Identification of high oxygen affinity hemoglobin variants in the investigation of patients with erythrocytosis

Erythrocytosis is a disorder of red cell production that can arise from several different causes. Familial cases have been classified into four groups (ECYT1-4) by Online Mendelian Inheritance in Man (OMIM) as listed at the website: *http://www.ncbi.nlm.nih.gov/sites/entrez?db=OMIM*. Erythrocytosis associated with mutations in the erythropoietin receptor belong to group ECYT1 and arise from a primary cause that is intrinsic to the red cell.[1] The other three categories of erythrocytosis, ECYT2-4, are secondary and are characterized by dysregulated erythropoietin (Epo) production resulting from defects in the oxygen sensing pathway.[1] OMIM classification does not consider other secondary causes of erythrocytosis that are related to abnormal oxygen delivery such as hemoglobin (Hb) variants or 2,3-biphosphoglycerate mutase (BPG) deficiencies. Approximately 100 hemoglobin variants with a high affinity for oxygen have been described that cause a decrease in the supply of oxygen to tissues.[2] These Hb variants are inherited in an autosomal dominant manner with an associated family history.[2] Both α and β globin genes can be affected[2] and the serum Epo would be inappropriately normal for the associated raised hematocrit.

During the differential diagnosis of erythrocytosis, a high oxygen affinity Hb can be excluded by studying the oxygen binding properties of freshly drawn blood.[2-4] Blood gas analyzers available in all hospitals will calculate P50 values. High affinity Hbs cause a left shift in the oxygen dissociation curve and thus lower the P50 value. Not all high affinity Hb variants are detected by routine Hb electrophoresis and isoelectric focusing in polyacrylamide and hence a number are misdiagnosed.

Over the last decade we have developed a data base of erythrocytosis patients referred from centers throughout the UK and Ireland.[5] Before patients can be included in the registry all secondary causes of erythrocytosis should be excluded. During an audit of the data base we decided it may be useful to confirm the absence of high oxygen affinity Hb variants. Patients with a strong family history and serum Epo within the reference range or above were selected for β globin gene sequencing. Those patients negative for a β chain variant were further screened for an alpha globin gene variant. Four different high oxygen affinity Hb variants, Olympia (β20Va→Met),[1,6,7] Pierre-Benite (β90Glu→Asp),[6,8] Santa Clara (β97His→Asn)[6,9] and Heathrow (β103Phe→Leu),[6,10] were detected in 6 families from a data base of 205 individuals giving a prevalence of 3%. All are electrophoretically silent and hence difficult to detect by routine laboratory tests.[6] Variants can be confirmed by high performance liquid chromatography or by mass spectrometry and identified by PCR-direct sequencing.[6] The hematological indices of the patients are listed in Table 1. In all cases the hematocrit and the hemoglobin level was raised, except for UPN224 which was found to be iron deficient. White blood cell and platelet counts were in the normal range. The serum Epo level was either elevated or inappropriately normal for the elevated Hb. Thrombotic events have been reported for only Patients 214 and 225.

The hemoglobin tetramer is composed of 2 chains of α and 2 chains of β globin arranged in two identical halves, containing one chain of each globin type. As Hb transfers from the low oxygen affinity state (known as T or tense) to the high affinity state (R or relaxed) there is an accompanying movement within the α1β2 subunit.[1] Many of the amino acid changes associated with a high affinity phenotype map to the α1β2 interface or the C-terminal of the β chain.[1] These mutations prevent the transition of the Hb molecule to the T state and hence are unable to release the bound oxygen molecules. The Santa Clara variant cannot achieve the T state[9] while Pierre-Benite is thought to stabilize Hb in the T quaternary structure[8,11] and Hb Olympia[12] affects the surface of the protein causing it to self-associate. Other mutations cause structural alteration of the hemepocket. The presence of several aromatic amino acids around the hememoiety is crucial for the correct orientation of the hemegroup. Replacement of Phe103 with Leu (Hb Heathrow) causes the affinity of the hemegroup for oxygen to be increased.[1]

During the differential diagnosis of erythrocytosis the first step would be the calculation of P50 using the routine blood gas analyzer. In those patients with a low value for P50 sequencing of the globin gene to establish

Table 1. Hematologic indices of erythrocytosis patients with hemoglobin variants.

Variant	UPN116 Olympia	UPN162 Olympia	UPN214 Pierre-Benite	UPN225 Pierre-Benite	UPN226 Santa Clara	UPN224 Heathrow
Age at presentation (years)	42	24	35	45	22	44
Affect family members	Father, paternal uncle	Maternal uncles	Mother, brother, daughter	Not known	Two brothers, maternal cousins	Mother, sister, nephew, niece
Hemoglobin (g/dL)	19.2	18.2	19.2	18.5	19.1	15.4
Hematocrit	0.59	0.532	0.561	0.52	0.581	0.48
MCV (fL)	94	85.8	86	91	101.7	72
Serum ferritin	34	218	ND	57	80.6	4
WBC ($\times 10^9$/L)	5.8	5.8	8.0	8.3	6.8	64
Platelets ($\times 10^9$/L)	223	186	416	153	171	334
Erythropoietin (mIU/mL)	9.9 (NR 4.2-16.3)	4.9 (NR 4.2-24.2)	23 (NR 5.5-16.5)	36 (NR 5.5-16.5)	8.4 (NR 2.6-18.5)	29.1 (NR 5-25)

the actual variant would be performed. In the absence of P50 values, as with patients in this study, it is important to eliminate the possibility of Hb variants by sequencing the globin genes, which confirmed 6 positive cases on the registry.

Melanie J. Percy,[1] Nauman N. Butt,[2] Gerard M. Crotty,[3] Mark W. Drummond,[4] Claire Harrison,[5] Gail L. Jones,[6] Matthew Turner,[4] Jonathan Wallis,[6] and Mary Frances McMullin[4,7]

[1]Department of Haematology, Belfast City Hospital, Belfast, Northern Ireland, UK; [2]Department of Haematology, Arrowe Park Hospital, Wirral, England, UK; [3]Regional Department of Haematology, Midland Regional Hospital at Tullamore, Tullamore, Ireland; [4]Department of Haematology, University of Glasgow, Glasgow, Scotland, UK; [5]Department of Haematology, St Thomas' Hospital, London, England, UK; [6]Department of Haematology, Royal Victoria Infirmary, Newcastle upon Tyne, England, UK; [7]Department of Haematology, Queen's University of Belfast, Belfast, Northern Ireland, UK

Correspondence: Melanie J. Percy PhD, Department of Haematology, Floor C, Tower Block, Belfast City Hospital, Lisburn Road, Belfast BT9 7AB, N. Ireland, UK. Phone: international +44.28.90263097. Fax: international +44.28.90263927. E-mail: melanie.percy@belfasttrust.hscni.net

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Pyruvate kinase M2 and prednisolone resistance in acute lymphoblastic leukemia

Treatment of childhood acute lymphoblastic leukemia (ALL) combines different classes of chemotherapeutic agents, such as *Vinca* alkaloids, anthracyclines and glucocorticoids (GCs). Although such therapy nowadays cures the majority of the patients, combination chemotherapy still fails in approximately 20%.[1] Most treatment failures can be explained by resistance to antileukemic agents,[2] and resistance to the glucocorticoids in particular has been shown to be related to an unfavorable event free survival.[3,4] Since the glucocorticoids prednisolone and dexamethasone play a crucial role in essentially all therapy protocols, the development of strategies to reverse resistance to these agents is important to improve ALL treatment efficacy. Recently, we have demonstrated that glucocorticoid resistance in pediatric ALL is associated with increased glucose metabolism, and that inhibition of glycolysis sensitizes prednisolone-resistant ALL cells to glucocorticoids.[5] Although it has been known for several decades that cancer cells shift their energy production from oxidative phosphorylation towards the less efficient glycolysis pathway (the so called Warburg effect)[6] it is still not clear how tumor cells establish this altered metabolic phenotype. Christofk *et al*. recently showed that altered expression of the glycolytic enzyme pyruvate kinase (PK), and more specifically the switch to the alternatively spliced isoform M2 (PKM2), is responsible for the increased rate of glycolysis observed in cancer cells.[7] Together, these findings suggest an upregulation of PKM2 in prednisolone resistant leukemia.

To investigate if pyruvate kinase M2 plays a role in glucocorticoid resistance in pediatric ALL, the expression of PKM2 was determined in leukemic cell samples of ALL patients and compared to PKM2 expression in normal peripheral blood and bone marrow samples. To distinguish between the different isoforms in real time quantitative PCR (RT Q-PCR), primers were designed that specifically amplify PKM2 or that recognize both isoform 1 and 2 of pyruvate kinase (PKM1/2, Figure 1A). The expression of PK isoforms was subsequently tested in different cell lines, confirming the specificity of the primer combinations (Figure 1B).

Next, the expression of the different PK isoforms was determined in normal peripheral blood lymphocytes, normal bone marrow and in leukemic samples from untreated children at initial diagnosis of ALL that were identified by an *in vitro* cytotoxicity assay (MTT)[8] as prednisolone-resistant (n=11, $LC_{50} \geq 150$ µg/mL), or sensitive to prednisolone (n=19, $LC_{50} \leq 0.1$ µg/mL).[6] In correspondence with the results of Christofk *et al*.,[7] a significant difference ($p<0.0001$) was found in the expression of PKM2 between normal and ALL cells (Figure 2A). However, PKM2 transcripts were found in all patient samples and no significant difference was observed between prednisolone-resistant or prednisolone–sensitive ALL cases