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## **Erythrocytosis associated with a novel missense mutation in the *BPGM* gene**

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**Running head:** BGPM mutation-associated erythrocytosis

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Erythropoiesis is stimulated by the oxygen-regulated glycoprotein, erythropoietin (Epo). Erythrocytosis is a condition characterized by increased red cell mass and characteristically elevated hematocrit (Hct) and hemoglobin (Hb) concentration<sup>(1)</sup>. Table 1 summarises its causes. When fully investigated (including genetic screening for known mutations), there remain a number of patients in whom no cause could be found, who are currently described as having idiopathic erythrocytosis. A large proportion of these have elevated or inappropriately normal (for the Hb level) serum Epo levels, suggesting an abnormality in oxygen sensing. Investigations have shown that several cases previously diagnosed as idiopathic erythrocytosis can now be attributed to mutations in genes involved in oxygen sensing<sup>(1)</sup>.

Here, we describe a patient who, following screening for known mutations in *VHL*, *EGLN1* and *EPAS1*, was classified as having sporadic idiopathic erythrocytosis with raised Epo levels. This Caucasian man presented at the age of 27 with fatigue. His past medical and family history were unremarkable. He was a non-smoker with a 16-unit alcohol intake per week. He was found to have a high Hb concentration of 193 g/l, a red cell count of  $6.53 \times 10^{12}/l$ , mean corpuscular volume (MCV) of 89.7 fl, mean cell hemoglobin (MCH) of 29.9 pg, MCHC of 333 g/l, Hct of 0.586 l/l, white cell count (WCC) of  $6.5 \times 10^9/l$  – with a normal differential – and platelets of  $167 \times 10^9/l$ . Red cell mass was 146% predicted. Vitamin B<sub>12</sub> was 380 ng/l, folate was 8.9 mcg/l and ferritin was 58 mcg/l. Liver function tests revealed a slightly raised AST of 61 U/l and ALT 101 U/l. These subsequently normalized following significant weight loss but the Hb and Hct remained elevated. An abdominal ultrasound was normal. Hb electrophoresis was normal and revealed: HbF 0.5% and HbA2 2.8%. Serum Epo was 15.9 mIU/ml (normal range: 2.5-10.5 mIU/ml). Arterial blood gas analysis found a carboxyhemoglobin of 1.2% and a P<sub>50</sub>, the partial pressure of oxygen at which 50% of Hb is saturated with oxygen, was calculated at 23.91 mmHg (normal range: 27-33 mmHg, control sample: 32.7 mmHg), suggesting a mild leftward shift in the Hb-oxygen dissociation curve. He commenced low-dose aspirin and was treated with venesection every three to six months, aiming for Hct of less than 0.5 l/l. Five years following his initial presentation, his disease etiology was revisited. In view of a high likelihood for a genetic etiology owing to his extreme phenotype of early onset, whole genome sequencing (WGS) was undertaken searching for causal mutations. Informed consent was obtained and procedures were performed in agreement with the Helsinki Declaration.

WGS was performed as part of the “WGS500 project”, whereby 500 individuals with a variety of medical conditions of suspected genetic origin were sequenced to assess the clinical utility of WGS. It was conducted on the Illumina HiSeq2000 at a 30x sequencing depth. Reads were mapped to the human reference genome (hg19) using STAMPY<sup>(2)</sup>, identification of alleles and variants was performed using the in-house software Platypus<sup>(3)</sup> and annotated using a pipeline based on

ANNOVAR<sup>(4)</sup>. We searched for heterozygous or homozygous variants in candidate genes: *HIF1A*, *EPAS1 (HIF2A)*, *HIF3A*, *ARNT (HIF1B)*, *HIF1AN (FIH)*, *EGLN1 (PHD2)*, *EGLN2 (PHD1)*, *EGLN3 (PHD3)*, *VHL*, *EPO*, *EPOR*, *JAK2*, *HBB*, *HBA1*, *HBA2* and *BPGM*; or for rare coding mutations in any gene. Given the large number of genes involved in oxygen sensing and the regulation of erythropoiesis, and the growing evidence of new genes interacting with the HIF factors, whole genome sequencing was considered an appropriate and most informative investigation, compared with traditional methods of DNA sequencing which are impractical, lengthy and labor-intensive.

A single novel heterozygous non-synonymous SNV was identified in the gene bisphosphoglycerate mutase (BPGM), at chr7:134346528. This was a G269A substitution, predicting the substitution of arginine at the evolutionarily conserved residue 90 with histidine (R90H). This mutation was not detected in any other samples sequenced in the WGS500 project, nor was it reported in the 1000 genomes analysis. No other candidate gene coding variants were found in the patient's genome. It was validated by Sanger sequencing. To establish whether this arose *de novo*, we screened the patient's parents and found that the mutation was inherited from his mother, who was also heterozygous (Figure 1). She had a [Hb] of 150 g/l and serum Epo level of 7.5 mIU/ml. A Polyphen score of 1.0 predicted that the R90H mutation is deleterious. Inspection of the 3D structure of BPGM suggests that replacement of arginine (R90) with histidine would potentially disrupt hydrogen bonds with backbone carbonyl groups, negatively affecting protein folding and stability (Figure 2). The functional effects of the mutation were confirmed by showing significantly lower levels of BPGM and its product 2,3-BPG in the red blood cells of both the patient and his mother (Table 2). BPGM was measured by the method Schröter and Kalinowsky, modified by Beutler<sup>(5)</sup>, and 2,3-BPG by a modification of the method of Krinsky<sup>(6)</sup>, as explained in Table 2.

BPGM is the enzyme responsible for the synthesis of 2,3-BPG (from 1,3-BPG) in red blood cells<sup>(7)</sup>. Normally 2,3-BPG is present at high concentrations (~5 mM) in red blood cells and binds the deoxy form of Hb to reduce its affinity for oxygen<sup>(8)</sup>. BPGM deficiency leads to reduced levels of 2,3-BPG, resulting in a leftward shift in the hemoglobin-oxygen dissociation curve. Less available oxygen induces Epo production to stimulate red cell production and hence the development of erythrocytosis. Defects in BPGM have been associated both with hemolytic anemia<sup>(9)</sup> and erythrocytosis<sup>(10, 11)</sup>. In 1978, Rosa *et al* reported the first case of a 42-year old man with complete deficiency of BPGM and erythrocytosis<sup>(10)</sup>. His Hb concentration was 190 g/l, his erythrocyte 2,3-BPG was below 3% of normal values and he had a low P<sub>50</sub>. His sister also had complete BPGM deficiency and excessive erythrocytosis and they were both compound heterozygous with a SNV on one allele at codon 89 (corresponding to codon 90 in the current reference genome) causing an arginine to cysteine substitution (R89C) resulting in low levels of an abnormal (inactive) BPGM protein and a deletion of

nucleotide 205 or 206 (codon 19) on the other allele, translating an aberrant product<sup>(12)</sup>. In contrast, his son and daughter, who were heterozygous only for the R89C mutation, had intermediate phenotypes with partial BPGM deficiency and modest erythrocytosis<sup>(12)</sup>. Garel *et al* reproduced the same functional defect in BPGM as the natural arginine-to-cysteine mutant in artificial mutants, Arg89Gly and Arg89Ser, produced by site-directed mutagenesis, giving evidence that indeed the arginine at this position is functionally important in the enzyme's function<sup>(13)</sup>. Only one other *BPGM* mutation, a homozygous Arg62Gln, was found in a male patient with erythrocytosis, who had concurrent G6-phosphate dehydrogenase deficiency and evidence of consanguinity in his family<sup>(11)</sup>. Indeed, mutations in *BPGM* are exceedingly rare. Recently, Bento *et al* sequenced 70 cases of idiopathic erythrocytosis, including sequencing of *BPGM* in cases with high Epo and no *BPGM* mutations were identified<sup>(14)</sup>.

It is of interest that the same amino acid residue (i.e. arginine at codon 90) is affected, as in previous cases of erythrocytosis<sup>(12)</sup>. However, in our patient a different base substitution occurs, resulting in a novel amino acid substitution of arginine to histidine as opposed to arginine to cysteine in previous cases. This reinforces the findings of Garel *et al*<sup>(13)</sup> that the arginine residue at 90 is important for the function/stability of BPGM. Our patient, initially thought to be a sporadic case, inherited this mutation from his mother, who is also heterozygous. This is of interest for two reasons. One is that all previous erythrocytosis cases associated with BPGM mutations at the arginine 90 residue were not sporadic but rather were found in families with an autosomal dominant pattern of inheritance. The second is that, while the mother is also heterozygous for this mutation she is not polycythemic and has Epo levels within the normal range. One explanation for this could be that there is a second "hit" in the patient leading to compound heterozygosity as in Lemarchandel *et al*<sup>(12)</sup>; however, WGS did not reveal any additional mutations/deletions in *BPGM*. Alternatively, the explanation could be that of variable penetrance of the disease-causing mutation, consistent with the phenotypes and patterns of inheritance of the family members in Lemarchandel *et al*. There, the index case and his sister, both compound heterozygotes, had complete BPGM deficiency and excessive erythrocytosis. His children, heterozygotes, had an intermediate phenotype, and his parents, at least one of whom was heterozygous for the arginine-to-cysteine mutation, were both healthy. Furthermore, while our patient's mother is not polycythemic, her [Hb] lies at the upper end of the normal range and there is evidence of dysfunction in her red blood cells with low BPGM activity and 2,3-BPG levels, reinforcing that this mutation is functional. Given that heterozygous individuals, both in our study and in Lemarchandel *et al*, have intermediate levels of BPGM activity and 2,3-BPG levels, i.e. much higher than compound heterozygotes, the "variable penetrance" observed may simply be the result of variable effects reduced 2,3-BPG has on hemoglobin and the induction of erythropoiesis. It is also possible that the

variable effects of the heterozygous BPGM mutation relates to different expression ratios of wild-type versus mutant BPGM in different individuals.

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## Authorship and Disclosures

All authors participated in the conception and design of the research and in the preparation of the manuscript. NP, RRC, TRJL, SEH, MJP and MFM performed research and analyzed the data. All authors approved the final version of this manuscript. The authors declare no conflicts of interest. This study was carried out as part of the WGS500 Consortium project, which was funded in part by Illumina, Inc.

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## Tables

**Table 1. Causes of Erythrocytosis.**

<b>PRIMARY ERYTHROCYTOSIS (typically low Epo levels)</b>	
<b>Congenital</b> Epo Receptor Mutations	<b>Acquired</b> Polycythemia vera (including <i>JAK2</i> mutations)
<b>SECONDARY ERYTHROCYTOSIS (high or normal Epo levels)</b>	
<b>Congenital (inherited)</b> 1. Defects in oxygen-sensing pathway: <ul style="list-style-type: none"> <li>• <i>VHL</i> mutations (e.g. Chuvash Polycythemia)</li> <li>• <i>EGLN1</i> (PHD2) mutations</li> <li>• <i>EPAS1</i> (HIF2A) mutations</li> </ul> 2. Other congenital defects: <ul style="list-style-type: none"> <li>• High-oxygen affinity Hb</li> <li>• Bisphosphoglycerate mutase deficiency</li> </ul>	<b>Acquired (Epo-mediated)</b> 1. Systemic hypoxia: <ul style="list-style-type: none"> <li>• Chronic lung disease</li> <li>• Right-to-left cardiopulmonary vascular shunts</li> <li>• Smoker's erythrocytosis</li> <li>• Nocturnal hypoventilation syndromes (e.g. obstructive sleep apnea)</li> <li>• High altitude</li> </ul> 2. Local hypoxia: <ul style="list-style-type: none"> <li>• Renal artery stenosis</li> <li>• Renal cysts (Polycystic kidney disease)</li> <li>• Post-renal transplant erythrocytosis</li> </ul> 3. Pathological Epo production (tumors) <ul style="list-style-type: none"> <li>• Brain (cerebellar hemangioblastoma, meningioma)</li> <li>• Renal cell carcinoma</li> <li>• Hepatocellular carcinoma</li> <li>• Phaeochromocytoma</li> </ul> 4. Drug-associated: <ul style="list-style-type: none"> <li>• Erythropoietin/androgen administration</li> </ul>

adapted from McMullin, M. F. (2008)

**Table 2. Results of BPGM and 2,3-BPG assays in red blood cells.**

	Patient (index case)		Patient's mother	Control sample with comparable [Hb]	Healthy control samples (n=10)
	At initial presentation	At time of functional studies			
Hb (g/l) NR: 130 – 180 (Male), 115 – 155 (Female)	193	159	155	157	126.60 ± 19.04*
BPGM (IU/g Hb)	NA	3.62	3.27	4.21	4.99 ± 0.59*
2,3-BPG (µmol/g Hb)	NA	14.5	11.3	18.7	27.60 ± 6.75*
RBC (x10 <sup>12</sup> /l) NR: 4.5 – 6.5 (Male), 3.9 – 5.6 (Female)	6.5	6.5	5.2	5.2	4.14 ± 0.64*
WBC (x10 <sup>9</sup> /l) NR: 4.0 – 11.0	6.5	4.2	7.0	6.3	7.99 ± 5.10*
Platelets (x10 <sup>9</sup> /l) NR: 150 - 400	167	210	230	177	307.30 ± 148.85*
Epo (miU/ml) NR: 2.5 – 10.5	15.9	NA	7.5	NA	NA

\* mean ± SD; Note that at the time of functional studies the patient's Hb is lower owing to the fact that he had already commenced therapy with venesection at three-monthly intervals.

BPGM was measured by the method Schröter and Kalinowsky, as modified by Beutler: Briefly, an equilibrium mixture of F-1,6-biP, GAP, DHAP, 1,3-BPG, NAD and NADH is established. Addition of hemolysate containing BPGM causes the removal of 1,3-BPG, displacement of the GAP/1,3-BPG equilibrium and the reduction of NAD to NADH, which is monitored by the increase in absorbance at 340 nm. The 2,3-BPG was measured by a modification of the method of Krinsky: Briefly, an equilibrium mixture of PEP, enolase, 2-PGA, PGAM-1 and 3-PGA is formed. Addition of red cell extract containing 2,3-BPG, the obligatory cofactor for PGAM-1 activity, causes depletion of PEP, which is monitored by the decrease in absorbance at 240 nm.

## Figure Legends

**Figure 1.** DNA sequence analysis detected a heterozygous G>A substitution at nucleotide c.269, in the patient and his mother but not his father. This predicts the substitution of arginine at residue 90 with histidine (R90H).

**Figure 2.** Structural effects. Inspection of the crystal structure of human BPGM (RCSB PDB id: 2HHJ, <sup>(15)</sup>) indicated that R90 is potentially involved in hydrogen bonds with carbonyl groups from E156 and W115, stabilizing the structure of the protein and the active site pocket (note proximity to phosphate group of 2,3-BPG). Figure produced using PyMOL (The PyMOL Molecular Graphics System, Schrödinger, LLC).



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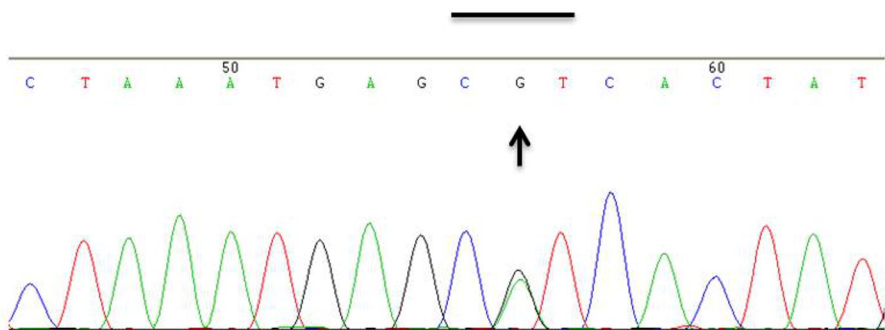
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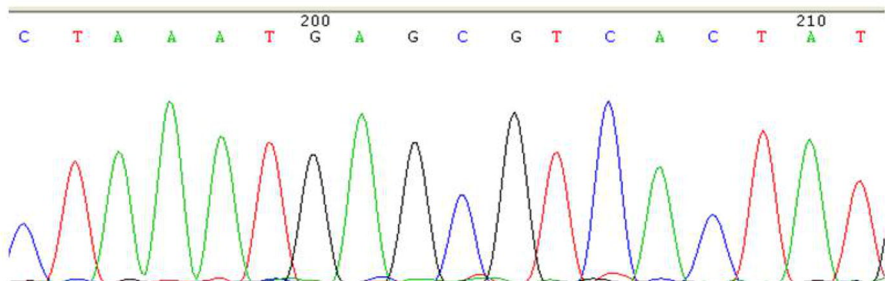
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Patient



Father



Mother



Figure 1

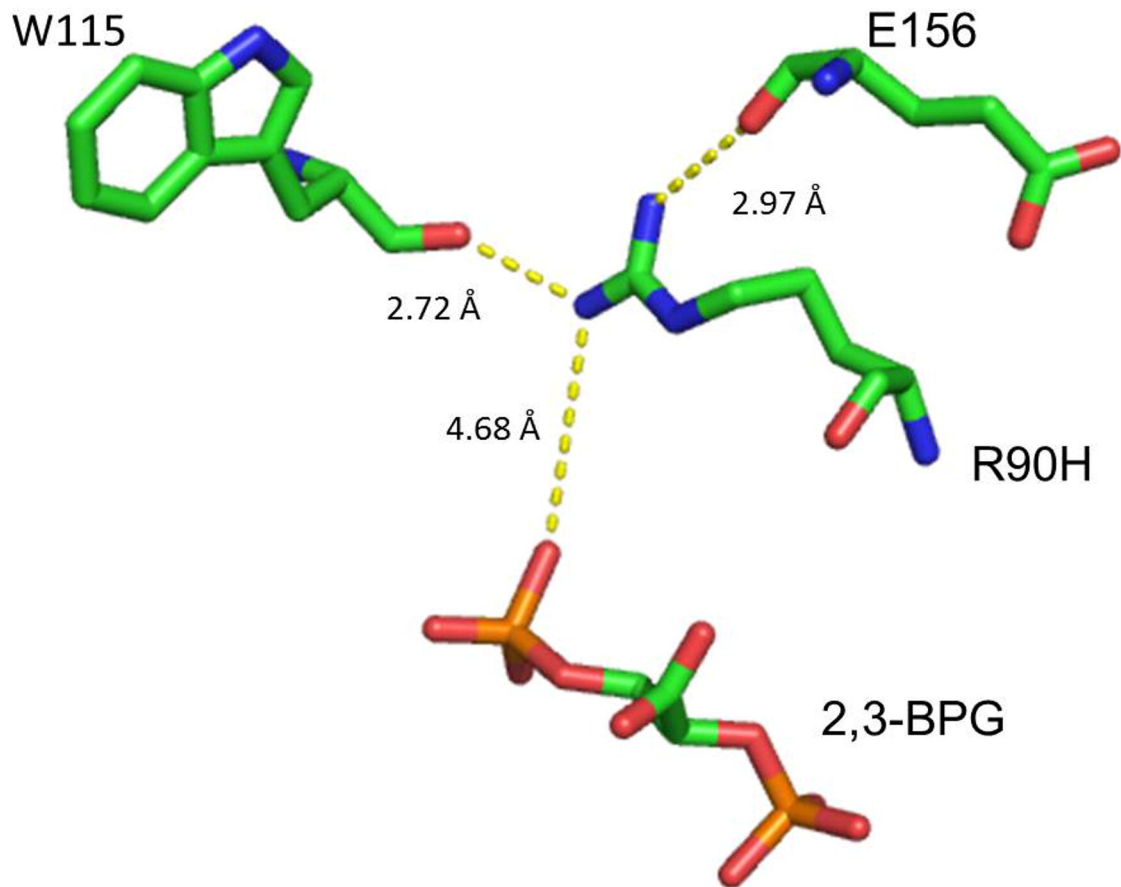


Figure 2