We herein present a case of congenital erythrocytosis caused by haemoglobin (Hb) Bethesda in a Japanese family. A 55-year-old asymptomatic man was referred to our hospital for the investigation of erythrocytosis, which was present in other members of his family. The patient’s serum erythropoietin level was normal, and the JAK2 V617F mutation was not detected. His P50 value was mildly decreased, thus we suspected the presence of an Hb variant with a high oxygen affinity. The high-performance liquid chromatography analysis showed an abnormal Hb, and by direct sequencing we identified the Hb Bethesda variant in this patient. For the differential diagnosis, we recommend the estimation of the P50 value as a practical and useful test.

Key words: haemoglobin Bethesda, high oxygen affinity haemoglobin variant, congenital erythrocytosis, P50

(Intern Med 54: 2389-2393, 2015)
(DOI: 10.2169/internalmedicine.54.4520)

Introduction

Patients with secondary erythrocytosis have a relative or absolute increase in the number of circulating red blood cells due to elevated or inappropriately normal serum erythropoietin (EPO) levels. Acquired secondary erythrocytosis is the most common form and can be caused by a high-altitude habitat, heavy smoking, or various disorders (e.g., chronic obstructive pulmonary disease, congenital heart disease, and EPO-producing tumour) (1-4). When these causes are excluded, a genetic cause involving haemoglobin (Hb) variants or the proteins of the oxygen-sensing pathway should be suspected, although these causes are rare (1-4). The presence of a high oxygen affinity Hb variant or 2,3-biphosphoglycerate mutase (BPGM) deficiency impairs oxygen release to the tissues. The oxygen dissociation curve (ODC) is shifted to the left and leads to inappropriately raised serum EPO levels with high Hb values (1-4). In this report, we describe a Japanese family who carry the Hb Bethesda variant, a haemoglobinopathy with a high oxygen affinity, and demonstrate the importance of determining the P50 value in the ODC for an accurate diagnosis.

Case Report

Our patient was a 55-year-old Japanese man living in Wakayama. During his adolescence, he occasionally suffered from headaches. Although he had had erythrocytosis detected in a health examination approximately 30 years previously, he had never visited a hospital. The patient had no history of thrombosis or pulmonary hypertension. He was a current smoker, but never consumed alcohol and his body mass index was 21.7.

Three months before being referred to our hospital, a health examination indicated severe diabetes (HbA1c 10.4 %). The patient visited his primary medical physician to treat and manage type 2 diabetes mellitus. At this time, he was recommended by the physician for a detailed investigation of erythrocytosis and was referred to our hospital. On
Hct, an anti-platelet drug was administered at the first visit.

The laboratory data showed that he had developed erythrocytosis with Hb level of 219 g/L and hematocrit (Hct) 0.61 and the platelet count was mildly decreased (130×10^9/L). The red cell morphology was normal. An echocardiogram revealed normal oxygen saturation, while the P50 value in the ODC showed a mild decrease (23.75 mmHg; reference range: 25-29 mmHg) (Table). According to these findings, we strongly suspected the presence of a Hb variant with a high oxygen affinity or 2,3-BPGM deficiency (1-5).

In our index case, an arterial blood gas analysis demonstrated normal oxygen saturation, while the P50 value in the ODC showed a mild decrease (23.75 mmHg; reference range: 25-29 mmHg) (Table). According to these findings, we strongly suspected the presence of a Hb variant with a high oxygen affinity or 2,3-BPGM deficiency (1-5).

This study was approved by the Institutional Review Boards of Kinan Hospital in accordance with the Declaration of Helsinki. Our patient and his son gave informed written consent. Blood samples were obtained from the patient and his son. An analysis by high-performance liquid chromatography (HPLC) (Tosoh G7 Automated HPLC Analyzer, Tosoh Bioscience, San Francisco, USA) (3, 6) revealed normal levels of both HbF (1.3 %) and HbA2 (2.4 %); however, an unknown peak (27.8%) was identified, thus suggesting the presence of an abnormal Hb variant (Fig. 2).

Therefore, we preformed direct Sanger sequencing of the \textit{Hb \beta-chain (HBB)} gene using DNA isolated from the blood samples of the present patient and his son (3, 4). A heterozygote mutation in exon 3 of the \textit{HBB} gene, c.436T>C, was identified in both cases (Fig. 3). In the former nomenclature, the first codon of the protein sequence (the initiation codon) in the HBB was not numbered; however, it is now considered to be the first codon. Thus, the nomenclature of the other codons has increased by one number. Therefore, this mutation in the present case results in the substitution

| Complete Blood Count | Chemistry | C-reactive Protein |
|----------------------|-----------|-------------------|
| White Blood Cells    | Creatinine| 0.07 mg/dL (NR: 0.0-0.3) |
| Neutrophil           | Blood Urea Nitrogen | 334 mg/dL (NR: 70-109) |
| Lymphocyte           | Sodium    | Hemoglobin A1c    | 10.4 % (NR: 4.6-6.2) |
| Monocyte             | Potassium | Ferritin          | 77 ng/mL (NR: 40-480) |
| Eosinophil           | Chloride  | Erythropoietin    | 20.9 mlU/mL (NR: 4.2-23.7) |
| Basophil             | Aspartate Transaminase | 15 IU/L (NR: 11-35) |
|                      | γ-glutamyl Transpeptidase | 9 IU/L (NR: 5-35) |
|                      | Alkaline Phosphatase | 104 IU/L (NR: 100-340) |
|                      | Lactate Dehydrogenase | 169 IU/L (NR: 120-231) |
|                      | Creatine Kinase | 57 IU/L (NR: 45-255) |
|                      | Total Protein | 6.3 g/dL (NR: 6.7-8.1) |
|                      | Albumin   |                   |

**Table.** Laboratory Data of the Patient at Visit to Our Hospital.

**Figure 1.** Pedigree of known family members, showing years, causes of death, and presence of erythrocytosis. The arrow indicates the present patient. Solid symbols represent family members with erythrocytosis. All the patients had no history of thrombosis.

this examination, the only findings were facial and hand plethora. His vital signs were the following: blood pressure 143/80 mmHg; pulse rate 80 beats/min; and body temperature 36.0°C. The laboratory data showed that he had developed erythrocytosis with Hb level of 219 g/L and hematocrit (Hct) 0.61 and the platelet count was mildly decreased (81×10^9/L) (Table). The white blood cell count, renal and hepatic function, and coagulation were normal. Additionally, the red cell morphology was normal. An echocardiogram revealed no thrombus in the pulmonary artery and deep vein. The serum EPO level was 20.9 mIU/mL (reference range: 4.2-23.7 mIU/mL), and the JAK2 V617F mutation was not detected. His bone marrow showed an increase in the number of erythroid cells (myeloid/erythroid ratio 0.4), with no blasts or fibrosis. There was no splenic enlargement on ultrasound and computed tomography. For the high value of Hct, an anti-platelet drug was administered at the first visit.

Erythrocytosis is defined by an Hb level above 185 g/L in men and 165 g/L in women or an Hct level above 0.52 in men and 0.48 in women (1, 2). We confirmed the laboratory data provided by the regular health examinations and primary care physicians of his affected family members. The present patient had a family history with an autosomal dominant mode of inheritance affecting at least 8 family members spanning 3 generations (Fig. 1). His family has no history of thrombosis or miscarriage. From the laboratory data obtained in our hospital, his son also exhibited erythrocytosis (Hb 201 g/L, Hct 0.60) and had a slightly low platelet count (130×10^9/L) with a serum EPO level in the normal range.

In our index case, an arterial blood gas analysis demonstrated normal oxygen saturation, while the P50 value in the ODC showed a mild decrease (23.75 mmHg; reference range: 25-29 mmHg) (Table). According to these findings, we strongly suspected the presence of a Hb variant with a high oxygen affinity or 2,3-BPGM deficiency (1-5).

This study was approved by the Institutional Review Boards of Kinan Hospital in accordance with the Declaration of Helsinki. Our patient and his son gave informed written consent. Blood samples were obtained from the patient and his son. An analysis by high-performance liquid chromatography (HPLC) (Tosoh G7 Automated HPLC Analyzer, Tosoh Bioscience, San Francisco, USA) (3, 6) revealed normal levels of both HbF (1.3 %) and HbA2 (2.4 %); however, an unknown peak (27.8%) was identified, thus suggesting the presence of an abnormal Hb variant (Fig. 2).

Therefore, we preformed direct Sanger sequencing of the \textit{Hb \beta-chain (HBB)} gene using DNA isolated from the blood samples of the present patient and his son (3, 4). A heterozygote mutation in exon 3 of the \textit{HBB} gene, c.436T>C, was identified in both cases (Fig. 3). In the former nomenclature, the first codon of the protein sequence (the initiation codon) in the HBB was not numbered; however, it is now considered to be the first codon. Thus, the nomenclature of the other codons has increased by one number. Therefore, this mutation in the present case results in the substitution

![Image](image.png)
of tyrosine by histidine at amino acid position 146 in the HBB protein product, a known high-affinity variant referred to as Hb Bethesda [beta 145(HC2) Tyr>His in the past] (7-12). We were able to reach the definitive diagnosis of congenital erythrocytosis due to the presence of a high affinity Hb variant, which, although rare, is the most frequent cause of congenital erythrocytosis (1-4, 12).

Discussion

The family history of Hb Bethesda presented herein is the eighth such familial case in the world (7-13). Moreover, it is the second familial case in Japan with the first reported 20 years ago (12). The reason for the limited number of reported cases with Hb Bethesda is that there are many unclear elements regarding its clinical presentation. The patients with a high oxygen affinity Hb variant, including Hb Bethesda, typically develop secondary erythrocytosis caused by the presence of a high affinity Hb variant, which, although rare, is the most frequent cause of congenital erythrocytosis (1-4, 12).

Figure 2. Cation-exchange HPLC of the hemoglobin fraction demonstrating HbF, HbA2, and the abnormal Hb variant. The abnormal Hb was eluted after HbA2 (arrow).

Figure 3. Identification of the c.436T>C mutation in the HBB gene by direct sequencing. The sequencing detected a heterozygous T to C change at base 436 in the present patient as indicated by the arrow. This mutation coincides with the Hb Bethesda variant, beta chain 146 Tyr replaced by His.

Hb Hiroshima is another form of high oxygen affinity Hb and cases are often concentrated in particular areas of Japan. The amino acid residue affected in Hb Hiroshima [HBB: c.439C>G; beta 146(HC3) His>Asp in the past] is located...
one amino acid downstream from the abnormal region of Hb Bethesda, both are located within the C-terminus tail of the β-globin chain (14, 15). The region of β-globin is an important site that controls oxygen affinity by stabilising the deoxy conformation of HbA. Patients with a genetic abnormality in these regions present with secondary erythrocytosis due to defects in oxygen dissociation (14, 15). The presence of a Hb variant may be diagnosed in the course of the determination of HbA1c, by unexpectedly low or high HbA1c levels, as in the case of Hb Hiroshima. Therefore, particular care must be taken in daily medical practice due to deviations between the HbA1c values and actual blood sugar levels (15-18). While the present patient had high levels of HbA1c, his fasting blood sugar was concurrently high and the patient’s HbA1c levels were mostly normalised after initiating oral medication for diabetes, leading to good glycaemic control. Hb Bethesda most likely does not affect the HbA1c levels because the abnormal variant was not present at the HbA1c melting point.

Secondary erythrocytosis due to high oxygen affinity Hb variants is generally believed to have a favourable prognosis. However, many aspects regarding the detailed treatment and follow-up still remain unclear. When the symptoms such as a headache are present, venesection therapy may be effective. Meanwhile, the limited number of reports makes it unequable to conduct a clinical trial and the true value of venesection is unclear (19, 20). Some reports have described cases of secondary erythrocytosis caused by high oxygen affinity Hb variants with hyperviscosity syndrome or thrombosis that required treatment (20-22). On the basis of the above cases, when a patient has symptoms associated with erythrocytosis or episodes of thrombosis, close follow-up on an outpatient basis is required, and treatments such as venesection therapy or anti-platelet therapy should be considered. For patients in a good general condition, a follow-up once every 6 to 12 months is adequate. Most cases of Hb Bethesda do not include reports of thrombocytopenia, although mild thrombocytopenia was seen in the present patient as in the first reported case of Hb Bethesda in Japan (7-13). In addition, his Hct was in excess of 60%, which is indicative of pre-stage hyperviscosity syndrome. The present patient was thus given an anti-platelet drug and is undergoing outpatient follow-up every two months.

We encounter many cases of erythraemia in routine clinical practice, but the majority of these are due to acquired causes, including polycythaemia vera. While these cases are broadly classified as EPO-dependent or -independent, a diagnostic method has now been established using molecular biology techniques to analyse mutations, for instance in the JAK2 gene (23). Meanwhile, algorithms for a definitive diagnosis are being established for congenital erythrocytosis, for which the number of cases is also small (1-4). Among these algorithms, a P50 measurement using an apparatus to analyse blood gases is a fast and simple test with proven efficacy that reflects a shift in the ODC (1-5, 24). In addition to a family history, a low P50 level in the present patient was observed using the automated blood gas analyser, which strongly suggested the presence of a high oxygen affinity haemoglobinopathy. HPLC was consequently performed, and we were able to confirm the presence of an abnormal Hb variant. Some previous cases with Hb Bethesda also showed a low P50 value (<13 mmHg), although the value was measured by in vitro experiments of the ODC (8-10, 12, 13). Hence, for the patients in whom acquired erythrocytosis is diagnosed by exclusion, a P50 measurement is recommended (1-5, 24). Despite this, patients suspected of having congenital erythrocytosis, in whom a definitive diagnosis is possible, account for approximately 30% of all cases (3, 4, 13). A definitive diagnosis may be expected in the patients where the cause of the disease is unknown by performing a genetic analysis using next-generation sequencing on an individual basis.

We herein successfully diagnosed an extremely rare Hb variant (Hb Bethesda) in a Japanese family. The P50 measurements served as an important step during the course of the definitive diagnosis (1-5). Many patients with erythrocytosis, whom we encounter in daily medical practice, may have hidden haemoglobinopathies, such as Hb Bethesda, highlighting the importance of differentiating various types of congenital erythrocytosis (1-5).

The authors state that they have no Conflict of Interest (COI).

Acknowledgement
This work was supported by the European Congenital Erythrocytosis Consortium (http://www.erythrocytosis.org/).

References
1. McMullin MF. The classification and diagnosis of erythrocytosis. Int J Lab Hematol 30: 447-459, 2008.
2. McMullin MF. Idiopathic erythrocytosis: a disappearing entity. Hematology Am Soc Hematol Educ Program 2009: 629-635, 2009.
3. Bento C, Almeida H, Maia TM, 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 91: 361-368, 2013.
4. Bento C, Percy MJ, Gardie B, et al. Genetic basis of congenital erythrocytosis: mutation update and online databases. Hum Mutat 35: 15-26, 2014.
5. Agarwal N, Mojica-Henshaw MP, Simmons ED, Hussey D, Ou CN, Prchal JT. Familial polycythemia caused by a novel mutation in the beta globin gene: essential role of P50 in evaluation of familial polycythemia. Int J Med Sci 4: 232-236, 2007.
6. Zurbiggen K, Schmugge M, Schmid M, et al. Analysis of minor hemoglobins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Clin Chem 51: 989-996, 2005.
7. Hayashi A, Stamatoyanopoulos G, Yoshida A, Adamson J. Haemoglobin Rainier: beta-145 (HC2) tyrosine leads to cysteine and haemoglobin Bethesda: beta-145 (HC2) tyrosine leads to histidine. Nat New Biol 230: 264-267, 1971.
8. Bunn HF, Bradley TB, Davis WE, et al. Structural and functional studies on hemoglobin Bethesda (αβ(viz)). a variant associated with compensatory erythrocytosis. J Clin Invest 51: 2299-2309, 1972.
9. Adamson JW, Hayashi A, Stamatoyanopoulos G, Burger WF.
Erythrocyte function and marrow regulation in hemoglobin Bethesda (β145 histidine). J Clin Invest 51: 2883-2888, 1972.

10. Olsen JS, Gibson QH. The functional properties of hemoglobin Bethesda (αβ145His). J Biol Chem 247: 3662-3670, 1972.

11. Schmidt RM, Jue DL, Ali MA, Lyonnais J, Moo-Penn WF. Hemoglobin Bethesda, beta 145 (HC2) Tyr replaced by His, in a Canadian family. Am J Clin Pathol 66: 449-452, 1976.

12. Kawashima I, Arima K, Hanada T, et al. Hemoglobin Bethesda causing polycythemia in a Japanese family. Intern Med 33: 242-247, 1994.

13. González Fernández FA, Villegas A, Ropero P, et al. Hemoglobinopathies with high oxygen affinity. Experience of Erythropathology Cooperative Spanish Group. Ann Hematol 88: 235-238, 2009.

14. Thom CS, Dickson CF, Gell DA, Weiss MJ. Hemoglobin variants: biochemical properties and clinical correlates. Cold Spring Harb Perspect Med 3: a011858, 2013.

15. Nishimori H, Harano K, Wada H, et al. A case of hemoglobin Hiroshima (β146 histidine to aspartic acid) with compensatory erythremia and undetectable HbA1c. Int J Hematol 95: 697-701, 2012.

16. Hamaguchi K, Harano K, Harano T, Abe N, Sakata T. Hb Ube-2 in a diabetic case with an abnormally low HbA1c value. Intern Med 38: 800-803, 1999.

17. Oshima Y, Ideguchi H, Takao M, et al. A patient with a hemoglobin variant (Hb JLome) unexpectedly detected by HPLC for glycated hemoglobin (HbA1c). Int J Hematol 68: 317-321, 1998.

18. Ohba Y, Miyaji T, Murakami M, et al. Hb himeji or β 140 (H18) ALA→ASP. A slightly unstable hemoglobin with increased β N-terminal glycation. Hemoglobin 10: 109-125, 1986.

19. Grace RJ, Gover PA, Treacher DF, Heard SE, Pearson PC. Venection in haemoglobin Yakima, a high oxygen affinity haemoglobin. Clin Lab Haematol 14: 195-199, 1992.

20. Fairbanks VF, Maldonado JE, Charache S, Boyer SH 4th. Familial erythrocytosis due to electrophoretically undetectable hemoglobin with impaired oxygen dissociation (hemoglobin Malmö, alpha 2 beta 2 97 gln). Mayo Clin Proc 46: 721-727, 1971.

21. Berreyer M, Francina A, Frenche P, Negrier C, Boneu B, Dechavanne M. Increased thrombosis incidence in a family with an inherited protein S deficiency and a high oxygen affinity hemoglobin variant. Am J Hematol 46: 214-217, 1994.

22. Hanss M, Lacan P, Aubry M, Lienhard A, Francina A. Thrombotic events in compound heterozygotes for a high affinity hemoglobin variant: Hb Milledgeville [α44(CE2)Pro→Leu (α2)] and factor V Leiden. Hemoglobin 26: 285-290, 2002.

23. Passamonti F. How I treat polycythemia vera. Blood 120: 275-284, 2012.

24. Percy MJ, Butt NN, Crotty GM, et al. Identification of high oxygen affinity hemoglobin variants in the investigation of patients with erythrocytosis. Haematologica 94: 1321-1322, 2009.