

Original Article

Screening for Uncommon Nondeletional α -Globin Gene Mutations Causing Severe Hb H Disease in a Thai Population Using High Resolution Melting Analysis

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Abstract:

A number of uncommon nondeletional α -thalassemia (*thal*) 2 mutations leading to severe forms of Hb H (α -thal 1/ α -thal 2) have been overlooked by routine screening techniques. High resolution melting (HRM) analysis protocols were developed to detect six known rare α -thal 2 alleles in this study including initiation codon del A, ATG>TG (*HBA2:c.1delA*; α 2 initiation codon Met>0), initiation codon del T, ATG>AG (*HBA2:c.2delT*; α 2 Initiation codon Met>0), alpha2 codon 30 deletion CAG (*HBA2:c.91_93delGAG*; α 2 30(B11) Glu>0), Hb Quong Sze (*HBA2:c.377T>C*; α 2 125(H8) Leu>Pro), donor splice site mutation at IVSI-I (*HBA1:c.95+1G>A*), and Hb Queens Park (*HBA1:c.98T>A*; α 1 32(B13) Met>Lys). These mutations were associated with severe forms of Hb H disease in Thailand. When the optimized HRM technique was performed in 100 random blood samples it showed heterozygous Hb Westmead (*HBA2:c.369C>G*; α 2 122(H5) His>Gln) in 3 samples and 5 samples carried *HBA2:c.43(G>C)* polymorphism differing from the normal controls. Therefore, HRM analysis is a useful technique that can help clinicians recognize several rare mutations in Hb H disease.

Keywords : ● α -Globin gene mutation ● Hb H disease ● High resolution melting (HRM) analysis
● Quantitative PCR, α -Thalassemia

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นิพนธ์ต้นฉบับ

การตรวจคัดกรองการกลยยพันธุ์ชนิดที่พบน้อยบนยีนอัลฟ่าชานิด Non-Deletion ที่ก่อให้เกิด Hb H Disease ชนิดรุนแรงในกลุ่มคนไทยด้วยวิธี High Resolution Melting (HRM) Analysis

สรวิภากรณ์ สุวรรณสิงห์ สุรีรัตน์ ลรร้อยโมรา และ สุมาลี จินดาดำรงเวช
ภาควิชาพยาธิวิทยา คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล

บทคัดย่อ

เนื่องจากการกลยยพันธุ์ของ α -thalassemia (thal) 2 ชนิดที่พบไม่ถูกค้นพบ หลายชนิดสามารถก่อให้เกิด Hb H disease ชนิดที่รุนแรงได้ แต่ไม่สามารถพบจากการตรวจวิเคราะห์ประจำวันทั่วไปได้ ดังนั้นในงานวิจัยครั้งนี้จึงได้มีการพัฒนาวิธี High resolution melting (HRM) analysis สำหรับการตรวจหา α -thal 2 อัลลิลชนิดที่พบยากที่ทราบชนิดแล้ว จำนวน 6 ชนิด ได้แก่ initiation codon del A, ATG>TG (HBA2:c.1delA; α 2 Initiation codon Met>0), initiation codon del T, ATG>AG (HBA2:c.2delT; α 2 initiation codon Met>0), alpha2 codon 30 deletion CAG (HBA2:c.91_93delGAG; α 2 30(B11) Glu>0), Hb Quong Sze (HBA2:c.377T>C; α 2 125(H8) Leu>Pro), donor splice site mutation at IVSI-I (HBA1:c.95+1G>A) และ Hb Queens Park (HBA1:c.98T>A; α 1 32(B13) Met>Lys) ซึ่งการกลยยพันธุ์เหล่านี้พบว่าเกี่ยวข้องกับการเกิด Hb H disease ชนิดที่รุนแรง ในประเทศไทย เมื่อใช้เทคนิค HRM ที่พัฒนาขึ้นเพื่อทดสอบในตัวอย่างเลือดแบบสุ่มจำนวน 100 ตัวอย่าง พบ heterozygous Hb Westmead (HBA2:c.369C>G; α 2 122(H5) His>Gln) จำนวน 3 ตัวอย่าง และ 5 ตัวอย่างมี HBA2:c-43(G>C) polymorphism แตกต่างจากตัวอย่างควบคุมปกติ ดังนั้น HRM analysis เป็นวิธีที่มีประโยชน์ซึ่งสามารถช่วยให้แพทย์ตรวจพบการกลยยพันธุ์ที่พบได้ยากอีกหลายชนิดที่ก่อให้เกิด Hb H disease ได้

คำสำคัญ : ● α -Globin gene mutation ● Hb H disease ● High resolution melting (HRM) analysis

● Quantitative PCR, α -Thalassemia

วารสารโลหิตวิทยาและเวชศาสตร์บริการโลหิต 2559;26:365-72.

Introduction

An alpha thalassemia (α -thalassemia) carrier inherits either a deletion of one (α -thal 2) or both duplicated (α -thal 1) α -globin genes.¹ A high prevalence of α -thalassemia has been reported in many countries in Southeast Asia and in Thailand with a prevalence of 30 to 40%.² Compound heterozygosity of α -thal 1 and α -thal 2 gives rise to Hb H disease, which usually presents mild or symptomless anemia.²⁻³ However, a severe form of Hb H disease can occur, with the occasional case of hydrops owing to the presence of nondeletional α -thal 2 allele.⁴⁻⁸ In Thailand, nondeletional mutation of Hb H disease is found mainly in Hb Constant Spring (HBA2:c.427T>C; α 2 142 Stop>Gln) and Hb Pakse (HBA2:c.429A>T; α 2 142 Stop>Tyr).⁹⁻¹¹ However, other rare types of α -globin gene mutations have also been reported, such as initiation codon del A, ATG>TG (HBA2:c.1delA; α 2 initiation codon Met>0),¹² initiation codon del T, ATG>AG (HBA2:c.2delT; α 2 initiation codon Met>0),¹³ alpha2 codon 30 deletion CAG (HBA2:c.91_93delGAG; α 2 30(B11) Glu>0),¹⁴ Hb Quong Sze (HBA2:c.377T>C; α 2 125(H8) Leu>Pro),¹⁵⁻¹⁶ donor splice site mutation at IVSI-I (HBA1:c.95+1G>A)¹² and Hb Queens Park (HBA1:c.98T>A; α 1 32(B13) Met>Lys).^{12,17} The presence of the causative Hb variant in severe Hb H disease is often missed in routine Hb screening due to their small contents and mutation identification generally relies on a number of DNA-based methods.¹⁸⁻¹⁹ However, reliance on primer-specific PCR-based methods allows only known common mutations to be recognized.²⁰⁻²⁴ Although techniques, such as denaturing high pressure liquid chromatography (DHPLC),²⁵ and DNA sequencing,²⁶ have been used to detect unknown mutations, these techniques suffer from such disadvantages as being labor intensive, inconvenient, complicated to perform,

time-consuming and costly; hence, not fit for large scale screening.

High resolution melting (HRM) analysis is one of the PCR-based techniques mainly used for genotyping and gene scanning of genetic variations.²⁷⁻²⁸ A melting profile is performed routinely following quantitative PCR amplification to confirm the unique presence of the desired amplicons with no spurious off-target amplifications.²⁹⁻³⁰ In this study, HRM analysis was optimized to detect six uncommon α -globin gene mutations presented in severe Hb H disease and the protocols were validated for use in mutation scanning of Thai individuals.

Materials and Methods

Samples

Plasmids and genomic DNA of six uncommon α -globin mutations coinherited with α^0 -thalassemia (α -thal 1)SEA deletion from individuals with severe Hb H disease were used in this study (Table 1).¹²⁻¹⁷ The exact sequences of these six mutations and five normal control DNA samples were determined by nucleotide sequencing (see below). In this study, 100 random DNA samples from the Blood Disease Diagnostic Laboratory, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand were analyzed for complete blood count (CBC) and routine α -thalassemia DNA analysis before screening for the presence of these six α -globin mutations using the optimized HRM analysis protocols. The study was approved by the Ethics Committee on Human Rights Related to Research Involving Human Subjects, Ramathibodi Hospital, Mahidol University.

PCR and HRM analysis

Three pairs of HRM primers (P1, P2 and P3) were designed to cover the regions of the six α -globin gene

Table 1 Primers used in HRM analysis of α -globin gene mutations

| Primer (5'- 3') | Mutation |
|-----------------------------|---|
| P1F (ATAAACCTGGCGCGCTC) | HBA2:c.1delA; α 2 Initiation codon Met>0 |
| P1R (GGTGGGAAAGGACAGGAACAT) | HBA2:c.2delT; α 2 Initiation codon Met>0 |
| P2F (ACCCTCTCTCTGCACAGCTCC) | HBA2:c.91_93delGAG; α 2 30(B11) Glu>0 |
| P2R (GGTCAGCACGGTGCTCACA) | HBA2:c.377T>C; α 2 125(H8) Leu>Pro |
| P3F (GCGAGTATGGTGCGGAGG) | HBA1:c.95+1G>A; donor splice site GT>AT |
| P3R (CTTCTTGCGTGGCCCTTA) | HBA1:c.98T>A; α 1 32(B13) Met>Lys |

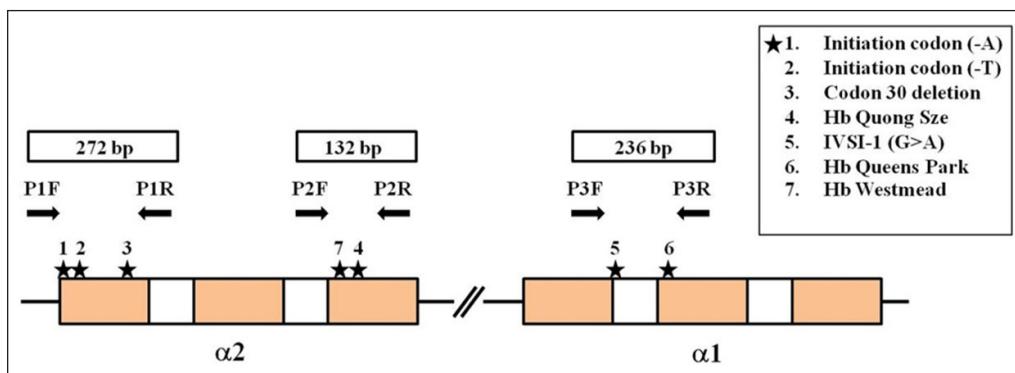


Figure 1 Location of primers used in HRM analysis of 6 α -globin gene mutations. Amplicon sizes were indicated in the boxes.

mutations of interest (Figure 1). The primers were designed using the Primer Express[®] Software Version 3.0 Program (Applied Biosystems, Thermo Fisher Scientific Inc., Foster City, CA, USA) and synthesized from Biodesign Co., Ltd, Thailand (Table 1). Nucleotide sequences of α -globin genes were acquired from NCBI reference sequence database, accession no. NG_000006.1 (<http://www.ncbi.nlm.nih.gov/>).

Results

HRM analysis of uncommon α -globin gene mutations associated with severe Hb H disease

DNA sequences of the six α -globin gene mutations were confirmed by DNA sequencing and then used to optimize HRM analysis protocols. Interestingly, all normal control samples ($n = 5$) had HBA2:c-43(G>C) polymorphism of exon 1 of the *HBA2* allele that differed from the NCBI reference sequence database (accession no. NG_000006). Each mutation, irrespective from genomic or plasmid DNA, manifested distinctive HRM plots that could be differentiated from each other and from normal controls (Figure 2). Mutations present in either *HBA1*(IVSI-1 (G>A) mutation and Hb Queens Park) or *HBA2* (initiation codon (-A) deletion, initiation codon (-T) deletion, codon 30 deletion, and Hb Quong Sze) allele could be detected. The great precision of repeatability and reproducibility of HRM protocols were gained. Each of six mutations and normal controls demonstrated its specific HRM patterns and was clustered in the correct group. Acceptable results were still obtained using DNA and $MgCl_2$ concentrations in the ranges of 40-60 ng and 1.0-1.5 mM, respectively.

Incorrect grouping and unreliable HRM patterns were found in high concentrations of DNA and $MgCl_2$ of 100 ng and 2.0 mM, respectively.

HRM screening for α -globin gene mutations in 100 random DNA samples

From 100 random DNA samples obtained from the Blood Disease Diagnostic Laboratory, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, 82 samples had normal CBC while 18 samples had slightly abnormal red blood cells with microcytosis and hypochromia (MCV = 62.9-79.7 fL, MCH = 19.9-26.4 pg). One case with low MCV (62.9 fL) was identified as homozygous Hb E coinherited with heterozygous α -thal 2 ($-\alpha^{37}$ deletion), the others involved three cases of heterozygous Hb E coinherited with heterozygous $-\alpha^{37}$ deletion, four cases of heterozygous Hb E coinherited with heterozygous Hb CS, seven cases of heterozygous α -thal 2 ($-\alpha^{37}$ deletion), and three cases of heterozygous Hb CS. All the 100 samples showed HRM profiles of normal α -globin gene using P3 primer sets. However, P1 and P2 primer sets identified nonnormal sequences by showing unknown the HRM pattern in five and three samples, respectively (Figure 2D and 2E). DNA sequencing revealed HBA2:c-43(G>C) polymorphism indicating that the five tested samples with unknown HRM pattern carried "G" the same as the reference sequence (NCBI reference sequence database, accession no. NG_000006). However, the five normal controls as well as the 95 tested samples carried "C" at this position. DNA sequencing of the latter three samples with unknown HRM patterns resulted in Hb Westmead (HBA2:c.369C>G; α 2 122(H5) His>Gln) (Figure 3).

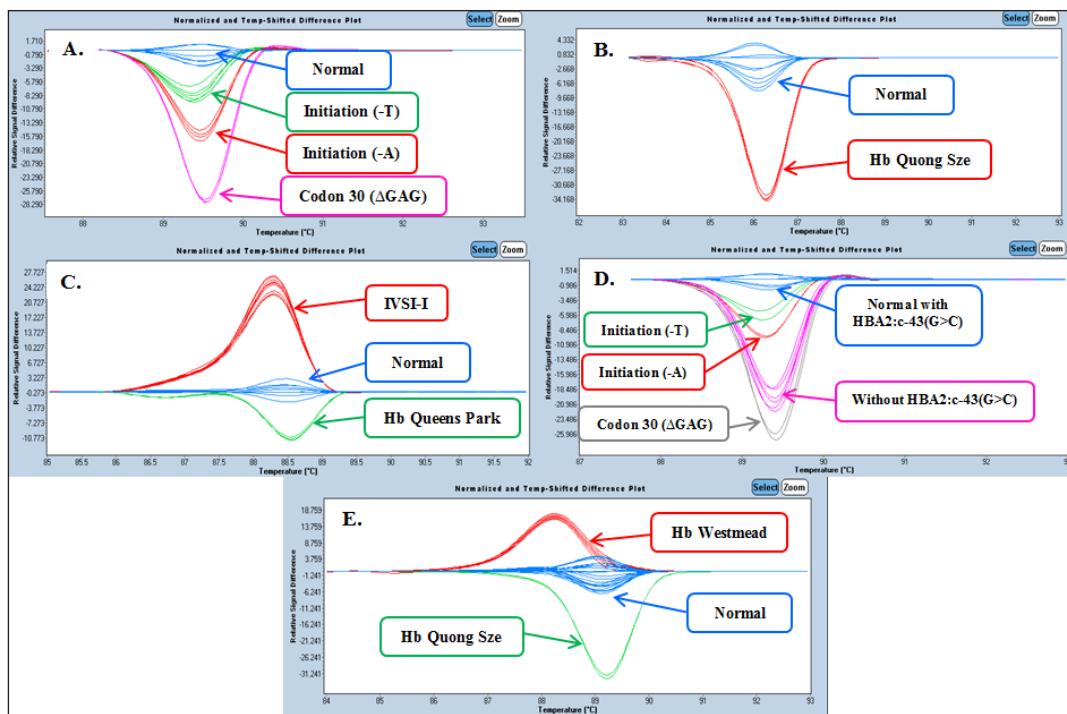


Figure 2 HRM profiles of α -globin gene mutations in Thai samples. Curves are different plots relative to normal controls. Panel **A**, amplicon generated by P1 primer set; panel **B**, amplicon generated by P2 primer set; panel **C**, amplicon generated by P3 primer set; panel **D**, amplicon generated by P1 primer set; panel **E**, amplicon generated by P2 primer set.

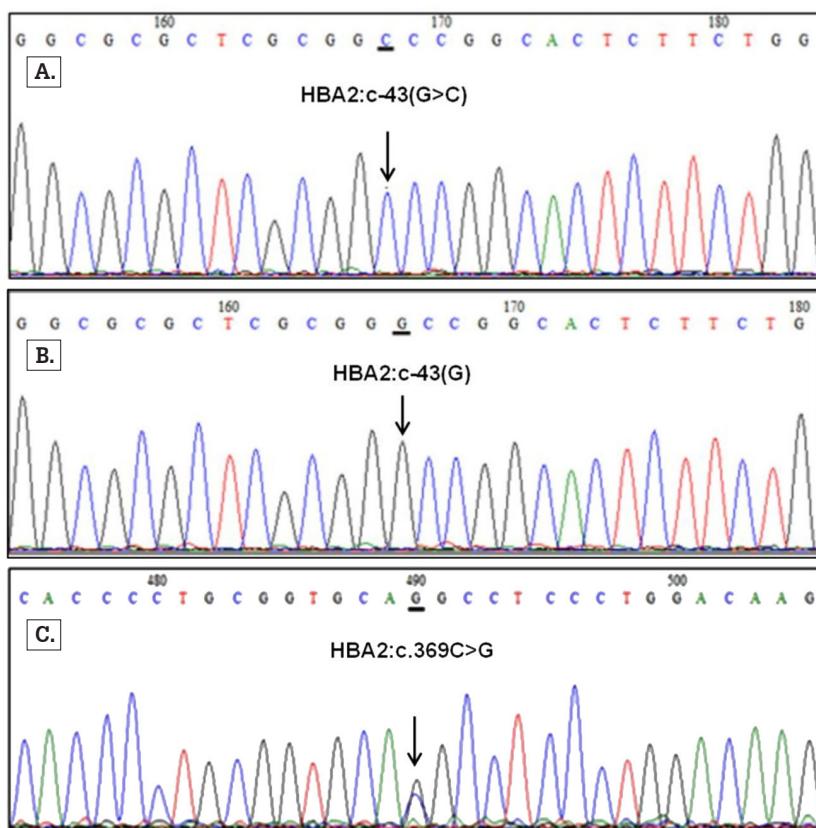


Figure 3 DNA sequencing chromatograms of polymorphisms at the position -43(G>C) of exon 1 of the *HBA2* gene [HBA2:c-43(G>C)] found in (A.) our normal control samples and 95 out of 100 random samples and (B.) normal sequences from the reference sequence (NCBI, NG_000006) and of 5 random samples positive for HRM unknown pattern. (C.) Heterozygosity of Hb Westmead (HBA2:c.369C>G; α 2 122(H5) His>Gln)

Discussion

HRM analysis provides a simple, rapid and sensitive means of screening or verifying genetic variations, including those present in the globin gene clusters.³¹⁻³⁴ This technique relies on the altered melting behavior of target DNA sequence combining to the ds-DNA binding dye, which is essential for the melting curve or melting profile production. In this study, three new primer sets were designed to apply HRM-based identification of six uncommon nondeletional α -thal 2 associated with severe forms of Hb H disease presented in Thailand.¹²⁻¹⁷ The protocol was validated for precision of repeatability and reproducibility. A difference in a single nucleotide sequence could be clearly distinguished even though the difference in T_m values was $<0.4^\circ\text{C}$, e.g., between initiation codon (-A) and initiation codon (-T) mutation.²⁷

The size of target PCR products was important for successful in HRM analysis in that short PCR products enhanced the resolution and efficiency of HRM results. In the recent study, sensitivity and specificity of 100% were achievable when target PCR product lengths were 300 bp or less, while sensitivity and specificity were reduced to 96.1% and 99.4%, respectively when PCR products were 400 to 1,000 bp.³⁵ In this study, all three HRM primers sets were designed to generated PCR product lengths less than 300 bp (272 bp, 132 bp, and 236 bp for P1, P2, and P3 primers, respectively) which was efficient for mutations or SNPs screening. Because shorter PCR products increased the differences of T_m values, the difference of HRM melting profiles of wild types and mutation types resulted in clearly differentiated groups of the different genotypes. To increase the efficiency of HRM analysis in detecting a single base pair difference, ensuring that the saturated DNA binding dye does not affect DNA polymerase activity or change melting temperature (T_m) value is necessary. No redistribution back to bind double strand DNA occurs again.³⁶ Moreover, any other mutation in the same amplicon could be discerned from the HRM different profiles. This was clearly exemplified by

the identification of HBA2:c-43(G>C) of exon 1 of the HBA2 gene in three samples and Hb Westmead in five samples from 100 random blood samples. Hb Westmead, commonly found in Guangxi Province, China, is rare in Thailand.¹² HBA2:c-43(G>C) polymorphism appears to be the common type among the Thai population, but more study is needed.

Conclusion

High resolution melting analysis (HRM or HRMA) is one molecular technique commonly used for scanning or screening variant mutations in target DNA sequences. Our new HRM scanning analysis protocols were successful in establishing the HRM melting pattern profiles of each known mutation set up. Moreover, other variant mutations in the vicinity of HRM primers could be detected such as Hb Westmead and HBA2:c-43(G>C) polymorphism.

The HRM method was originally devised to identify uncommon nondeletional α -thal 2 alleles. However, when they present together with α -thal 1 chromosome giving rise to a more severe Hb H disease, it has been proven to be useful for screening α -globin gene mutations in general. The ease and robustness of the HRM technique will make this type of gene mutation analysis a valuable tool in genetic counseling among patients with α -thalassemia and other hereditary diseases.

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