Diagnosis of Compound Heterozygous Hb Tak/ β -Thalassemia and HbD-Punjab/ β -Thalassemia by HbA₂ Levels on Capillary Electrophoresis

Sitthichai Panyasai¹ · Supachai Sakkhachornphop² · Sakorn Pornprasert³

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Abstract A misdiagnosis of β -thalassemia carrier in samples with Hb Tak and HbD-Punjab, the β -variants, can be a cause of inappropriate genetic counseling thus having a new case of β -thalassemia major. A capillary electrophoresis (CE) is very efficient in separating and quantifying HbA₂. In this study, HbA₂ levels of samples which were doubted for compound heterozygous Hb Tak/ β -thalassemia or heterozygous HbD-Punjab/ β -thalassemia were measured and compared between CE and high performance liquid chromatography (HPLC). The molecular confirmation for Hb Tak, HbD-Punjab and β -thalassemia codons 17 (A > T), 41/42 (-TCTT), 71/72 (+A) and IVSI-nt1 (G > T) mutations and 3.4 kb deletion were also performed. Based on DNA analysis, 3 cases were diagnosed as compound heterozygous Hb Tak/ β -thalassemia and one for HbD-Punjab/ β -thalassemia. The elevated HbA₂ levels were found in all 4 samples with ranges of 4.6–7.3% on CE while those were not found on HPLC. Thus, the elevated HbA₂ measured by CE can be used as a screening parameter for differentiating the homozygote of Hb Tak and HbD-Punjab from the compound heterozygote of these hemoglobinopathies and β -thalassemia.

Keywords Capillary electrophoresis · HbA₂ · HbD-Punjab · Hb Tak · HPLC

Introduction

Thalassemia and hemoglobinopathy are inherited hemoglobin (Hb) disorders that result in a production of structurally abnormal Hb variants or a reduction in the synthesis of structurally normal Hb, consequently, to red cell pathology. There are over 1000 abnormal hemoglobins which resulting from alterations in the α -, β -, γ - and δ -globin chains [1]. Many Hb variants are unstable or have an altered oxygen affinity and also in the heterozygous form may be associated with clinical and hematological manifestations (i.e. hemolytic anemia, or erythrocytosis). Furthermore, the homozygous form or co-inheritance together with other thalassemia and hemoglobinopathies can result in severe disease. Moreover, the homozygous form of hemoglobinopathy must be carefully differentiated from the co-inheritance form of α - or β -thalassemia and hemoglobinopathy. Because the misdiagnosis, especially during premarital screening, can be a cause of having a child with thalassemia major as reported by Belhoul et al. [2]. Generally, the hemoglobin analysis for β -thalassemia and hemoglobinopathy is performed by high performance liquid chromatography (HPLC), low pressure liquid chromatography (LPLC) and capillary electrophoresis (CE) [3]. On CE, common hemoglobinopathies including HbS [β 6(A3)Glu $\rightarrow$ Val; HBB: c.20A > T], HbC [β 6(A3)Glu $\rightarrow$ Lys; HBB: c.19G > A], HbD-Punjab [β 121(GH4)Glu $\rightarrow$ Gln; HBB: c.364G > C], and HbE [β 26(B8)Glu $\rightarrow$ Lys; HBB: c.79G > A] migrate separately from HbA₂ and they do not interfere the HbA₂ quantification [3, 4]. Thus, CE is very efficient in separating and

✉ Sakorn Pornprasert
sakornmi001@gmail.com

¹ School of Allied Health Sciences, University of Phayao, Phayao, Thailand

² Research Institute for Health Sciences, Chiang Mai University, Chiang Mai, Thailand

³ Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, 110 Intawaroros Road, Chiang Mai 50200, Thailand

quantifying HbA₂. The aim of this study is to demonstrate that whether the HbA₂ measured by CE can be used as a screening parameter for differentiating between the homozygotes of HbD-Punjab or Hb Tak (HBB:c.441_44 2insAC) and the compound heterozygotes of these hemoglobinopathies and β -thalassemia.

Materials and Methods

Blood Samples and Hematologic Analysis

This study was approved by the Institutional Review Board (IRB) of the University of Phayao, Phayao, Thailand (Ethical approval number HE5403010001). The ethylenediamine tetraacetic acid (EDTA) anti-coagulated blood samples were collected from 4 Thai subjects at the Associated Medical Sciences Clinical Service Center, Chiang Mai University, Chiang Mai, Thailand for thalassemia and hemoglobinopathy diagnosis. The Hb, packed cell volume (PCV), and red blood cells indices including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were estimated with an automated blood counter (Sysmex KX-21, Sysmex Corporation, Kobe, Japan). Quantification of HbA₂ (for detection of β -thalassemia) and identification of hemoglobinopathies were performed using CE (CapillarysTM 2 Flex Piercing, Sebia, Norcross, Georgia, USA) and HPLC (VARIANT II, β -thalassemia Short Program, Bio-Rad Laboratories, Hercules, California, USA).

Molecular Analysis

The genomic DNA was extracted from the blood sample by using the NucleoSpin[®] kit (Macherey–Nagel, KG., Duren,

Germany) according to manufacturers' instructions. The DNA was stored at -20°C until used. The α -thalassemia-1 South-East Asian (SEA) and Thai type deletions were detected by using real-time PCR with SYBR Green1 high resolution melting (HRM) analysis as previously described [5, 6]. In addition, the α -thalassemia-2 ($-\alpha^{3.7}$ and $-\alpha^{4.2}$ kb deletions), Hb Constant Spring (HBA2:c.427T > C) and Hb Paksé (HBA2: c.429A > T) mutations were analyzed by using the PCR methods as described elsewhere [7, 8]. The allele-specific (AS)-PCR for detection of HbD-Punjab, Hb Tak and the common β -thalassemia in South-East Asia including the codons 17 (A > T), 41/42 (-TCTT), 71/72 (+A) and IVSI-nt1 (G > T) mutations and the Gap-PCR for detection of β -thalassemia 3.4 kb deletion were also performed, according to protocols described previously [9–11].

Results

The 290 bp amplified fragment from Hb Tak allele (Fig. 1a) and the 329 bp amplified fragment from HbD-Punjab allele (Fig. 1b) were observed in the 3 and 1 samples, respectively. In addition, the 445 and 286 bp amplified fragments from β -thalassemia condons 41/42 and IVSI-nt1 mutations, respectively (Fig. 2a) and 496 bp amplified fragments from β -thalassemia 3.4 kb deletion (Fig. 2b) were found in these samples. The DNA sequencing demonstrated gene mutations for Hb Tak, Hb HbD-Punjab and β -thalassemia at codons 41/42 and IVSI-nt1 mutations and 3.4 kb deletion (data not shown). The negative analysis results for α -thalassemia-1 and -2 deletions and Hb CS and Hb Pakse' mutations were observed in all samples. Based on the DNA analysis, 3 samples were therefore diagnosed as having a compound heterozygous

Fig. 1 The agarose gel electrophoresis for: **a** The allele-specific (AS)-PCR for detection of the Hb Tak. The produce fragments of 290 and 676 bp specific for Hb Tak and internal control fragment, respectively, are indicated. Lane 1 Hb Tak carrier; lane 2 normal control; lanes 3–6 the subjects. M represents the GeneRuler 100 bp

DNA ladder. **b** The AS-PCR for detection of the HbD-Punjab. The produce fragments of 329 and 578 bp specific for HbD-Punjab and internal control fragment, respectively, are indicated. Lane 1 HbD-Punjab carrier; lane 2 normal control; lanes 3–6 the subjects. M represents the GeneRuler 100 bp DNA ladder

Fig. 2 The agarose gel electrophoresis for: **a** The AS-PCR for detection of β -thalassemia mutations. The 578 bp internal control fragment and 445 and 286 bp amplified fragments from β -thalassemia codons 41/42 and IVSI-nt1 mutations, respectively, are indicated. Lanes 1 and 2 positive control for β -thalassemia codons 41/42 and IVSI-nt1 mutations, respectively. Lane 3 normal control; lanes 4–7 the subjects. M represents the GeneRuler 100 bp DNA ladder. **b** The

Gap-PCR for detection of β -thalassemia 3.4 kb deletion. The produce fragments of 496 and 578 specific for β -thalassemia 3.4 kb deletion and internal control fragment, respectively, are indicated. Lane 1 positive control for β -thalassemia 3.4 kb deletion, lane 2: normal control; lanes 3–6 the subjects. M represents the GeneRuler 100 bp DNA ladder

Table 1 The characteristics and hematological parameters of the samples

Characteristics and parameters	Sample no. 1	Sample no. 2	Sample no. 3	Sample no. 4
Gender-age (years)	Female-32	Male-17	Male-33	Female-7
RBC ($10^{12}/L$)	8.3	5.7	9.1	6.3
Hb (g/L)	190	141	209	115
PCV (L/L)	0.60	0.41	0.66	0.33
MCV (fL)	72.0	72.4	73.1	52.5
MCH (pg)	22.8	24.7	23.0	18.1
MCHC (g/L)	317	341	315	345
<i>Hb analysis by CE</i>				
Hb Tak or HbD-Punjab (%)	91.6	94.9	90.2	81.9
HbA (%)	0.0	0.0	0.0	10.8
HbA ₂ (%)	6.5	4.6	7.0	7.3
HbF (%)	0.0	0.5	0.0	0.0
<i>Hb analysis by HPLC</i>				
Hb Tak or HbD-Punjab (%)	86.1	79.8	86.5	80.7
HbA (%)	1.3	5.8	2.9	5.1
HbA ₂ (%)	3.4	1.7	3.3	3.2
HbF (%)	7.3	8.6	4.2	4.9
β -globin genotype	$\beta^{\text{Tak}}/\beta^{41/42}$	$\beta^{\text{Tak}}/\beta^{41/42}$	$\beta^{\text{Tak}}/\beta^{\text{IVSI-nt1}}$	$\beta^{\text{D-Punjab}}/\beta^{3.4 \text{ kb}}$

Normal ranges for the measurements elaborated in the table are as follows: red blood cell counts $4.2\text{--}6.1 \times 10^{12}/L$; hemoglobin 120–180 g/L; packed cell volume 0.37–0.52 L/L; MCV 80–100 fL; MCH 27–31 pg; MCHC 320–360 g/L; HbA > 85%; HbA₂ 1.5–3.5%; HbF 0–1%

Hb Tak/ β -thalassemia (2 samples with codons 41/42 and 1 with IVSI-nt1 mutations) and 1 sample with a compound heterozygous HbD-Punjab/ β -thalassemia (3.4 kb deletion). Their characteristics and hematological parameters are shown in Table 1. Their MCV and MCH were lower than the reference ranges. The erythrocytosis with increased RBC (8.3 and $9.1 \times 10^{12}/L$) and PCV (0.60 and 0.66 L/L) were found in 2 samples with Hb Tak/ β -thalassemia. The

sample with HbD-Punjab/ β -thalassemia had the lowest levels of Hb, PCV, MCV, and MCH when compared to those of samples with Hb Tak/ β -thalassemia. On CE electrophoregram, the increased HbA₂ levels (>4%) were observed in all 4 samples while those were not found on the HPLC chromatograms. On the other hand, the elevated HbF levels (>1%) were found on HPLC chromatogram but not on CE electrophoregram (Table 1).

Fig. 3 Representative HPLC chromatogram and CE electrophoregram of samples with compound heterozygous Hb Tak/β-thalassemia (a, b respectively) and compound heterozygous HbD-Punjab/β-thalassemia (c, d, respectively)

Discussion

The secondary polycythemia with increased RBC and PCV was found in 2 of 3 (67%) samples with Hb Tak/β-thalassemia while the patient with HbD-Punjab/β-thalassemia had a lower level of PCV than the reference range. Hb Tak is a high oxygen affinity thus it has been known as an uncommon cause of secondary erythrocytosis. The secondary polycythemia was found not only in samples with Hb Tak/β-thalassemia but also in samples with homozygous Hb Tak and compound heterozygous Hb Tak/HbE [12, 13].

The phenotypic findings of cases with compound heterozygous Hb Tak/β-thalassemia or HbD-Punjab/β-thalassemia have been fairly consistent with a mild anemia (Hb values between 80 and 120 g/L), occasionally splenomegaly, and typical thalassemic indices and morphological changes of the red cells [14]. The compound heterozygous Hb Tak/β-thalassemia and HbD-Punjab/β-thalassemia must be carefully differentiated from homozygous form of these hemoglobinopathies during premarital screening, especially when the partner has β-thalassemia trait as there is a 25%

risk for having a child with β-thalassemia major [2]. The HbA₂ level is used as a diagnostic marker for β-thalassemia trait. On HPLC, Hb Tak and HbD-Punjab elute close to HbA₂ and they generally lead to underestimated HbA₂ and elevated HbF levels (Fig. 3a, c, respectively) while these hemoglobinopathies migrate separately from HbA₂ on CE (Fig. 3b, d, respectively). Thus, they do not interfere the HbA₂ and HbF quantification [3, 4]. The elevated HbA₂ levels (>4%) quantified by microchromatographic analysis had been reported in three cases of compound heterozygous HbD-Punjab/β-thalassemia in Kuwait [15]. The consistently raised HbA₂ levels (4.0–5.6%) by manual column chromatography were also reported in three males with compound heterozygous HbD-Punjab/β-thalassemia from three unrelated families in Iran [16]. However, some cases of compound heterozygous HbD-Punjab/β-thalassemia had HbA₂ levels measured by column chromatography or HPLC lower than 4.0% [17, 18]. In addition, the normal value of HbA₂ (1.7%) and the raised HbF (4.3%) on HPLC was also reported in the 7 year-old Thai boy who has compound heterozygous Hb Tak/β-thalassemia [19]. Therefore, these studies suggest that HbA₂ measured by column

chromatography or HPLC is not a reliable parameter for differentiating the homozygote of Hb Tak or HbD-Punjab from the compound heterozygote of these Hb Tak or HbD-Punjab with β -thalassemia. Das et al. [20] compared HbA₂ levels measured by the CE and the four HPLC devices in samples with compound heterozygous HbD-Punjab/ β -thalassemia and found that only the CE showed a reliable HbA₂ values. In the present study, the elevated HbA₂ levels (>4%) were found in all samples when they were analyzed by CE. Therefore, the CE can be used as a screening method for differentiating the homozygote of Hb Tak or HbD-Punjab from the compound heterozygote of these hemoglobinopathies and β -thalassemia.

In conclusion, the misdiagnosis of β -thalassemia trait in samples with Hb Tak and HbD-Punjab can be a cause of inappropriate genetic counseling and having a new case of β -thalassemia major. The CE was proved useful in quantifying HbA₂ level, a reliable parameter for differentiating the homozygote of Hb Tak and HbD-Punjab from the compound heterozygote of these hemoglobinopathies and β -thalassemia.

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Compliance with Ethical Standards

Conflict of interest The authors report no conflicts of interest.

Ethical Standard This study was approved by the Institutional Review Board (IRB) of the University of Phayao, Phayao, Thailand (Ethical Approval Number HE5403010001).

Informed Consent The informed consent was obtained from the subjects included in the study.

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