Original Article

Molecular Characterization of α^0-Thalassemia in Patients with Hemoglobin H (Hb H) Disease and in Hb Bart's Hydropic Fetuses

Chantawat Kantamool, Siriwan Ong-chai* and Torpong Sanguansermsri**

Department of Biochemistry, Phramongkutklao College of Medicine, Bangkok, *Department of Biochemistry and **Pediatrics, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

Abstract: *The most common α^0-thalassemia deletion in Thai populations is the so-called Southeast Asian ($-^{SEA}$) deletion. In addition, there have been several reports among Thai and Southeast Asian populations involve two other deletions, the Thai ($-^{THAI}$) and Filipino ($-^{FIL}$) deletions. But, in Thailand, the detection of α^0-thalassemia gene carrier in a routine clinical laboratory was only ($-^{SEA}$) deletion. Thus carriers of non-SEA deletion cannot be identified using the routine PCR of the ($-^{SEA}$) deletion. In this report, we developed multiplex PCR-based protocols for rapid and reliable DNA diagnosis for the ($-^{SEA}$), ($-^{THAI}$), and ($-^{FIL}$) deletions. The multiplex PCR assays were carried out on 114 genomic DNA samples from patient with Hb H disease. 113 of 114 samples were heterozygous for ($-^{SEA}$) deletion while the other one was heterozygous for ($-^{THAI}$) deletion. Furthermore, we analyzed genomic DNA of 100 Hb Bart's hydropic fetuses. Only two out of 100 cases were of compound heterozygote for the ($-^{SEA}$) and ($-^{THAI}$) deletions: ($-^{SEA}/-^{THAI}$), the remaining 98 cases were homozygous for the common ($-^{SEA}$) deletion: ($-^{SEA}/-^{SEA}$). None of them has the ($-^{FIL}$) deletion. From this study, we expected that this method shouldefficiently facilitate the genetic screening and molecular diagnosis of these deletions in Thai populations.*

Key Words: ● *α^0-Thalassemia* ● *Hb H disease* ● *Multiplex PCR* ● *Southeast Asian deletion*

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Introduction

The frequency of alpha zero (α^0)-thalassemia in Thai population is one of highest in the world. The most common deletion is the so-called Southeast Asian ($-^{SEA}$) deletion. This deletion spans approximately 20.5 kilobases, removing both functional α-globin genes while leaving the zeta ($\zeta 2$)-globin gene intact. Definitive diagnosis of the ($-^{SEA}$) deletion is usually based on polymerase chain reaction (PCR) using primers that flank the deletion breakpoints. Although the ($-^{SEA}$) deletion is by far the most prevalent in cis deletion in Thailand, there have been several reports among Thai and Southeast Asian populations

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involve two other deletions, a complete deletion of the $\zeta\alpha$ complex on one chromosome. They are called the Thai ($—^{THAI}$) and Filipino ($—^{FIL}$) deletions. Since these deletions extend beyond the α-genes at the 5' end and beyond the α-genes at the 3' end of the α-globin complex, they cannot be positively identified in heterozygotes ($—/\alpha\alpha$) by using the routine PCR of the ($—^{SEA}$) deletion and may therefore be missed during genetic counseling and prenatal diagnosis. In particular, the ($—^{THAI}$) deletion that was first reported in Thai individual since 1984[1-5]. But, in Thailand, the detection of α^0-thalassemia gene carrier in a routine clinical laboratory was only ($—^{SEA}$) deletion. Thus carriers of non-SEA deletion cannot be identified using the routine PCR of the ($—^{SEA}$) deletion.

Although of carriers of these deletions have mild microcytosis, with or without anemia, but otherwise are healthy. However, if both couples are carriers, they could be at reproductive risk for hemoglobin (Hb) Bart's hydrops fetalis syndrome, a condition that generally results in fetal demise in utero or shortly after birth. Alternatively, they also could be a less severe condition known as Hb H disease[6]. Therefore, it is important to develop a simple and reliable molecular genetic diagnosis test for these deletions.

In this study, we have developed a multiplex PCR for rapid and reliable DNA diagnosis of the ($—^{SEA}$), ($—^{THAI}$) and ($—^{FIL}$) deletions and confirmed the deletion breakpoints of these deletions by the DNA sequencing technique. Furthermore, we have investigated whether Thai individuals have other deletion, over ($—^{SEA}$) deletion type of α^0-thalassemia or not, and identified whatthe non-Southeast Asian deletion type of α^0-thalassemia are found in Thai individuals.

Materials and Methods

Patient samples

This study was carried out with human and accepted by ethics committee of Faculty of Medicine, Chiang Mai University. The peripheral venous blood were collected from 114 confirmed cases of patients with Hb H disease (January-December, 2002) and the fetal blood were collected from 100 confirmed cases of Hb Bart's hydropic fetuses who submitted to the Pediatrics Thalassemia Clinic, Maharaj Nakorn Chiang Mai Hospital, Department of Pediatrics, Faculty of Medicine at Chiang Mai University.

Genomic DNA extraction

Genomic DNA was extracted from peripheral blood or fetal blood using the Chelex method[7]. Briefly, 1-mL of 0.5% Triton X-100 was added to 50-μL of whole blood in a 1.5-mL microcentrifuge tube, vortexed and centrifuged at 14,000 rpm for 1 min. The supernatant was removed by suction and 1-mL of distilled water was added. After centrifugation as above, the supernatant was removed again. From a Chelex-100 aqueous suspension settled Chelex beads were added so that they cover the nuclei pellet with a 2-mm thick layer. After adding 120-μL of distilled water, the samples were incubated for at least 3 h (but usually overnight) at 56°C. After vortexing and centrifugation the samples were boiled for 8 min and again vortexed and centrifuged. The extracts were store at 4°C until they were used as templates in PCR.

Strategy for multiplex PCR

To screen for severe α^0-thalassemia deletions, primers were designed for ($—^{SEA}$), ($—^{THAI}$) and ($—^{FIL}$) deletions by utilizing the gap-PCR approach[8,9] (Figure 1). Each 25-μL reaction of PCR contained 200-μM of each dNTP; 1.5-mM $MgCl_2$; 1x Q-solution (Qiagen, GmbH, Germany); 1.25 U Hotstar Taq DNA Polymerase (Qiagen); 0.4-μM of each primer (Table 1) and 100-200 ng genomic DNA. Reactions were carried out in a GeneAmp PCR system-2400 or -9600 thermal cycler

Table 1 Primer sequences for α^0-thalassemia multiplex PCR and expected amplicon sizes

Name	5'⟶3' sequence	GenBank ID:Nucleotides	Amplicon (size)
FIL-F	CTGCCCTTCACACCTCAGACA	Z84721:11961⟶11981	(—^{FIL}) jxna fragment
FIL-R	GCAATCTTGGCTCACTGCAGG	Z69706:278⟶258	(597-bp)
THAI-F	TGACTGCATCATAATTCCAGCAG	Z84721:10504⟶10523	(—^{THAI}) jxn fragment
THAI-R	TGAGGCAGGAGATTCGCTTGA	Z69706:1478⟶1458	(480-bp)
SEA-F	GCGATCTGGGCTCTGTGTTCT	Z84721:26119⟶26139	(—^{SEA}) jxn fragment
SEA-R	ACTGCAGCCTTGAACTCCTG	Z69706:2662⟶2643	(195-bp)
SEA-F	As above	As above	control fragment
α-R	GTTCCCTGAGCCCCGACACG	Z84721:26435⟶26416	(314-bp)

jxn, junction

(Perkin-Elmer Co., NJ): initial denaturation at 95°C for 15 min; followed by 40 cycles of 94°C for 40 s, 60°C for 20 s, and 72°C for 2 min; with a final extension of 72°C for 10 min. A 10-μL amount of each amplification reaction product was analyzed on a 1% agarose gel in 1x Tris-borate-EDTA buffer (TBE).

Results

Figure 2 shows the multiplex PCR results of various α-globin genotypes and the control. The multiplex PCR assays were carried out on 114 genomic DNA samples from patients with Hb H disease (—/-α). Out of these 114 cases, 113 cases were heterozygous for (—^{SEA}) deletion and only one case was heterozygous for (—^{THAI}) deletion. None of them has the (—^{FIL}) deletion. Furthermore, we analyzed DNA of 100 Thai Hb Bart's hydropic fetuses (—/—), it was found that only two out of 100 cases were of compound heterozygotes for the (—^{SEA}) and (—^{THAI}) deletions: (—^{SEA}/—^{THAI}). The remaining 98 cases were homozygous for the

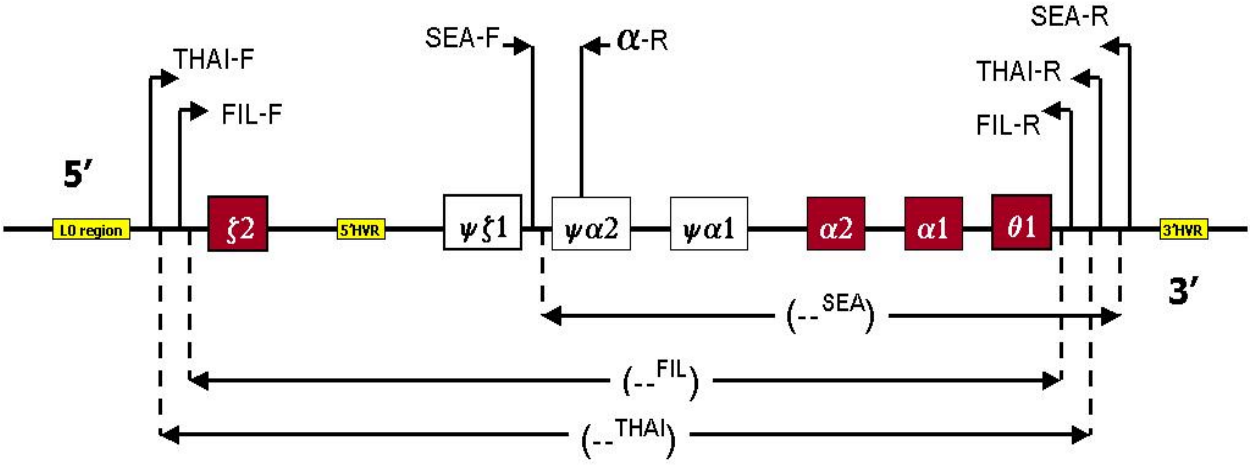

Figure 1. Schematic representation of location of multiplex PCR primers in α-globin cluster. Genes are represented as filled boxes and pseudogenes as open boxes. Hypervariable regions (HVR) are denoted as small boxes. Forward (F) and reverse (R) primers are shown for the (—^{SEA}), (—^{THAI}), and (—^{FIL}) deletions. The SEA-F primer also serves as the forward primer for the normal control α-globin amplification reaction.

Table 2 The results from the detection of α^0-thalassemia deletion in 114 cases of patient with Hb H disease and 100 cases of Hb Bart's hydropic fetuses by multiplex PCR technique

Genotype	Cases
* Hb H disease:	114
- $(—^{SEA})/(-\alpha$ or $\alpha^T\alpha)$	113
- $(—^{THAI})/(-\alpha$ or $\alpha^T\alpha)$	1
* Hb Bart's hydropic fetuses	100
- $(—^{SEA})/(—^{SEA})$	98
- $(—^{SEA})/(—^{THAI})$	2
Total	**214**

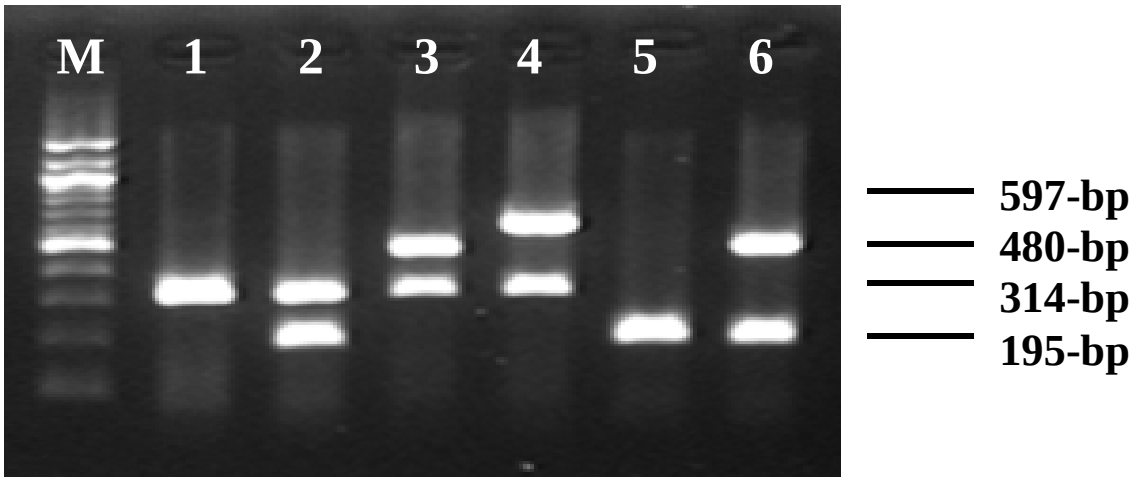

Figure 2 Multiplex PCR results for various α-globin genotypes. Specially sized PCR products were obtained for the normal [314-bp], $(—^{SEA})$ [195-bp], $(—^{THAI})$ [480-bp], and $(—^{FIL})$ [597-bp] genotypes. Lane M, 100-bp DNA ladder (New England Biolabs), lane 1, ($\alpha\alpha/\alpha\alpha$); lane 2, $(—^{SEA}/\alpha\alpha)$; lane 3, $(—^{THAI}/\alpha\alpha)$; lane 4, $(—^{FIL}/\alpha\alpha)$; lane 5, $(—^{SEA}/—^{SEA})$; lane 6, $(—^{SEA}/—^{THAI})$

common $(—^{SEA})$ deletion: $(—^{SEA}/—^{SEA})$. None of them has the compound heterozygotes for the $(—^{SEA})$ or $(—^{THAI})$ and $(—^{FIL})$ deletions. The results were summarized in Table 2. We sequenced the PCR products corresponding to these deletions and confirmed the multiplex PCR results (data not shown).

Discussion

α-thalassemia is common throughout Southeast Asia. In Thailand between 15% and 30% of the population are carriers. The severe α-thalassemia syndromes (Hb H disease and Hb Bart's hydrops fetalis syndrome) both occur in Thailand[10]. The most fre-

quently encountered defects are the ($—^{SEA}$) and ($-\alpha^{3.7}$) deletions (Hb Bart's hydrops fetalis: ($—^{SEA}/—^{SEA}$) and Hb H disease: ($—^{SEA}/\alpha^{3.7}$)). Nevertheless there have been reports of Thai individuals with α^0-thalassemia in whom there is a complete deletion of the $\zeta\alpha$ complex on one chromosome. We have called these the ($—^{THAI}$) and ($—^{FIL}$) deletions. Because of the detection of α^0-thalassemia gene carrier in a routine clinical laboratory was the only ($—^{SEA}$) deletion. Thus carriers of non-SEA deletion cannot be identified using the routine PCR of the ($—^{SEA}$) deletion. Therefore, the missing of genetic determination may lead to fault during genetic counseling and prenatal diagnosis and couples with these deletions are at risk of having a hydrops baby or offspring with Hb H disease[11].

We have developed a multiplex PCR technique by optimizing various parameters of the multiplex PCR capable to detect the α^0-thalassemia ($—^{SEA}$), ($—^{THAI}$), and ($—^{FIL}$) deletions. Especially important for a successful multiplex PCR assay are the cycling, the concentration of the PCR buffer, and the balance between the dNTP and $MgCl_2$ concentrations were titrated to provide the best result. A clear single band of each specially sized PCR products were obtained for the normal: 314 basepair (bp), ($—^{SEA}$): 195 bp, ($—^{THAI}$): 480 bp, and ($—^{FIL}$): 597 bp, genotypes had been observed without any complications neither non-specific background bands nor the formation of primer-dimer.

In addition, we have overcome the inherent problem of PCR amplification of the GC-rich α-globin locus by using enhancers (Q-solution) and Hotstart Taq DNA Polymerase[12], are supplied in an inactive state that has no polymerase activity at ambient temperatures. These prevent extension of non-specifically annealed primers and primer-dimers formed at low temperatures during PCR setup and the initial PCR cycle.

From the pilot studies, the optimal multiplex PCR technique was employed to detect α^0-thalassemia in 114 cases of patient with Hb H disease and 100 Hb Bart's hydropic fetuses. Although, only one and two cases of Hb H disease group and Hb Bart's hydrops fetalis group, respectively, were heterozygous for ($—^{THAI}$) deletions. These limited data suggest that, compared with the ($—^{SEA}$), ($—^{THAI}$), and ($—^{FIL}$) deletions are either relatively uncommon or under represented in subjects with Hb H disease and the Hb Bart's hydrops fetalis syndrome. More extensive studies will be required to determine their true prevalence in these populations.

Furthermore, the prevalence of non-Southeast Asian deletion type of α^0-thalassemia ($—^{THAI}$) and ($—^{FIL}$) deletions in normal population should be further determined to increase the importance of these types. Although only three α^0-thalassemia deletion types are included in the multiplex PCR assay at present, other α-thalassemia deletions, such as α^+-thalassemia: ($-\alpha^{3.7}$) and ($-\alpha^{4.2}$) deletions or another α^0-thalassemia: ($—^{MED}$), should be easily incorporated into the assay if required. In application, the further DNA-based planning and preventive program of α^0-thalassemia should be more concerned in these types to efficiently facilitate the improvement of medical services such as carrier screening and prenatal diagnosis for pregnancies at risk for Hb Bart's hydrops fetalis syndrome in the future[13].

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คุณลักษณะเฉพาะในระดับโมเลกุลของโรคอัลฟ่าศูนย์ธาลัสซีเมียในผู้ป่วยโรคฮีโมโกลบินเอช และในทารกที่เป็นโรคฮีโมโกลบินบาร์ทสไฮดอร์บฟีทัลลิส

ฉันทวัฒน์ กันธะมูล, ศิริวรรณ วงค์ไชย* และ ต่อพงศ์ สงวนเสริมศรี**

ภาควิชาชีวเคมี กองการศึกษา วิทยาลัยแพทยศาสตร์พระมงกุฎเกล้า

*ภาควิชาชีวเคมี และ **ภาควิชากุมารเวชศาสตร์ คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

บทคัดย่อ: *โรคอัลฟ่าศูนย์ธาลัสซีเมียในประชากรไทยที่พบบ่อยคือชนิดเซาธ์อีสท์เอเชียน ดีลีชั่น นอกจากนี้มีรายงานว่าพบโรคอัลฟ่าศูนย์ธาลัสซีเมียที่ไม่ใช่ชนิดเซาธ์อีสท์เอเชียน ดีลีชั่นอีก 2 ชนิดในกลุ่มคนไทยและประชากรในแถบเอเชียตะวันออกเฉียงใต้ได้แก่ ชนิดไทยและฟิลิปิโน ดีลีชั่น แต่ในประเทศไทยการตรวจหายีนพาหะของโรคธาลัสซีเมียในห้องปฏิบัติการโดยเทคนิคพีซีอาร์จะทำเฉพาะชนิดเซาธ์อีสท์เอเชียน ดีลีชั่นเท่านั้น ดังนั้นยีนพาหะชนิดอื่นที่ไม่ใช่เซาธ์อีสท์เอเชียน ดีลีชั่นจะไม่สามารถตรวจหาได้ ในการศึกษาครั้งนี้เราได้พัฒนาเทคนิคมัลติเพล็กพีซีอาร์ที่มีความรวดเร็วและแม่นยำในการตรวจหาโรคอัลฟ่าศูนย์ธาลัสซีเมียทั้ง 3 ชนิด คือ เซาธ์อีสท์เอเชียน ไทย และฟิลิปิโน ดีลีชั่นพร้อมกัน โดยนำเทคนิคดังกล่าวมาใช้ตรวจหาชนิดของโรคอัลฟ่าศูนย์ธาลัสซีเมียในผู้ป่วยโรคฮีโมโกลบินเอชจำนวน 114 ราย จากตัวอย่างทั้งหมดพบผู้ที่เป็นพาหะชนิดเซาธ์อีสท์เอเชียน ดีลีชั่นจำนวน 113 ราย และชนิดไทย ดีลีชั่นจำนวน 1 ราย นอกจากนี้เราได้ตรวจวิเคราะห์ดีเอ็นเอของทารกที่เป็นโรคฮีโมโกลบินบาร์ทสไฮดอร์บฟีทัลลิสจำนวน 100 ราย พบว่ามีเพียง 2 รายที่สาเหตุของโรคเกิดจากการรวมตัวกันของยีนแบบเฮเทอโรไซกัสระหว่างชนิดเซาธ์อีสท์เอเชียน ดีลีชั่นกับชนิดไทย ดีลีชั่น ส่วนอีก 98 รายเกิดจากการรวมตัวกันของยีนแบบโฮโมไซกัสของชนิดเซาธ์อีสท์เอเชียน ดีลีชั่น โดยการศึกษาครั้งนี้ไม่พบพาหะของยีนชนิดฟิลิปิโน ดีลีชั่นแต่อย่างใด จากการศึกษาครั้งนี้เราคาดหวังว่าเทคนิคนี้น่าจะช่วยเพิ่มประสิทธิภาพในกระบวนการตรวจคัดกรองและการวินิจฉัยโรคก่อนคลอดของโรคอัลฟ่าศูนย์ธาลัสซีเมียในประชากรไทยให้ดียิ่งขึ้น*

Key Words: ● *โรคอัลฟ่าศูนย์ธาลัสซีเมีย* ● *โรคฮีโมโกลบินเอช* ● *มัลติเพล็ก พีซีอาร์* ● *เซาธ์อีสท์เอเชียน ดีลีชั่น*

เวชสารแพทย์ทหารบก 2548;58:301-7.