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CASE REPORT

Challenges in Interpreting Capillary Electrophoresis in the Co-existence of Non-deletional Alpha Thalassaemia with Increased Haemoglobin F

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Abstract

Haemoglobin (Hb) analysis by Capillary electrophoresis (CE) able to diagnose most cases of beta thalassaemia. However, for alpha thalassaemia, only limited cases could be diagnosed by CE, such as HbH, Hb Barts and haemoglobinopathy (i.e. Hb Constant Spring). All alpha thalassaemia traits will be missed by Hb analysis. Here we report a case of non-deletional alpha with raised Hb F. A 10-year-old, Indonesian boy presented with moderate anaemia, and a complete blood count showed Hb of 7.84 g/dL; MCV 83 fL; MCH 24.8 pg; MCHC 29.9 g/dL; Hct 26.2% and an increase in ferritin level of 1,548.88 ng/mL. The result of CE showed decreased percentage of Hb A (92.9%), HbA2 (2.1%), the presence of abnormal Hb variant eluted at zone 12 (0,4%), and zone 2 (0.8%) and raised in HbF (3.8%) level led to a presumptive of Hb H disease. Molecular analysis confirmed the diagnosis of a compound heterozygous of non-deletional alpha thalassaemia (Hb Adana and Constant Spring). Beta thalassaemia mutations analysis for common beta gene mutations by Multiplex Amplification Refractory System (MARMRS) PCR were normal. Increased HbF in this case is not inherited from both parents as confirmed by family study and this might be due to marrow response to the anaemic state or the possibility of concomitant presence of delta beta thalassaemia abnormalities.

Keywords: Molecular Genetics, Thalassaemia, Public Health

Introduction

Thalassaemia is a quantitative disorder of the globin chain due to impaired synthesis of one or more normal globin chains. This causes an imbalance in the formation of haemoglobin (Hb) due to compensatory excessive production of other globin chains (Schrier 2015). Clinical presentations in thalassaemia patients vary from being asymptomatic to varying degrees of symptomatic anaemia (Surapon 2011; Schrier 2013; Weatherall 2000; Furachoen 2002). Alpha thalassaemia is caused by decreased or absent production of alpha globin chains, resulting in an excess of gamma globin chains (in foetuses and newborns) or beta globin chains (in children and adults). Excessive beta globin chains can form soluble tetramers (β4 or HbH) which are unstable and easily precipitate in cells, causing various clinical manifestations. Meanwhile, beta thalassaemia is caused by a decrease in the production of beta globin chains resulting in an excess of alpha globin chains (Weatherall 2000, Schrier 2013).

Screening for thalassaemia or hemoglobinopathies is performed by capillary electrophoresis (CE), a widely used analytical separation technique. The CE system can be used to separate and quantitate normal Hb such as Hb A2 and Hb F (Ramli et.al, 2023) as well as abnormal Hb such as Hb E or Hb Constant Spring. Beta thalassaemia trait analysed on CE showed decreased in Hb A (α2β2), increased in Hb A2 (α2δ2), with normal or increased percentage of Hb F(α2γ2) (Weatherall 2000, Schrier

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2013). An increase of HbF in alpha thalassaemia is rare. Here we report a case of non-deletional alpha thalassaemia patient with increased in HbF and abnormal variant eluted at zone 12 and zone 2 that had contributed to the difficulties in interpreting the CE result.

Case Report

A 10-year-old Indonesian boy was referred to the outpatient clinic with pallor and aches in both lower limbs for 1-week duration. There was no history of frequent fever, nausea or vomiting, or bleeding. His development milestone is at the appropriate age. On physical examination, the patient was pale. However, there were hepato- and splenomegaly findings, thalassaemia facies without icterus, cyanosis and dyspnoea.

The results of full blood count revealed Hb of 7.84 g/dL; MCV 83 fL; MCH 24.8 pg; MCHC 29.9 g/dL; Hct 26.2%) and an increase in ferritin (1548.88 ng/mL). The result of CE revealed a decrease in Hb A (92.9%), low A2 (2.1%), an increase of Hb F (3.8%), a small abnormal peak at zone 12 (0.4%) and another abnormal peak at zone 2 /C zone (0.8%)(most likely Hb CS) (Picture 1a). This CE analysis was performed 2 weeks after the patient received the last transfusion.

The patient was diagnosed with thalassaemia in another hospital. The result of haemoglobin electrophoresis test by HPLC at diagnosis before receiving any transfusions was a decrease of HbA2 1.9%, an increase of HbF 7.4% and Hb Var 1.8%. This patient was suspected for alpha thalassaemia, but since there was an increase of Hb F and other Hb variants, molecular diagnostic was performed to confirm the diagnosis. The result of molecular test for alpha thalassaemia showed a compound heterozygous state of α2 codon 59 (GGC > GAC) Hb Adana (NG 000006.1:g.34071G>A) and α2 codon CD142 (TAA > CAA) Hb Constant Spring (Hb CS) (NG 000006.1:g.34461T>C). In view of high Hb F level in this case, Multiplex ARMS-PCR for β-thalassaemia was done to exclude common beta mutations and showed no mutation was found in gene IVS1-5, Codon 26, IVS2-654. There was no other molecular analysis done for uncommon beta-thalassaemia mutations or delta gene mutations.

The patient had received regular transfusions every two 2 weeks, folic acid 2x5 mg, vitamin E, and Deferiprone 500 mg. There is no history of similar problems in the other family members. The patient is the third child with two older brothers. CE analysis was done to confirm the inheritance pattern in his family, and the result is shown in the following pictures (Figure 1: picture 1b-d). Molecular analysis was not performed on all family members due to the limitation of resources.

Haemoglobin Electrophoresis Test of Patients (Minicapillary electrophoresis)

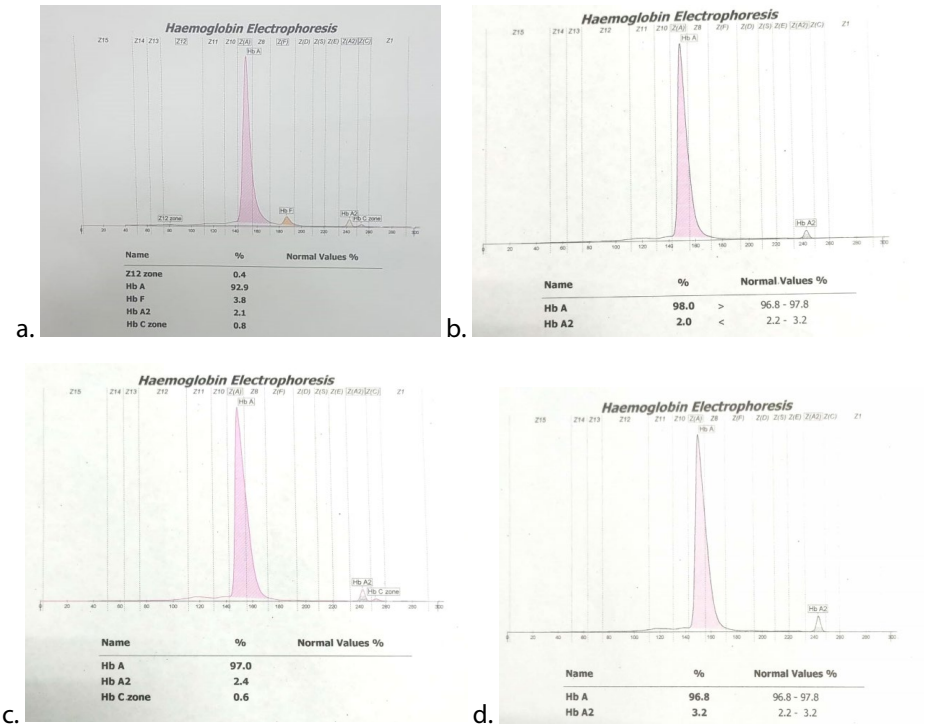

Figure 1. Haemoglobin electrophoresis test showing: a. patient; b. patient's mother; c. patient's father; d. patient's brother.

Table 2. Haematological parameter profile of the patient and his family

Erythrocyte index	Patient	Patient's mother	Patient's father	Patient's brother	Reference value
Hb (g/dL)	7.84	6.7	13.4	12.9	Male: 13.3 - 16.6 P: 11.0 -14.7
RBC (x10^6/uL)	3.16	3.99	5.16	4.56	3.69 -5.46
HCT (%)	26.2	23.2	41.2	38.2	L: 41.3 -52.1 P: 35.2 -46.7
MCV (fL)	83.0	58.1	79.8	83.8	86.7 -102.3
MCH (pg)	24.8	16.8	26.0	28.3	27.1 -32.4
MCHC (g/dL)	29.9	28.9	32.5	33.8	29.7 -33.1
RDW (%)	26.2	39.6	12.5	11.7	41.2-53.6
Hb A	92.9	98	97	96.8	96.8-98
Hb A2	2.1	2	2.4	3.2	2.2-3.2
Hb F	3.8	-	-	-	
Hb CS	0.8	-	0.6	-	
Z12 zone	0.4	-	-	-	

Based on haematological parameters and CE results of the family members, this patient's mother had very low haemoglobin in view of moderate hypochromic microcytosis with a decrease of Hb A2 in the electrophoresis result suggested for alpha thalassaemia, might be Hb Adana. However, the severity of anaemia should be further investigated in his mother.

The father could have Hb CS in view of the abnormal variant at zone 2/C zone suggestive of Hb CS, the result of the Hb 2/C zone in this patient might be inherited from his father.

Discussion

The diagnosis of thalassaemia in this patient was based on haematological parameters, which include the complete blood counts (CBC), haemoglobin analysis and molecular testing. Alpha thalassaemia generally shows varying degrees of anaemia depending on the chain affected. Our patient, diagnosed with transfusion-dependent thalassaemia (TDT) due to non-deletional Hb H disease presented with moderate to severe hypochromic anaemia with an increase in RDW indicating variation in the shape and size of erythrocytes. The CE showed a decrease in HbA, HbA2 with an increase in the Hb F fraction and the presence of Hb variants peak at zone2/C and zone 12 suggestive of Hb CS and Hb Bart's, which led to difficulties in interpreting the CE result.

Beta thalassaemia on Hb electrophoresis test showed decreased or even absent Hb A, increased Hb F, and normal or increased percentage of Hb A2. Molecular analysis is needed for diagnosis, especially for confirmation of carrier status, and genetic counselling. The synthesis of globin in normal individuals is regulated by four globin genes, two on each copy of chromosome 16 written as αα/αα genotype. Most alpha thalassaemia occur due to the deletion of one (-α) or both genes (--) on chromosome 16 (Harteveld 2010). While non-deletion alpha thalassaemia causes decreased chain synthesis and a more severe phenotypic appearance than abnormalities due to chromosomal deletions. Point mutations affecting the α-2 gene cause more severe abnormalities, because it normally expresses 2-3 times more -globin chains than the α-1 gene (Grosso 2012, Harteveld 2010).

An unexpected finding of non-deletional HbH disease without an H peak (zone 15), and an increase of Hb F posed a challenge for an accurate diagnosis based on interpretation from the CE result. Molecular testing was performed to determine the cause, either this patient suffered from alpha thalassaemia or the co-inheritance with beta thalassaemia. The Multiplex Amplification Refractory Mutation System (MARMS)-PCR technique uses different PCR multiplexes consisting of five MARMS-A, B, C, D, E and one ARMS-F also done to confirm there was no possibility of alpha beta thalassaemia mutations. Each type of MARMS PCR uses a different specific primer. Six regions of interest beta-globin globin gene in a reaction containing common oligoprimers and normal oligonucleotides or mutations for some of the more

common mutations found such as IVS-1 nucleotide 1 or IVS-1 nucleotide 5, codon 26, and IVS-2 nucleotide 654 region. Multiple primers were used in different sizes of PCR products and enable detection after the amplification process. This method simplifies mutation detection and allows for automation with the use of fluorescent-tagged primers and can detect 20 different mutations in the β-globin gene. Direct sequencing can be performed as confirmation for negative results (Hanafi et al, 2014). Molecular test results of this patient showed a compound heterozygous state of α2 codon 59 (GGC > GAC) Hb Adana (NG 000006.1:g.34071G>A) and α2 codon CD142 (TAA > CAA) Hb Constant Spring (Hb CS) (NG 000006.1:g.34461T>C). Multiplex ARMS PCR for diagnosis of beta thalassaemia confirmed no common beta thalassaemia mutation.

A study suggested that CE will give a peak at Zone 2 for Hb CS. Another variant that shares the same peak as Hb CS (and with similar clinical presentation) are Hb Paksé, Hb C, Hb F Texas, Hb C-Harlem, and variant Hb A2 'Setif (Ramli et.al, 2023). Haemoglobin C is a hereditary hemoglobinopathy. It is an autosomal recessive disorder that results from the biparental inheritance of the allele that codes for Haemoglobin C (Bachir and Galactors, 2004; Karna et al,2023). The abnormal haemoglobin C in this patient and his father was obtained with a percentage of <1%, so it is unlikely an Hb C. However, HbC (Z2) zone in this patient and his father might be caused by Hb CS. Meanwhile, the Z12 zone represented Hb Bart (Zhang et.al, 2018). The most common manifestation of α-thalassaemia (α-thal) syndrome is Hb H disease, even though it is basically caused by deletional mutations but it may also arise from coinheritance of α0- thalassaemia with non-deletional mutations or with abnormal Hb variants such as Hb Constant Spring. HbH disease patients usually present with mild symptoms, normal growth and development, and bearable anaemia without requiring regular blood transfusions, but patients with non-deletional α-thal mutations almost always present with a more severe phenotypic expression than those with large deletion ones (Tampaki et.al,2020). Patients of Hb Adana in combination with non-deletional mutations mostly showed more severe symptoms than those who had it with single gene deletion. It is seen from the blood transfusion status and earlier age at onset of clinical manifestation (Nainggolan et. al, 2013)

A high level of HbF is mainly due to pathological conditions such as β-thalassaemia major, δβ-thalassaemia (as γ globin chains compensate for the lack of functional β-globin chains), erythropoietic stress and bone marrow malignancies, or nonpathological conditions, known as HPFH, such as large deletions within the β-globin gene cluster (deletional HPFH), promoter variants of γ globin genes (non-deletional HPFH) or pregnancy (Curcio, 2019). An earlier study showed that high levels of Hb F tend to be inherited but the genetic inheritance pattern is not clearly known and does not correlate with beta-globin gene clusters. Persistent Hb F and F cells in the population show that the variation is not derived from one locus but comes from a combination of several genes from different chromosomes, indicating that it does not always follow Mendel's law (Carrocini et. al., 2011). It was also reported that the combination of alpha thalassaemia and high Hb F levels led to the S/S phenotype (Milner et. al., 1986). A previous study showed mutations in the KLF1 gene trigger a series of benign human red blood phenotypes, such as an increase in HbA2 and HBF in α-thalassaemia patients (Satta et.al, 2017; Keikhaei et.al, 2018). Testing of KLF1 gene mutation may be beneficial for confirming the cause of increased HBF in alpha thalassaemia. Molecular analysis for other uncommon beta gene mutations or delta beta gene by MLPA or sequencing would be beneficial for confirming the cause of Hb F increase in this patient, but this was not done due to the limitation of facilities to perform the tests in Indonesia.

Conclusion

Our patient presented with transfusion-dependent thalassaemia with low Hb A and Hb A2 levels, abnormal fraction eluted at zone 2 and zone 12 on CE and this has led to a presumptive diagnosis of non-deletional (Hb Adana and Constant spring) Hb H disease. Increased Hb F in this non-deletional alpha thalassaemia patient was not inherited from both parents. It might be caused by marrow response to anaemia or the MARMS PCR for the common beta gene was negative but the possibilities of concomitant presence of delta beta thalassaemia abnormalities need to be further confirmed with a deletional study by MLPA.

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