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Short Communication

A novel nine base deletion mutation in *NADH*-cytochrome b5 reductase gene in an Indian family with recessive congenital methemoglobinemia-type-II

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ABSTRACT

Recessive hereditary methemoglobinemia (RCM) associated with severe neurological abnormalities is a very rare disorder caused by NADH- cytochrome b5 reductase (cb5r) deficiency (Type II). We report a case of 11 month old male child who had severe mental retardation, microcephaly and gross global developmental delay with methemoglobin level of 61.1%. The diagnosis of NADH-CYB5R3 deficiency was made by the demonstration of significantly reduced NADH-CYB5R3 activity in the patient and intermediate enzyme activity in both the parents. Mutation analysis of the *CYB5R* gene revealed a novel nine nucleotide deletion in exon 6 leading to the elimination of 3 amino acid residues (Lys173, Ser174 and Val 175). To confirm that this mutation was not an artifact, we performed PCR-RFLP analysis using the restriction enzyme *Drd* I. As the normal sequence has a restriction recognition site for *Drd* I which was eliminated by the deletion, a single band of 603-bp was seen in the presence of the homozygous mutation. Molecular modeling analysis showed a significant effect of these 3 amino acids deletion on the protein structure and stability leading to a severe clinical presentation. A novel homozygous 9 nucleotide deletion (p.K173–p.V175del3) is shown to be segregated with the disease in this family. Knowing the profile of mutations would allow us to offer prenatal diagnosis in families with severe neurological disorders associated with RCM — Type II.

1. Background

Hereditary methemoglobinemia is a rare autosomal recessive disease that results from the deficiency of the enzyme NADH cytochrome b5 reductase (NADH-CYB5R3). There are two isoenzymes of NADH-CYB5R in mammals: the membrane-bound and the water soluble forms of CYB5R and each one has a specific function. Two distinct clinical manifestations of NADH-CYB5R deficiency have been observed. In type I, cyanosis is the only clinical presentation and the enzyme defect is limited to red blood cells whereas in type II, cyanosis is associated with severe neurological impairment including mental retardation, microcephaly and movement disorders and the enzyme defect also exists in non-erythroid cells, such as fibroblasts and lymphocytes. [1–2].

NADH-CYB5R is encoded by a single *CYB5R3* gene and only 58 mutations have been described in patients with type I and type II RCM, Some mutations are common to both types of recessive hereditary methemoglobinemia [1,3] So far only 6 clinical reports of recessive congenital methemoglobinemia type-II have been reported from India. Among these, 3 cases had mental retardation and 3 cases were presented with RCM associated with neurological problems since birth. [3]. However, a molecular diagnosis of RCM has only recently begun to appear from India. P.Trp236X mutation associated with type II methemoglobinemia has also been reported by us earlier in three Indian patients [3,4]. Three dimensional structure of NADH-CYB5R3 has been resolved recently for the rat and the human enzymes. X-ray crystallographic studies have shown that the CYB5R enzyme is composed of two functional lobes linked by a flexible hinge region, which is important for the maintenance of the critical protein architecture required for enzyme activity [3,5].

In this report, we describe the clinical, neurological, hematological, biochemical and molecular findings in 11 month old child with recessive congenital methemoglobinemia type II. Using a combination of different PCR-based methods (restriction enzyme digestion, single strand conformation analysis and direct sequencing) we identified a novel nine-nucleotide deletion in exon 6 of the *CYB5R3* gene that leads to the elimination of thee amino acid residues (Lys173, Se174 and Val175) leaving the message inframe. The three-dimensional structural alterations induced in the enzyme by novel nine-nucleotide deletion have been elucidated by molecular graphic analysis using the crystal

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structure of the human NADH-cytochrome b5 reductase as a reference model. These studies showed that the elimination of Lys173, Ser174 and Val175 perturbs the correct orientation of adjacent catalytic residues such as Cys203 and His204.

2. Methods

10 ml peripheral blood was collected in EDTA from the patient and his parents after an informed consent. Hematological indices were measured on an automated cell counter (Sysmex-K-1000, Japan). The methemoglobin (Hi) levels, Hb-M Variant and erythrocyte NADH-CYB5R3 activity measured as described earlier using the Specord 205 spectrophotometer (Analytical Jena, Germany) [3]. Genomic DNA was isolated by standard methods. Polymerase chain reaction (PCR) and DNA sequencing were done using standard protocol for characterization of mutations [1]. Mutations have been named according to the recommendations for the description of sequence variations (www.hgvs.org/mutnomen). Gene Bank reference sequences are: NC_000022.10 and NP_000389.1. The project was approved by the Ethic committee of National Institute of Immunohaematology.

2.1. Molecular modeling of the NADH-CYB5R3 mutant

Modeling of human NADH-CYB5R was carried out using the structure of human NADH-CYB5R at 2.4 A° resolution (Protein Data Bank, code 1UMK). Molecular graphics were generated using the program PyMOL (W.L. DeLano, The 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.

3. Results

3.1. Clinical case

The proband was an 11 month old male child, delivered by Cesarean section (LSCS) with a birth weight of 3 kg. The parents originated from South India and there was a history of consanguinity in the family (Fig. 1A). There was no history of cyanosis at the time of birth. Central cyanosis was noted at 6 months of age when assessed for developmental delay. On examination, the head circumference was 39 cm and weight was 7.2 kg. The child cried excessively, had a mild fixed upward gaze and showed developmental delay, failure to thrive and delayed milestones also short neck, central cyanosis were observed. The central nervous system evaluation showed that the child was moving all four limbs symmetrically, followed light briefly and had head lag and hypotonia. The cardiovascular system was normal (S1S2 + and no murmur). The proband had severe mental retardation, microcephaly and gross global developmental delay. There was no history of a similar illness in any other family member. Thyroid function test and karyotyping study was normal. 2D Echocardiogram and X-ray chest was normal. There was no history of any drug exposure and G6PD activity was also normal. Pulse oximetry showed SpO_2 was 80.0% whereas arterial blood gas analysis showed pH — 7.357, pCO_2 — 30.0 mm Hg, pO_2 — 54.9 mm Hg, HCO_3 — 16.4 mmol/L, and sO_2 — 96.2%. The Hb level was 16.7 g/dl and the peripheral blood smear showed a normochromic and normocytic picture.

Patient was sent to us for investigating the cause of cyanosis associated with severe neurological impairment. After an informed consent, blood samples were collected from the proband and his parents. The methemoglobin level of the patient was significantly increased i.e. 61.1% (N.R. ≤ 1.5%) whereas parents showed a slight increase in the

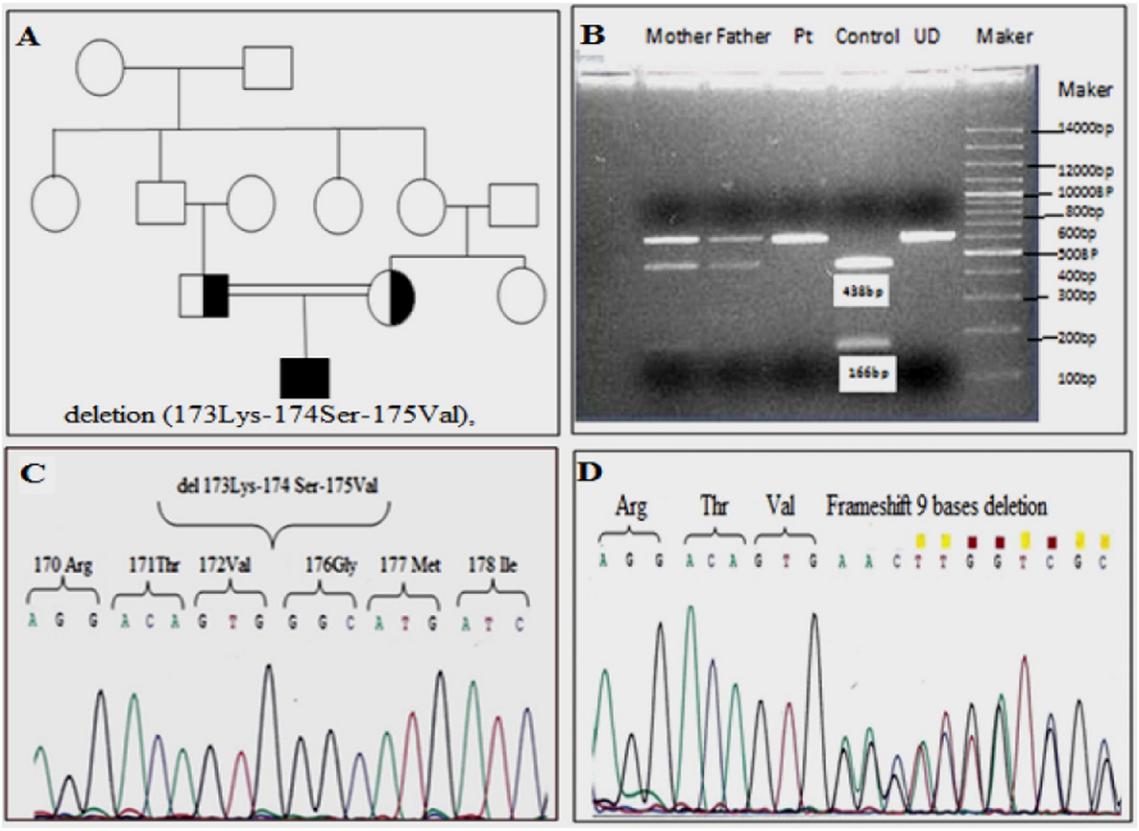

Fig. 1. (A) Consanguineous pedigree of patient. (B) Diagnostic restriction digest of an CYB5R gene amplicon from patient showing loss of *Drd* I site due to the homozygous deletion and shows a single fragment of 603 bp. (C) Sequence traces from exon 6 of CYB5R gene of the patient showing the novel homozygous 3 amino acid deletion (173Lys-174Ser-175Val) and (D) sequence of one of the parents showing heterozygous for the same mutation.

Table 1
Hematological and biochemical data of the proband and his family.

Parameters	Proband	Father	Mother
WBC ($\times 10^3$/μl)	10.2	7.2	5.9
RBC ($\times 10^6$/μl)	6.27	5.51	4.49
HB (g/dl)	15.7	16.5	12.5
HCT (%)	50.1	46.1	37.2
MCV (fl)	79.9	84.2	82.9
MCH (pg)	25.0	29.9	27.8
MCHC (g/dl)	31.3	35.6	33.6
PLT (10^3/μl)	506	302	225
RDW (%)	19.9	12.3	12.9
Meth-Hb level (normal level — <1%)	61.1	2.05	2.80
Hb M variant	Absent	Absent	Absent
NADH-CYB5R activity (normal range — 35 ± 5 IU/g Hb)	15.05	24.01	25.65
Mutation analysis	173Lys-174Ser-175Val Deletion homozygous	173Lys-174Ser-175Val Deletion heterozygous	173Lys-174Ser-175Val Deletion heterozygous

methemoglobin level. Spectrophotometric screening for Hb M variants showed two normal absorption peaks at 502 nm and 632 nm suggesting the absence of Hb M variants. The diagnosis of NADH-CYB5R3 deficiency was made by the demonstration of a reduction in NADH-CYB5R3 activity by 60.0% whereas both the parents showed intermediate enzyme activity. Both the parents were asymptomatic. The hematological, biochemical and molecular finding of the family are shown in Table 1.

3.2. Genetic analysis

DNA sequencing of the *CYB5R3* gene using the ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) in the proband showed a nine base deletion corresponding to three amino acid deletions (173Lys-174Ser-175Val). This was a novel mutation in exon 6 and the parents were also heterozygous for the same mutation leaving to a frameshift in the downstream coding region (Fig. 1C and D).

PCR amplified genomic fragments containing exon 6 were then digested with the restriction enzyme *Drd* I and the products were analyzed by electrophoresis on a 3.0% agarose gel and visualized after ethidium bromide staining. As the normal sequence has a restriction recognition site for *Drd* I which was eliminated by the deletion, a single band of 603-bp was seen in the presence of the homozygous mutation (Fig. 1B).

3.3. Enzyme structure–function implications

Molecular modeling using the human crystal structure of NADH-CYB5R3 as a model was done to determine how this mutation could affect enzyme structure and function. Ribbon diagram of the 3 dimensional model of the human NADH-CYB5R3 was generated using the program PyMOL (Fig. 2). The FAD binding domain is drawn in blue (residues 31–146 AA), the NADH binding domain is drawn in magenta color (residue 174–301 AA) and Linker domain is drawn in green color (residue 147–173 AA). The NADH-CYB5R3 molecular architecture corresponds to an (α/α) 8 barrel with the active site in a deep pocket at the C-terminal end. The pocket is lined primarily by charged amino acids that interact with the substrate. Among others, Gly147 and Met187 are directly bound to NADH, the product of the catalytic reaction. [5]. In NADH-CYB5R3 Lys173 belongs to a loop connecting the α-helix D with the α-strand 4 (Fig. 2B) and Ser174 is the first residue of strand 4. Val174 links to His204 and Cys203, which are located in the near α-strand 5, through two hydrogen bonds. Therefore, the N-terminal ends of strands 4 and 5, which are two adjacent staves off the α-barrel, are maintained close to each other by these hydrogen bonds. To explore the structural alterations resulting from the deletion, we carried out a systematic search for closure α-helix D with the β-strand 5, after omitting residues 173Lys-174Ser and 175Val various possible closures were found with satisfactory stereochemistry, indicating that the mutated enzyme should be able to fold. However, to rebuild

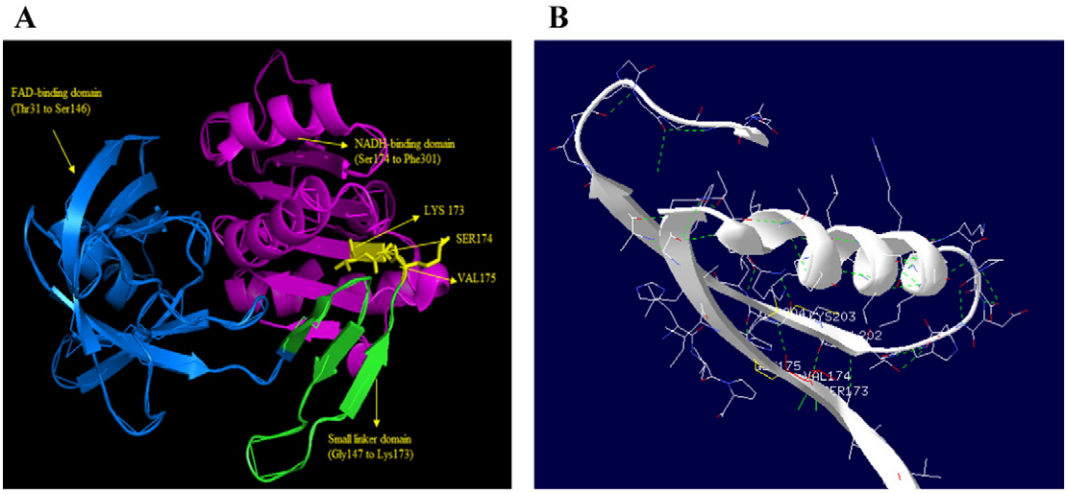

Fig. 2. Molecular modeling: enzyme structure–function implications of NADH-CYB5R deficiency: Ribbon representation of three-dimensional structure of human CYB5R3. Alpha helices are shown in magenta; anti-parallel β-sheets are shown in blue, the FAD cofactor is shown. A) Residues in yellow indicate the locations of mutation found in this study. B) Close-up view of hydrogen bonding between Lys 173, Ser174 and Val 175 links to Val 202, Cys203 and His204 resp. which are located in the near α-strand 5, through three hydrogen bonds.

this connection, a variation of the backbone conformation of at least three residues (amino acids 202 to 204) is required. The mutation produces, therefore, the unwinding of the C-cap amino acids 202–204 of the α-helix and the weakening of the contacts between N-ends of strands 4 and 5. This might severely alter the helix/strand connection. Furthermore, it is noteworthy that the catalytic residues are located in NADH binding domain and are not far from the deletion site. This proximity may induce major local changes to side chains of 173Lys-174Ser and 175Val and these variations may explain the remarkable reduction in enzyme activity also these three amino acids [173Lys-174Ser-175Val] are located at the junction of the linker domain and the NADH binding domain. Hence, these 3 amino acids are important for the maintenance of the critical protein architecture required for enzyme activity. Due to the loss of these amino acids, the entire structure of the enzyme would be destroyed; resulting in decreased catalytic activity and this would have a significant effect on the protein structure and stability (Fig. 2). Therefore, the analysis of altered protein conformation by molecular graphic modeling contributes to the understanding of the proper functioning at the enzyme active site.

4. Discussion

NADH–CYB5R3 deficiency (Type II) is a very rare cause of congenital methemoglobinemia associated with severe neurological abnormalities. There are only six cases of recessive congenital methemoglobinemia (RCM) type II reported from India in the last 10 years. Their genotype–phenotype relationships were discussed in our earlier paper [3,6]. Out of these six cases, molecular characterization was done in four cases where nonsense mutations and splice-site mutations were shown. In this paper, for the first time we described a deletion of nine nucleotides corresponding to the deletion of three amino acids [173Lys-174Ser-175Val] which are highly conserved.

In this report, we diagnosed the proband with RCM type II at the age of 11 months and the cyanosis and neurological impairment was the key to diagnosis. Our earlier cases were diagnosed only after the age of 3–5 years [3–4]. In an earlier report, a Tunisian patient was diagnosed at 8 years of age, when cyanosis became noticeable because of a pulmonary infection. [2]. Failure to detect cyanosis may delay the diagnosis. The cyanosis is mainly increased during stressful events such as birth and during episodes of infection but otherwise may remain unnoticed for a long period of time. We suggest that any child with unexplained severe neurological impairment should also be tested for RHM type II.

We have also confirmed this mutation (173Lys-174Ser-175Val amino acids deletion) of the *CYB5R3* gene by restriction enzyme (*Drd* I) digestion. The PCR product of 603 bp (lane 3) is cut into two fragments, 438 bp and 166 bp in the wild type genomic DNA (control), whereas the PCR product in the proband's genomic DNA remains uncut due to abolition of the restriction enzyme site due to the homozygous deletion and shows a single fragment of 603 bp (proband lane-3) (Fig. 1B). The newly identified mutation was not a polymorphism since it was absent in 25 unrelated individuals by restriction analysis and further confirmed by DNA sequencing.

Cyanosis can be well treated with 200–500 mg of Ascorbic acid daily, but there is no effective therapy for the neurological problems. The patient had improvement in cyanosis that may be due to the initiation of daily ascorbic acid therapy. It has been hypothesized that a possible response to ascorbic acid may be related to the effect of making additional ferrous iron available for its role as a cofactor in carnitine synthesis [7]. Our patient had a methemoglobin level of 61.1% and was treated with methylene blue (0.5 mg/kg body weight). This resulted in reduction in the methemoglobin levels but no improvement in mental performance. The patient has severe mental retardation, microcephaly and gross global developmental delay. Presently there is no way of treating the neurological deterioration associated with RHM type II and even the pathophysiology of RCM Type II is still not clear.

The most frequent mutations in the Indian population appear to be p.Ala179Thr followed by p.Arg50Trp and p.Gly76Ser. The p.Ala179Thr mutation has been reported previously in two Asian Indian siblings [3]. Due to the severity of the disease (RHM Type II) and a 25.0% chance of recurrence in every pregnancy, prenatal diagnosis could now be offered to the parents in a subsequent pregnancy. Molecular prenatal diagnosis is more reliable and can be done only when the mutation in the *CYB5R3* gene has been identified in families with severe neurological abnormalities [8].

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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