Detection of Compound Heterozygous of Hb Constant Spring and Hb Q-Thailand by Capillary Electrophoresis and High Performance Liquid Chromatography

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Received: 20 February 2014 / Accepted: 22 May 2014 / Published online: 13 June 2014
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Abstract A capillary electrophoresis (CE) has proven to be superior to a high performance liquid chromatography (HPLC) in the detection of hemoglobin Constant Spring (Hb CS). Thus the aim of this study was to analyze the efficacy of CE and HPLC for the detection of Hb CS in samples with compound heterozygous of Hb CS and Hb Q-Thailand. Hemoglobin analysis was performed in blood samples of 2 patients with compound heterozygous of Hb CS and Hb Q-Thailand by using HPLC and CE. The HPLC chromatogram and CE electrophoregram of the two techniques were compared. Hb CS was not found on HPLC chromatogram while Hb QA₂ ($\alpha_2^{\text{QT}}\delta_2$), a derivative of Hb Q-Thailand, was presented at the retention time of 4.70–4.80 min and it was close to the retention time of Hb CS. On CE electrophoregram, Hb CS was presented at zone 2 (Z2) and it was distinctly separated from Hb QA₂ which was presented at Z1. Therefore, CE was more efficient to the HPLC for diagnosis of compound heterozygous of Hb CS and Hb Q-Thailand.

Keywords Capillary electrophoresis · Hemoglobin typing · Hb CS · Hb Q-Thailand · High performance liquid chromatography

Introduction

Hemoglobin Constant Spring (Hb CS) is the most prevalent nondeletional α -thalassemia among the Southeast Asian

population. This abnormal hemoglobin results from point mutation at the stop codon of $\alpha 2$ -globin gene (TAA > CAA) which leads to the addition of 31 amino acids to a normal α -globin sequence [1, 2]. The heterozygous of Hb CS is clinically and hematologically normal however the homozygous of Hb CS shows a clinical picture as thalassemia intermedia with mild anemia, jaundice and hepatosplenomegaly. Moreover, co-inheritance of Hb CS with α -thalassemia-1 leads to Hb H-CS disease ($-\alpha^{\text{CS}}\alpha$) that may have clinical manifestation like β -thalassemia major [3]. Hb Q-Thailand is another α -globin chain variant that results from a point mutation (Asp → His, GAC > CAC) at codon 74 of the $\alpha 1$ -globin gene on chromosome 16p with a leftward single α -globin gene deletion ($-\alpha^{4.2}$). Because of the linked ($-\alpha^{4.2}$) α -thalassemia-2 allele, heterozygous or homozygous of Hb Q-Thailand usually shows slight anemia with a few red blood cell microcytosis [4]. Co-inheritance with α -thalassemia-1 leads to a clinical phenotype of the Hb Q-H disease with clinical features similar to the deletional Hb H disease [4, 5].

The prevalence of Hb CS and Hb Q-Thailand is high among the Thai population with a frequency of 1–8 % and 0.35 %, respectively [6, 7]. Therefore, a compound heterozygous of both abnormal genes can be found in this area. However, it is difficult to diagnose the compound heterozygous of Hb CS and Hb Q-Thailand because Hb CS mRNA is unstable and it causes the rate of α -globin chain synthesis to decrease [8, 9]. Moreover, some of the variants can co-elute and get masked on HPLC chromatogram and capillary electrophoresis (CE) electrophoregram [10, 11]. A CE has proven to be superior to a HPLC in detecting Hb CS [12]. Thus the aim of this study was to analyze the efficacy of CE and HPLC for detecting and quantifying Hb CS in samples with compound heterozygous of Hb CS and Hb Q-Thailand.

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Materials and Methods

Subject and Hemoglobin Analysis

Blood sample of two Thai women were collected in vacutainers with ethylenediamine tetraacetic acid (EDTA) as anticoagulant and submitted for thalassemia diagnosis, to the Associated Medical Sciences Clinical Service Center, Chiang Mai University, Chiang Mai, Thailand. Hb, hematocrit (Hct) and the mean corpuscular volume (MCV) of red blood cells were measured with an automated blood counter (Sysmex KX-21; Sysmex Corporation, Kobe, Japan). Quantification of Hb A₂ (for detection of β -thalassaemia) and identification of hemoglobinopathy was performed by using the HPLC (VARIANT II, β -thalassaemia Short Program, Bio-Rad Laboratories, Hercules, CA, USA). Furthermore, Hb Q-Thailand and Hb CS were analyzed in these blood samples within 24 h by using CE (CAPILLARY 2, Sebia, Norcross, GA, USA).

DNA Preparation and Molecular Analysis

Genomic DNA was extracted from the whole blood samples by using a NucleoSpin[®] kit (Macherey–Nagel, KG., Duren,

Germany) according to manufacturers' instructions. The DNA was then stored at -20°C until used. The molecular analysis for detection of α -thalassaemia-1 Southeast Asian (SEA) type deletion and Hb CS was performed by using real-time PCR with SYBR Green1 high resolution melting (HRM) analysis as previously described [13, 14]. The multiple allele specific polymerase chain reaction (ASPCR) was also performed for molecular diagnosis of Hb Q-Thailand as previously described [4, 15].

Results

The HPLC chromatogram and CE electrophoregram of two patients are shown in Fig. 1. The HPLC chromatogram and CE electrophoregram between the two patients were similar. On HPLC, a specific peak of Hb Q-Thailand was presented at the retention time of 4.63–4.65 min while Hb QA₂ ($\alpha_2^{\text{QT}}\delta_2$), a derivative of Hb Q-Thailand, was presented at the retention time of 4.70–4.80 min. Peaks of Hb CS which are usually seen at least one peak at the retention time of 4.73, 5.07 or 5.25 min, was not found on the HPLC chromatogram (Fig. 1a, c). On CE, Hb Q-Thailand co-migrated with Hb F ($\alpha_2\gamma_2$) and appeared at zone 7 (Z7) while Hb QA₂

Fig. 1 The HPLC chromatogram and CE electrophoregram of patients with compound heterozygous of Hb CS and Hb Q-Thailand. **a** and **b** presented the HPLC chromatogram and CE electrophoregram of the first patient while **c** and **d** presented those of the second patient

was present at Z1 (Fig. 1b, d). Moreover, a peak of Hb CS was found at the Z2 of CE electrophoregram. The levels of Hb, Hct and MCV of the first patient were 107 g/L, 0.34 L/L and 74.0 fL while those of the second patient were 108 g/L, 0.35 L/L and 66.0 fL, respectively. The ASPCR analysis and agarose gel electrophoresis showed a specific band for amplified fragment of Hb Q-Thailand (data not shown). Furthermore, the real-time PCR with SYBR Green1 HRM analysis showed the specific melting curve of Hb CS while it displayed a negative result for thalassemia-1 SEA type deletion (data not shown).

Discussion

On HPLC, Hb Q-Thailand and its derivative (Hb QA₂) were not distinctly separated but they were clearly observed on CE. Moreover, on HPLC chromatogram, Hb QA₂ was presented at the retention time of 4.70–4.80 min and it was close to a retention time of Hb CS. Thus, one peak of Hb CS at the retention time of 4.70 min may be co-eluted with Hb QA₂. However, other peaks of Hb CS at the retention time of 5.07 and 5.25 min were not found. On the other hand, a peak of Hb CS was clearly observed in Z2 of CE electrophoregram and it was distinctly separated from a peak of Hb QA₂ which was presented in Z1.

There are three α -globin gene defects including α^{QT} , $-\alpha^{4.2}$ and α^{CS} , in the compound heterozygous of Hb CS and Hb Q-Thailand. However, it does not result in the Hb H disease. Peak of Hb H and Hb Bart's were not found on HPCL and CE analysis. The previous studies showed that patients with compound heterozygous of Hb CS and Hb Q-Thailand had mild hypochromic microcytic anemia [4, 15] and it was consistent with the present study which showed that a patient had low levels of Hb and MCV. However, patients in the present study had a higher MCV than the patients with the compound heterozygous of Hb CS and Hb Q-Thailand who were reported by Singsanan et al., and it was quite similar to the MCV of 26 patients with heterozygous of Hb Q-Thailand [4]. Thus, a lower MCV might be due to the iron deficiency. Interestingly, a patient with compound heterozygous of Hb CS and Hb Q-Thailand had a level of Hb Q-Thailand >40.0 % which is higher than the heterozygous of Hb Q-Thailand [4]. A higher level of Hb Q-Thailand in the patient with compound heterozygous of Hb CS and Hb Q-Thailand could be explained by the defect of the three α -globin gene synthesis. Thus, a high level of Hb Q-Thailand (>40 %) on HPLC is useful in the detection of an uncharacterized mutation. However, it could not clarify the compound heterozygous of Hb CS and Hb Q-Thailand from that of α -thalassemia-2 deletion and Hb Q-Thailand. The decrease of ability to produce Hb A in compound heterozygous of Hb

CS and Hb Q-Thailand might generate a prominent peak of Hb CS. However, the Hb CS is unstable and some peaks of Hb CS may be co-eluted with Hb QA₂ on HPLC analysis.

The number of samples of heterozygous of Hb CS and Hb Q-Thailand used in this study is two, which is inadequate to conclude that CE had better efficacy than HPLC without conducting comprehensive diagnostic study. However, the similar patterns of HPLC chromatogram and CE electrophoregram were observed in those two samples. Thus, the CE might be a preferable method for detection of compound heterozygous of Hb CS and Hb Q-Thailand. In areas where the CE analysis is not available, the level of Hb Q-Thailand >40.0 % analyzed by HPLC may be an alternative method for screening of this combination and the confirmation test by DNA analysis for detection of Hb CS is recommended to be performed in these cases. Moreover, the parental hemoglobin analysis by HPLC or CE might facilitate the investigational direction for appropriate molecular analysis and genetic counseling.

In conclusion, the CE had a high efficacy for detecting and quantifying of Hb CS. It was superior to the HPLC for detection of the compound heterozygous of Hb CS and Hb Q-Thailand. Therefore, this method is essential for providing accurate diagnosis, genetic counseling, prevention and control programs of thalassemia and hemoglobinopathy.

Conflict of interest The authors report no conflict of interest.

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