Four New Mutations in the NADH-Cytochrome b5 Reductase Gene From Patients With Recessive Congenital Methemoglobinemia Type II

By Luisa Mota Vieira, Jean-Claude Kaplan, Axel Kahn, and Alena Leroux

Recessive congenital methemoglobinemia (RCM) due to NADH-cytochrome b5 reductase (cytb5r; E.C.1.6.2.2) deficiency leads to two different types of diseases. In the type I form, cyanosis is the only symptom, and the soluble enzyme is defective in red blood cells. In the type II form, cyanosis is associated with severe mental retardation and neurologic impairment; the enzymatic defect is systemic, involving both soluble and membrane-bound isoforms. We characterized mutations responsible for cytb5r deficiency in three unrelated patients with severe RCM type II. The first patient presented a homozygous exon 5 skipping. The only mutation detected was a homozygous G to C transversion at position +8, downstream from the 5' splice site of exon 5. We suggest that this unusual mutation might be responsible for the abnormal splicing of the primary transcripts, resulting in frameshift with premature STOP codon. The second mutation found corresponds to a homozygous C to T transition changing the Arg-218 codon to a premature STOP codon in exon 8. The third case was a compound heterozygote, carrying two different mutant alleles in the cytb5r gene. One allele presented a frameshift mutation with replacement of Cys-203 (TGC) by Arg (CGC) in exon 7. The second allele carried a 3-bp deletion (TGA) of nucleotides 815 to 817, modifying two contiguous codons in exon 9 of the cDNA with loss of Met-272. These results confirm the genetic polymorphism of cytb5r gene mutations identified in RCM type II, as observed for the mutations described in the RCM type I, and shed light on the molecular bases of the two different diseases associated with cytb5r deficiency.

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R E CESSIVE CONGENITAL methemoglobinemia (RCM; McKusick no. 250800) is due to NADH-cytochrome b5 reductase (cytb5r; E.C.1.6.2.2) deficiency. Two forms of this enzyme are known, a membrane-bound form mainly found in microsomes of all investigated tissues1,2 and a soluble form present in erythrocytes.3 In red cells the soluble enzyme is involved in the reduction of methemoglobin,4,5 whereas in others cells the membrane-bound microsomal enzyme participates in the desaturation and elongation of fatty acids,6,7 as well as in cholesterol biosynthesis8 and drug metabolism.9 Structurally, the soluble form (275 amino acid residues) lacks a hydrophobic segment at the NH2-terminus, present in the membrane-bound enzyme (300 amino acid residues).10,11 Both isoforms are produced by a single gene.12,13 In rats, according to recent reports from Pietrini et al14 and our group,15 the soluble erythrocYTE form is produced by an erythroid-specific transcript. These results, completed by our findings of two other cytb5r transcripts responsible for the production of the ubiquitous soluble enzyme, reflect regulation complexity of cytb5r gene in rats. In humans, such a complex mechanism has not yet been established, and the biogenetic relationship between soluble and membrane-bound enzymes remains unclear.

Two clinical types of RCM have been defined: (1) RCM type I,16 in which cyanosis is the single clinical symptom, and (2) RCM type II,12 in which cyanosis is associated with severe mental retardation and neurologic impairment. The enzyme deficiency is restricted to the red cell soluble cytb5r in RCM type I, whereas in the type II form, the enzyme defect is generalized to all tissues, involving both soluble and microsomal forms of enzyme. Identification of the different mutations occurring at different positions within the NADH-cytochrome b5 reductase gene might account for the phenotypic heterogeneity of this disease. In the past 3 years, five homozygous mutations in the cytb5r gene have been reported: three missense mutations (Arg57Gln/exon 3, Val105Met/exon 4, and Leu148Pro/exon 5) in cases of RCM type I,17-19 a missense mutation (Ser127Pro/exon 5), and an in-frame 3-bp deletion (del Phe-298/exon 9) in RCM type II.20

We have analyzed cDNA and genomic DNA from three unrelated patients with RCM type II, while only one mutation associated with this severe form of RCM was known when we started this investigation. The second 3-bp deletion determined in RCM type II was published later.20 In the three investigated patients, whose observations and enzyme defects have been previously reported,21,22 we report four new mutations in the cytb5r gene: a homozygous exon skipping, a homozygous nonsense mutation, and a compound heterozygote carrying a new missense mutation in one allele and an out-of-frame 3-bp deletion in the other allele.

MATERIALS AND METHODS

Case reports. Three patients with RCM type II were investigated. Patients BEN and BOU have been described in previous reports.21,22 Briefly, both presented the clinical symptoms of RCM type II (profound mental retardation, microcephaly, and bilateral athetosis) with abolished enzyme activities in erythrocytes, lymphocytes, and lymphoblastoid cell lines. Both of them were from Algerian families with known consanguinity. Whole-blood samples were obtained from BOU's parents and from normal controls. Patient LAM presented cyanosis at birth. The diagnosis of RCM type II was established at 15 days, according to a persistent cyanosis and neurologic disorders associated with a complete enzyme deficiency in erythrocytes and lymphocytes. The deficiency was confirmed in established continuous lymphoblastoid cell line. Heterozygous levels of methemoglobin reductase (cytb5r) were detected in both parents’
and the maternal uncle’s blood cells. The parents were not related: the mother was of Spanish ancestry, and the father was French. A lymphoblastoid cell line was established from a normal control.

General procedures. Total RNA was prepared from lymphoblastoid cells using the guanidium thiocyanate procedure.27 Genomic DNA was isolated from the same cells or from peripheral white blood cells by standard techniques.28 Southern blotting of polyacrylamide gels was performed as previously described29 using a nylon Hybond-N membrane (Amersham, Aylesbury, UK). Blots were hybridized with oligonucleotides 5'-OH labeled with [32P]-adenosine triphosphate (ATP; Amersham) using T4 polynucleotide kinase.

Analysis of the cytb5r mRNA by reverse transcription-polymerase chain reaction (RT-PCR). The synthesis of cDNA was performed from 5 μg of total RNA using random hexanucleotide primers and MoMLV-reverse transcriptase, as described.13 The PCR was performed by 30 or 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and primer extension at 72°C for 1 minute in 100 μL of 67 mmol/L Tris-HCl (pH 8.8) buffer, 17 mmol/L (NH4)2SO4, 10 mmol/L 2-mercaptoethanol, 5 μmol/L EDTA, 0.5 μmol/L each of deoxynucleoside triphosphates (dNTPs; deoxyadenosine triphosphate [dATP], deoxycytidine triphosphate [dCTP], deoxyguanosine triphosphate [dGTP], and deoxythymidine triphosphate [dTTP]), 50 pmol of upstream and downstream primer, 10% (by volume) dimethyl sulfoxide, and 2.5 U of Thermus aquaticus (Taq) DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). For each pair of primers, the optimal concentration of MgCl2 in the assay was determined to be in the range of 1.5 mmol/L to 9 mmol/L. Taq-DNA polymerase was added after heating the reaction mixture at 80°C for 10 minutes. The PCR products were analyzed by 8% (mass/volume) polyacrylamide gel electrophoresis.

PCR amplification of genomic DNA. DNA fragments were amplified from 500 ng of genomic EcoRI-digested DNA by 40 cycles of PCR as described above. Taq-DNA polymerase was added after denaturing the reaction mixture at 95°C for 10 minutes.

Sequencing. The RT-PCR or genomic DNA-PCR fragments were purified by electrophoresis through a 3% (mass/volume) low melting point agarose gel (NuSieve; FMC, Rockland, ME) run with 1× TAE buffer, pH 7.6 (40 mmol/L Tris-acetate/2 mmol/L EDTA, pH 8.0), recovered by phenol extraction of the agarose slices, and ethanol-purified. Purified DNA fragments were sequenced directly as described80 or after cloning into a TA cloning vector (Invitrogen, San Diego, CA) using Sequenase version 2.2 sequencing kit (US Biochemical, Cleveland, OH) with [32P]-dATP (Amersham) and the appropriate oligonucleotide for each fragment as the sequencing primer.

Restriction site analysis. About 500 ng PCR-amplified genomic DNA fragments were ethanol-precipitated before digestion with 5 to 10 U of the appropriate restriction enzyme, using buffers and digestion temperatures recommended by the manufacturers. The restriction fragments were analyzed on 8% (mass/volume) polyacrylamide gels.

Oligonucleotides. The oligonucleotides (Institut Pasteur or GenSet-France, Paris, France) used for PCR, hybridization, or sequencing are listed in Table 1.

RESULTS

Analysis of patients’ cDNA. PCR amplification of two overlapping cDNA fragments was performed with two sets of primers (primers 1 and 8; primers 6 and 14; Table 1) on total RNA extracted from lymphoblastoid cell lines from patients BEN, BOU, and LAM and from a normal control. Amplified fragments designated as 5′ and 3′ cDNAs were analyzed on a polyacrylamide gel, Southern blotted, and hybridized with internal specific oligoprobes (primers 2 and 11; Fig 1A and Table 1). Amplified 5′ cDNA fragments from patients BOU and LAM presented a normal size in comparison with the control (530 bp), while the 5′ cDNA fragment amplified from patient BEN exhibited an abnormal, approximately 130 bp lower size (Fig 1B). In this patient, the expected normal-sized 5′ cDNA was found in extremely low levels. The trace of correctly spliced RNA detected only by specific hybridization (oligoprobe 2; Table 1) of 40 cycles of PCR product was not functional, as no cytb5r activity was detected in this patient’s cells. According to hybridization with intronic oligoprobe proximal to donor splice site of intron 5, no signal was found (data not shown), suggesting that the faint upper band does not correspond to partial intron 5 retention. Thus, this additional band is most likely a heteroduplex with slower electrophoretic mobility.

As shown in Fig 1B, no difference in size was found for 3′ cDNA fragments for both patients BEN and BOU in comparison with the normal size of this fragment (407 bp). In contrast, the 3′ cDNA fragment amplified from patient

Table 1. List of Primers Used

<table>
<thead>
<tr>
<th>Primer No.</th>
<th>Primer Sequence (5' → 3')</th>
<th>Orientation</th>
<th>Location of Primer</th>
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Abbreviations: S, sense; A, antisense.
Total RNA from the lymphoblastoid cell lines was used for synthesis of cDNA by random primer technique, as described in Materials and Methods. Each cDNA sample (5 μL) was subjected to PCR amplification with two sets of primers (primers 1 and 8, and primers 6 and 14) able to amplify the cDNA in two overlapping fragments (5’ cDNA and 3’ cDNA, respectively). 10% of each RT-PCR product was separated by 8% (mass/volume) polyacrylamide gel electrophoresis. The Southern blots were hybridized with internal specific oligoprobes: primer 2 and primer 11 for 5’ and 3’ cDNA fragments, respectively. The sequence of primers are shown in Table 1. (A) Schematic representation of amplified cytb5r cDNA in two overlapping fragments: 5’ cDNA and 3’ cDNA. The size of amplified fragments, the location and orientation (−, sense; −, antisense) of PCR primers, and the internal radiolabeled specific oligoprobes (asterisk) are shown. (B) Southern blot analysis of the RT-PCR products: hybridization with internal radiolabeled specific oligoprobes. The size of specific hybridized cDNA fragments amplified from a normal control and patients BOU, LAM, and BEN are indicated on the right side for both 5’ and 3’ cDNA fragments. The 5’ cDNA fragment from patient BEN and the 3’ cDNA fragment from patient LAM exhibit abnormal patterns.

Fig 1. Analysis of cytb5r cDNAs of patients BOU, LAM, and BEN. Total RNA from the lymphoblastoid cell lines was used for synthesis of cDNA by random primer technique, as described in Materials and Methods. Each cDNA sample (5 μL) was subjected to PCR amplification with two sets of primers (primers 1 and 8, and primers 6 and 14) able to amplify the cDNA in two overlapping fragments (5’ cDNA and 3’ cDNA, respectively). 10% of each RT-PCR product was separated by 8% (mass/volume) polyacrylamide gel electrophoresis. The Southern blots were hybridized with internal specific oligoprobes: primer 2 and primer 11 for 5’ and 3’ cDNA fragments, respectively. The sequence of primers are shown in Table 1. (A) Schematic representation of amplified cytb5r cDNA in two overlapping fragments: 5’ cDNA and 3’ cDNA. The size of amplified fragments, the location and orientation (−, sense; −, antisense) of PCR primers, and the internal radiolabeled specific oligoprobes (asterisk) are shown. (B) Southern blot analysis of the RT-PCR products: hybridization with internal radiolabeled specific oligoprobes. The size of specific hybridized cDNA fragments amplified from a normal control and patients BOU, LAM, and BEN are indicated on the right side for both 5’ and 3’ cDNA fragments. The 5’ cDNA fragment from patient BEN and the 3’ cDNA fragment from patient LAM exhibit abnormal patterns.

Identification of an exon 5 skipping. To further understand the precise genomic defect responsible for abnormal ctb5r mRNA in patient BEN, the 5’ cDNA PCR product was directly sequenced using one of the PCR primers (primers 1 or 8; Table 1) or the internal primer 2 (Table 1). The cDNA was found to be completely devoid of exon 5 and to have exon 4 precisely abutted to exon 6 (Fig 2). Consequently, the translational reading frame of the resulting mRNA was shifted by one base, and a new STOP codon, TGA, appears 52 nucleotides downstream from the junction point, within exon 6. The predictive translated product of mutated mRNA is a hybrid truncated polypeptide, concordant with the enzymatic inactivity that had been demonstrated in our previous studies.21,22

To determine the gene defect responsible for this loss of exon 5, we amplified genomic DNA fragments encompassing exon 5 (primers 3 and 5; Table 1 and Fig 3A) for both patient BEN and the normal control. The amplified genomic DNA fragment from patient BEN exhibits the same size (679 bp) as the normal control (Fig 3B), confirming the presence of exon 5 in the patient’s ctb5r gene. We then looked for splice mutations in the neighboring donor or acceptor sites. The sequences of patient BEN’s genomic fragment at the exon-intron junctions of exons 4, 5, and 6, as well as the complete sequence of exon 5, were investigated. The only mutation detected was a G to C transversion, located in intron 5 at position +8 downstream to the exon-intron junction of exon 5 (Fig 3C) and out of the canonical +6 donor splicing site.

As the G to C mutation generates a new restriction Msp I site, we have confirmed the presence of this base change on both alleles of patient BEN’s ctb5r gene by restriction enzyme analysis of the genomic 679-bp PCR product, amplified between intron 4 and exon 6 (Fig 3D). The patient’s digestion pattern confirmed the presence of a new additional Msp I site: the 279-bp fragment was detected only in the affected patient, instead of the 323-bp fragment present in normal subjects (Fig 3D). For further confirmation of this homozygous mutation in patient BEN and to exclude the possibility that this mutation is a common variant in the population, identical genomic fragments were amplified from 10 unaffected subjects of different ethnic origins. Digestion with Msp I showed the normal pattern (data not shown), suggesting that the G to C transversion found for patient BEN, at out of donor splice consensus position, is...
probably involved in the loss of exon 5 in RNA from this patient.

Characterization of an Arg 218 STOP nonsense mutation. The apparently normal lengths of 5' and 3' cDNA PCR products (Fig 1B) from patient BOU indicated that neither an important nucleotide deletion nor an insertion exists in mRNA of this patient. Both amplified cDNA fragments were directly sequenced, using one of the PCR primers (primer 1, 8, 6, or 14; Table 1) or an internal primer (primer 2 or 11; Table 1). The nucleotide sequence showed a unique C to T transition, located at nucleotide position 655 of coding sequence. This mutation leads to the substitution of a CGA codon (Arg) by a TGA codon (STOP codon) at residue 218 within exon 8 (Fig 4A).

As a new Mae III restriction site is created by this alteration, we used it to digest 201-bp genomic fragments, encompassing exon 8, amplified with primers 10 and 12 (Table 1) from DNA of patient BOU, both parents, and a normal control (Fig 4B). The genomic restriction enzyme analysis, shown in Fig 4C, confirmed the C to T nonsense mutation (Arg218STOP) detected in the cDNA sequence of patient BOU and also the identical heterozygous mutation in both parents, indicating that patient BOU is actually homozygous for this mutation. The presence of a premature STOP codon results in the production of truncated cytb5r and should explain the complete enzyme deficiency observed in this patient in our previous studies.21,22

Identification of compound-heterozygous mutations: Cys-203Arg and an out-of-frame 3-bp deletion, associated with loss of Met-272. The nucleotide sequence of the 5' cDNA fragment from patient LAM showed only one deviation from the normal sequence: a heterozygous G to A transition at nucleotide position 132 within exon 2 of the cDNA sequence. This base change is silent, as it occurs in the third position of the Pro-43 codon (CCG to CCA; data not shown).

Direct sequencing of a 3' cDNA fragment amplified from
Fig 4. Identification of homozygous nonsense mutation in patient BOU (A) Nucleotide sequence of the 3' cDNA fragment from patient BOU. The figure shows the comparison of control and patient BOU's nucleotide sequence of the 3' cDNA fragment. The arrow indicates the single homozygous C to T transition in the cDNA of the affected patient. This mutation generates a premature STOP codon: Arg218STOP within exon 8. (B) Diagram of genomic restriction enzyme analysis. The 201-bp genomic fragment including exon 8 was amplified with primers 10 and 12. Digestion of the mutated allele possessing a new unique restriction enzyme Mae III site produces two fragments (66 bp and 135 bp). (C) Restriction enzyme analysis of the Arg218STOP mutation in patient BOU's family. The 201-bp genomic PCR fragments from patient BOU, both parents, and the normal control were digested with Mae III, separated by 8% polyacrylamide gel electrophoresis, and stained with ethidium bromide. The patient is homozygous for the mutation, whereas both of his parents are heterozygous. The faint 201-bp band observed in the propositus is presumably due to incomplete digestion of abundant PCR product. M, φX174 Hae III DNA; ○, normal control; □ and ●, father and mother carriers; ■, homozygous patient BOU.

patient LAM allowed us to detect a heterozygous T to C transition at nucleotide 610, located in exon 7 (Fig 5A). This nucleotide change results in a novel missense mutation with the substitution of cysteine by arginine (TGC to CGC) at residue 203 (Cys203Arg). As this mutation creates an additional restriction enzyme Hha I site, the presence of this allelic base change was tested in patient LAM’s cytb5r gene by restriction enzyme analysis of exon 7 containing a genomic 262-bp PCR product (primers 7 and 9; Fig 5B). The patient’s digestion pattern confirmed the heterozygous missense mutation in genomic DNA by the presence of an additional 135-bp fragment beside the 161-bp and 101-bp bands produced from the normal allele (Fig 5C).

As the Cys203Arg mutation was found in a heterozygous state and the direct sequencing of the 3' cDNA fragment was difficult in the region of exon 9, we cloned this fragment. The clones containing the mutated allele at Arg-203 (CGC) were eliminated after digestion with Hha I. The sequence of remaining clones showed a normal sequence for the codon Cys-203 (TGC) but an out-of-frame 3-bp deletion (TGA) between nucleotides 815 and 817 of cDNA (815delTGA). The C/TG A/TG sequence at position 814 to 819 in exon 9, coding for Leu-271 and Met-272, was replaced by the CTG triplet, with conservation of Leu-271 and loss of Met-272 (Fig 6A). The 3-bp deletion was further confirmed by direct sequencing of a 220-bp genomic fragment, including exon 9, amplified from affected patient LAM and a normal control (primers 13 and 14; Table 1). The comparison of nucleotide sequences of exon 9 from a normal control and patient LAM showed the presence of two nucleotides on the same line, due to the 3-bp deletion in only one allele of patient LAM (Fig 6B). Identification of these two mutations in patient LAM can explain the observation of electrophoretically slower 3' cDNA fragments. This pattern is most likely due to the presence of the two heteroduplexes with a mismatch at residue 203 and a deletion on one strand at residue 272, resulting in a partly melted conformation slowing electrophoretic migration.

**DISCUSSION**

Recessive congenital methemoglobinemia due to cytb5r deficiency is classified into two types: restricted to erythro
Fig 5. cDNA and genomic DNA nucleotide sequence analysis of a heterozygous missense mutation in patient LAM. (A) Nucleotide sequence of the 3' cDNA fragment from patient LAM. The comparison of nucleotide sequence of normal and mutated alleles is shown. The arrow indicates the allelic T to C transition at codon 203 (Cys to Arg) located within exon 7. (B) Diagram of restriction enzyme analysis. The 262-bp genomic PCR fragment including exon 7 was amplified with primers 7 and 9. Digestion with Hha I generates two fragments from the normal allele (101 bp and 161 bp) and three fragments from the mutated allele (101 bp, 37 bp, and 135 bp). (C) Restriction enzyme analysis of the Cys203Arg mutation in patient LAM. The 262-bp genomic PCR fragments from patient LAM and a normal control were restricted with Hha I and separated by 8% (mass/volume) polyacrylamide gel stained with ethidium bromide. The presence of the 124-bp restriction Hha I fragment in patient LAM confirms the heterozygous T to C transition.

Fig 6. Sequence analysis of cDNA and genomic DNA of a heterozygous out-of-frame 3-bp deletion in patient LAM. (A) Nucleotide sequence of the cloned 3' cDNA fragment. The comparison of the nucleotide sequences of normal and mutated alleles is shown. In the mutated allele, the dots indicate the position of the TGA deletion (nucleotides 815 to 817 of the cDNA), boxed in the normal allele. This 3-bp deletion spans two codons, Leu-271 and Met-272, and is associated with the loss of Met-272. (B) Partial nucleotide sequence of exon 9 amplified from genomic DNA. Patient LAM's nucleotide sequence presents in some positions, marked by asterisks, two nucleotides on the same line, due to the 3-bp allelic deletion [TGA] located in exon 9.
cytes (type I) and generalized (type II). The identification and characterization of mutations of the cytb5r gene should provide insight into the understanding of the heterogeneity of the two types of RCM and also into the correlation of structure with activity of mutated enzymes.

We report four different mutations in three unrelated patients with severe RCM type II. In patient BEN, we have detected an exon 5 skipping, with the precise junction of exon 4 to exon 6 in all cytb5r mRNA, which is consistent with this patient's being born from consanguineous parents. This exon skipping creates a frameshift that results in a premature termination of protein synthesis. Consequently, soluble and membrane-bound cytb5r proteins with only 102 and 127 residues (normal sizes, 275 and 300 amino acids, respectively) are devoid of the nicotinamide adenine dinucleotide (NAD) domain and, thus, cannot bind NADH.

The most common causes of RNA splicing defects in human pathology are mutations located at the exon-intron junctions. In patient BEN, we did not detect any mutations in the donor or acceptor splice site canonical sequences. Only one homozygous G to C transition was found in exon 8 of the cytb5r gene, leading to a premature termination of protein synthesis. Consequently, soluble and membrane-bound cytb5r proteins with only 102 and 127 residues (normal sizes, 275 and 300 amino acids, respectively) are devoid of the nicotinamide adenine dinucleotide (NAD) domain and, thus, cannot bind NADH.

Two new allelic mutations were identified for patient LAM. One allele presented a T to C transition that changed Cys-203 to Arg in exon 7. The second allele showed an out-of-frame 3-bp deletion (815delTGA) in exon 9, resulting in a remaining CTG triplet, with conservation of the Leu-271 and loss of the Met-272. Therefore, this patient was a compound heterozygote for two molecular defects probably affecting the enzyme conformation, which could explain the enzyme inactivity and/or lability.

The Cys-203, located in the β2-sheet of the NAD domain, is one of four cysteines (Cys-203, Cys-273, Cys-283, and Cys-297) found in the last third of cytb5r protein, at the COOH-terminus. The Cys203Arg mutation introduces a positive charge and may eventually abolish a thiol group critical to enzyme functionality. The second allelic mutation identified in patient LAM corresponds to the 3-bp deletion associated with the loss of Met-272. The latter, located in the β4-sheet of the NAD domain, is immediately followed by Cys-273 and Gly-274, which have been identified in the multiple alignments as invariant residues in the ferredoxin-nicotinamide adenine dinucleotide phosphate (NADP+) reductase family, including the cytb5r. The role of the Cys-273 in facilitating the rate of catalytic activity of cytb5r has been well documented by site-directed mutagenesis.

In summary, the nine mutations in the cytb5r gene described herein indicate that the three missense mutations detected in RCM type I affect the 5' part of the gene, while the six mutations identified in RCM type II were located in the 3' part of the gene (Fig 7). These two regions correspond
roughly to the binding domains for flavine adenine dinucleotide (FAD) and NAD at the NH2 and COOH termini, respectively. On the other hand, functional studies of type I mutant enzymes have pointed out a molecular instability of protein with a relatively normal enzymatic activity\textsuperscript{18,19}, while type II mutant enzymes seem to be affected in their catalytic efficiency.\textsuperscript{20,26}

Six mutations for both types I and II of RCM occurred in consanguineous families from Japan and Algeria; the consanguinity of the Italian patient\textsuperscript{18,23} was not clearly reported. According to patient LAM’s family genealogy (French and Spanish ancestry for father and mother, respectively), consanguinity can be excluded, and, thus, this patient represents the first case of compound heterozygosity for the cyt b5 gene defect. At present, each different mutation has been found in only one single family, except for the Arg57Gln mutation, which has been detected in three unrelated Japanese patients with RCM type I.\textsuperscript{18,37} The study of additional mutations for both types of RCM are under investigation to complete the present observations.

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