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RESEARCH ARTICLE

Prenatal diagnosis in a high-risk pregnancy for severe neurological disorders due to Type II Recessive Congenital Methemoglobinemia (RCM) in Indian families

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Abstract

Objective: The nicotinamide adenine dinucleotide (NADH)- cytochrome *b*5 reductase (CYB5R) enzyme deficiency is one the most common cause of recessive congenital methemoglobinemia. The RCM is classified into two types, mild to moderate cyanosis is general occure in type I whereas, in type II, cyanosis is accompanied by severe neurological disorders, brain dysfunction, dystonia, choreoathetosis, microcephaly, intellectual disability. This study aimed to diagnose and offer a prenatal diagnosis to RCM II high-risk families.

Method: Five patients were diagnosed with NADH-CYB5R deficiency associated with the RCM Type II having neurological dysfunction. After informed consent, c*horionic villus sampling (CVS)* was done at 11 weeks gestation. The molecular characterization was done by DNA sequencing.

Results: NADH-CYB5R deficiency causing RCM II were showed a 35% to 70% loss of NADH-CYB5R activity, causing RCM II associated with neurological problems. A genetic study of these patients identified pathogenic homozygous p.Gly76Ser variant in three cases, and the remaining two cases showed, p.Gln77X variant and novel p.Arg92Trp variant, respectively. In the subsequent pregnancy, the prenatal diagnosis of fetal DNA revealed one homozygous, three heterozygous, and one no mutations.

Conclusions: This study reported the genetic analysis of RCM II families having pathogenic homozygous variants p.Gly76Ser, p. Gln77X, and novel p.Arg92Trp in the *CYB5R3* gene, which is vital for assessing the mutation status of fetuses Thus, providing a prenatal diagnosis in RCM II high-risk families helps in the prevention of severity and recurrence of type II RCM disease in affected families

Keywords: *CYB5R3 gene, mMethemoglobinemia; Developmental disorders; inherited recessive diseases; Prenatal diagnosis, the Indian population*

Introduction

Metahemoglobinemia is a condition characterized by an elevated amount of hemoglobin in which heme-iron is oxidized to ferric (Fe3+), making it an inefficient carrier of oxygen, which results in varying degrees of cyanosis. This condition may result from genetic aberrations in the structure of hemoglobin or RBC metabolism. Still, it can also be acquired through exposure to different oxidant drugs or toxins [1]. Recessive Congenital Methemoglobinemia (RCM) occurs when there is part of or complete absence of enzyme activity of reduced nicotinamide adenine dinucleotide (NADH) cytochrome b5 reductase (CYB5R) in the body. This enzyme deficiency disorders are classified into two types. Type I (erythrocyte) patients suffer from specific symptoms including cyanosis at birth and shortness of breath as a result of hypoxia in their tissues [2]. There are severe neurological disorders, brain dysfunction, dystonia, choreoathetosis, microcephaly, and intellectual disability in the case of type II (generalized) cyanosis [3,4]. Two different isoforms of NADH-CYB5R exist, each with a different function. The membrane-associated isoform is a 35 kDa protein of 301 amino acids. This enzyme contains a FAD and NADH binding domain which are linked by a hinge region [5]. The CYB5R3 gene encodes Type II RCM, which has been associated with 82 mutations, some of which are common to Type I and Type II mutations. Mutations associated with Type II occur in the splicing process, disrupt the active site, or result in protein truncation. We have diagnosed five patients with RCM who have a clinical history of type II RCM. Efficient prenatal screening is essential to prevent this disease [6-8]. So we aimed to deliver normal healthy children to the families at risk of severe neurological disorders due to common pathogenic variants causing type II RCM in 5 Indian families. In this study, an efficient diagnostic method for prenatal screening for the absence or presence of type II RCM mutation in 5 CVS samples with a family history of RCM type II was performed.

Material and methods

Case study

The study was performed at the ICMR National Institute of Immunohematology Mumbai. The clinical history of five patients of RCM type II includes bluish discoloration of the nail beds, lips, and tongue at birth, and they later develop severe neurological disorders, brain dysfunction, dystonia, choreoathetosis, microcephaly, and intellectual disability were included in the present study. Genetic analysis was performed in the index cases and parents as well as their siblings. The Ethical Committee of the ICMR-National Institute of Immunohematology approved this research work, and the parents of the patients gave their informed written consent. The analytical procedures and examinations were all conducted in accordance with the Helsinki Declaration of 1975. The methemoglobin level was instantly evaluated by potassium ferricyanide protocol on freshly collected blood in EDTA, and erythrocyte NADH -CYB5R enzyme activity was determined after subsequent NADH oxidation at 340nm a Spectrophotometer by Analytic JENA (Analytical JENA, Germany) at 30^0C for 10 minutes [10]. Genomic DNA was extracted from each sample, and PCR was performed using primer sets specific to genomic sequences of nine exons of the *CYB5R3,* which were retrieved using the GenBank database (accession IDs M28705 to M28713). All PCR assays were performed using the Taq DNA polymerase and Premix Taq DNA Polymerase Kit (Cat. #R006A) (TAKARA Japan), and PCR products were resolved on an agarose gel and extracted by QIAquick PCR purification kit (cat # 28104 and 28106). The sequencing of the PCR amplicons was performed using a 3730 DNA analyzer and Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc, www.thermofisher.com) [6-8].

Structural analysis of the NADH-CYB5R3 mutant

The structural interactions of identified mutant protein were studied using the three-dimensional (3D) crystal structure of pig liver b5R at 2.4 Å resolution (Protein Data Bank file: 1UMK) [11]. Molecular graphics were generated using PyMOL Molecular Graphics System (www.pymol.org). Molecular modeling of human NADH-CYB5R was done by side-chain substitution using Deep View Swiss-PDB viewer (http://expasy.org/spdbv/) and PyMOL (http://pymol.sourceforge.net/) software.

Prenatal diagnosis

Due to the severe clinical presentation and neurological abnormality present in the index cases, we have provided a prenatal diagnosis to five families. The mothers of the five index cases were in pregnancy of the first trimester, and these five pregnant women were involved in the prenatal diagnosis of RMC type II. The workflow reflected the patient management procedures in our study (Figure 1). During the mother's 2nd pregnancy, *chorionic villus sampling* (*CVS*) was sampled at 11 weeks' gestation following proper ethical guidelines. DNA was extracted from the CVS sample using standard techniques, and sequencing of the respective exon of the CYB5R3 gene mutation was performed. Maternal contamination was excluded by examining STR markers in the CVS material and maternal DNA. Four chorionic villus samples (CVS) and one amniotic fluid were referred to the department of Hematogenetics at NIIH Mumbai for prenatal diagnosis [12].

Results and Discussion

Table 1 described the hematological, biochemical, molecular, and clinical assessments of all five patients. Figure 2 explains the pedigree of five RCM Type II families referred for prenatal diagnosis.

In family-1, the parents belonging to the Gujarat state of India had a non-consanguineous marriage. Their first 2 yrs old male child was diagnosed with Type II RCM. The child had shown a methemoglobin level of 12% and NADH-CYB5R activity of 16.5%. The clinical presentation showed severe mental retardation, global developmental delay, and gross failure to thrive at two years. Further molecular analysis of the affected child showed homozygous mutation at c.226G>A (p. Gly76Ser), changing glycine to serine at 76 codons in the index case, and parents showed the heterozygous mutation.

In family-2 from the Gujarat state of India had a consanguineous marriage, their first female child died after three days of birth due to unknown reasons, and the second two and half-year-old female child had Type II RCM. The child had shown a methemoglobin level of 39.4% and NADH-CYB5R activity of 9.2%. The symptoms included severe mental retardation, global developmental delay, and gross failure to thrive. Molecular analysis showed c.226G>A (p. Gly76Ser) homozygous mutation in the index case and heterozygous state in the parents.

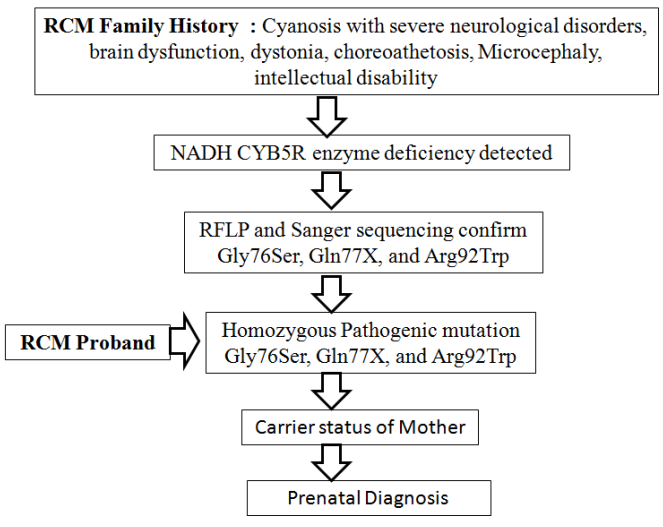

Figure 1: Flowchart for prenatal diagnosis of RCM

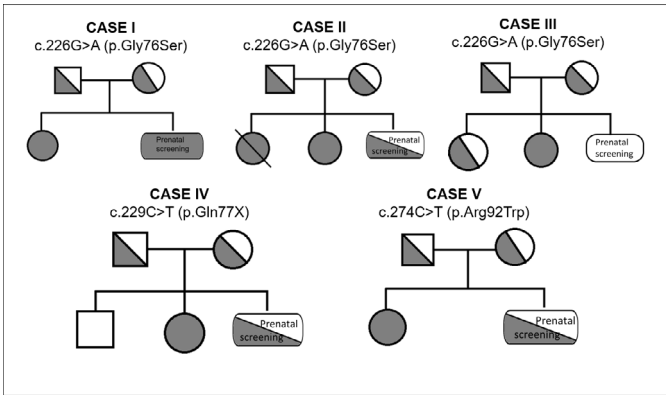

Figure 2: Pedigree of five Families of Recessive Congenital Methemoglobinemia (RCM) Type II in India with Prenatal diagnosis

Family 3, who was referred from Kerala state (India), had a non-consanguineous marriage. Their first twin female children of age three years referred to us as an index case were diagnosed with Type II RCM. The children had shown a methemoglobin level of 63% and 72% and NADH-CYB5R activity of 14.5% and 9.2%, respectively. The symptoms were the same as in the earlier cases, and both had severe mental retardation with global developmental delay. Genetic analysis confirmed c.226G>A (p.Gly76Ser) mutation in the homozygous state in both children and heterozygous in the parents.

In Family-4, the first four-year-old male child was normal, and the second 10 months old female child was born to non-consanguineous parents belonging to Gujarat (India). She was referred to us as an index case and was diagnosed with Type II RCM. The first and the second child had shown a methemoglobin level of 1.2% and 39% and NADH-CYB5R activity of 22% and 9%, respectively. The primary symptoms of the second child had severe mental retardation, global developmental delay. The Sanger's sequencing detected c.229C>T (p. Gly77X) stop codon mutation in the homozygous state in the second child and heterozygous state parents and the first child on further molecular analysis.

In Family-5, the two-year-old patient from south India with a history of severe neurological condition and cyanosis since birth was referred to rule out the cause of RCM. Proband detected 36% methemoglobin level and 25 % NADH-CYB5R activity (enzyme activity: 7.5 IU/g Hb). A molecular study identified novel homozygous mutation c.274C>T (p. Arg92Trp) and parent's DNA confirmed c.274C>T (p. Arg92Trp) in heterozygous conditions.

Prenatal analyses of the CVS of the fetus from family 1 demonstrated p. Gly76Ser in the homozygous state and was subsequently advised to terminate the pregnancy to avoid fetal abnormalities while it was found to be a heterozygous state in family 2. In the case of the third family, a normal wild-type CYB5R3 genotype was detected in fetus DNA. In family 4, stop codon (p.Gln77X) heterozygous mutation was detected in the fetus, and CVS of family five showed heterozygous novel mutation (c.274C>T (p.Arg92Trp) (Table 1). The fetuses were a simple heterozygote for the paternal mutation. The pregnancy of three families was allowed to continue, and healthy babies were born.

The structural model of soluble CYB5R protein suggests two specific domains. In the ribbon representation of the 3D structural model of human NADH-Cyb5R shown in figure 3, the NADH

Table 1: Clinical history, biochemical and molecular characteristics of RCM II patients and fetus status in NADH-cytochrome *b5* reductase deficient family

No. of Family studied		Origin	Age	Gender	Clinical History/Symptoms	Meth-Hb level	NADH-CYB5R activity
Family I NM_000398.7(CYB5R3) c.226G>A (p. Gly76Ser)	Mother	Gujrat, India	31Y	F	No Consanguinity	1.6%	24.5 IU/g Hb
	Father		35Y	M		2.5%	23.8 IU/gHb
	1st Child		25M	F	Severe neurological and developmental failure	12.50%	16.5 IU/g Hb
	2nd child	**Prenatal Screening**			**Fetus status: Homozygous for p. Gly76Ser** (Medically terminated)		
Family II NM_000398.7(*CYB5R3*) c.226G>A (p. Gly76Ser)	Mother	Gujrat, India	32Y	F	Consanguinity	1.9%	21.2IU/g Hb
	Father		29Y	M		1.5%	24.3IU/gHb
	1st Child		3 D	F	Died after three days of birth	ND	ND
	2nd child		22 M	F	Severe neurological and developmental failure	39.4%	9.2 IU/g Hb
	3rd	**Prenatal Screening**			**Fetus status: Heterozygous for p. Gly76Ser**		
Family III NM_000398.7(*CYB5R3*) c.226G>A (p. Gly76Ser)	Mother	Kerala, India	32Y	F	No Consanguinity	1.8%	22.8IU/g Hb
	Father		43Y	M		2.7%	22.0IU/gHb
	1st Child		3Y	F	Normal	1.3.0%	24.5 IU/g Hb
	2nd child		3Y	F	Severe neurological and developmental failure	72.00%	9.2 IU/g Hb
	3rd	**Prenatal Screening**			**Fetus status: Normal**		
Family IV NM_000398.7(*CYB5R3*) c.229C>T (p. Gly77X)	Mother	Kerala, India	32Y	F	NO Consanguinity	1.8%	23.16 IU/g Hb
	Father		33Y	M		2.0%	21.08 IU/g Hb
	1st Child		4Y		Normal	1.2%	22.01 IU/g Hb
	2nd child		10 M	F	Severe neurological and developmental failure	39.4%	9.2 IU/g Hb
	3rd	**Prenatal Screening**			**Fetus status: Heterozygous for p. Gly77X**		
Family V NM_000398.7(*CYB5R3*) c.274C>T (p. Arg92Trp)	Mother	Kerala, India	34Y	F	NO Consanguinity	1.2%	15.07 IU/g Hb
	Father		36Y	M		1.3%	12.63IU/g Hb
	1st Child		2Y	F	Severe neurological and developmental failure	36.2%	7.5 IU/g Hb
	2nd	**Prenatal Screening**			**Fetus status: Heterozygous for p. Arg92Trp**		

Normal Range: Meth-Hb level- uptp1.5% and NADH-cytochrome *b5* reductase activity: 18-25.01 IU/g Hb

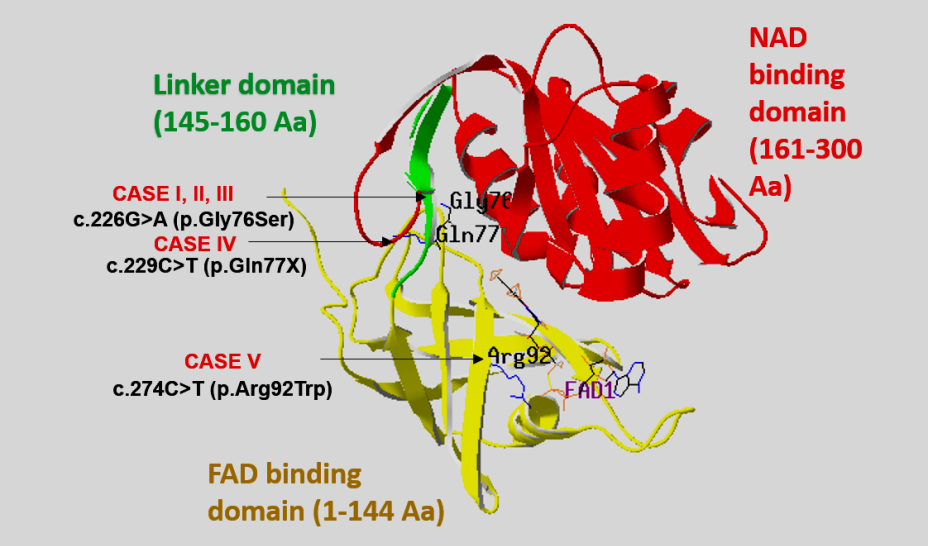

Figure 3: Ribbon representation of the three-dimensional structure of human *CYB5R3* shown NADH binding domain are shown in red, anti-parallel ß-sheets are shown in yellow the FAD-binding domain is joined by green color linkage domain. Residues in red indicate the locations of novel mutations found in this study. All mutations associated with Type 2 RCM are shown as a side chain in the FAD domain

binding domain (161-300AA) is represented in red, anti-parallel ß-sheets are represented in yellow FAD-binding domain (1-144 AA) is joined by green color linkage domain (145-160AA). The side chain residues indicate the positions of the identified mutations in this study. The binding site for the FAD prosthetic group is present in the FAD-binding domain at the N-terminal end of the protein (Ile34 to Arg143, Pfam ID PF00970), while others are in the NADH domain (residues Lys173 to Phe301, Pfam Id PF00175). A hinge region which is a large interdomain cleft separated the two domains mentioned above [5,11]. All the mutations found in the current study associated with Type II RCM are located in the FAD-binding domain (p.Gly75Ser, p.Gln76Term, and p.Arg92Trp), as shown in figure 3. There are few reports of type II RCM mutations that were found in the earlier report provided a prenatal diagnosis in Type II RCM are c.82C>T(Gln27STOP), c.136 C>T(Arg45Trp), and c.721A>G(Arg241Gly) [13-15]. However, no reports have been yet published on prenatal screening in India by the CYB5R3 gene variants p.Gly76Ser, p.Gln77X, and p.Arg92Trp. The most common mutation found in India is p.Gly76Ser in which glycine at 76 positions is present in the hinge region of the protein and may thus be crucial for proper orientation of the NAD domain, which would affect the binding capacity of the protein. However, the exact

role of this splice-site mutation in the protein's functional activity is unknown. However, the p.Gln77X mutation will lead to a truncated protein which explains the severity of this mutation. Analysis of the protein's active site (Figure 3) shows that amino acids Arg92 are crucial in forming hydrogen bonds with the Cofactor FAD molecule. Asada *et al*. suggested that Arg92 and Arg143 are also involved in the bonding with the electron transfer complex [16]. The Arg92Trp in the FAD-binding domain led to a change in the aliphatic side chain to the aromatic side chain, which might significantly affect the conformation and stability of protein structure, resulting in reduced enzyme activity. We have reported several novel missenses, nonsense, and frameshift mutations in the *CYB5R3* gene in an Indian family with recessive congenital methemoglobinemia causing Type I RCM. Previously have reported an unusual family with recurrent early pregnancy loss (REPL) due to RCM type II due to NADH-cytochrome b5 reductase deficiency in an Indian family [6-8,17,18].

We reported prenatal diagnosis in a pregnancy at risk of severe mental retardation and neurological disabilities due to RCM II in five Indian families due to homozygous p.Gly76Ser, p.Gln77X, and novel p.Arg92Trp pathogenic variants. Characterization of mutation profiles in these families with severe neurological disorders allowed us to offer a prenatal diagnosis for RCM Type II for these families. Thus, prenatal diagnosis can help to prevent the severity and recurrence of type II RCM in affected families.

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