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

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.[1] Table 1 summarizes 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.[1]

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 6.53×10^{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 6.5×10^{9}/L (with a normal differential) and platelets of 167×10^{9}/L. Red cell mass was 146% predicted. Vitamin B_{12} 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_{50}, 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 3-6 months, aiming for Hct of less than 0.5 l/L. Five years following his initial presentation, his disease etiology was re-visited. 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 Declaration of Helsinki.

Whole-genome sequencing 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,[2] identification of alleles and variants was performed using the in-house Platypus software[3] and annotated using a pipeline based on ANNOVAR.[4] 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

Table 1. Causes of erythrocytosis.

Primary erythrocytosis (typically low Epo levels)	
Congenital	Acquired
Epo receptor mutations	Polycythemia vera (including *JAK2* mutations)
Secondary erythrocytosis (high or normal Epo levels)	
Congenital (inherited)	Acquired (Epo-mediated)
Defects in oxygen-sensing pathway:	Systemic hypoxia:
VHL mutations (e.g. Chuvash polycythemia)	Chronic lung disease
EGLN1 (PHD2) mutations	Right-to-left cardiopulmonary vascular shunts
EPAS1 (HIF2A) mutations	Smoker's erythrocytosis
	Nocturnal hypoventilation syndromes (e.g. obstructive sleep apnea)
Other congenital defects	High altitude
High-oxygen affinity Hb	Local hypoxia:
Bisphosphoglycerate mutase deficiency	Renal artery stenosis
	Renal cysts (polycystic kidney disease)
	Post-renal transplant erythrocytosis
	Pathological Epo production (tumors)
	Brain (cerebellar hemangioblastoma, meningioma)
	Renal cell carcinoma
	Hepatocellular carcinoma
	Phechromocytoma
	Drug-associated:
	Erythropoietin/androgen administration

Adapted from McMullin.[1]

the growing evidence of new genes interacting with the HIF factors, WGS 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; this 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

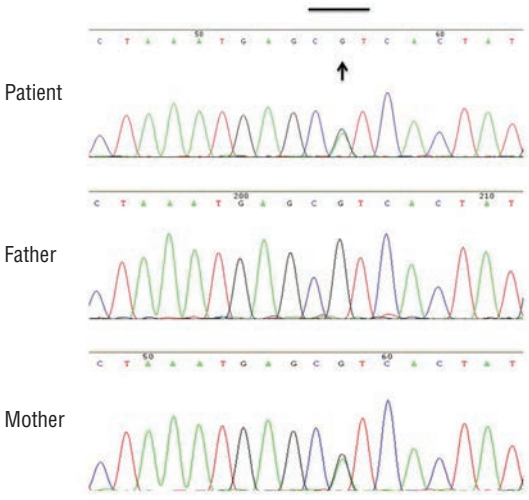

Figure 1. DNA sequence analysis detected a heterozygous G>A substitution at nucleotide c.269, in the patient and his mother but not his father. This predicts the substitution of arginine at residue 90 with histidine (R90H).

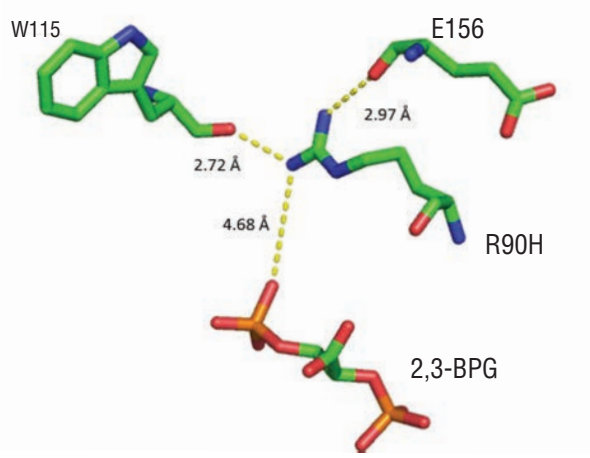

Figure 2. Structural effects. Inspection of the crystal structure of human BPGM (RCSB PDB id: 2HHJ[5]) indicated that R90 is potentially involved in hydrogen bonds with carbonyl groups from E156 and W115, stabilizing the structure of the protein and the active site pocket (note proximity to phosphate group of 2,3-BPG). Figure produced using PyMOL (The PyMOL Molecular Graphics System, Schrödinger, LLC).

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 ($x10^{12}$/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 ($x10^{9}$/L) NR: 4.0 – 11.0	6.5	4.2	7.0	6.3	7.99 ± 5.10*
Platelets ($\times10^{9}$/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 3-monthly intervals. BPGM was measured by the Schröter and Kalinowsky method, 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 Krimsky. 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 co-factor for PGAM-1 activity, causes depletion of PEP, which is monitored by the decrease in absorbance at 240 nm.*

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,[6] and 2,3-BPG by a modification of the method of Krimsky,[7] as explained in Table 2.

Bisphosphoglycerate mutase is the enzyme responsible for the synthesis of 2,3-BPG (from 1,3-BPG) in red blood cells.[8] 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.[9] 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[10] and erythrocytosis.[11,12] In 1978, Rosa *et al.* reported the first case of a 42-year old man with complete deficiency of BPGM and erythrocytosis.[11] His Hb concentration was 190 g/L, his erythrocyte 2,3-BPG was below 3% of normal values and he had a low P_{50}. His sister also had complete BPGM deficiency and excessive erythrocytosis and they were both compound heterozygous with an 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.[13] In contrast, his son and daughter, who were heterozygous only for the R89C mutation, had intermediate phenotypes with partial BPGM deficiency and modest erythrocytosis.[13] 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.[14] 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.[12] 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.[15]

Interestingly, the same amino acid residue (i.e. arginine at codon 90) is affected, as in previous cases of erythrocytosis.[13] 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.*[14] 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.*;[13] however, WGS did not reveal any additional mutations/deletions in *BPGM*. Alternatively, the explanation could be that of a variable penetrance of the disease-causing mutation, consistent with the phenotypes and patterns of inheritance of the family members in Lemarchandel *et al.*[13] 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 the view that this mutation is functional. Given that heterozygous individuals, both in our study and in that of Lemarchandel *et al.*[13] 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.

Nayia Petousi,[1] Richard R. Copley,[2,3] Terence R.J. Lappin,[4] Sally E. Haggan,[5] Celeste M. Bento,[6] Holger Cario,[7] Melanie J. Percy,[5] The WGS Consortium,[+] Peter J. Ratcliffe,[1] Peter A. Robbins,[8] and Mary Frances McMullin[4]

[1]Nuffield Department of Medicine, University of Oxford, UK; [2]Wellcome Trust Centre for Human Genetics, University of Oxford & NIHR Biomedical Research Centre, UK; [3]CNRS, Sorbonne Universités, UPMC Univ Paris 06, Laboratoire de Biologie du Développement de Villefranche-sur-mer, Observatoire Océanographique, France; [4]Centre for Cancer Research and Cell Biology, Queen's University, Belfast, UK; [5]Department of Haematology, Belfast City Hospital, Belfast, UK; [6]Department of Haematology, Centro Hospitalar e Universitário de Coimbra, Portugal; [7]Department of Paediatrics and Adolescent Medicine, University Medical Center Ulm, Germany; and [8]Department of Physiology, Anatomy and Genetics, University of Oxford, UK;

[+]A full list of members is provided at the end of the manuscript

Correspondence: M.McMullin@qub.ac.uk or nayia.petousi@ndm.ox.ac.uk

doi:10.3324/haematol.2014.109306

WGS500 Consortium: names and affiliations of authors. Steering Committee: Peter Donnelly (Chair),[1] John Bell,[2] David Bentley,[3] Gil McVean,[1] Peter Ratcliffe,[1] Jenny Taylor,[1,4] Andrew Wilkie.[4,5] Operations Committee: Peter Donelly (Chair),[1] John Broxholme,[1] David Buck,[1] Jean-Baptiste Cazier,[1] Richard Cornall,[1] Lorna Gregory,[1] Julian Knight,[1] Gerton Lunter,[1] Gil McVean,[1] Ian Tomlinson,[1,4] Andrew Wilkie.[4,5] Sequencing & Experimental Follow up: David Buck (Lead),[1] Christopher Allan,[1] Moustafa Attar,[1] Angie Green,[1] Lorna Gregory,[1] Sean Humphray,[3] Zoya Kingsbury,[3] Sarah Lamble,[1] Lorne Lonie,[1] Alistair Pagnamenta,[1] Paolo Piazza,[1] Guadelupe Polanco, and Amy Trebes.[1] Data Analysis: Gil McVean (Lead),[1] Peter Donnelly,[1] Jean-Baptiste Cazier,[1] John Broxholme,[1] Richard Copley,[1] Simon Fiddy,[1] Russell Grocock,[3] Edouard Hatton,[1] Chris Holmes,[1] Linda Hughes,[1] Peter Humburg,[1] Alexander Kanapin,[1] Stefano Lise,[1] Gerton Lunter,[1] Hilary Martin,[1] Lisa Murray,[3] Davis McCarthy,[1] Andy Rimmer,[1] Natasha Sahgal,[1] Ben Wright,[1] and Chris Yau;[6] [1]The Wellcome Trust Centre for Human Genetics, Oxford, UK; [2]Office of the Regius Professor of Medicine, University of Oxford, UK; [3]Illumina Cambridge Ltd., Little

Chesterford, Essex, UK; [4]NIHR Oxford Biomedical Research Centre, UK; [5]Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK; and [6]Imperial College London, London, UK

Funding: this work was supported in part by a Wellcome Trust Core Award (090532/Z/09/Z) to the Wellcome Trust Centre for Human Genetics. NP is supported by a NIHR (National Institute for Health Research) Academic Clinical Lectureship.

Key words: BGPM mutation-associated erythrocytosis.

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. McMullin MF. The classification and diagnosis of erythrocytosis. Int J Lab Hematol. 2008;30(6):447-59.
2. Lunter G, Goodson M. Stampy: a statistical algorithm for sensitive and fast mapping of Illumina sequence reads. Genome Res. 2011;21(6):936-9.
3. Rimmer A, Mathieson I, Lunter G, McVean G. Platypus: An Integrated Variant Caller. 2012.
4. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 2010;38(16):e164.
5. Wang Y, Liu L, Wei Z, Cheng Z, Lin Y, Gong W. Seeing the process of histidine phosphorylation in human bisphosphoglycerate mutase. J Biol Chem. 2006;281(51):39642-8.
6. Beutler E. Red-cell metabolism. A manual of biochemical methods. 3rd ed: Grune and Stratton; New York, 1984.
7. Krimsky I. D-2,3-Diphosphoglycerate: Academic Press; New York, 1965.
8. Sasaki R, Ikura K, Sugimoto E, Chiba H. Purification of bisphosphoglyceromutase, 2,3-bisphosphoglycerate phosphatase and phosphoglyceromutase from human erthrocytes. Three enzyme activities in one protein. Eur J Biochem. 1975;50(3):581-93.
9. Benesch R, Benesch RE. Oxygenation and ion transport in red cells. Science. 1968;160(3823):83.
10. Travis SF, Martinez J, Garvin J Jr., Atwater J, Gillmer P. Study of a kindred with partial deficiency of red cell 2,3-diphosphoglycerate mutase (2,3-DPGM) and compensated hemolysis. Blood. 1978;51(6):1107-16.
11. Rosa R, Prehu MO, Beuzard Y, Rosa J. The first case of a complete deficiency of diphosphoglycerate mutase in human erythrocytes. J Clin Invest. 1978;62(5):907-15.
12. Hoyer JD, Allen SL, Beutler E, Kubik K, West C, Fairbanks VF. Erythrocytosis due to bisphosphoglycerate mutase deficiency with concurrent glucose-6-phosphate dehydrogenase (G-6-PD) deficiency. Am J Hematol. 2004;75(4):205-8.
13. Lemarchandel V, Joulin V, Valentin C, Rosa R, Galacteros F, Rosa J, et al. Compound heterozygosity in a complete erythrocyte bisphosphoglycerate mutase deficiency. Blood. 1992;80(10):2643-9.
14. Garel MC, Lemarchandel V, Prehu MO, Calvin MC, Arous N, Rosa R, et al. Natural and artificial mutants of the human 2,3-bisphosphoglycerate as a tool for the evaluation of structure-function relationships. Biomed Biochim Acta. 1990;49(2-3):S166-71.
15. Bento C, Almeida H, Maia TM, Relvas L, Oliveira AC, Rossi C, et al. Molecular study of congenital erythrocytosis in 70 unrelated patients revealed a potential causal mutation in less than half of the cases (Where is/are the missing gene(s)?). Eur J Haematol. 2013;91(4):361-8.