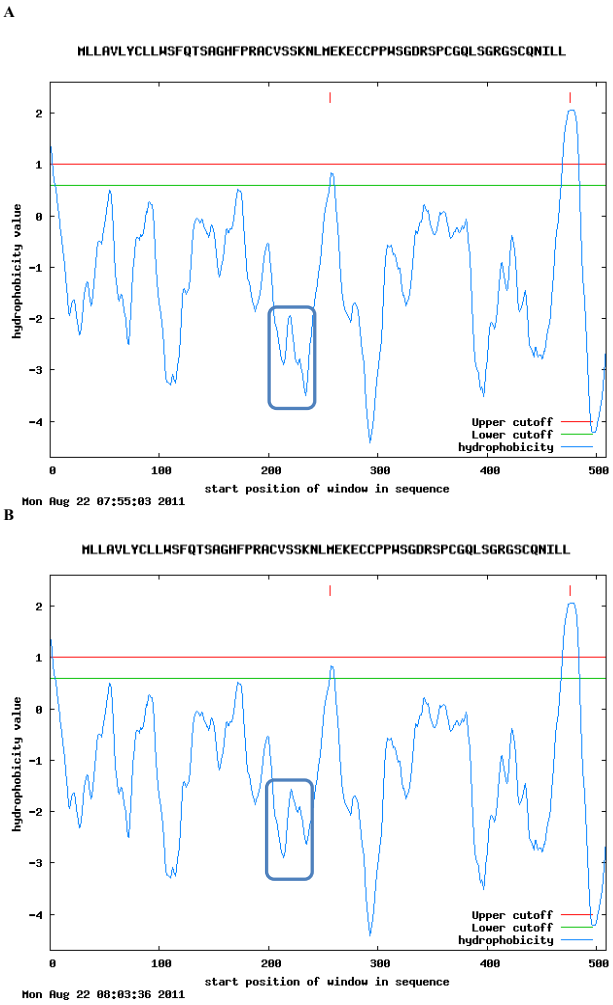

Figure 5: Physicochemical property-Hydrophobicity analysis.
Comparison of Hydropathy plot between wild-type and mutated TYR gene. TopPred online software predict the effect of substitution of R239W on TYR protein hydrophobicity. The x-axis represents the position of amino acids. The y-axis represents the hydropathy value in a default core window size: (-n) 10; wedge window size: (-q) 5.
A: Hydrophobicity of wild type protein.
B: Hydrophilicity of mutant protein. The difference in the hydrophobicity at the point of mutation is highlighted with rectangle box.

Here, we report the theoretical models of tyrosinase which predict the potential structural variation in substitution of R239W.

Analysis of physico-chemical properties for wild and mutant human tyrosinase: Structure prediction and computational analysis of human tyrosinase in 239th (R-W) position revealed the valuable information on the structural differences in the mutant tyrosinase. The physico-chemical properties such as molecular weight, pI, Instability Index, Aliphatic Index and Grand average of hydropathy (GRAVY) were analyzed for the WT and mutant (Table 2). A single amino acid substitution of R239W has increased the molecular weight from 60393 kDa to 60423kDa and the Grand Average of hydropathy has also increased 0.006 (Figure 5A and Figure 5B). As the changes are trivial, the reason behind the deficiency of melanin pigments might be due to some other factors.

Structural analysis of wild and mutant tyrosinase: The function of the protein is determined by the protein structure, position of amino acids and its orientation. A change in the three dimensional structure of the protein would lead to loss of function. We anticipated that the substitution at 239th position might cause some structural changes that would be the rationale behind the malfunctioning of tyrosinase. Hence, the structural properties including the number of hydrogen bonds, distance between copper ion and 239th residue and also the distance between copper ions with the histidine residues were measured in both wild and mutant theoretical models. In wild type, Arg forms three intramolecular hydrogen bonds with Asp 237 and Arg 311 which are important for the protein stability (Figure 6A). Arg

Table 2: Physicochemical property comparison between wild and R239W mutant Tyrosinase.

Features	Normal	Mutant
Molecular Weight	60393.2kDa	60423.3kDa
Theoretical Pi	5.71	5.64
Instability Index	56.76	56.76
Aliphatic Index	71.76	71.76
Grand average of hydropathy (GRAVY)	0.356	0.350
Hydropathy	-2.29	-2.14
	(Hydrophobicity)	(Hydrophilicity)

Sequence based comparison of WT and mutant form

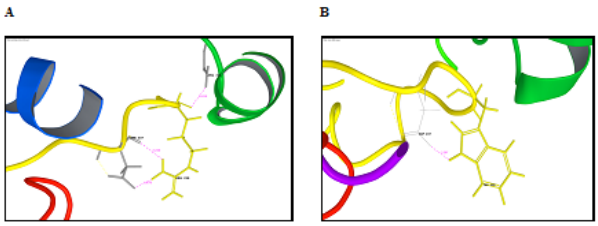

Figure 6: Comparison of number and length of hydrogen bonds between wild and mutant types.
A: Hydrogen bond between 239th Arginine residue to Asp 237 (backbone and side chain groups) and Arg 311 in Wild type.
B: Hydrogen bond length between 239th Tryptophan residue to Asp 237 in mutant type.

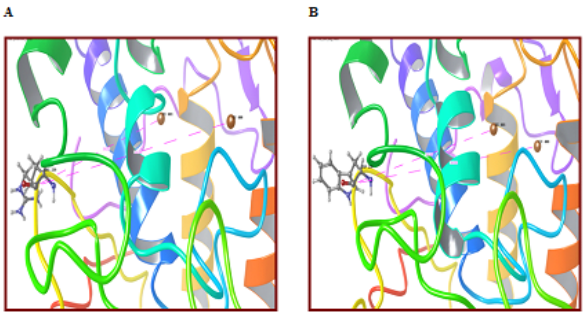

Figure 7: Distance between the Dicopper atom and 239th residue.
A: Distance between the 239th Arginine residue to copper atom in wild type.
B: Distance between the 239th Tryptophan residue to copper atom in mutant type.

239 interacts with both the backbone oxygen and side chain oxygen of Asp 237 and also with the NH group of Arg 311. Mutant model showed only one hydrogen bond with the oxygen of Asp 237. The hydrogen bond length of NH...O for wild type is 2.246Å whereas in mutant type, it is 2.297Å (Figure 6B) (Table 3).

As the copper atoms in the tyrosinase maintain the protein integrity, the distance between the mutated residue and the copper atoms were measured. In the case of wild type, the distance between Arg 239 and CuA and CuB is 16.22Å and 22.33Å respectively, whereas in mutant type the distance between Trp 239 with CuA and CuB is 18.1Å and 24.35Å respectively (Figure 7A and Figure 7B). As histidine residues play a vital role in metal binding protein, the histidine residues were also analyzed with respect to di-copper

Table 3: Intramolecular hydrogen bond Interactions of Tyrosinase at 239th position.

Sl.No	Type	Residue	Interaction	H-bond length (Å)	Prime Energy (kcal/mol)
1.	Wild	Arginine	NH…O (Asp 237)[a]	2.246	-10784.5
			NH…O (Asp 237)[b]	1.675	
			O…NH (Arg 311)	2.015	
2.	Mutant	Tryptophan	NH…O (Asp 237)	2.297	-13403.1

a. Backbone oxygen atom
b. Side chain groups

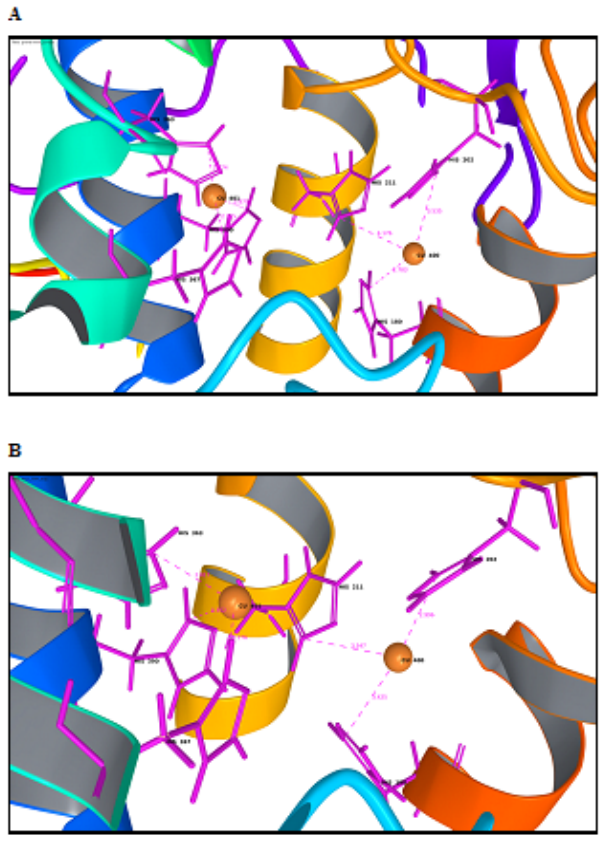

Figure 8: Distance between the Di-Copper centers and histidine residues.
A: Di-Copper centers surrounded by histidine residues in wild type.
B: Di-Copper centers surrounded by histidine residues in mutant type.

centers. CuA is coordinated by His180, His202, and His211 whereas the second copper ion (CuB) is coordinated by His363, His367, and H390 residues which are illustrated in Figure 8A and Figure 8B. So, the distance between copper ions and histidine residues were measured. It was found that the distances are not identical in wild and mutant models. The differences are in the range of 0.143Å to 0.777Å (Table 4) which might be the reason for loss of function of human tyrosinase. The molecular surface analysis of wild and mutant tyrosinase revealed the reduction in positively charged regions in the tyrosinase which alters the surface of the protein that would probably affect the protein-protein interactions (Figure 9A and 9B). Overall, the structural analysis of wild and mutant models revealed that there

Table 4: Distance between two copper atoms with six histidine residues in the tyrosinase active site.

No. of Cu Atoms	Histidine Residues	Distance from Cu to Histidine Residues (Å)	
		Wild	Mutant
CuA	180	2.783	3.425
		3.535	2.996
		4.176	3.547
CuB	363	2.674	2.817
		2.715	3.276
		5.879	5.102

Comparison of the distance between the copper atoms (CuA & CuB) to histidine residues of wild and mutant model of TYR was measured.

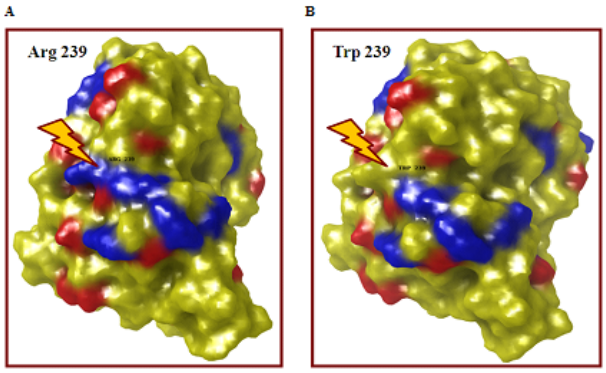

Figure 9: Molecular Surface of the human tyrosinase of wild and mutant models.
A: The 3D structure of wild type.
B: The 3D structure of R239W mutant. The blue color surface indicates positively charged residues and yellow color surface indicates negatively charged residues. The 239th position changed from positive to negative charge by the replacement of tryptophan in arginine at 239th position.

are no dramatic changes in the three dimensional structure and secondary structural elements. Nevertheless, it alters the distances at the di-copper centers and reduces positively charged regions, which might be the possible reason for the abnormality in tyrosinase activity.

Carrier detection of other families

Family 3: Mother has an ongoing pregnancy of 13 weeks during this study period. Molecular analysis of OCA1 candidate gene (TYR) revealed one novel synonymous change c.168G>A (p.Gln56Gln) and one novel stop codon c.324G>A (p.Trp108X) in the heterozygous form on both of the parent samples but they belongs to non-consanguineous marriage (Figure 10A). In Mother's DNA sample alone two reported (Oetting et al. [42], Oetting et al. [43]) polymorphisms c.-301C>T (rs4547091) and c.-199C>A (rs1799989) were also observed in heterozygous form, but both participants showed the normal phenotypes.

Family 4: In this Proband, previously reported homozygous polymorphisms c.-301C>T (rs4547091) and c.-199C>A (rs1799989) were detected in the proximal promoter region (up to 379bp from the coding region) of *TYR*. Though the parents belong to non-consanguineous marriage, the heterozygous form of c.-301C>T and

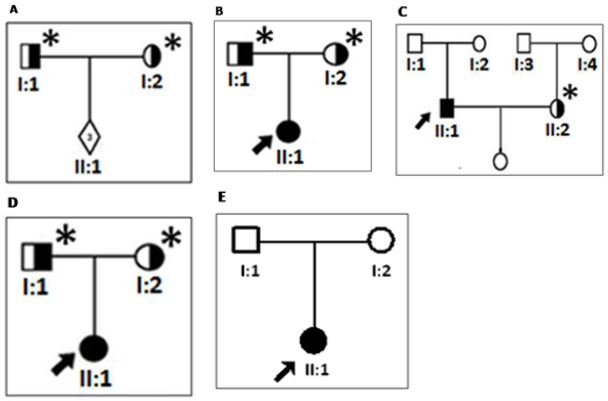

Figure 10: Family Pedigrees participated in carrier detection analysis.

c.-199C>A polymorphisms (Figure 10B) were observed on both the individuals.

Family 5: Since this family belongs to familial category (diseased generation not mentioned in the pedigree), the second generation non consanguineous couple was willing for molecular diagnosis of OCA. Two reported *TYR* polymorphisms c.-301C>T (rs4547091) and c.-199C>A (rs1799989) were observed as heterozygous condition in proband and his wife (Figure 10C). Similar to the fourth family, the same promoter variation was observed as heterozygous form in both of the parent's sample, but their baby was phenotypically showing normal pigmentation.

Family 6: In this case, one *TYR* novel variation c.-33G/T was observed in the promoter region (5' UTR); One previously reported polymorphism c.1205G>A (p.Arg402Gln) was observed in exon-4 of Proband's DNA sample. These two variations in the proband (Figure 10D) were observed as the heterozygous form. Though her parents are phenotypically normal, the Mother's DNA sample showed heterozygous conditions for c.-33G/T variation. In her father, the sequence pattern was normal for c.-33G/T and heterozygous pattern was observed for p.Arg402Gln variation.

Family 7: In this proband, the reported stop codon mutation c.832C>T (p.Arg278 Stop codon) was identified in exon-2 region and confirmed as OCA type 1-A-negative pattern (Figure 10E). The parents were not willing to participate in this study.

Discussion

Genetic test provides the knowledge of risk incidence in a family and assist to prevent further abnormal birth by early diagnosis. For a recessive genetic disease, the only prevention involves carrier detection with the parent samples along with the first affected baby sample. This R239W mutant was previously reported [10,31,32] in a patient with tyrosinase-negative OCA1. In this study, we have analyzed the *TYR* (OCA1) coding region in the DNA of CVS collected from the proband's mother during the third month of pregnancy. This CVS procedure for the analysis of fetal genomic tyrosinase DNA is a rapid and reliable approach to the prenatal diagnosis of oculocutaneous albinism at a relatively early stage of pregnancy, and is safer and less invasive than other methods such as fetal skin biopsy.

This genetic analysis was extended to 20 individuals from the same family I, the mutant sequence pattern of R239W was observed in two

affected individuals, wild type was observed in 10 individuals, and heterozygous (carrier) type in eight individuals. DNA extraction from the fetal (CVS) sample during the second trimester of pregnancy is the first successful method for the prenatal diagnosis of oculocutaneous albinism in southern region. Subsequent direct DNA sequencing of the fetal DNA provided more practical and reliable information for diagnosing tyrosinase-negative oculocutaneous albinism prenatally. The recent elucidation of the specific gene mutation of tyrosinase in the affected individual now allows DNA-based prenatal diagnosis of tyrosinase-negative oculocutaneous albinism in the first trimester of pregnancy. Supporting these molecular findings, the woman delivered a baby boy without any features of albinism.

Thus, analysis of the fetal tyrosinase gene by PCR amplification enables the prenatal diagnosis of tyrosinase-negative OCA in an earlier stage of gestation. These investigations provide new insight into the diagnosis of recessive genetic disease, and thus have implications for the development of carrier detection and prenatal care. Moreover, this is a first kind of report with Indian albinism case analyzed for its structural features based on computational approach. The study enabled to correlate, pathogenesity of the mutant with wild type. The molecular model of both wild type and mutant tyrosinase are generated based on the tyrosinase structure of *Streptomyces castaneoglobisporus*.

In previous reports the protein was modeled using both 1WX2 and 3NM8 structures for different purpose. 1WX2 was used in the study of C-terminusglycans role for maturation of secretary glycoproteins [33] whereas in another study, 3NM8 was used to model human tyrosinase to compare the protein-protein interactions [34].

Hydrophobicity is found to be increased in the mutant tyrosinase at 239th position. As the polar amino acid (Arg) changed to non-polar amino acid (Try), it is obvious that the substitution increases the hydrophobicity. The difference in the grand average of hydropathicity index (GRAVY) is –0.006 when comparing the GRAVY of wild and mutant tyrosinase. Overall, there is no dramatic variation in its hydropathicity as the mutation in a single residue.

Generally, the energy of the model determines the stability of the protein. The Prime energy is also found to be increased in mutant form which is calculated with Prime module in Schordinger, LLC. Copper ion maintain the structural network of tyrosinase and it contains (type 3) di-copper ions at the catalytic site that participates in the formation of pigment melanin by catalyzing the hydroxylation of tyrosine to l-3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to DOPA-quinone [35]. Both copper atoms (CuA and CuB) are coordinated by three conserved histidine residues [36]. The role of Cu ions were analysed in many diseases and reported as an important element to maintain the pigmentation. In Menkes disease, the patients are known to have abnormal pigmentation, which can be partially treated by subcutaneous copper injections [37]. The proper folding nature of tyrosinase polypeptide appears to be necessary for the suitable copper binding, and a variety of human OCA1 mutations affects the copper binding properties and thus leads to improper catalytic function of tyrosinase.

Mutational studies of tyrosinase also suggested that both CuA and CuB sites are important for its copper binding and catalytic activity [38]. Therefore, the distance between 239th residues with two Cu atoms were measured to emphasis the structural variation in wild and mutant models. The distance between each Cu ions with Arg 239 is increased in mutant compared to wild type which may affect the function of the protein. The two copper ions (CuA and CuB) in the crystal structures of bacterial tyrosinase, *Streptomyces castaneoglobisporus* and *Bacillus megaterium* are coordinated by six histidine residues in its active site [19,39]. It alters the tyrosinase active center formed by dinuclear coppers and slight difference in the distance was noted. Recently, the enzyme was reported to be linked to Parkinson disease and other neurodegenerative diseases [40,41].

Since the positively charged amino acid is replaced with aromatic, non-polar amino acid, it may affect the folding properties of tyrosinase and that ultimately leads to changes in the functional characters. Moreover, tryptophan contains more reactive atoms. This biochemical features may decline the melanin synthesis. Analysis of mutation(s) affecting the catalytic activity of tyrosinase is valuable for further characterization of its structure and reaction mechanisms to study the possible pattern of folding properties. Overall this study provides the structural implications of mutant tyrosinase compared to wild type tyrosinase. In the post genomic era, structure prediction and comparison of human tyrosinase is most important to understand the function of pathogenic mutations. Developing the structural model of tyrosinase would help in understanding the effect of mutation in the structure and function of the protein. This study reports the structural model of tyrosinase in both wild and mutant. Overall, the structure of wild and mutant is altered. Though, there are slight differences in the physico chemical and structural features of wild and mutant tyrosinase, but it leads to complete absence of tyrosinase activity in the melanocytes and thus cause OCA1A-negative form in patients. This substitution may affect proteins that interact with tyrosinase which is not reported in this study.

Carrier Detection

In third family, phenotypically normal couples showed novel synonymous c.168G>A (p.Gln56Gln) and a novel stop codon c.324G>A (p.Trp108X) change in the heterozygous form. Though both the parents belong to the carrier sequencing pattern for these two mutants, they were not willing for molecular diagnosis of their fetal sample. The report concluded, the fetus may get 50% chance of occurrence of the OCA1 disease (though, this p.Gln56Gln polymorphism won't produce any effects) mainly due to the heterozygous pattern of pathogenic mutant (p.Trp108X) observed in parents, will lead to the homozygous pattern in the fetus and thus suggested, it may produce OCA1 in the fetus.

In this family, the previously reported Caucasian, and Oriental populations [42,43] *TYR* promoter variants rs4547091 and rs1799989 were observed in the proband. These two polymorphisms were present in the 5' upstream region of promoter segment. Though these variations do not show any effects on coding regions, it may alter the structural folding properties and thus delivered OCA type 1-A phenotype conditions in the proband. These two heterozygous patterns were inherited to their baby, and confirmed as homozygous pattern and these two promoter variations may be the disease causative pattern in the proband.

In this family, the couple was interested to analyze their DNA samples before getting pregnant. Since these two reported c.-301C>T (rs4547091) and c.-199C>A (rs1799989) [42,43] polymorphisms were observed in the promoter region in both of these two individuals as heterozygous and thus, concluded the frequency of OCA1A in the fetus may be at 50% of risk. The mother delivered a phenotypically normal baby but the genotypic status was not observed as they were not interested in further analysis.

In sixth family, the proband had five *TYR* variations in heterozygous forms with different maternal and paternal inheritance. All these compound heterozygous variations may produce the partially active or completely inactive form of tyrosinase enzyme that condition affects the biosynthesis of the melanin pathways. The variation c.1205G>A (p.Arg402Gln) was previously reported in Caucasians-[44-47] and also in South Indian patient [3]. Several reports have shown that the allele containing glutamine at codon 402 produces thermo labile enzymatic activity. This has led to the association of the R402Q mutation with oculocutaneous albinism when found with a pathologic tyrosinase gene mutation on the homologous allele. The Q402 allele has been associated with autosomal recessive ocular albinism when it is in trans with a tyrosinase gene mutation associated with oculocutaneous albinism type 1.

In seventh family, the proband was diagnosed as OCA type 1-A (negative pattern) due to the pathogenic effects of the reported stop codon mutation c.832C>T (p.Arg278 Stop codon) which was observed as homozygous form. Since, parents were not willing to participate in the study concluded that this homozygous mutant in the proband might be inherited from both of the carrier parents. The pathogenesity of R278X was reported in different populations, indicates its hot spot specificity.

Recently, there has been increased awareness and attention paid to genetic diseases. The outcome of this research application using a basic diagnostic molecular method helps toward creating a healthy society. Since oculocutaneous albinism type 1 (*TYR*) is the most common form in India. This study provides the need for genetic testing, especially for the affected individuals from familial cases, to avoid the occurrence of OCA diseases in future generations. This kind of molecular testing is newly emerging procedure for early carrier detections to develop the prenatal diagnosis for eye disorders. The majority of affected individuals have been compound heterozygotes with different maternal and paternal alleles. This broad investigation of carrier diagnosis should, in principle, build prenatal diagnosis in near future. Our research in prenatal screening will reveals rapid development of a molecular diagnostic tool to improve the implementation of genetic testing for other eye disorders. These strategies ultimately contribute to the progress of genetic counseling of inherited ophthalmic diseases associated with ocular defects. Moreover, this is the first successful DNA-based strategy conducted in India for the prenatal diagnosis of the specific R239W mutation of tyrosinase (OCA1A) in a proband with carrier parents for the diagnosis of a fetal sample. This finding reiterates the need for increased awareness of molecular diagnosis and public health intervention in order to better address the medical, psychological and social needs of these albinism populations in worldwide.

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