Original Article

Screening for Rare and Novel β-Globin Gene Mutations by High Resolution Melting Analysis

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Abstract:

Rare and novel β-globin gene mutations could be misdiagnosed among patients with α- and β-thalassemia using routine Hb typing and DNA analysis. In coinheritance of α- and β-thalassemia, Hb A_2 might be lower than normal level and thus arouses no suspicion for the presence of β-thalassemia. Although the common β-globin gene mutations in a given region are detected in routine DNA tests, cases of co-inherited α-thalassemia can be missed in detecting β-thalassemia without prior indication. High resolution melting (HRM) analysis was used to scan β-globin genes in 140 samples. Rare and novel β-globin gene mutations were identified in three cases of β-thalassemia trait, namely, HBB: c.2T>G, Hb Monroe [HBB:c.92G>C] together with nt-42 [HBB: c.-92C>G] mutation and 14-nucleotide (+AGGGCAATAATTTC) insertion downstream of IVSII-561 together with IVSI-1 [HBB:c.92+1G>T] mutation. In addition, Hb Agenogi [HBB:c.271G>A] was present in one case of α-thalassemia trait, and among three cases of Hb H disease one co-inherited Hb Korle-bu [HBB:c.220G>A] and two nt-28 [HBB:c.-78A>G] mutations. Thus, HRM scanning for β-globin gene mutations provides a useful tool in providing information for counseling at risk couples.

Keywords : ● *High resolution melting (HRM) analysis* ● *α-Thalassemia* ● *β-Thalassemia* ● *Hemoglobin variant* ● *β-globin gene mutation*

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นิพนธ์ต้นฉบับ

การตรวจหาการกลายพันธุ์ชนิดหายากและชนิดใหม่บนยีนเบต้าโกลบินด้วยวิธี High Resolution Melting Analysis

ภัทรียา เหล่าชินชาติ และ สุมาลี จินดาดำรงเวช
ภาควิชาพยาธิวิทยา คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล

บทคัดย่อ

แม้ว่าในงานประจำวันจะสามารถตรวจการกลายพันธุ์ของยีนเบต้าโกลบินชนิดที่พบบ่อยได้ แต่เป็นการตรวจเพื่อยืนยันชนิดของการกลายพันธุ์ ที่มีการวินิจฉัยเบื้องต้นแล้วว่าเป็นเบต้าธาลัสซีเมีย ในผู้ป่วยที่มีแอลฟาและเบต้าธาลัสซีเมียร่วมกัน ค่า HbA_2 อาจลดลงจนอยู่ในช่วงค่าปกติ ทำให้พลาดการวินิจฉัยเบื้องต้นว่ามีเบต้าธาลัสซีเมียได้ และการตรวจดีเอ็นเอในงานประจำวันไม่ได้รองรับการกลายพันธุ์ของยีนเบต้าโกลบินชนิดหายากและชนิดใหม่ การวิจัยนี้ใช้เทคนิค High resolution melting (HRM) analysis ตรวจหาการกลายพันธุ์ของยีนเบต้าโกลบินใน 140 ตัวอย่าง พบว่ามีการกลายพันธุ์ของยีนเบต้าโกลบินชนิดหายากและชนิดใหม่ใน 3 ตัวอย่าง ของกลุ่มพาหะเบต้าธาลัสซีเมีย คือ HBB:c.2T>G, Hb Monroe [HBB:c.92G>C] พบร่วมกับ nt-42 [HBB:c.-92C>G] และการกลายพันธุ์ชนิดมีเบสเพิ่มขึ้น 14 เบส (+AGGGCAATAATTTC) ที่ IVSII-561 พบร่วมกับ IVSI-1 [HBB:c.92+1G>T] ใน 1 ตัวอย่าง ของพาหะแอลฟาธาลัสซีเมีย พบการกลายพันธุ์ของยีนเบต้าโกลบินชนิด Hb Agenogi [HBB:c.271G>A] ส่วนใน 3 ตัวอย่างของกลุ่มโรค Hb H พบว่ามี 1 ตัวอย่างพบ Hb Korle-bu [HBB:c.220G>A] และ 2 ตัวอย่างพบการกลายพันธุ์ชนิด nt-28 [HBB:c.-78A>G] ดังนั้นการใช้เทคนิค HRM เพื่อตรวจหาการกลายพันธุ์ของยีนเบต้าโกลบินมีประโยชน์ด้านการป้องกัน และควบคุมการเกิดธาลัสซีเมียชนิดรุนแรงในกลุ่มประชากรที่ต้องการวางแผนครอบครัว

คำสำคัญ : ● *High resolution melting (HRM) analysis* ● *α-Thalassemia* ● *β-Thalassemia* ● *Hemoglobin variant* ● *β-globin gene mutation*

วารสารโลหิตวิทยาและเวชศาสตร์บริการโลหิต 2560;27:241-50.

Introduction

Beta (β)-thalassemia is the most common hematological disease in Southeast Asia, including Thailand, with a prevalence of 3-9%.[1,2] Almost all are caused by point mutations resulting in reduced or the absence of β-globin chain synthesis.[3] β-Thalassemia diagnosis plays an important role in providing counseling information to couples in this region of the world.

Currently, diagnosis of β-thalassemia is based on clinical and hematological data including hemoglobin (Hb) typing; however, confirmation requires genotyping.[4] In routine Hb typing, relatively elevated HbA_2 (> 3.5%) level is indicative of β-thalassemia trait.[5] This may not be the case when a subject is a carrier of both β- and α-thalassemia. As rare and novel mutations are not included in routine DNA analysis of β-globin gene, this can lead to false-negative results, impacting correct pre-natal counseling. This problem can be avoided by sequencing across the whole β-globin gene region, but this technique remains too costly on a routine basis. This report describes the application of high resolution melting (HRM) analysis of amplicons across the whole β-globin gene[6-8] allowing detection of putative rare or novel mutations identified by subsequent DNA sequencing. This strategy allowed us to identify 6 rare β-globin mutations and one novel β-thalassemia mutations among 140 samples.

Materials and Methods

Samples

Surplus blood samples from routine thalassemia screening were obtained from the Blood Disease Diagnostic Laboratory, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand.

Thalassemia screening consisted of complete blood count (CBC) using a Sysmex XS-1000i automated blood cell counter (Kobe, Japan), and Hb typing using Capillarys 2 capillary electrophoresis (CE) (Sebia, Lisses, France). Genotyping of common β- and α-thalassemia present in Thailand were identified by multiplex PCR as previously described.[9, 10] Thirty presumptive non-thalassemia, 30 identified β-thalassemia trait, 40 identified α-thalassemia 1 trait and 40 Hb H disease samples were subjected to HRM analysis. Of 140 cases, 91 were females and 49 were males with ages ranging from 1-79 years (median = 30 years).

The study protocol was approved by the Ethics Committee of Ramathibodi Hospital, Mahidol University (MURA2012/23).

Methods

HRM analysis was conducted using six primer sets (P1-P6) (Table 1) to amplify regions of known common mutations found in the Thai population,[9] viz. nt-87[HBB:c.-137C>G], nt-31[HBB:c.-81A>G], nt-28[HBB:c.-78A>G], codon17[HBB:c.52A>T], codon19[HBB:c.59A>G], IVSI-1[HBB:c.92+1G>T], codon41/42[HBB:c.126_129delCTTT], codon71/72[HBB:c.216_217insA], IVSII-654[HBB:c.316-197C>T], and codon126[HBB:c.380T>G] (Figure 1). The 20-ml PCR mixture consisted of 50 ng of genomic DNA, 4 μL of 5X Colorless Go*Taq* Flexi Buffer (Promega, Madison WI, USA), 1.2-2.9 mM $MgCl_2$, 0.2mM dNTPs, 0.3-0.8 μM each primer pair, 0.65 U Go*Taq* DNA polymerase and 0.5 μL LightCycler 480 ResoLight Dye (Roche Diagnostics, Penzberg, Germany). PCR amplification was performed in a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Penzberg, Germany) using the following thermocycling conditions: 94° for 5 minutes; followed by 40 cycles of 94° for 20 seconds, annealing temperature appropriate for each primer set (Table 1) for 60 seconds and 72°C for 45 seconds. HRM analysis was conducted by heating at 95°C for 1 minute, then at 40°C for 1 minute, followed by 60 - 90°C at 25 acquisitions/°C for fluorescence signal detection. Data were analyzed using LightCycler 480 Gene Scanning Software, Version 1.5 (Roche Diagnostics) and presented as difference plots.

All 140 samples were analyzed using HRM analysis. Samples positive by HRM scanning (i.e. having different HRM pattern from wild type and common mutation controls) were subsequently identified by DNA sequencing at the U2Bio (Thailand) Co., Ltd, Thailand.

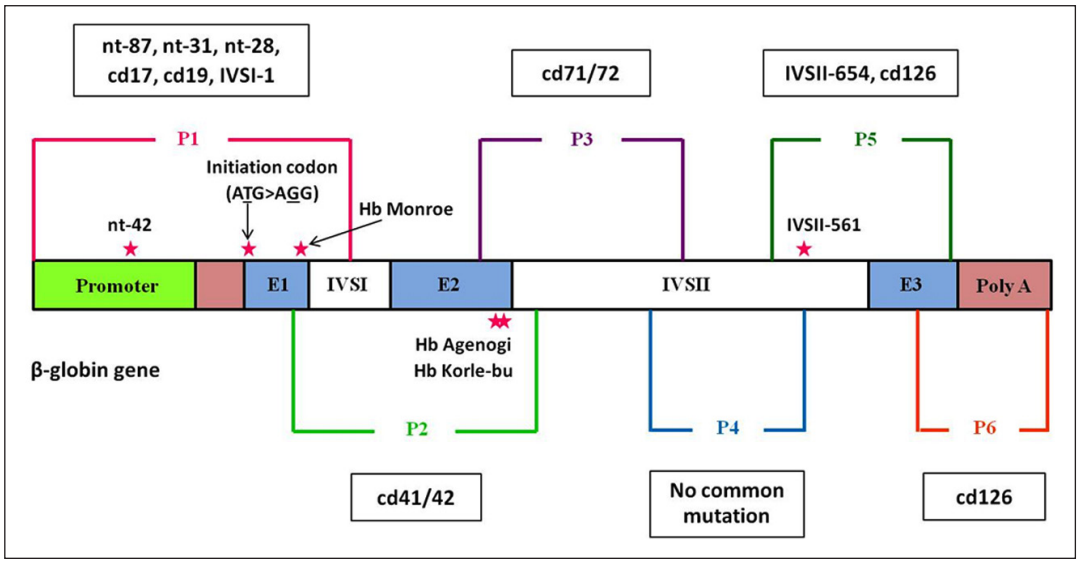

Figure 1 Diagram indicating the locations of primers used in high resolution melting profiling of the β-globin gene. Box indicates location and common β-thalassemia mutations in Thailand[10]. *denotes rare and novel β-thalassemia mutations identified in this study.

Table 1 Primers used in the study

Primer	Sequence 5'- 3'	Amplicon size (bp)	$T_{annealing}$ (°C)
P1-F	CTGTCATCACTTAGACCTCACCCTG	336	57
P1-R	GAGTCTTCTCTGTCTCCACATGCC		
P2-F	TGGTATCAAGGTTACAAGACAGGTT	373	57
P2-R	CATCAAGCGTCCCATAGACTCAC		
P3-F	GCACCTTTGCCACACTGAG	406	66
P3-R	CCAAATAGTAATGTACTAGGCAGACTG		
P4-F	ACTTTACACAGTCTGCCTAGTACATTA	317	57
P4-R	CTTTAGAATGGTGCAAAGAGGCAT		
P5-F	CTTTCAGGGCAATAATGATACAATG	366	65
P5-R	ACCACTTTCTGATAGGCAGCCT		
P6-F	TGCTGGCCCATCACTTTG	297	65
P6-R	TGCACTGACCTCCCACATTC		

F, forward; R, reverse. Reference sequence NG_000007.3.

Results

HRM analyses were consistent with multiplex PCR-based β-globin genotyping of all 30 nonthalassemia, 25/30 β-thalassemia trait, 39/40 α-thalassemia 1 trait and 37/40 Hb H disease samples (Table 2). DNA sequencing of amplicons with unidentified HRM profiles, having a different HRM pattern from the wild type and common mutation controls, (Figure 2) revealed among the five β-thalassemia trait samples, one α-thalassemia one trait sample and three Hb H disease samples. Regarding the β-thalassemia trait samples, two cases had compound heterozygosity 14-nucleotide (+AGGGCAA TAATTTC) insertion downstream of IVSII-561[HBB:c. 316-290_316-289insAGGGCAATAATTTC] with IVSI-1 [HBB:c.92+1G>T] mutation (sample U1), and Hb Monroe [HBB:c.92G>C] with nt-42 [HBB: c.-92C>G] mutation (sample U3)], a case of HBB:c.2T>G initiation codon mutation (sample U2), a case of SNP at IVSII-657 [HBB:

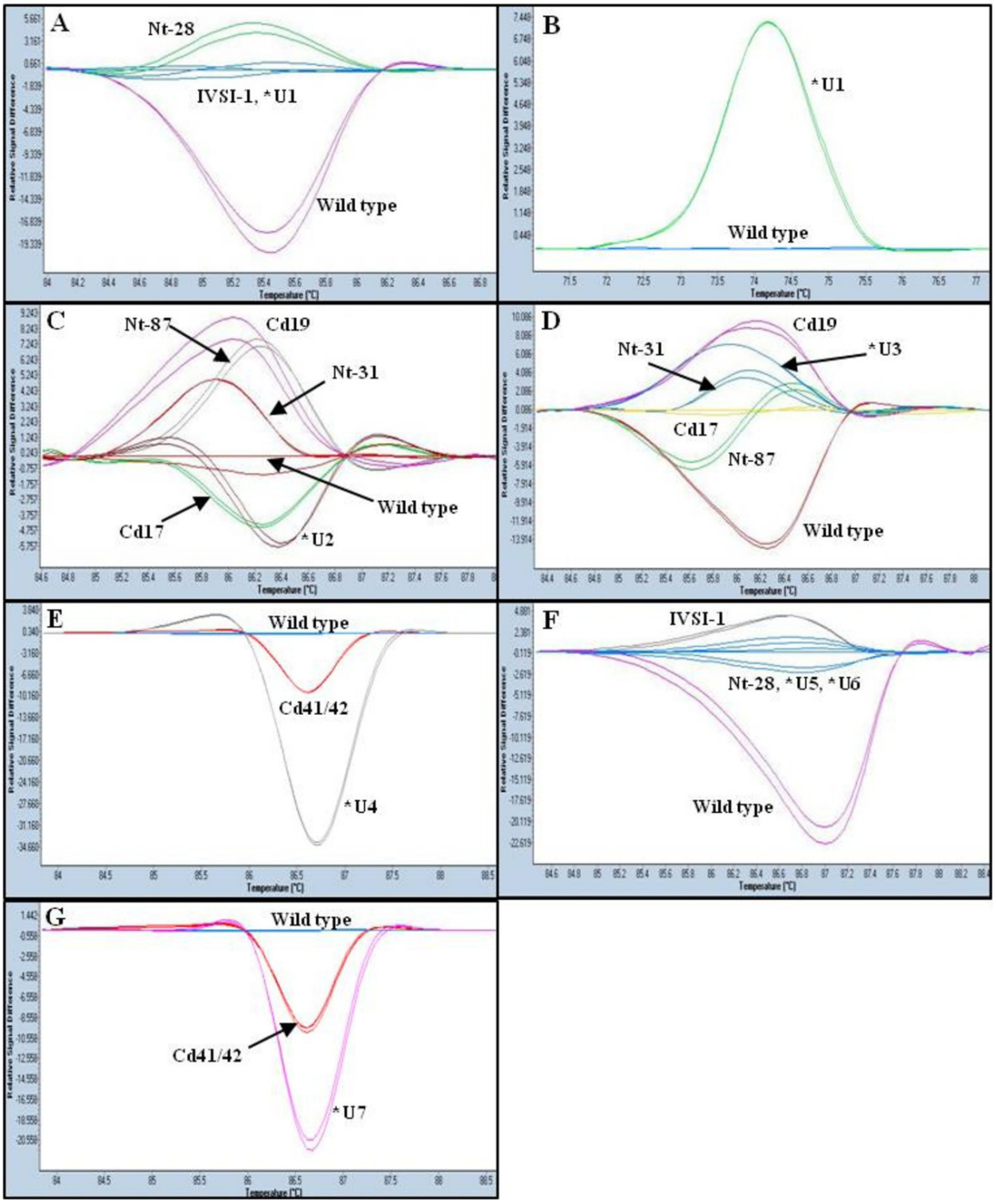

Figure 2 High resolution melting (HRM) profiles of samples U1-U7 with β-thalassemia mutations unidentified from comparison with HRM profiles of known common mutations in Thailand

Table 2 High resolution melting (HRM) analysis of 140 samples

Sample (n)	HRM analysis	
	Known profile (n)	**Unidentified profile* (n)**
Nonthalassemia (30)	Wild type (30)	None
β-thalassemia trait (30)	Cd41/42 (11)	Initiation codon (ATG>AGG) (1)
	IVSII-654 (5)	Hb Monroe (1)
	Nt-28 (2)	IVSII-561 (+14 bp) (1)
	Cd19 (2)	SNP at IVSII-657 (T>C)(1)
	IVSI-1 (2)	SNP rs1609812 (1)
	Nt-28 (1)	
	Nt-31 (1)	
	Nt-92 (1)	
	Cd17 (1)	
	IVSI-5 (1)	
	Cd71/72 (1)	
	Cd126 (1)	
	Wild type[a] (1)	
α-thalassemia 1 trait (40)	Wild type (38)	Hb Agenogi (1)
		SNP rs713040 (T>C) (1)
Hb H disease (40)	Wild type (37)	Hb Korle-bu (1)
	Nt-28 (2)	

*Genotyped by sequencing: [a]No β-globin gene mutation was found by PCR-based genotyping and DNA sequencing

c.316-194T>C] and SNP rs1609812 [HBB: c.316-185C>T] present with codon 71/72 [HBB:c.216_217insA] mutation (sample U4), and one nonthalassemia case, previously identified as β-thalassemia trait by Hb typing (sample U5). The single α-thalassemia one trait sample had Hb Agenogi [HBB:c.271G>A] together with SNP rs713040 [HBB:c.9T>C] (sample U6) and among the three Hb H disease samples had nt-28 [HBB:c.-78A>G] trait (samples U7and U8) and Hb Korle-bu [HBB:c.220G>A] (sample U9) (Figure 3). Hematological data of these nine cases are shown in Table 3.

Discussion

In this study, HRM analysis of amplicons covering the entire β-globin gene region in 140 samples allowed us to detect 8 β-globin mutations not identified by multiplex PCR method. This demonstrated the utility of conducting HRM analysis to confirm identification of the β-globin mutations and to reveal any false negative results, thereby obviating the need to sequence all samples under investigation. Among the nine samples one novel mutation (samples U1), three rare Hb variants (samples U3, U6, and U9) and six known β-thalassemia mutations (samples U1, U2, U3, U4, U7, and U8) were found. The misidentification and false negative results from PCR-based technique (without confirmation by DNA sequencing) highlight the sensitivity of HRM profiling in screening for β-thalassemia traits.

High Hb A_2 level (> 3.5%) is used as a presumptive diagnosis of β-thalassemia trait.[5] Notably, no false negatives were present among the 30 nonthalassemia samples. However, co-inheritance of α-thalassemia trait or Hb H disease negates this criterion. In addition, genotyping for β-thalassemia and Hb variants, preferably uses HRM profiling as it detects common mutations present in the data set as well as unknown mutations,

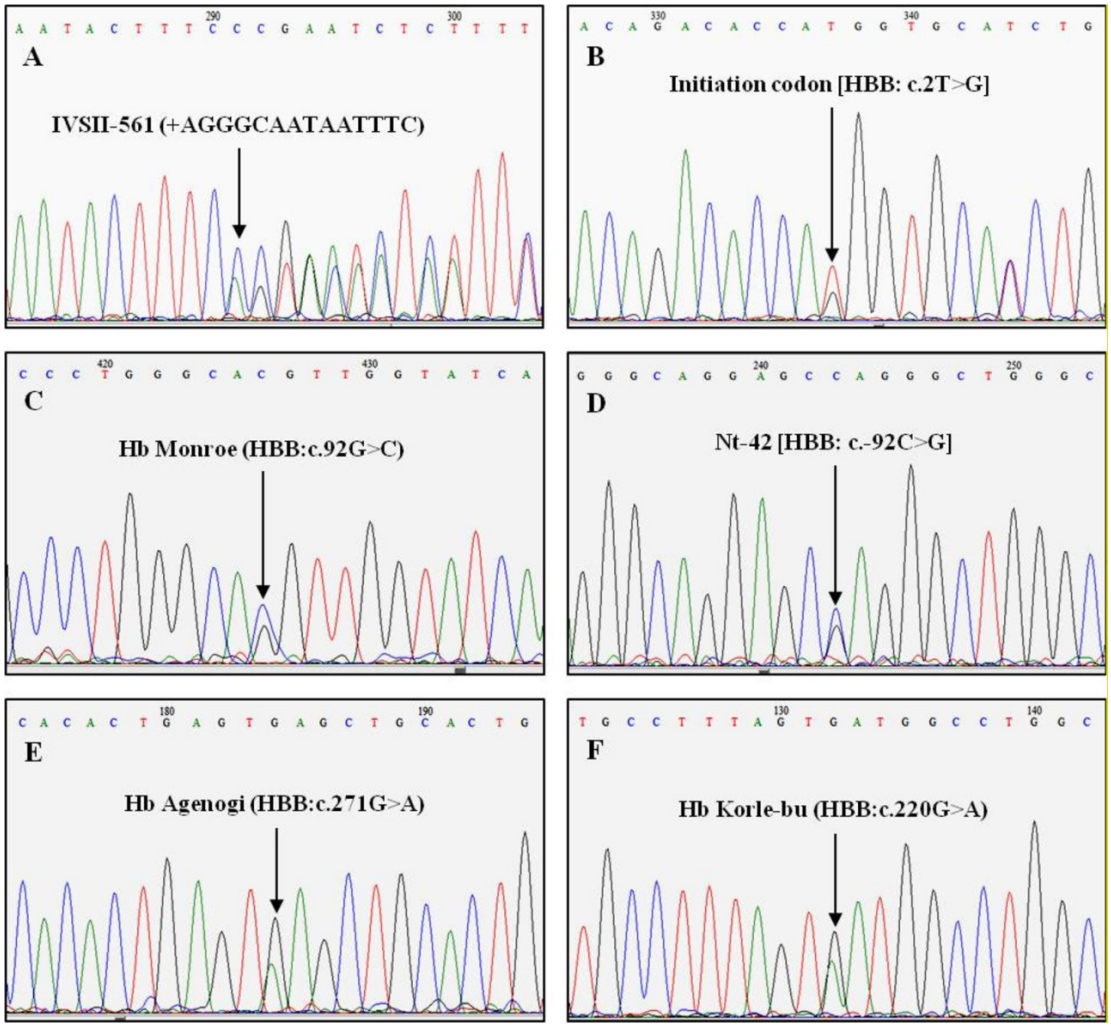

Figure 3 DNA sequencing chromatogram of amplicons of samples U1-U7 unidentified by high resolution melting analysis: Panel A, U1; panel B, U2; panel C and D, U3; panel E, U4; panel F, U7.

which can subsequently be identified by DNA sequencing, as exemplified in this study.

The novel insertion mutation in IVSII of case U1 was predicted to generate a cryptic acceptor splice site at IVSII-593 using the Splice View Program of WebGene (http://www.itb.cnr.it/webgene/) with score of 92 and using the NNSPLICE 0.9 version of Splice Site Predictor (http://www.fruitfly.org/seq_tools/splice.html) with a score of 0.97, producing a β^+-thalassemia phenotype. Moreover, the patient subsequently received a diagnosis of iron deficiency anemia with a ferritin level of 2.2 ng/mL and serum iron level of 21 mg/dL. Thus, the two mutations of β^0 and β^+ were suspected to occur in *cis* consistent with β-thalassemia trait Hb typing results, whereas anemia in case U1 was caused by iron deficiency (Table 3).

The rare initiation codon (case U2) leads to a β^0-thalassemia phenotype and has been reported in Chinese, Korean and Thai families.[11-14] Hematological data of case U2 was more consistent to thalassemia intermedia than β-thalassemia trait, though exhibiting extremely low Hb F, but no other mutation was found suggesting the other conditions such as severe iron deficiency anemia were present. Unfortunately, the patient was lost to follow-up. Hb Monroe (case U3), also known as Hb Kairouan, is an unstable hemoglobin previously reported in African-American, Bangladeshi, Indian, Iranian, Tajikistan and Tunisian families,[15-19] but this is the first case reported in Thailand. The related report in the Tajikistan subject homozygous Hb Monroe is co-inherited with heterozygous nt-42, indicating that HBB:c.92G>C is present with nt-42 in *cis*.[19] Whether

Table 3 Hematological data and thalassemia typing of samples U1-U9

Samples	Sex	Age (years)	Parameters								
			RBC (10^6cell/uL)	Hb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	RDW (%)	Hb typing	α-thalassemia genotype	β-globin genotype
U1	F	26	5.33	8.1	26.6	49.9	15.2	20.7	β-thal trait (A_2 = 4.8%, F < 1%)	Negative	IVSI-1 [HBB:c.92+1G>T] and IVSII-561[HBB:c.316-290_316-289ins AGGGCAATAATTTC]
U2	F	27	5.95	9.9	29.6	49.7	16.6	20.0	β-thal trait (A_2 = 6.4%, F < 1%)	Negative	Initiation codon [HBB: c.2T>G]
U3	F	ND	6.07	11.1	41.4	68.2	18.3	18.4	β-thal trait (A_2 = 5.8%, F < 1%)	Negative	Nt-42 [HBB: c.-92C>G] and Hb Monroe [HBB: c.92G>C]
U4	F	41	4.59	9.4	28.0	61.0	20.5	14.2	β-thal trait (A_2 5.8 = %, F = 1.3%)	Negative	Cd71/72 [HBB:c.216_217insA], SNPs at IVSII-657 [HBB:c.316-194T>C] and rs1609812 [HBB: c.316-185C>T]
U5	M	ND	5.31	13.9	43.9	82.7	26.2	13.7	β-thal trait (A_2 3.9 =%, F = 3.2%)	Negative	Negative
U6	M	42	6.23	13.1	41.2	66.1	21.0	14.9	A_2A (A_2 = 2.6%, F < 1%)	α-thal 1 trait	Hb Agenogi [HBB: c.271G>A] and SNP rs713040 [HBB:c.9T>C]
U7	M	1	6.76	10.1	29.4	43.5	14.9	22.6	Hb H disease (A_2 = 4.3%, F = 4.7%)	Hb H disease	Nt-28 [HBB: c.-78A>G]
U8	M	1	7.44	10.6	31.8	42.7	14.2	23.1	Hb H disease (A_2 = 3.4%, F = 15.4%)	Hb H disease	Nt-28 [HBB: c.-78A>G]
U9	F	ND	5.03	8.7	28.7	57.1	17.3	24.8	Hb H disease with abnormal Hb (A_2 = 1.1%, abnormal Hb = 46.9%)	Hb H disease	Hb Korle-bu [HBB: c.220G>A]

F: female; M: male; ND: no data

this represented the situation with case U3 is unknown; however, the hematological picture of β-thalassemia trait suggested only one allele of β-globin gene was affected. Mild anemia in case U4 was identified as β-thalassemia trait because the patient was HIV positive.

Co-inheritance of β-thalassemia with α-thalassemia trait or Hb H disease results in normal percent Hb A_2 level, and HRM profiling provides a convenient means of detecting and identifying this genotype. Hb Agenogi (case U6) did not appear as an abnormal peak during routine Hb typing using Capillarys-2 CE (data not shown). Hb Agenogi has previously been reported in Japanese and Sicilian families[20,21] but this poses the first reported case in Thailand. Heterozygosity of this Hb variant shows normal hematological data and clinically asymptomatic presentation. Moreover, the abnormal hematological parameters of one case U6 is probably due to the presence of α-thalassemia 1 trait ($--^{SEA}$). However, Hb Korle-bu (case U9) appeared as an abnormal Hb peak in Hb typing (data not shown). This Hb variant has previously been described in English and Thai families.[22,23] The presence of Hb Korle-bu did not affect the Hb H disease condition of case U9 (Table 3). On the other hand, co-inheritance of nt-28 (A>G), a β^+-thalassemia, with Hb H disease (cases U7 and U8) resulted in less anemia with higher total hemoglobin and hematocrit (Table 3).

In conclusion, HRM profiling to detect β-globin gene mutations has proven to be a sensitive and reliable method and should be considered the technique of choice in such screening programs.

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Declaration of conflict of interests: The authors report no conflicts of interest.

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