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Accuracy of Hemoglobin E Screening Test Using Allelic Discrimination Assay

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ABSTRACT

Objectives: To determine the accuracy of allelic discrimination (AD) assay for hemoglobin E (Hb E) screening test in Chiang Mai Strategy for thalassemia prevention and control.

Materials and Methods: This study evaluated the AD assay compared with conventional Hb E screening tests used in Chiang Mai Strategy of Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand. In this assay, two TaqMan probes were designed to discriminate heterozygous and homozygous by detecting normal and mutant nucleotides of Hb E gene.

Results: From 55 blinded DNA samples, the AD assay revealed the results with 100% sensitivity, specificity, positive predictive value, negative predictive value and efficiency when compared to the conventional Hb E screening tests of the Chiang Mai Strategy.

Conclusion: The AD assay is effective as an Hb E screening test in the thalassaemia prevention and control program. Moreover, AD assay can distinguish heterozygous from homozygous genotypes.

Keywords: allelic discrimination, E screening test, hemoglobin E, real-time PCR.

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ความถูกต้องของการทดสอบคัดกรองฮีโมโกลบินอีโดยวิธี Allelic Discrimination

สุปราณี อุปนันท์, สมเดช ศรีชัยรัตนกูล, ศิริวิภา ปิยะมงคล, วีรวิทย์ ปิยะมงคล

บทคัดย่อ

วัตถุประสงค์: *เพื่อหาความถูกต้องของวิธี allelic discrimination (AD) สำหรับการตรวจคัดกรองฮีโมโกลบินอี (Hb E) ใน Chiang Mai Strategy สำหรับการป้องกันและควบคุมธาลัสซีเมีย*

วัสดุและวิธีการ: *การศึกษานี้เป็นการประเมินวิธี AD เทียบกับการตรวจคัดกรอง Hb E แบบเดิมที่ใช้ใน Chiang Mai Strategy ของโรงพยาบาลมหาราชนครเชียงใหม่ จังหวัดเชียงใหม่ ในวิธีนี้ TaqMan probe จำนวน 2 เส้น ได้ถูกออกแบบเพื่อใช้แยกเฮเทอโรซัยกัส (heterozygous) และโฮโมซัยกัส (homozygous) โดยตรวจหานิวคลีโอไทด์ปกติและผิดปกติ (mutant) ของยีน Hb E*

ผลการศึกษา: *จากตัวอย่างดีเอ็นเอ จำนวน 55 ตัวอย่าง พบว่า วิธี AD แสดงผลการวิเคราะห์ด้วยร้อยละ 100 ของค่าความไว (sensitivity) ค่าความจำเพาะ (specificity) ค่าทำนายผลบวก (positive predictive value) ค่าทำนายผลลบ (negative predictive value) และประสิทธิภาพ (efficiency) เมื่อเปรียบเทียบกับการตรวจคัดกรอง Hb E แบบเดิมของ Chiang Mai Strategy*

สรุป: *วิธี AD มีประสิทธิภาพสำหรับการตรวจคัดกรอง Hb E ในโปรแกรมป้องกันและควบคุมธาลัสซีเมีย นอกจากนี้วิธี AD สามารถแยกแยะยีนชนิดเฮเทอโรซัยกัสจากโฮโมซัยกัสได้*

คำสำคัญ: *allelic discrimination, การตรวจคัดกรองอี, ฮีโมโกลบินอี, Real-time PCR*

Introduction

Thalassemia syndrome and hemoglobinopathy are the most common single gene disorder and costs significant health and economic burden worldwide. Hemoglobin E disease is the most common hemoglobinopathy in Thailand with the average prevalence of 13%. It is an inherited hemoglobin disorder with a point mutation within β-globin gene (codon 26, G→A). β-Thalassemia/Hb E is one of prime targets in the public health policy to prevent and control severe thalassemias(1-4). Therefore, effective laboratory tests for Hb E screening to identify Hb E gene carriers are very important. Since the types of thalassemias are very diverse and complicated, a single laboratory test is not sufficient for every type of thalassemia diagnosis. This study thus aims to develop and determine the accuracy of allelic discrimination (AD) assay for Hb E screening in comparison to the conventional Hb E screening tests (i.e. hemoglobin typing (%A2/E) or E screen test) in Chiang Mai Strategy of Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand(1, 4-8).

Following the instruction in the allelic discrimination guide of Applied Biosystem company for ABI 7500™ real-time polymerase chain reaction (PCR) machine (Applied Biosystems, California), TaqMan allelic discrimination is high-throughput for genotyping of single nucleotide polymorphisms(9). This assay is a multiplexed real-time PCR reaction with end-point detection. The reaction mix contains deoxynucleoside triphosphate (dNTP) substrates, Taq deoxyribonucleic acid (DNA) polymerase, a forward primer, a reverse primer and two TaqMan probes detecting normal (G) and mutant (A) nucleotide in one tube. Each TaqMan probe consists of different fluorescent reporter dye (e.g. VIC® and FAM®) at 5' end and nonfluorescent quencher (e.g. NQF) at 3' end offering lower background signal. The specific site of probes was between forward and reverse primer sites. To detect variants of a single nucleic acid sequence at the codon 26 (G→A), a green dye (VIC®) TaqMan probe was designed to specific guanine (G) for normal allele while a blue dye (FAM®) TaqMan probe was designed to specific to adenine (A) for mutant allele of Hb E mutation. The primers and probes would anneal with their own matching complementary sequences. Taq DNA polymerase added dNTPs to the 3' end of primer for polymerization. Subsequently, the hybridization probe was cleaved by the 5' nuclease activity of Taq DNA polymerase and released a fluorescent signal due to separation of the reporter dye from the quencher dye; only specific sequence was completely amplified. The AD assay combines PCR and mutation detection in a single step by measuring an increase in the fluorescence intensity of the reporter dye and performing allelic discrimination on the post-PCR product using SDS software presenting the data as graph plot of heterozygosity and homozygosity (Fig. 1) normal (VIC® dye fluorescent signal only), homozygous Hb E (FAM® dye fluorescent signal only) and heterozygous Hb E (both VIC® and FAM® dye fluorescent signals).

Indeed, in addition to wildtype allele the AD assay can distinguish between homozygous and heterozygous Hb E gene mutations. Therefore, it would be an alternative accurate, rapid and sensitive test for Hb E screening in the thalassemia prevention and control program.

Materials and Methods

Blinded DNA samples (N=55) from 3 normal individuals and 52 thalassemia patients: Hb E trait (N=9), Hb E trait/α1-trait (N=1), homozygous Hb E (N=2), β-major (N=2), β-trait (N=25), β-trait/α1-trait (N=2) and α1-trait (N=11) were obtained from Thalassemia Center, Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand. The study protocol was approved by the Research Ethics Committee, Faculty of Medicine, Chiang Mai University, Thailand (Study Code: OBG-2559-03835/Research ID: 3835). The patients were diagnosed Hb E disorder using conventional Hb E screening tests, including %A2/E or E screen test, according to Chiang Mai Strategy of Maharaj Nakorn Chiang Mai Hospital(1, 4-8). The sample size was calculated using statistic descriptive studies.

Primer and probe sequences for real-time PCR were designed using Primer3 software and were synthesized by Applied Biosystems, California. The

forward and reverse primers are 5'-GCA AGG TGA ACG TGG ATG AAG T-3' and 5'- GTC TCC TTA AAC CTG TCT TGT AAC CT-3', respectively. TaqMan probes for normal and mutant alleles were designed as 5'-VIC®-AGG GCC T[C]A CCA CCA-NFQ-3' and 5'- FAM®-CAG GGC CT[T] ACC ACC A-NFQ-3', respectively. DNA samples (1 μl) were mixed with the reaction mixture (9 μl) containing 2x TaqMan® Mix (5 μl), 40x Primer-Probe Mix (0.25 μl) and nuclease-free water (3.75 μl). The real-time PCR profile (95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min) and the AD assay were operated on the ABI 7,500™ real-time PCR machine (Applied Biosystems, California) according to the manufacturer's instructions. The validity of the AD assay was determined by diagnostic indices using a two-by-two table in comparison to the conventional Hb E screening tests (%A2/%E/E screen) of Chiang Mai Strategy in order to evaluate sensitivity, specificity, positive predictive value, negative predictive value and efficiency. Chi square was employed for correlation analysis.

Results

From a total of 55 blinded DNA samples from normal cases and various types of thalassemia patients (Hb E, β-Thalassemia and α-Thalassemia), the AD assay interpreting from the allelic discrimination plot could discriminate 43 normal, 10 heterozygous Hb E and 2 homozygous Hb E samples (Fig. 1). The results of the AD assay were positive in 21.8% (12/55) and negative in 78.2% (43/55) (Table 1). In addition, the validity of the AD assay was evaluated in comparison to the conventional Hb E screening tests (%A2/%E/E screen) in the Chiang Mai Strategy. The diagnostic indices from chi square analysis were shown in Table 1. Interestingly, the AD assay showed 100% sensitivity, specificity, positive predictive value, negative predictive value and efficiency. This implies that this test can identify all Hb E traits and homozygotes with no false-negative or false-positive result.

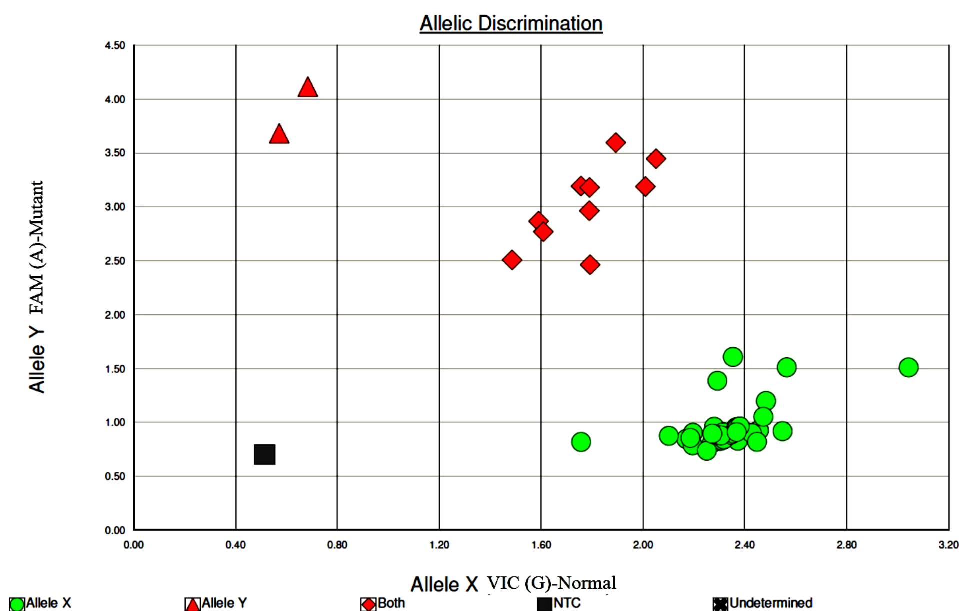

Fig. 1. Allelic discrimination plot of the AD assay of samples from subjects with normal, heterozygous and homozygous Hb E (β-globin gene codon 26, G→A) genotypes (N = 55). The x-axis is VIC (green) dye fluorescence signaling for normal allele with G nucleotide; the y-axis is FAM (red) dye fluorescence signaling for mutant allele with A nucleotide. Red triangles indicate homozygous Hb E. Orange diamond-shaped quadrangles indicate heterozygous Hb E. Green circles indicate normal allele. Grey square indicates no-template control (NTC).

Table 1. Two-by-two table showing the diagnostic indices of the allelic discrimination (AD assay results of DNA samples of normal, heterozygous and homozygous Hb E comparing with those using conventional Hb E screening methods (N = 55).

AD assay	Conventional Hb E screening tests (%A2/%E/Hb E screen)		
	Positive	**Negative**	**Total**
Positive	12	0	12
Negative	0	43	43
Total	**12**	**43**	**55**

Discussion

In order to prevent and control severe thalassemias, in particular β-thalassemia/Hb E disease according to public health strategies in Thailand, population screening for Hb E homozygotes or carriers is very crucial[1-4]. In the Chiang Mai Strategy, the assays from blood samples including Hb typing (%A2/E) or E screen were employed as the conventional Hb E screening tests for thalassemia diagnosis due to the cost of the analysis[4, 6, 10]. In the former, a single assay could not diagnose all types of thalassemias due to the limitations of the assays and the wide variety of different mutations. For instance, Hb typing using high performance liquid chromatography (HPLC) or electrophoresis is too expensive and complicated even though it can distinguish heterozygous and homozygous Hb E[5, 11]. Dichlorophenolindophenol (DCIP) test is cheap but possesses high false-positive results with blue color detection[1, 8, 12, 13]. DCIP and E screen cannot differentiate between heterozygous and homozygous Hb E genotypes[1, 7, 8, 12, 13]. Therefore, rapid and accurate molecular assays detecting the point mutation of Hb E gene with heterozygote and homozygote were developed. From only one reaction tube containing primers and probes specific for normal (G) and mutant (A) Hb E gene, the AD assay is one of such assays showing the allelic discrimination plot which can indicate the number of normal, heterozygous and homozygous Hb E samples. This demonstrated that the AD assay is not complicated for handle and analysis even though the ABI 7500 real-time PCR machine is really required and specific for this assay. Although Kho and colleagues had developed the AD assay for detection of Hb E gene mutation[9], the improved newly designed primers, probes and real-time PCR conditions in this study were developed and tested for its validity comparing with the conventional Hb E screening tests (i.e. hemoglobin typing (%A2/E) or E screen test) in the Chiang Mai Strategy. The AD assay in this study also showed 100% sensitivity and specificity without any false-negative and false-positive results. Therefore, it was demonstrated that the AD assay in this study could be used as an alternative test for Hb E screening as in the Chiang Mai Strategy. Obviously, it is a time-effective system using a reaction mixture and provides an easier result interpretation presenting heterozygous and homozygous genotype identification. Interestingly, the applications of this assay for dry blood spot and a non-invasive test such in DNA samples from buccal swabs is also possible.

Conclusion

In comparison to the conventional Hb E screening tests (i.e. hemoglobin typing (%A2/E) or E screen test) in Chiang Mai Strategy, the AD assay is a rapid and accurate assay with 100% sensitivity and specificity for Hb E screening. Additionally, it can discriminate Hb E genotypes (normal, heterozygous and homozygous Hb E) using one reaction tube and an easy interpretation plot. Therefore, the AD assay can be an alternative test for Hb E screening in the Chiang Mai Strategy for thalassemia prevention and control.

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Potential conflicts of interest

The authors declare no conflict of interest.

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