Familial Idiopathic Methemoglobinemia Revisited: Original cases reveal 2 novel mutations in NADH-cytochrome b5 reductase

Short title: Mutations in DIA 1 gene explain methemoglobinemia

Melanie J. Percy¹, Matthew J.S. Gillespie², Geraldine Savage², Anne E. Hughes³, Mary Frances McMullin⁴ and Terry R. J. Lappin⁴

¹Department of Haematology, Belfast City Hospital, Belfast, Northern Ireland;
²Department of Haematology, Royal Victoria Hospital, Belfast, Northern Ireland;
³Department of Medical Genetics, Queen’s University, Belfast, Northern Ireland,
⁴Department of Haematology, Queen’s University, Belfast, Northern Ireland.

This research was supported by the Northern Ireland Leukaemia Research Fund.

Corresponding Author: Dr Melanie J. Percy, Department of Haemtology, Floor C, Belfast City Hospital, Lisburn Road, Belfast, BT9 7AB, Northern Ireland.
Phone Number: +44-28-9032-9241   Ext 3361
Fax Number: +44-28-9026-3527
Email: melanie.percy@bll.n-i.nhs.uk

Word Counts: Abstract - 152      Text – 1320

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Abstract

In 1943 the first description of familial idiopathic methemoglobinemia in the United Kingdom was reported in 2 members of one family. Five years later, Quentin Gibson correctly identified the pathway involved in the reduction of methemoglobin in the family, thereby describing the first hereditary trait involving a specific enzyme deficiency. Recessive congenital methemoglobinemia (RCM) is caused by deficiency of NADH-cytochrome b5 reductase. One of the original propositi with the Type I disorder has now been traced. He was found to be a compound heterozygote harboring 2 previously undescribed mutations in exon 9, a point mutation G873A predicting a G291D substitution, and a 3bp in-frame deletion of codon 255 (GAG) predicting loss of glycine. A brother and surviving sister are heterozygous, each bearing one of the mutations. Thirty-three different mutations have now been recorded for RCM. The original authors’ optimism that RCM would provide material for future genetic studies has been amply justified.

Email: melanie.percy@bll.n-i.nhs.uk

ACKNOWLEDGEMENTS

We thank Dr Quentin Gibson for making available his original notebooks and Mrs Elsie Hutchinson for providing family histories.
Introduction

In an era dominated by functional genomics it is salutary to reflect that the first hereditary disorder involving an enzyme deficiency was discovered just over half a century ago by Quentin Gibson\(^1\). In 1943 Dr Deeny a general practitioner described two brothers, Russell and Fred Martin from Banbridge in Northern Ireland, who had a blue appearance\(^2\). When Russell was treated with the vitamin C he turned pink, and Dr Deeny assumed that he had corrected an underlying heart condition. However the cardiologists in Belfast were more sceptical and were unable to find any abnormality in either brother. The conundrum attracted the attention of the physiologist Henry Barcroft who carried out a detailed study of Dr Deeny's cases during treatment and found raised levels of methemoglobin in two members of the Martin family \(^3\). Gibson correctly defined the pathway involved in the reduction of methemoglobin in the family and, in so doing he described the first hereditary trait involving a specific enzyme deficiency\(^1\).

The disorder recessive congenital methemoglobinemia (RCM: McKusick no 250800) is caused by a deficiency of NADH-cytochrome b5 reductase (cytb5r: E.C.1.6.2.2). Two forms of cytb5r are known, a soluble form and a membrane-bound membrane one, localised in different cellular compartments. The soluble form is present mainly in red cells\(^4\) and is involved in the reduction of methemoglobin\(^5\). The membrane-bound form is found mainly in the endoplasmic reticulum, and outer mitochondrial membrane\(^6\) where it participates in the desaturation and elongation of fatty acids, the biosynthesis of cholesterol and P-450–mediated drug metabolism. The cytb5r gene is 31 kb in length, contains nine exons and has been localised to chromosome 22q 13-qter. Both forms of the enzyme are generated from tissue-specific alternative
transcripts (see Figure 1) which give rise to the 275 amino acid soluble form and the 300 amino acid membrane-bound form. They have an identical hydrophilic catalytic domain but differ at the N-termini where the membrane bound form has an additional 25 hydrophobic amino acids. There are two distinct clinical forms of cytb5r deficiency. Type I is characterised clinically by a single symptom, cyanosis, and biochemically by a deficiency of the red cell soluble form of the enzyme. In Type II the cyanosis is accompanied by severe mental retardation and neurological impairment involving both the soluble and membrane bound forms of the enzyme.

Figure 1
Organisation of the cytb5r gene showing the arrangement of the nine exons and the two mRNA transcripts which code for the membrane bound and soluble forms.
We were curious to know the actual mutations involved in Gibson’s landmark discovery, and eventually had the opportunity to analyse blood from one of the propositi, Fred, who had emigrated to Australia in 1968, and two surviving siblings.

**Materials and Methods**

*Case history*

The pedigree for the family originally reported by Deeny et al and Barcroft et al ²,³ is shown in Figure 2. The three surviving family members were available for study, one of the original propositi (#6, FM) who has RCM Type I, a brother (#3, BM) and a sister (#5, EH). Unfortunately, during the course of this study both Fred Martin and his brother BM died.

Figure 2.

The family with methemoglobinemia as reported by Barcroft et al (1945). Affected males are represented by black squares, carriers by shaded squares (male) and shaded circles (females). Three members of the family were available for the present study, #3, #5 and #6. Cytb5r activity and phenotype are indicated for each subject. The status of the remaining family members could not be established as they were deceased at the time of study.
PCR amplification of genomic DNA.

Genomic DNA was isolated from buffy coats by the Nucleon BACC 1 DNA extraction kit (Nucleon Biosciences, UK). The nucleotide sequences of the nine exons were obtained from the GenBank database (Accession numbers M28705 to M28713) and the following primers were designed: EX1F, 5’-gagcgagagcgagcgagc-3’; EX1R, 5’-gtcacccggcaagcagc-3’; EX1SF, 5’-cattctgagccagctctgg-3’; EX1SR, 5’-ctgtcttaacccggaggaag-3’; EX2F, 5’-ctttctggatgagggtggt-3’; EX2R, 5’-gaggcagtgagtgtggttca-3’; EX3F, 5’-gtttctggccaccttgtt-3’; EX3R, 5’-ccttcccactctcattccaa-3’; EX4F, 5’-caggccaggttgttaatgtg-3’; EX4R, 5’-ccctctggagcctggag-3’; EX5F, 5’-gtacagccctgtttggtg-3’; EX5R, 5’-agctggcctgagagtc-3’; EX67F, 5’-cctctacccacacacca-3’; EX67R, 5’-gtcatccccagaatactcag-3’; EX8F, 5’-gagtcctcctctgaaagctc-3’; EX8R, 5’-gaaaggactctgtctgga-3’; EX9F, 5’-gggatcagctctctctatt-3’; EX9R, 5’-gggagagcgtactctgaag-3’.

RNA isolation, cDNA synthesis and cloning.
Mononuclear cells were separated from venous blood using Ficoll-Paque (Life Technologies, Paisley, UK). RNA was prepared using Trizol reagent (Life Technologies). A nested PCR protocol involving two rounds of PCR was used to amplify the full length cytb5r cDNA. First round PCR reactions were set up using forward (5′-gcgacagagcgcggcg-3′) and reverse (5′-ggtggccgttgtgaccgggtc-3′) primers. The second round of PCR used forward (5′-gcatggatccatggggcccagctcagcacg-3′) and reverse (5′-gcatggaattccctcaagacgaacgcgcgc-3′) primers located inside the first round primers. The Bam HI and EcoRI sites located in the nested forward and reverse primers respectively (shown underlined) permitted directional cloning of the full length cytb5r cDNA into the pGEM -T Easy vector (Promega, Southampton, UK). Plasmid DNA containing the appropriate size of insert upon restriction with Bam HI and EcoRI sites was subjected to DNA sequencing.

**Sequencing**

PCR products were purified using Concert Rapid PCR Purification System (Life Technologies, Paisley, UK) and were sequenced using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit Version on ABI 3100 DNA Genetic Analyzer (Applied Biosystems, Warrington, UK).

**Results and Discussion**

The cytb5r activity, measured using the NADH-ferricyanide method was < 0.1 U/g Hb in FM, 6.37 IU/g Hb in BM and 7.75 IU/g Hb in EH (normal range 11.51 to 29.9 IU/g Hb). These results suggested that BM and EH were heterozygote carriers of a
cyt5r mutation. DNA sequencing of PCR products of the nine exons revealed two mutations in exon 9 of FM, one in each allele. The first was a point mutation of G to A at codon 291 which would cause a change of glycine to aspartic acid. The second was a 3 base pair in-frame deletion resulting in the loss of codon 255 and the deletion of a glycine. The siblings each carry one mutation: G to A at codon 291 in the case of BM and the 3bp deletion causing loss of codon 255 in the case of EH. This was confirmed by cloning and sequencing cDNA from all three subjects. Thus both the enzymic activity analysis and DNA sequencing indicate that FM is a compound heterozygote and his siblings BM and EH are heterozygotes for cyt5r mutations.

In the late 1940’s when Gibson was investigating methemoglobinemia Warburg and his colleagues had just shown that sugar is oxidized to pyruvate as methemoglobin is reduced to hemoglobin in intact red cells. It was also known that methylene blue accelerates the rate of reduction of methemoglobin. Gibson\textsuperscript{1} showed that methylene blue could reduce methemoglobin in the patients’ red cells but incubation of the cells with sugar alone had little effect. He postulated that these patients had an enzymatic defect. Later he showed that iodoacetic acid, an inhibitor of glyceraldehyde phosphate dehydrogenase inhibited the reduction of methemoglobin when sugar, but not lactate, was added as substrate and deduced that the proposed enzyme defect affected the reaction between Coenzyme I (\textit{i.e.,} NAD) and methemoglobin. This deduction was confirmed in many subsequent investigations. An intriguing account of this early work has been recorded by Gibson\textsuperscript{11}.

These two hitherto unidentified mutations in the family originally studied by Gibson bring to 33 the number of different mutations described in patients with RCM ( see
Previously 15 mutations had been associated with the Type I disorder \(^{12-19}\) and 16 with Type II RCM \(^{16, 19, 20-26}\). Mutations have been found in all exons, except 1 and 1S. The mutations which cause exon skipping and those predicted to give truncated proteins with severe impairment of function have only been described in Type II except in one case where the child did not show neurologic abnormalities at the age of one year \(^{18}\).

**Figure 3**

The location of the mutations reported to date in the cytb5r gene of patients with Types I and II RCM. The mutations are shown in relation to the nine exons of the 31 kb gene. Novel mutations found in the present study are indicated in the shaded boxes.
Since the structure of cytb5r has recently been resolved by X-ray crystallography it is now possible to interpret the effects of mutations on enzyme function. Although the two novel exon 9 mutations are located outside the FAD and NADH binding sites both would be expected to have a deleterious effect on enzyme activity (see Figure 4). Loss of L255 would affect the packed hydrophobic environment formed by I177, L206, A208, T237, M256, M278 and C283 and the presence of aspartic acid at codon 291 instead of the non-polar glycine would also lead to perturbation of the secondary structure.
Figure 4

The predicted translation, demarcation of the soluble and membrane forms and the location of the secondary structure elements of cytb5r.

MGAQLSTLGMLVFLPVPVFLLYLMMKLFQRSTPAILTEPDIYKPLRLIDREIISHDT
Membrane bound form only
RRFRFALPSPQHILGPVGQHIYLSARIDGNLUNVRVPYCPISDDDKGFVDLVIKVVYF
FAD binding domain
KDTHPKFPAGKMSQYLESMQIGDTIEFRGSPGLLVYQKGKFAIRPDPDKKNPII
NADH binding domain
RTVKSVGMIAGGTGPMLQVIRAIMKDPPDHVTCVHLFANQTEKDLRPEEELR
NKHSARFKLWYTLDRAPEAWDYGGFVNEEMIRDRHPPPEEPLVLMCGPPPMIQA
CLPNLDHVGHPTERCVF

In 1943 Deeny et al reported the first case of RCM in the United Kingdom and it was hoped that the mode of inheritance would be defined and genetic basis of the disorder established. Over 40 years later the cytb5r gene was cloned thereby allowing the genetic basis of the disorder to be defined and a further 20 years before the mutations in the original family to be identified.

References


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