Three Novel Mutations in *CYB5R3* Gene Causing NADH-cytochrome b5 Reductase Enzyme Deficiency Leads to Recessive Congenital Methaemoglobinemia

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Research Article

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Abstract

Two types of recessive congenital methaemoglobinemia (RCM) is caused by NADH-dependent cytochrome b5 reductase enzyme deficiency encoded by *CYB5R3* gene. RCM-I is characterized by higher methaemoglobin levels (>2 g/dL), causing only cyanosis, whereas RCMR-II is associated with cyanosis with neurological impairment. 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 are 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 frame-shift of termination codon are associated with neurological impairment. This study can help to conduct genetic counselling and, subsequently, prenatal diagnosis of high-risk genetic disorders.

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, demonstrating specific 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]. In RBCs, it converts 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 $\leq 1.5\%$ 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. In other cells, NADH-CYB5R participates in fatty acid desaturation 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 31kb gene also known as *DIA* (*diaphorase gene*). It contains nine exons and is located on chromosome 22. It codes for both soluble and microsomal forms of NADH-cytochrome b5 reductase [7]. The exon 2 encodes the sequence joining the membrane-binding domain with the catalytic domain of NAHD-CYB5R, suggesting that protein undergoes post-translational processing for producing two different forms of CYB5R [8-9]. The hydrophobic, non-polar C-terminal is later inserted into the lipid bilayer of the membranes of the golgi apparatus, microsomes, and mitochondria [10]. The membrane-bound enzyme is absent in RBCs due to the absence of these organelles[11]. A total of 75 pathogenic variants of *CYB5R3* have been reported so far around the globe [12]. 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, blueish 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 a lack of social interactions indicating neurological symptoms.

Biochemical, Haematological, and clinical assessment

Routine haematological investigations were done as per Dacie & Lewis [13]. The methaemoglobin level was evaluated by the potassium cyanide-ferricyanide method [14] 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 [15]. The kinetic properties of NADH-cytochrome b5 reductase were determined in I.U./gm Hb by measuring the oxidation of NADH at 340nm at 37⁰C 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, 2mM K₃Fe(CN)₆, 1mM Tris-HCl pH 8.0, 0.5mM EDTA and 0.2mM NADH [16]. 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.) [17](Mannino et al., 2018). 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 (FI 5'ACGGTCTCGGCGGCGGC 3' and RI 5'GTACTTGATGTCCGGGCTCT 3'), (FII 5'CTGGTTCCTGTACAGTCTGC 3', RII 5'GTCCTTCTCGGTCTGGTT 3'), (F III 5'ATCCGACCTGACAAAAAGTC 3' RII 5'GCGTACTGGATCATGGGTG 3'), (FIV 5'CAGGAACAAACATTCTGCAC 3' RIV 5'TGCTCAGCCAGGTGATT3') 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 [18] SDM [19] PROVEAN [20], PolyPhen [21], and SIFT score [22]. The *CYB5R3* frame-shift variant's protein modeling was performed with the I-TASSER server [23].

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 10.2g/dL and 14.75g/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-1 and 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 in 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.1 a**). In this patient of p.Phe157Ser, aliphatic amino acid replaced the aromatic amino acid (**Fig. 1 b**). 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 along with cyanosis at the age of 5 years and showed a methaemoglobin level of 63 g/dL and having NADH-CYB5R activity of 9.3 IU/g Hb. The heterozygous mother presented a methaemoglobin level of 12.33 g/dL and reduced NADH-CYB5R activity (20.79 IU/g Hb), while the heterozygous father showed 3.27% methaemoglobin level and reduced NADH-CYB5R activity (18.03 IU/g Hb) **Table-1**. Molecular characterisation of patient-3 showed homozygous variant g.25679_25679delA at the splice site in IVS 8 with deletion of A from Splice acceptor site AG_3'. Both maternal and paternal DNA showed a heterozygous form of an identified pathogenic variant (**Fig. 2a**). The resultant transcript 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 typical 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 [24]. 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 10g/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 15.93g/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 15.78 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, c.824_825insC [(p.Pro278ThrfsTer367)] in *CYB5R3* gene. Both maternal and paternal DNA contains a heterozygous form of the identified frame-shift variant (**Fig.**

2c). The single nucleotide insertion in the coding sequence of *CYB5R3* resulted in a frame-shift variant starting from the replacement of Proline at 278 positions 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 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 $F > 0.0156$ [25]. 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 are 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 to *CYB5R3*.

Conclusion

In this study we have identified three novel mutation in *CYB5R3* gene causing very severe RCM due to NADH-CYB5R deficiencies in northern, southern and western part of India. As India has ethnically and geographically diverse population with a high percentage of endogamous groups, there are chances of heavy load of genetic disorders. And, RCM is one of the most common genetic disorder with neurological impairment at risk. Extensive research is required to estimate of 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 frame-shift 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.

Declarations

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Conflict of interest: The authors declare that they have no conflict of interest.

Availability of data and material: All data generated or analyzed during this study are included in this published article.

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

Ethics 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 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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Tables

Table 1: Haematological, Biochemical, and molecular data of three novel pathogenic variants of CYB5R3 gene causing Type-I and II Recessive Congenital Methaemoglobinemia (RCM).

Cases	Normal Range	I	II	III	IV
Age	-	15 years	15 years	5 years	1 year
Gender	-	M	M	M	M
Consanguinity	-	Yes	Yes	Yes	Yes
Haematological, Biochemical and Molecular parameters					
Methaemoglobin level (%)	<1.5	10.29	14.75	63.32	15.93
CYB5R Activity IU/g Hb	35±5	10.5	8.02	9.3	6.2
WBC (X 10 ³ /μl)	4.5- 11.0	8.90	7.60	5.60	10.90
RBC (X 10 ⁶ /μl)	Men: 4.7- 6.1 Women: 4.2 -5.4	7	6.02	5.58	5.19
HGB g/dl	Men: 13.5- 17.5 Women:12.0- 15.5	19.6	15.6	11.3	12.3
HCT (%)	Men: 41-50 Women: 36- 48	58.2	47.4	39	40
MCV (fl)	80- 100	83.1	78.7	69.9	77.1
MCH (pg)	29 ± 2	28	25.9	20.3	23.7
MCHC (g/dl)	34 ± 2	33.7	32.9	29	30.8
PLT (X 10 ³ /μl)	150- 450	190	211	390	462
RDW (%)	12.2-16.1	13.6	14.4	22.4	12
RCM.		Type I	Type I	Type II	Type II
Nucleotide change		c.175C>T	c.470T>C	n.634-2delA	c.824_825insC
Amino acid Change		p. (Arg59Cys) Novel	p. (Phe157Ser) (Reported)	(Novel)	p.(Pro278Thr fsTer367) (Novel)
Domain of NADH-CYB5R		FAD	Hinge region	NAD	NAD

Pathogenicity predictions of <i>CYB5R3</i> variants (UniProt accession ID: P00387)		
Grantham Variation	0.00	0.00
Grantham Deviation	179.53	154.81
Grantham Prediction	C65-most likely to interfere with the function	C65-most likely to interfere with the function
Provean score	-6.88	-7.27
Provean prediction	Deleterious	Deleterious
SIFT Score	0.005	0
SIFT Prediction (cutoff=0.05)	Damaging	Damaging
PolyPhen Score	1	1
PolyPhen Prediction	Probably Damaging	Probably Damaging

Note: METH HB-Methaemoglobin, WBC: White Blood Cells, RBC; Red Blood Cells, PLT; Platelet, HGB; Haemoglobin, HCT; Haematocrit, MCV; Mean Corpuscular Volume, MCH; mean Corpuscular Haemoglobin, MCHC; Mean Corpuscular Haemoglobin Concentration, RDW; Red Cell Distribution Width

Figures

Figure 1

DNA sequencing chromatogram and Protein modeling of novel CYB5R3 pathogenic variants (a) Novel Variant in patient-1: c.175C>T (p.Arg59Cys) in exon 3 (b) Reported variant in patient-2: c.470T>C (p. Phe157Ser) in exon 6.

Figure 2

(a) DNA sequencing chromatogram of Novel Variant in patient-3: [g.25679_25679delA] (b) DNA sequencing chromatogram of cDNA of propositus and parents with splice-site mutation n.634 -2delA (c) DNA sequencing chromatogram of the Novel homozygous variant of patient-4 with heterozygous parents and Protein modelling of novel variant: c.824_825insC [(p.Pro278ThrfsTer367)]