

## Heterogeneity of the molecular biology of methemoglobinemia: a study of eight consecutive patients

**Congenital methemoglobinemia can be caused by mutations involving five different genes. We studied the etiology and molecular biology of eight consecutive patients with methemoglobinemia. Four had *b5R* mutations; two were novel. A novel intronic mutation caused markedly reduced mRNA resulting in type II methemoglobinemia. Three patients had acquired methemoglobinemia without any *b5R* mutations.**

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We studied eight consecutive patients with methemoglobinemia. Methemoglobin levels were quantified by spectrophotometry as described by Evelyn and Malloy.<sup>1</sup> One subject with abnormal spectral absorbance had hemoglobin electrophoresis and high performance liquid chromatography performed as previously described.<sup>2</sup> *B5R* activity (NADH methemoglobin reductase) was quantified in erythrocytes, lymphocytes, granulocytes and platelets in patients and available parents.<sup>3</sup> *B5R* activity in Epstein-Barr virus (EBV)-transformed lymphocytes was assayed in two subjects. Genomic DNA was evaluated by amplifying the exons with intron/exon boundaries of *b5R* gene using 6 sets of primers. Mononuclear cell RNA was reverse transcribed to cDNA using random primers to amplify the coding region of the *b5R* gene using 4 sets of overlapping primers. The polymerase chain reaction (PCR) products were purified and sequenced. All identified mutations were confirmed by sequencing in both directions, sequencing of parental DNA or cDNA and, when feasible, by restriction endonuclease digestion. In patients with multiple transcripts, the relative proportion of each transcript was determined by a semi-quantitative densitometric analysis.<sup>4</sup> *B5R* mRNA was measured by real-time reverse transcription (RT)-PCR using EBV-transformed lymphocyte-derived cDNA in subjects with splice site mutations and a commercially available Assay-on-Demand gene expression kit (Assay ID: Hs00240921, Applied Biosystems) according to published methods.<sup>5</sup> The results are depicted in Table 1. Patients 1 and 4 had multiple *b5R* mRNA transcripts as depicted in Figure 1.

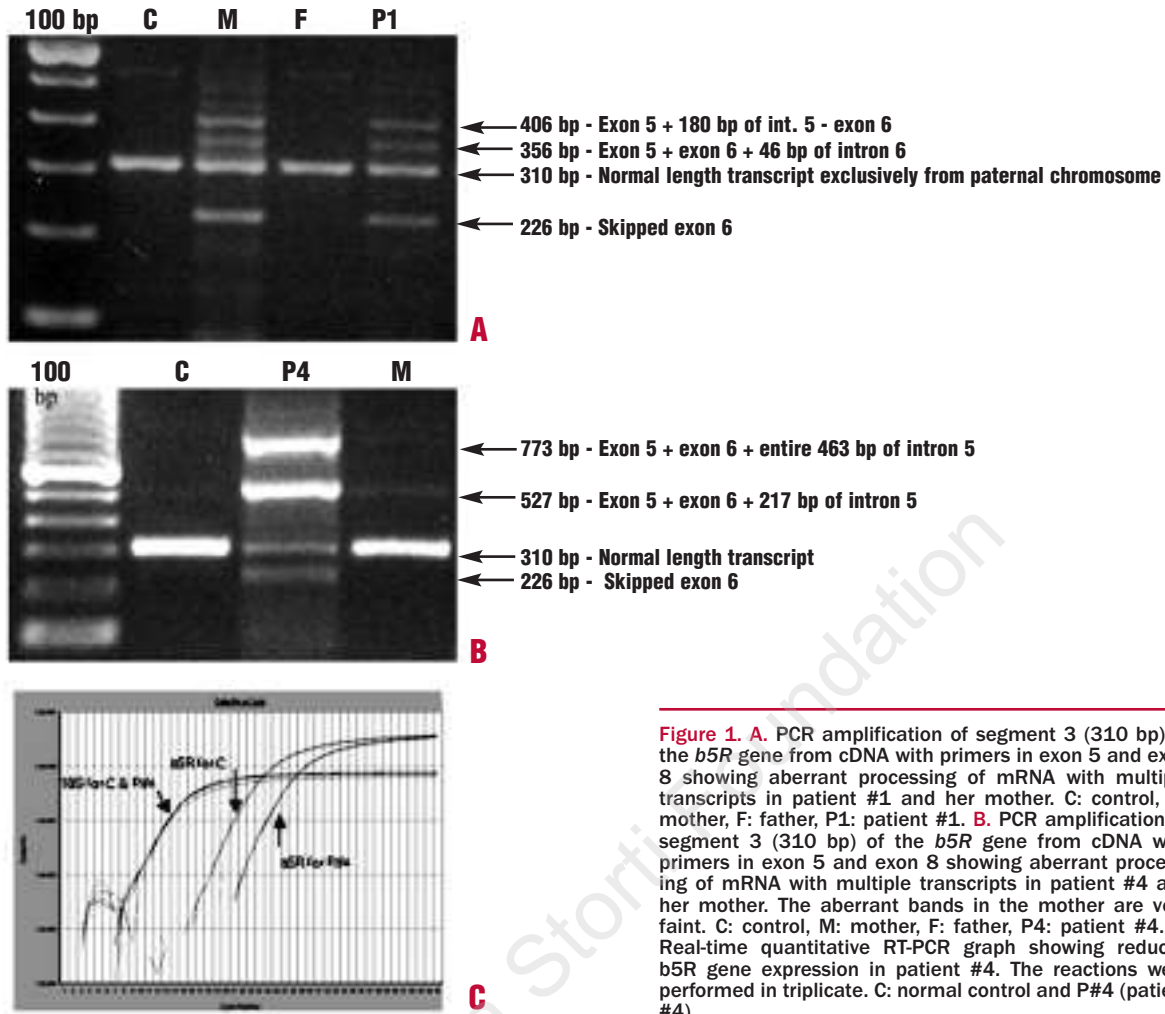
The paternal mutation of patient 1 has been described as type I recessive congenital methemoglobinemia (RCM).<sup>6</sup> Her maternally inherited G→A transversion in the first nucleotide of intron 6 (IVS 6+1) at the *b5R* nucleotide 22249 is a novel mutation and is the first identified mutation in intron 6 of this gene. This mutation alters the consensus sequence of the splice donor site causing aberrant processing of mRNA with no normal transcripts. A splice site mutation with aberrant mRNA processing resulting in multiple transcripts has not been previously described in this gene. Real-time RT-PCR showed near normal amounts of mRNA demonstrating that there is minimal if any transcriptional interference by the *b5R* isoforms associated with this mutation. The mutation in patient 2 is a C→T transversion at nucleotide 29951 which substitutes tryptophan for arginine in codon 258 of exon 9. The secondary structure of *b5R* includes 3 sheets, 15 strands and 10 helices as seen by X-

**Table 1. Summary of results.**

Pt#	Diagnosis	MHb	Cell type & <i>B5R</i> activity	<i>B5R</i> gene mutation	Consequences of mutation and remarks
1	Type I RCM	12%	RBC-20%	1.22236 G→A Exon-6 2.22249 G→A* (IVS 6+1)	178 Alanine→Threonine Aberrant mRNA processing with 3 different transcripts: a) in-frame skipping of exon 6; b) all exons +46 bp v intron 6; c) skipped exon 6 with 179 bp of intron 5. No normal transcript. Normal expression by real time qPCR
2	Type I RCM	NA	RBC-25% PLT, GNC & MNC-100%	29951 C→T* Exon-9	258 Arginine→Tryptophan
3	Type II RCM	30%	RBC-50% EBV-25%	22526 T→C Exon-7	203 Cysteine→Arginine
4	Type II RCM	20%	RBC-2% EBV- 25%	22163 A→C (IVS 5-2)	Aberrant mRNA processing with 4 different transcripts: a) in-frame skipping of exon 6; b) normal transcript; c) 217 bp of intron 6 included d) entire intron 6 (463 bp) included. Decreased mRNA expression by real time qPCR (only 7% of normal)
5	Infant Toxic	35%	60%	None	One week of diarrheal illness. Needed several administrations of methylene blue.
6	Toxic		12%	100%	None Lidocaine exposure.
7	Toxic	44%	100%	None	Unidentified toxin or infection
8	Hb.M-HP	NA	NA	C→T β globin codon 92 CAC→TAC	β92 Histidine→Tyrosine

MHb, level of methemoglobin as a percentage of total hemoglobin concentration. NA – not available. The mutations marked by \* are novel. RCM-recessive congenital methemoglobinemia, GNC-granulocytes, MNC-mononuclear cells, EBV-EBV transformed lymphocytes, PLT-platelets, qPCR-quantitative real-time PCR, *b5R* gene mutations are numbered as in NCBI accession AY341030.

ray crystallographic structure of rat *b5R* (PDB=1IBO). Codon 258 is in helix 7 (254–260), which has a helix-helix interaction with helix 9 in the NADH binding domain. This mutation substitutes the non-polar neutral tryptophan for a basic positively charged arginine in helix 7. This is likely to disrupt the secondary structure and the stability of the enzyme consistent with a type 1 recessive congenital methemoglobinemia (RCM) phenotype with 25% *b5R* enzyme activity in red blood cells while platelets, granulocytes and mononuclear cells capable of continuous enzyme synthesis have ~100% activity. The mutation in patient 3 was previously reported as type II RCM.<sup>7</sup> His *b5R* activity in erythrocytes was 50% of normal which is higher than expected, demonstrating that reliance on enzyme assays alone is not sufficient to exclude or diagnose RCM. Congenital methemoglobinemia due to reduced mRNA of the *b5R* gene has not been



**Figure 1. A.** PCR amplification of segment 3 (310 bp) of the *b5R* gene from cDNA with primers in exon 5 and exon 8 showing aberrant processing of mRNA with multiple transcripts in patient #1 and her mother. C: control, M: mother, F: father, P1: patient #1. **B.** PCR amplification of segment 3 (310 bp) of the *b5R* gene from cDNA with primers in exon 5 and exon 8 showing aberrant processing of mRNA with multiple transcripts in patient #4 and her mother. The aberrant bands in the mother are very faint. C: control, M: mother, F: father, P4: patient #4. **C.** Real-time quantitative RT-PCR graph showing reduced *b5R* gene expression in patient #4. The reactions were performed in triplicate. C: normal control and P#4 (patient #4).

previously described. The intronic *b5R* mutation at nucleotide 22163 in patient #4 results in reduced *b5R* mRNA, which was 7% of normal in freshly harvested exponentially growing EBV cells ( $\delta C_T = -3.9$ ). Only a small proportion of this will encode for fully functional protein as our probe, located in exon 7/exon 8 junction, also detects the aberrant transcripts. Interestingly the mother, who is heterozygous, would be expected to have at least 50% of mRNA. But her mRNA, on repeated testing was 28% of normal ( $\delta C_T = -1.84$ ). We can speculate that the low mRNA level may be due to the fact that a nonsense mutation causes ribosomes to detach from mRNA, with a consequent decreased half-life of the latter.<sup>8</sup> We could only demonstrate these abnormal transcripts in immediately prepared mRNA derived from exponentially growing cells and not from the cells that were processed after overnight shipment, suggesting that these transcripts are unstable.

Patients #5, 6 and 7 had acquired toxic methemoglobinemia. Patient #5 had an age-related reduced activity of *b5R*. Patients #6 and 7 had normal red cell *b5R* activity. Comprehensive evaluation of the *b5R* DNA and cDNA of patients #5, 6 and 7 failed to show any mutations. It has been reported that heterozygosity for *b5R* deficiency, while asymptomatic in steady state, predisposes to acute toxic methemoglobinemia<sup>9</sup> but this was not found in the

patients we report here. Many patients who develop toxic methemoglobinemia may not have any apparent inherited predisposition to this sometimes fatal disorder.

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## Red Cell Disorders

### Low frequency of *VHL* gene mutations in young individuals with polycythemia and high serum erythropoietin

**In one out of six young individuals with polycythemia and high erythropoietin levels we found a heterozygous *VHL* gene mutation (430G→A; Gly144Arg). The man's unaffected mother and sister carry the same mutation. No other *VHL* genomic or expression alterations were found. In one other patient different genetic conditions were found.**

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The molecular basis of congenital and familial polycythemia are largely unknown. Chuvash polycythemia (CP) is an autosomal recessive polycythemia with increased serum erythropoietin (Epo) caused by bi-allelic mutations of the von Hippel-Lindau (*VHL*) gene.<sup>1</sup> *VHL* mutations are also responsible for von Hippel-Lindau disease, an autosomal dominant syndrome predisposing to the development of multiple tumors.

Since 50% of sporadic cases of congenital CP with high serum Epo are attributed to bi-allelic *VHL* gene mutations,<sup>2</sup> we searched for *VHL* gene mutations in 4 previously described children with congenital polycythemia and unexplained high levels of serum Epo<sup>3</sup> and in 2 other individuals with a similar phenotype. While in 5 of the cases polycythemia appeared to be congenital, in 1 case it was not possible to date the age of onset of the disease precisely. All patients were Italian from the Venetian region; none of them was of Chuvashian origin. None had splenomegaly or detectable causes of secondary erythrocytosis. In one patient neurofibromatosis type 2 was found in association with the polycythemia. The main clinical and molecular data of our patients are summarized in Table 1.

Serum Epo was measured with a commercially available immunoassay kit (Erythropoietin, Nicols Institute Diagnostics, CA, USA). We carried out the burst-forming unit-erythroid colony assay (EEC) on peripheral blood

mononuclear cells using standard methods.<sup>1</sup>

Genomic DNA was extracted from peripheral blood leukocytes by conventional methods after signed informed consent, according to the Declaration of Helsinki. Mutation analysis was conducted on the entire coding sequence and intron-exon boundaries of the *VHL* gene by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis, followed by direct sequencing of the amplicons, as previously reported.<sup>4</sup> Total cytoplasmic RNA was extracted from peripheral blood with RNazol (RNA-Bee, Tel-Test, Inc.) and retro-transcribed by random-priming.

The *VHL*-specific cDNA was amplified with primers 1CF (5'-GTGCTGCGCTCGGTGAATC-3') and 3R (5'-CAAGACTCATCAGTACCATCAAAGCTG-3')<sup>5</sup> which obtained amplification of two different isoforms, due to the normal alternative splicing of exon 2. The *VHL* isoform, including exon 2 was then sequenced with primers 2Fi (5'agg tca cct ttg gct ctt cag a 3') and 3R.

One out of six patients was heterozygous for a missense mutation involving the *VHL* amino acid residue in position 144 (430 G→A; Gly144Arg); molecular analysis of the mRNA confirmed the expression of the transcript from both *VHL* alleles. The same mutation was present in the man's 49 year-old mother as well as in one of his sisters (30 years old); neither had signs of polycythemia. A complete clinical work-up (ophthalmological and audiological evaluations, brain, spinal and abdominal CT scan/MRI and blood and urine measurement of catecholamines) failed to identify possible undetected *VHL*-related manifestations. Molecular analysis of the *VHL* gene in the other members of the proband's family was negative. The *VHL* mutation reported here was not detected in over 200 unrelated cases.

CP has been demonstrated to be associated with homozygosity for the 598C→T *VHL* mutation.<sup>6</sup>

Some non-Chuvash individuals with polycythemia and bi-allelic homozygous<sup>7</sup> or compound heterozygous<sup>2,7,8</sup> *VHL* gene mutations have also been described.

None of the individuals we have tested carried mutations on both *VHL* alleles and we have not detected the 598C→T mutation reported as the most important cause of congenital polycythemia with inappropriately high Epo serum levels.<sup>8</sup> The *VHL* alteration found in our patient is a new mutation, never described in *VHL* disease or in patients with polycythemia, although *VHL* codon 144 has been previously found to be altered by missense mutations in patients with *VHL*-related tumors ([www.umd.be:2020/](http://www.umd.be:2020/) and HGMD).

The Gly144Arg mutation does not seem to cause *VHL* disease in the patient and his relatives. While the proband has congenital erythrocytosis, both his mother and sister carrying the mutation show no signs of polycythemia, and hence the mutation itself does not correlate with either phenotype. No other *VHL* mutation was detected at the DNA level; the transcripts of both *VHL* wildtype and mutated allele were documented at mRNA level. A similar situation has been previously observed (Table 2), although mRNA has not always been studied.

Since CP is considered an autosomal recessive condition, we hypothesize that when the heterozygous *VHL* mutation is present without other structural or transcriptional alterations, other as yet unknown factors might contribute to determining the polycythemia phenotype. However, the molecular mechanism of erythrocytosis in these cases remains to be elucidated.

It is worth noting that we found the association of