Three novel mutations in CYB5R3 gene causing NADH-cytochrome b5 reductase enzyme deficiency leads to recessive congenital methaemoglobinemia

Anuradha Deorukhkar1 · Anuja Kulkarni1 · Prabhakar Kedar1

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Abstract

Background Methemoglobin is the reduced form of haemoglobin that is normally found in the blood in levels < 1%. Methemoglobinemia can occur as a congenital or acquired disease. Two types of recessive congenital methaemoglobinemia (RCM) are caused by the NADH-dependent cytochrome b5 reductase enzyme deficiency of the CYB5R3 gene. RCM-I is characterized by higher methaemoglobin levels (> 2 g/dL), causing only cyanosis, whereas RCM-II is associated with cyanosis with neurological impairment.

Methods Routine haematological investigations were done by standard method. The methaemoglobin level was evaluated by the potassium ferricyanide assay. NADH-cytochrome b5 reductase (cytb5r) enzyme activities were measured by standard methods, and molecular analysis was performed by polymerase chain reaction (PCR) followed by DNA sequencing. The interpretation of mutation effect and the molecular modeling were performed by using specific software DEEP VIEW SWISS-PDB VIEWER and Pymol molecular graphics program.

Results The present study discovered three novel homozygous pathogenic variants of CYB5R3 causing RCM I and II in four unrelated Indian patients. In patient-1 and patient-2 of RCM type I caused due to novel c.175C>T (p.Arg59Cys) and other reported c.469T>C (p.Phe157Ser) missense pathogenic variants respectively, whereas patient-3 and patient-4 presented with the RCM type II are related to developmental delay with cyanosis since birth due to a novel homozygous (g.25679_25679delA) splice-site deletion and novel homozygous c.824_825insC (p.Pro278ThrfsTer367) single nucleotide insertion. The CYB5R3 transcript levels were estimated by qRT-PCR in the splice-site deletion, which was 0.33fold of normal healthy control. The insertion of nucleotide C resulted in a frameshift of termination codon are associated with neurological impairment.

Conclusions Molecular diagnosis of RCM can help to conduct genetic counselling for novel mutations and, subsequently, prenatal diagnosis of high-risk genetic disorders.

Keywords CYB5R3 · DIA1 · Methaemoglobinemia · Neurological impairment · Developmental disorders · Inherited recessive diseases · RCM I and II

Introduction

The genetic aberrations in the CYB5R3 gene (OMIM 613213) result in Recessive Congenital Methaemoglobinemia (RCM) (OMIM 250800), a recessively inherited disorder caused by the partially non-functional enzyme or complete loss of enzyme activity of NADH-dependent cytochrome B5 reductase (NADH-CYB5R) [1]. RCM is classified into two categories: symptoms like cyanosis and shortness of breath related to hypoxia in tissues due to increased methaemoglobin levels categorized as type I RCM. Type II (generalized) RCM is characterized by cyanosis along with severe...
neurological disorders, brain dysfunction, dystonia, choreoathetosis, microcephaly, intellectual disability [2].

NADH-CYB5R (P00387) encoded by CYB5R3 plays an essential role in several oxidation–reduction reactions by transferring reducing equivalents NADH via NAD domain to the small molecules of cytochrome B5[3]. RBCs convert methaemoglobin to the haemoglobin with heme–iron oxidized to the ferric (Fe$^{3+}$) state, back to the normal haemoglobin with Fe$^{2+}$. Methaemoglobin is not an efficient oxygen carrier, which leads to a varying level of cyanosis, the normal level of methaemoglobin in whole blood is maintained at ≤ 1.5% or ≤ 0.2 g/dL [4]. Enzyme activity of NADH-dependent cytochrome b5 reductase is within the range of 35 ± 5 IU/g of Hb. NADH-CYB5R participates in fatty acid desaturation in other cells by providing the electrons to desaturate and the cholesterol biosynthesis pathway enzyme—4-methyl sterol oxidase, respectively [5]. Siendones et al. 2018 suggest CYB5R3 deficiency imparts pleiotropic tissue effects since it is an essential modulator of the intracellular NAD+/NADH ratio and therefore plays a vital role in aerobic metabolism and managing the oxidative stress of fibroblasts, neurons, and cardiomyocytes [6].

The CYB5R3 is a 31 kb gene also known as DIA (diaphorase gene). It is split into nine exons and is located on chromosome 22 (22q13.2). It codes for both soluble and membrane-bound isoforms of NADH-cytochrome b5 reductase [7]. The soluble isoform, only expressed in erythrocytes and a membrane-bound isoform is expressed in all cells. Both human isoforms are produced from a single gene locus, DIA1 [8, 9]. Human CYB5R3 of the non-erythroid cells encodes for an isoform that exhibits an additional exon (M) upstream of the first exon of the soluble protein present in erythrocytes [10]. Therefore, the two enzymes are identical, but the membrane-bound isoform contains a short amino acid sequence (MGQLSTL), which is a myristoilated anchor at its N-terminus [10, 11]. NADH-CYB5R deficiency can occur as a result of mutations in the CYB5R3 gene and has two clinical phenotypes based on defects in either the soluble or the membrane-bound isoforms. Soluble CYB5R3 is expressed explicitly in erythrocytes for methemoglobin (MetHb) reduction, and its deficiency is responsible for type I RCM, which is a benign condition with mild cyanosis, fatigue, and shortness of breath upon exertion, and increased level of MetHb. The membrane-bound CYB5R3 isoform is anchored to the outer mitochondrial membrane (OMM), endoplasmic reticulum (ER), and plasma membrane (PM) of all cells, and its deficiency, causes type II RCM, an incurable encephalopathy with permanent mild cyanosis and severe brain developmental deficiency is present in all type II RCM patients, which can develop severe neurological impairments [12–16].

A total of 75 pathogenic variants of CYB5R3 have been reported so far around the globe [17]. Here we describe the molecular basis of type I and type-II methemoglobinemia due to three novel mutations in the CYB5R3 gene causing NADH-CYB5R deficiency in four patients from three distinct regions in India. We have also described the genotype–phenotype correlation of all three novel variants in the CYB5R3 gene.

**Methods**

**Patient history**

Patient-1 and Patient-2: The two patients aged 15 years at the time of diagnosis referred to us from Uttar Pradesh (UP), North India, had cyanosis, bluish discoloration of lips, nails, no cardiac problem, no hepatosplenomegaly. Patient-3: This patient is a 5-year-old male child from Uttar Pradesh, India, born to a consanguineous family was reported with cyanosis and severe neurological symptoms with a developmental delay, underdeveloped limbs leading to complete dependency. This patient has two healthy female siblings. Patient-4: This patient is a 1-year male child born to a consanguineous family from the Nagpur region of Maharashtra, Central India, who was reported with cyanosis since birth with type II RCM symptoms such as encephalopathy, mental retardation, microcephaly, generalized dystonia, and/or movement disorders, and beyond a lack of social interactions indicating neurological disorder.

**Biochemical, haematological, and clinical assessment**

Routine haematological investigations were done as per Dacie and Lewis procedure [18]. The methaemoglobin level was evaluated by the potassium cyanide-ferricyanide method [19] modified from Evelyn and Malloy et al. method instantly after blood withdrawal, and informed consent from the parents of the patient was taken for further analysis [20]. The kinetic properties of NADH-cytochrome b5 reductase were determined in I.U./gm Hb by measuring the oxidation of NADH at 340 nm at 37 °C for 10 min on a spectrophotometer (Analytical JENA, Germany). The reaction rate was determined from the linear portion of the curve, and rates were calculated using a linear regression model. Each reaction contained hemolysate, 2 mM K$_3$Fe(CN)$_6$, 1 mM Tris–HCl pH 8.0, 0.5 mM EDTA and 0.2 mM NADH [21]. All the analytical procedures and examinations were done as per the Helsinki Declaration of 1975.
Molecular characterization of RCM patients by Sanger sequencing of DNA and RNA

Genomic DNA was extracted from leukocytes using a Qiagen DNA extraction kit. Nine exons of the CYB5R3 (GenBank database accession IDs M28705 to M28713) were amplified, and the PCR amplicons were sequenced using a 3730 DNA analyzer and Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc.) [22]. RNA was extracted from whole blood lymphocytes by TRIzol™ Reagent (Thermo Fischer Scientific). The quality of extracted RNA was checked using a Nanodrop spectrophotometer. The corresponding cDNA was synthesized from 30 ng of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and amplified using four sets of primers for Sanger sequencing 3′UTR from ATG (F I 5′ACCGGTC TCGCCGCGCCG3′ and RI 5′GTACTTGATGTCCGGCCT CT3′), (F II 5′CTGGTCTCTGTAACACTGTC3′, RI 5′GTCT TCTCTCTGCTGTGTT 3′), (F III 5′ATCCCAACCTGAC AAAAAGTC3′ RII 5′GCCTACTGATCATGGGTG3′), (F IV 5′CAGGAAACAAAACATTTCGCAC3′ RIV 5′TGCTCA GCCAGGTGATT3′) up to 3′UTR.

Gene expression of CYB5R3 by qRT-PCR

The cDNA synthesized from 30 ng of RNA was amplified with a Step-one real-time-PCR ABI 7700 Sequence Detector (Applied Biosystems, USA.) Applied Biosystems using TaqMan™ Gene Expression Master Mix and TaqMan® FAM-MGB probes for gene expression assays of CYB5R3. GAPDH was used as an internal control, and 15 healthy adults from the age group of 20 to 50 years were taken as normal control. The gene expression was estimated by $2^{-\Delta\Delta CT}$ method (Livac’s methods) in comparative fold expression of CYB5R3 in RCM patients concerning healthy controls.

Bioinformatics analysis

Novel RCM variant was subjected to protein modeling using a Swiss PDB viewer. The pathogenic variants of CYB5R3 identified were tested for their effect on the structural and functional integrity of respective proteins using Grantham Score for change in polarity [23] SDM [24] PROVEAN [25], PolyPhen [26], and SIFT score [27]. The CYB5R3 frame-shift variant’s protein modeling was performed with the I-TASSER server [28].

Results and discussion

Patient one and patient two from north India were referred to us with primary symptoms like cyanosis and Bluish discoloration of lips and nails for the first time in 15 years. The haematological, biochemical, and molecular data are shown in Table 1 indicate the methaemoglobin level in Patient-1 and Patient-2 was 10.2 g/dL and 14.75 g/dL (Normal Methaemoglobin level < 2 g/dL), whereas NADH-CYB5R activity was reduced to 10.5 and 8.02 IU/g Hb (normal range: 35 ± 5 IU/g Hb), respectively. Sanger sequencing of the exons of the CYB5R3 of Patient-1and Patient-2 led to the identification of novel substitution variants c.175C>T (p.Arg59Cys) in exon three and second variant c.470T>C (p.Phe157Ser) in exon 6 of CYB5R3 gene. The protein modeling using the Swiss-PDB viewer predicted the putative formation of a new hydrogen bond of cysteine 59 with an amino acid from the β sheet in close proximity (Fig. 1a). In this patient of p.Phe157Ser, aliphatic amino acid replaced the aromatic amino acid (Fig. 1b). These protein conformation changes have likely changed NADH-cytochrome b5 reductase activity in RBC, causing only cyanosis. Grantham score prediction for p.Arg59Cys and p.Phe157Ser is of the C65 category assumed to be significantly affected protein function of NADH-CYB5R. Bioinformatics tools such as Provean, SIFT, PolyPhen algorithms have also predicted c.175C>T (p.Arg59Cys) and c.470T>C (p.Phe157Ser) as most likely pathogenic variants (Table 1).

The patient-3 presented with developmental delay and cyanosis at the age of 5 years showed a methaemoglobin level of 63 g/dL and NADH-CYB5R activity of 9.3 IU/g Hb. The heterozygous mother has presented a methaemoglobin level of 12.33 g/dL and reduced NADH-CYB5R activity (20.79 IU/g Hb), heterozygous father showed 3.27% methaemoglobin level and reduced NADH-CYB5R activity (18.03 IU/g Hb) shown in Table 1. Molecular characterisation of patient-3 showed homozygous variant g.25679_25679delA at the splice site in IVS 8, which was anticipated to be affected due to splice-site deletion when the cDNA was amplified, demonstrated a typical sequence (Fig. 2A). The potential alternative splice-site sequence of g.25679_25679delA was determined by cDNA sequencing. The junction sequence of exons 7 and 8, which was anticipated to be affected due to splice-site deletion when the cDNA was amplified, demonstrated a similar sequence (Fig. 2B). The potential alternative splice-site was analyzed for splice site variant in patient-3 using Human Splicing Finder—Version 3.1. We further estimated the gene expression of CYB5R3 in propositus and parents. The CYB5R3 expression was found to be 0.33 folds in g.25679_25679delA novel homozygous variant when compared to normal healthy control, indicating low transcript levels owing to Nonsense-mediated mRNA decay (NMD) mutant transcripts [29]. Mother and Father of patient-3 with heterozygous g.25679_25679delA showed 3.4 folds and 5.73 folds of the transcript. The absence of 1 out of 2 normal alleles of CYB5R3 might have triggered NMD of mutant transcripts in heterozygous parents leading to insufficient
CYB5R3 mRNA, which resulted in over-expression of CYB5R3 in Type II heterozygous parents. The mother was reported to have incidences of mild cyanosis and difficulties in breathing in contrast to the asymptomatic father of patient-3. Although methaemoglobin levels are recommended in the normal range of 0.0–1.5 g/dL, unprecedented levels of cyanosis cannot be identified below the concentration of 10 g/dL. Hence, heterozygous patients with an identical variant have a variable presentation of phenotype. The patient-4 was a one-year-old child with RCM II presentation, was found to have a methaemoglobin level of 15.93 g/dL and NADH-CYB5R activity of 6.02 IU/g Hb. His heterozygous mother presented a methaemoglobin level of 5.34 g/dL, and NADH-CYB5R activity was 6.2 IU/g Hb, while the heterozygous father showed 6.05 g/dL methaemoglobin level and 14.07 IU/g Hb NADH-CYB5R activity, and both were reported to be asymptomatic patients. The exon sequencing of the patient-4 presented with Type II RCM symptoms identified novel homozygous variant,
Both maternal and paternal DNA contains a heterozygous form of the identified frameshift variant (Fig. 2C). The single nucleotide insertion in the coding sequence of CYB5R3 resulted in a frameshift variant starting from the replacement of Proline at 278 position to Threonine substitution of 24 amino acids at 3'end and shift of the termination codon adding 66 amino acids on 3'end that culminated in an extended protein (Fig. 2c). The protein tertiary structure of p.Pro278ThrfsTer367 was predicted by I-TASSER.
(C-score -1.71, Normal range -5 to 2) showed alteration in native secondary and tertiary conformation of protein from Amino acid 278 to 367, severely affecting enzyme activity lead to Type II RCM. In clinical genetics, the first degree of consanguinity is defined as a union between a couple related as second cousins or closer from, equivalent to a coefficient of inbreeding in their progeny of F > 0.0156 [30]. Parents of patient-3 and patient-4 with RCM II were reported to have third-degree consanguineous marriage in a community that practices austere endogamy spanning several generations. The presence of a homozygous pathogenic variant of CYB5R3 has again highlighted that consanguinity is the most acceptable factor in preserving the deleterious mutations in populations. These data further elaborate that type I and II recessive methemoglobinemia (RCM) is due to the instability of the NADH-dependent cytochrome b5 reductase enzyme regardless of the specific causative mutation in CYB5R3.

Conclusion

In this study, we have identified three novel mutations in CYB5R3 gene causing very severe RCM due to NADH-CYB5R deficiency in northern southern and western parts of India. As India has an ethnically and geographically diverse population with a high percentage of endogamous groups, there are chances of a heavy load of genetic disorders. And RCM is one of the most common genetic disorders with neurological impairment at risk. Extensive research is required to estimate the distribution of pathogenic genetic variations in RCM genes in all groups. The Splice-site deletion g.25679_25679delA (n.634-2delA) at the splice-acceptor site interfered with the post-transcriptional splicing mechanism leading to CYB5R3 under-expression causing RCM II with severe neurological symptoms and single nucleotide insertion p.Pro278ThrfsTer367 resulted in a frameshift of coding sequence affecting the original termination codon of CYB5R3 gene, causing structural and functional changes in native protein which affect neurological abnormalities. This study can help to conduct genetic counselling and, subsequently, prenatal diagnosis of high-risk genetic disorders.

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Author contributions PK: Conceived ideas and initiated the experimental work and supervision of work. AK: Contributed to the findings of the work. AD: Further experimental work and statistical analysis for expansion of idea were carried under the supervision of PK, AD, and PK: Manuscript was written by AD in consultation with PK.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interests The authors declare that they have no conflict of interests.

Ethical approval This study was approved by the Ethics Committee of the Institutional Ethical Committee of ICMR-National Institute of Immunohaematology, Mumbai. All procedures performed in studies involving human participants were as per the ethical standards of the institutional review board of the Institute and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent to participate Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

Consent for publication The written informed consent for publication was obtained.

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