ORIGINAL ARTICLES

Detection and haplotype differentiation of Southeast Asian α-thalassemia using polymerase chain reaction and a piezoelectric biosensor immobilized with a single oligonucleotide probe

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DNA-based diagnosis of α-thalassemias routinely relies on polymerase chain reaction (PCR) and gel electrophoresis. Here, we developed a new procedure for the detection and haplotype differentiation of Southeast Asian (SEA) α-thalassemia using a 3-primer system for PCR coupling with a DNA-based piezoelectric biosensor. PCR products amplified from genomic DNA were differentiated directly by using a quartz crystal microbalance immobilized with a single oligonucleotide probe. The frequency changes after hybridization of the PCR products amplified from a representative sample of normal α-globin, SEA α-thalassemia heterozygote, and homozygote were 206 ± 11, 256 ± 5, and 307 ± 3 Hz, respectively. The fabricated biosensor was evaluated through an examination of 18 blind specimens. It could accurately discriminate between normal and SEA α-thalassemic samples, which suggests that this biosensor system is a promising alternative technique to detect SEA α-thalassemia because of its specificity and less hazardous exposure as compared with conventional methods. (Translational Research 2008;151:246–254)

Abbreviations: CV = coefficient of variation; Hb = hemoglobin; HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PCR = polymerase chain reaction; QCM = quartz crystal microbalance; SD = standard deviation; SEA = Southeast Asian.

The thalassemia syndromes are inherited disorders because of either decreased production or absence of α- or β-globin chains of the erythrocyte hemoglobin, which results in α-thalassemia and β-thalassemia, respectively. These genetic diseases cause major public health as well as socioeconomic problems in many regions, particularly in Southeast Asian countries.[1] Two common α-thalassemia alleles exist, namely α-thalassemia-1 and α-thalassemia-2 genes. The former results from deletion of both α-globin genes (−−/α α), whereas the latter results from deletion of single α-globin gene. Among α-thalasse-

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AT A GLANCE COMMENTARY

Background

Southeast Asian (SEA) α-thalassemia is the most prevalent genetic disorder in this region. Screening of thalassemia carriers in routine clinical laboratories is conducted using several methods, which include blood cell analysis, hemoglobin typing, and DNA-based testing.

Translational Significance

We developed a new method that couples polymerase chain reaction (PCR) with a piezoelectric biosensor device for the detection of SEA α-thalassemia in both carrier and disease forms. The system required only 3 primers and a single oligonucleotide probe. The biosensor can be reused 3 times without adverse effect on their results. This study is a pilot project that uses a DNA-based biosensor for the rapid and accurate diagnosis of SEA α-thalassemia.

mia-1, the Southeast Asian (SEA) deletion ($--^{SEA}$) is the most prevalent mutation.[1,2] Homozygous α-thalassemia-1 ($--/--$) leads to the most severe thalassemia called hemoglobin (Hb) Bart's hydrops fetalis. Loss of α-globin chains together with the dysfunction of Hb Bart's is lethal either *in utero* or soon after birth.[2] Accurate identification of SEA α-thalassemia carriers is an essential step to prevent birth of severe thalassemia. Screening of thalassemia carriers in routine clinical laboratories is conducted using several methods, including blood cell analysis, hemoglobin typing, and DNA-based testing.[2] The latter method can diagnose specific genotypes of α-thalassemia. A variety of DNA-based diagnosis for α-thalassemia has been developed. Polymerase chain reaction (PCR)[3-6] and Southern blot hybridization[2,7] have been applied to detect the deletion types of α-thalassemia, whereas molecular analysis using denaturing gradient gel electrophoresis, single-strand conformation analysis, and direct DNA sequencing are effective strategies for detecting nondeletion forms.[8] These molecular techniques are time-consuming and technically difficult. PCR coupling with electrophoresis seems to be widely used in clinical laboratories. Carcinogenic ethidium bromide is, however, the commonly used dye for the visualization of DNA targets in most laboratories.

Recently, a potential application of biosensors has occurred in many biologic assays. A biosensor is a device that combines a recognition component of biologic origin with a physicochemical transducer. A wide range of transduction modes, including electrochemical, optical, magnetic, thermal and piezoelectric means, has been developed.[9-11] Biosensor technologies therefore offer great possibilities for use in DNA analysis by nucleotide hybridization with high selectivity.[10,12-15] Piezoelectric biosensors created from quartz crystal microbalance (QCM) are one of the most promising types of biosensors. They can monitor the frequency changes that result from a change in the mass on the surface of crystal because of the formation of biomolecule complexes. Hybridization between the target DNA and a specific nucleotide probe immobilized on the surface of the piezoelectric crystal can be measured.[11,12,16-18] The coupling of this biosensor with PCR has been used successfully for the detection of α-thalassemia.[19,20] To our knowledge, no report has occurred regarding the genetic detection of α-thalassemia using a DNA-based biosensor. Because the incidence of α-thalassemia is relatively high in Thailand together with the fact that the most common SEA β-thalassemia can cause hemoglobin Bart's hydrops fetalis, a DNA detection tool for SEA type was developed using the quartz crystal microbalance biosensor device immobilized with a single oligonucleotide probe.

METHODS

Blood samples. The samples were human EDTA blood obtained through a material transfer agreement from the Hematological Unit, Rajavithi Hospital, where hemoglobin typing and molecular diagnosis of the α-globin gene were analyzed. A genetically known specimen from normal individual as well as heterozygous and homozygous SEA α-thalassemia was processed to set up the biosensor system. Seven genetically normal α-globin, 8 heterozygous, and 3 homozygous (from the cord blood of hemoglobin Bart's hydrops fetalis fetuses) SEA α-thalassemic samples were analyzed as blind samples for evaluation of the developed biosensor.

DNA preparation. Genomic DNA was prepared from EDTA blood using phenol chloroform extraction as described elsewhere.[21] The concentration of DNA was determined by measuring the absorbance at 260 nm before diluting to final concentration of 50 μg/mL.

DNA amplification and agarose gel electrophoresis. A new 3-primer set (SEA-F: 5′ CGATCTGGGCTCTGTGTTCTC3′, SEA-R: 5′AGCCCACGTTGTGTTCATGGC3′, and A1B: 5′GTTCCCTGAGCCCCGACACG3′) were modified from previous studies.[3,5,6] The primer location on the globin gene is depicted in Fig 1, *A*. The PCR was conducted in a 25-μL reaction mixture that contained 50 ng of DNA sample, 5 pmol of each primer, and the reaction mixture (Qiagen, Gaithersburg, Md). The cycling reaction was started with a 15-min denaturation at 95°C and followed by 30 cycles of 98°C for 45 s, 60°C for 90 s, and 72°C for 135 s. The final cycle was followed by an extension at 72°C for 5 min. The sizes of the PCR products were estimated after electrophoresis on a 1.5% agarose gel.

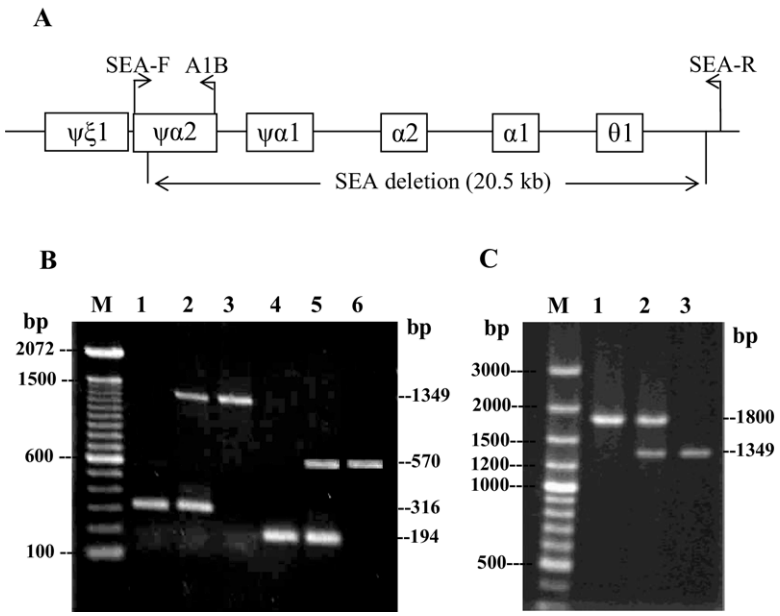

Fig 1. The location of primers on the globin gene locus[3, 6] and PCR products using different sets of primers. **A**, The position and orientation of primers on the globin genes are shown. An arrow indicates the orientation of primer. **B**, The PCR products were separated on agarose gel electrophoresis. The products from the new 3-primer set (lane 1–3) show a band of 316 bp for normal α-globin and 1349 bp for SEA α-thalassemia, and from a conventional 3-primer set (lane 4 – 6) that gives a band of 194 bp for normal α-globin and 570 bp for SEA α-thalassemia. Lane 1 and 4, 2 and 5, and 3 and 6 represent PCR products from normal, heterozygous, and homozygous SEA α-thalassemia samples, respectively. M represents molecular weight markers (100-bp DNA ladder; Invitrogen, Carlsbad, Calif). **C**, PCR product from the 4-primer system that generated a band of 1800 bp for normal α-globin and 1349 for SEA α-thalassemia. Lanes 1, 2, and 3 represent PCR products from normal, heterozygous, and homozygous SEA α-thalassemia samples, respectively. M represents molecular weight markers (100-bp DNA ladder plus; Fermentas, Vilnius, Lithuania).

Designation of oligonucleotide probe. The 5′ biotinylated 20-mer oligonucleotide probe, 5′CTCTGTGTTCTCAGTATTGG3′, was designed from the nucleotide sequence that flanked the SEA region. Its sequence is partially complementary to the SEA-F primer, which thereby allows hybridization with the PCR products from both genetically normal and SEA thalassemia samples.

QCM system. The DNA-based piezoelectric biosensor system was developed from a 5-MHz AT-cut quartz crystal (2.54 cm in diameter) with a gold electrode (1.33 cm^2 area). The quartz crystal was housed inside a polyvinyl chloride cell with a Viton O-ring. The oscillation frequency shift from piezoelectric property of the quartz crystal after mass deposition onto the crystal surface was measured by a frequency counter (PM-710; Maxtex Inc., Alpharetta, Ga). The frequency response of the biosensor system was stable within ±1.6 Hz. The Data-Log Software (Maxtex Inc.) was used for data acquisition and monitoring. The QCM system was prepared as described previously.[10,16,18,20] The gold surface of the quartz crystal was cleaned with hot Piranha solution (1 volume 30% H_2O_2 in 4 volumes of concentrated H_2SO_4) for 5 min, rinsed with water and absolute ethanol, then soaked in 5 mmol/L mercaptopropionic acid for 20 min, and rinsed once with water before it was placed in the polyvinyl chloride cell. The surface of the crystal was activated with a solution that contained 50-mmol/L *N*-hydroxysuccinimide and 200-mmol/L 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide before covalent coupling with 200 μL of optimal concentration of avidin in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (0.05 M HEPES, 0.5 M NaCl), pH 7.5 for 20 min. Excess avidin was removed by washing with buffer. The residual reacting sites were blocked with 1 M ethanolamine hydrochloride, pH 8.6. Next, 200 μL of an optimal concentration of a 5′-biotinylated probe in HEPES buffer was added and incubated for 20 min before being washed with buffer to remove the excess probe. The double-stranded PCR products were denatured by boiling for 5 min and immediately chilled in an ice bath. An appropriate amount of the heat-denatured PCR products (diluted to a total volume of 200 μL with HEPES buffer) was loaded into the device and allowed to hybridize with the immobilized oligonucleotide probe for 20 min and then washed twice with HEPES buffer to remove an unbound PCR product. The frequency shift was recorded and analyzed.

Statistical analysis. The significance of differences among the groups was statistically determined using a one-way analysis of variance and post hoc pairwise comparison with Scheffe test. A significant difference was observed at *P* less than 0.05.

Table I. PCR products amplified from each primer set

Primer set	PCR products (bp)		
	Normal α-globin gene	SEA α-globin gene	Size difference (bp)
Conventional 3-primer*	194	570	376
4-primer†	1800	1349	451
New 3-primer‡	316	1349	1033

*The conventional 3-primer consists of A4 (5′GGGGCGCCTTGGGGCGGTTC3′), A9 (5′ATATATGGGTCTGGAAGTGTATC3′) and A1B primers.[3]
†The 4-primer consists of α2/3.7F (5′CCCCTCGCCAAGTCCACCC3′), α2-R (5′AGACCAGGAAGGGCCGGTG3′), SEA-F and SEA-R.[5,6]
‡The new primer set consists of SEA-F, SEA-R, and A1B primers.

RESULTS

A new 3-primer set for normal α-globin and SEA α-thalassemia amplification by PCR. The principle of a piezoelectric biosensor is to discriminate samples based on their masses. Thus, PCR products with similar sizes may be difficult to differentiate. A PCR-based diagnosis of the SEA α-thalassemia gene was reported previously using conventional 3-primer,[3] and 4-primer systems.[5,6] The PCR products from normal α-globin and SEA α-thalassemia using those primer sets, however, are not much different in their molecular sizes. Therefore, we designed a new 3-primer set that consisted of SEA-F, SEA-R, and A1B that yields large differences in the amplified DNA from normal α-globin (316 bp) and SEA α-thalassemia (1349 bp) as illustrated in Table I and Fig 1, *B and C*. DNA amplification of normal α-globin gene generated a PCR product from SEA-F and A1B primers corresponding to the size of 316 bp. A deletion of 20.5-kb DNA, which contains A1B primer region, in SEA α-globin gene (Fig 1, *A*) gave a PCR product of 1349 bp from SEA-F and SEA-R primers.

Development and optimization of a piezoelectric biosensor for SEA α-thalassemia detection. A single biotinylated probe was coated onto the gold electrode through the specific interaction between biotin and avidin. Because the number of molecules of avidin or biotinylated probe bound to the surface of the electrode can affect the frequency change signal of the biosensor, concentrations of avidin and probe were optimized. Avidin at concentration of 0.1 mg/mL has been shown to be the optimal dose for immobilizing avidin on the QCM electrode.[16] Hence, the concentration of avidin was first fixed at 0.1 mg/mL, and the amount of probe was optimized. Various probe concentrations (0.2, 0.5, 0.8, 1.0, and 2.0 μmol/L) were added (200 μL) after avidin was immobilized on the gold electrode surface. The mean frequency changes were 26, 70, 100, 120, and 160 Hz for 0.2, 0.5, 0.8, 1.0, and 2.0 μmol/L probe concentrations, respectively (Fig 2, *A*). Then, the DNA samples derived from a PCR product of a normal α-globin amplified using the new 3-primer system was loaded into the device. We started by adding 10 μL of the PCR product (adjusted to total 200 μL with HEPES buffer) to each probe concentration. High-frequency changes after target DNA and probe hybridization were observed for 1.0-μmol/L and 2.0-μmol/L oligonucleotide probes (Fig 2, *B*). Therefore, 1.0 μmol/L of probe, which generated a frequency change of 198 ± 15 Hz with 7.6 % coefficient of variation (CV), was selected for the next set of experiments.

Next, the concentration of avidin was optimized using various amounts of avidin (200 μL of 0.01, 0.05, 0.1, and 0.2 mg/mL). The frequency change detected was increased depending on the avidin concentration used with a mean frequency change of 89, 112, 175, and 288 Hz for avidin concentrations of 0.01, 0.05, 0.1, and 0.2 mg/mL, respectively (Fig 2, *C*). The biotinylated probe (1.0 μmol/L) was then applied on each avidin concentration and allowed to hybridize with 10 μL of PCR product from a normal α-globin sample. The results showed a direct correlation between the frequency change and the avidin concentration, and the maximal change was observed at an avidin concentration of 0.1 mg/mL (Fig 2, *D*). At 0.2 mg/mL of avidin, the frequency change was comparable with that of 0.1 mg/mL, which suggests that an excess amount of avidin was used. Therefore, 0.1-mg/mL avidin was chosen for the following experiments. At this avidin concentration, the frequency change value after target DNA was bound to the probe was 198 ± 15 Hz with a 7.6% CV.

The optimal volume of the DNA targets loaded into the piezoelectric biosensor was determined. Aliquots of 10, 20, and 40 μL of PCR products amplified from a representative sample from the genetically normal as well as heterozygote and homozygote SEA α-thalassemia groups were diluted in HEPES buffer to a total volume of 200 μL before being added into the biosensor system. As shown in Fig 2, *E*, the 316-bp target PCR product of normal sample gave the greatest frequency change at a volume of 20 μL (308 ± 2 Hz). Similar patterns of frequency changes were observed for the PCR product from SEA α-thalassemia heterozygote samples that contained the mixture of 316-bp and 1349-bp target DNA (277 ± 16 Hz for 20 μL PCR product). Notably, only the frequency change signal using 10 μL of PCR product from the heterozygous sample (256 ± 5 Hz) was greater than that of the DNA product from

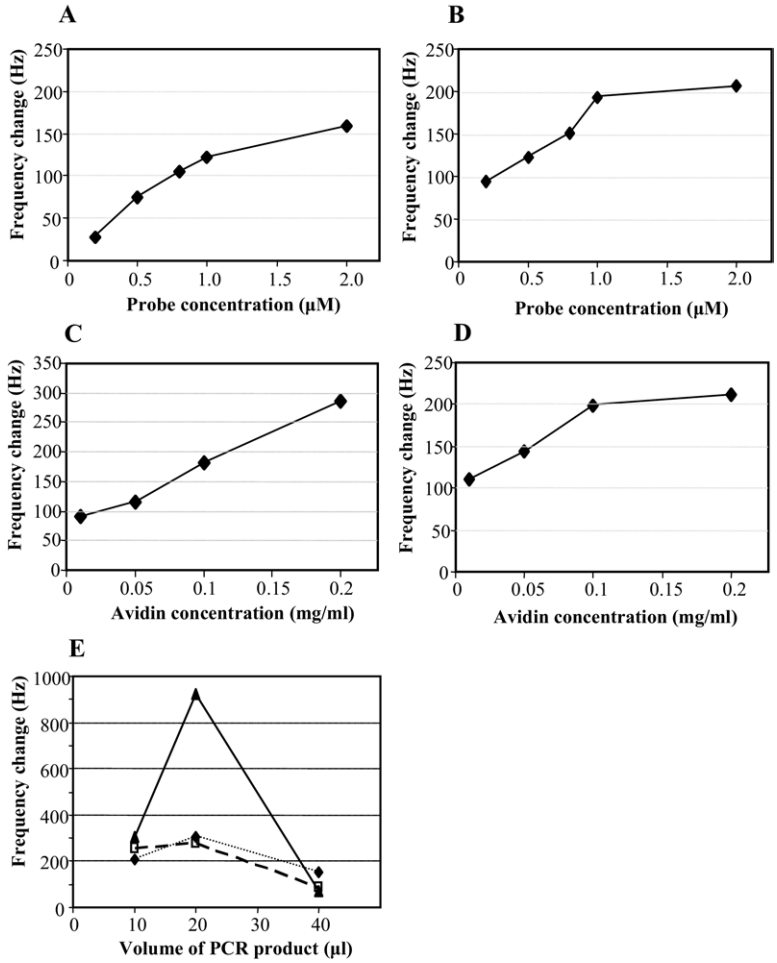

Fig 2. Optimization of biosensor. The target DNA was prepared from PCR amplification of a normal α-globin sample using the new 3-primer set. **A**, The frequency changes were monitored after various concentrations of oligonucleotide probe bound to avidin (0.1 mg/mL) coated on the surface of the crystal and (**B**), after target DNA was hybridized with the immobilized probe. **C**, The frequency changes were monitored after various concentrations of avidin were covalently bound on the surface of the crystal and (**D**), after the target DNA hybridized with the probe. All experiments were repeated independently 3 times, and the representative data are shown. **E**, The frequency changes were monitored after target DNA from normal (◆), heterozygous (□), and homozygous (▲) SEA α-thalassemia at indicated volumes were used. The data represented are the means from 3 independent experiments.

the normal sample (206 ± 11 Hz), whereas the other volumes of the PCR products used showed lower frequency changes (Fig 2, *E*). The PCR product of the SEA α-thalassemia homozygote sample that consisted of only 1349-bp DNA showed an extremely high-frequency change signal at 925 ± 88 Hz when 20 μL of the target DNA was used in the hybridization (Fig 2, *E*). The frequency change was substantially greater than those observed in genetically normal and heterozygote samples when using 10 μL of PCR product (307 ± 3 Hz). Inversely, the frequency change using 40 μL of the homozygote samples was lesser than the other types (Fig 2, *E*). All sample types showed low frequency signal changes when 40 μL of PCR products was used, which suggests that this volume was not suitable to be used in this biosensor detection system. Although using 20 μL of PCR product showed the greatest frequency change, the signals of the normal and heterozygote samples were not much different and were difficult to distinguish. To avoid this problem, 10 μL of PCR product was selected for the sample volume loaded into the biosensor device. Under the optimized conditions, the frequency changes [mean ± standard deviation (SD)] for genetically normal, SEA α-thalassemia heterozygote, and homozygote were 206 ± 11, 256

± 5, and 307 ± 3 Hz, respectively. The results indicated that the fabricated piezoelectric biosensor could be used in screening and diagnosis of SEA α-thalassemia.

Evaluation of the new 3-primer set coupling with piezoelectric biosensor for SEA α-thalassemia detection in clinical specimens. To evaluate the DNA piezoelectric biosensor device coupled with PCR using the new 3-primer set in diagnosis of SEA α-thalassemia, 18 blood samples were PCR amplified as blind samples. The known normal and SEA α-thalassemia DNA samples were used as internal controls. The PCR products were loaded into the established piezoelectric biosensor device. As shown in Fig 3, *A*, the frequency change signals obtained could be distinguished among those for normal α-globin as well as for heterozygous and homozygous SEA α-thalassemic samples. The frequency changes were 200 ± 9, 256 ± 5, and 308 ± 2 Hz, respectively. The results from each sample group could be separated clearly by their 3SD ranges (Fig 3, *A*). The 3SD ranges of frequency change values were 174–227, 241–271, and 302–314 Hz for normal, heterozygous, and homozygous SEA α-thalassemic groups, respectively. DNA diagnosed by this PCR coupling with the piezoelectric biosensor was inline with that from conventional PCR and gel electrophoresis.

Reusability of the quartz crystal. The reusability of the quartz crystal was evaluated. After monitoring the frequency change of the first target DNA-probe hybridization, the target DNA was removed from the immobilized probe by washing with 1-mmol/L HCl.[16] The used biosensor was then rehybridized with the DNA samples and monitored for frequency changes. As shown in Fig 3, *B*, for genetically normal, heterozygous, and homozygous α-thalassemia, the biosensor could be reused for 3 times with less than 8% CV. Experiments were extended to reuse the biosensor with different types of samples. The new biosensor was first used with one genetic type and was subsequently reused with the different genetic types. As shown in Fig 3, *C*, the reused biosensor gave frequency changes within the acceptable ranges in all types of samples.

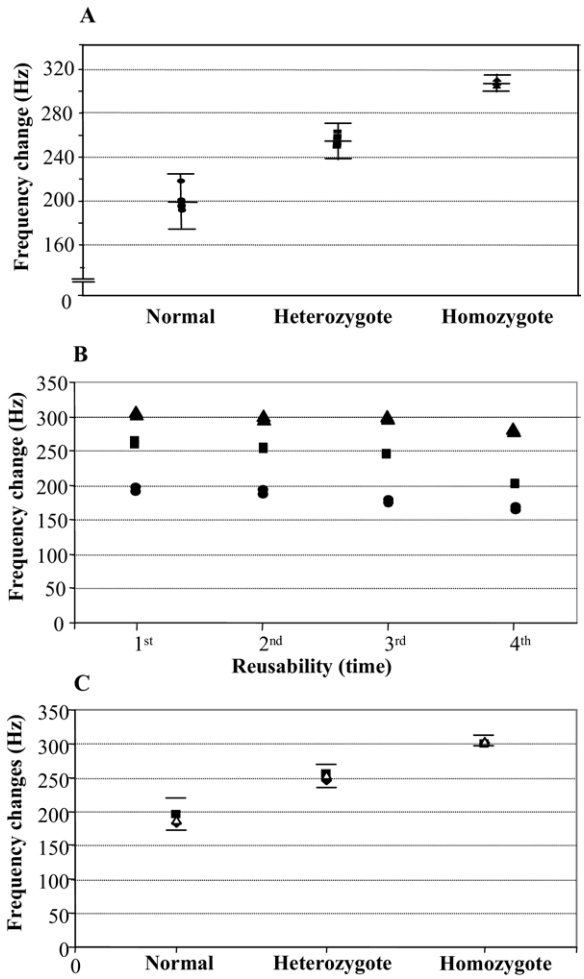

Fig 3. Evaluation of the fabricated piezoelectric biosensor. **A**, DNA samples from 18 patients that consisted of 7 normal α-globin, 8 hetrozygous, and 3 homozygous SEA α-thalassemia were amplified using the new 3-primer set. Ten microliters of the PCR products were diluted with HEPES buffer to a final volume of 200 μL, which was loaded into the biosensor device. The frequency changes were scored. **B**, The used biosensors were reused within the group of samples. (●), normal; (■), SEA heterozygote; and (▲), SEA homozygote. **C**, The used biosensors were reused with different groups of samples. The new biosensors were used randomly to monitor 1 genetic type before being reused with other distinct genetic types. The data represented are the means from 3 independent experiments. Bars represent means and 3 SD ranges of the frequency change from each group of samples.

DISCUSSION

DNA-based biosensors are generating increasing interest because they offer the potential advantage for rapid, real-time solution monitoring of DNA hybridization, as well as high sensitivity and specificity without the requirement of radioisotope or fluorophore labeling systems.[16] Application of biosensors for the detection of β-thalassemia gene mutations and deletions has been reported using either surface plasmon resonance or piezoelectric.[14-16,19,20,22] Unlike β-thalassemia, in which most abnormalities develop from gene mutations, α-thalassemia is caused by gene deletion; the most common in the Southeast Asian region is the SEA deletion type.[23] Several PCR-based techniques have been developed to diagnose α-thalassemia using primers that flank the deletion regions on the α-globin gene. The differences in the size of PCR products amplified from the nor-

mal and abnormal α-globin gene is observed by gel electrophoresis. Here, a piezoelectric biosensor was applied to discriminate between PCR products obtained from normal individuals and SEA α-thalassemia patients. The advantage of this biosensor is caused by the specific hybridization between the target DNA and the immobilized oligonucleotide probe. As frequency change signals detected by piezoelectric biosensor are correlated directly with the size of the hybridized DNA, the target DNA in PCR products of similar size is not distinguished easily. Moreover, because the detection system of the biosensor is based on the DNA hybridization, the secondary structure of the target DNA should be taken into consideration when developing the detection system. In this study, a new PCR primer set was designed to optimize the size of DNA products to allow discrimination using the developed biosensor and to minimize the number of primers in the PCR amplification. Our new 3-primer set generated DNA products that were much different in their molecular sizes between the normal (316 bp) and SEA (1349 bp) α-globin gene (Table I). A single oligonucleotide probe (20-mer) with 12 nucleotides complementary to the SEA-F primer was designed and used in the detection system of the developed biosensor. The biotinylated oligonucleotide probe was immobilized on the gold electrode via an avidin-biotin coupling reaction. Avidin molecules were bound covalently to the surface of the electrode by chemical reactions. The amount of avidin and oligonucleotide probe was optimized, and the most favorable concentrations were found to be 0.1-mg/mL avidin and 1.0-μmol/L oligonucleotide probe. Based on Sauerbrey's equation,[24] established for an AT-cut QCM, $\Delta f = -2f_0^2(\rho_q\mu_q)^{-1/2}\ \Delta m/A$, where Δf is the frequency shift in hertz from the mass added, f_o is the fundamental oscillation frequency of dry crystal, Δm is mass added to the crystal, A is the active area of the crystal, ρ_q is the quartz density (2.65 g/cm^3), and μ_q is the shear modulus of quartz (2.95×10^{11} dyne/cm^2). Our calculations suggest that a frequency change of 1 Hz corresponds to a mass increase of 23.09 ng/cm^2. When 0.1-mg/mL avidin was used, the observed frequency change was 175 Hz, which corresponds to 3.6×10^{13} avidin molecules immobilized on the gold surface of the electrode. Theoretically, an avidin molecule occupies a 33-nm^2 surface area.[16] The gold surface area of the crystal used is 1.33 cm^2. Therefore, 4.0×10^{12} avidin molecules were expected to bind on the surface. More avidin molecules calculated from Sauerbrey's equation might be the result of nonspecific binding or accumulation of avidin molecules on the gold surface. The calculated biotinylated oligonucleotide probes bound on the gold surface at concentrations of 1.0 μmol/L using Sauerbrey's equation is 2.6×10^{14} molecules/cm^2 developing an avidin/biotin ratio of 7, which is greater than theoretical ratio of 4. This suggests the presence of nonspecific binding of the biotinylated probes to the electrode. Nevertheless, the nonspecific binding caused no adverse effects on the ability of this detection system to discriminate between normal and SEA thalassemia.

The PCR product from patients with the SEA globin gene was 4 times larger than that of the normal globin gene product. Therefore, the measured frequency change after probe-target DNA hybridization from a SEA homozygote should be 4-fold greater. However, the maximal frequency change observed was only 3-fold greater when 20 μL of PCR product were used (Fig 2, *E*). This change may result from the lowered binding affinity caused by a larger molecular mass of the PCR product from SEA homozygote. Steric hindrance of the long DNA strand also lowers the binding capacity to the probe. The frequency changes monitored from the SEA α-thalassemia heterozygote were closed to the signals of normal samples when 20 μL of PCR product were used. This finding implied that the PCR products from the normal α-globin gene (316 bp) were more competitive in their binding capacity than those from SEA α-globin, which generated larger PCR products (1349 bp) because both DNA fragments contained 12 nucleotides complementary to the immobilized oligonucleotide probe. Moreover, binding inhibition was observed when excess target DNA (40 μL) was loaded. Therefore, a 10-μL volume of PCR product was suitable for SEA α-thalassemia diagnosis in this system. Thus, for cost reduction, the PCR reaction could be reduced to the final volume of 10 μL instead of 25 μL. Zhou et al[16] suggested that the target DNA at a concentration of 50–70 μg/mL was the optimal dose to be used for probe-target DNA hybridization of β-thalassemia in their QCM piezoelectric system. We found that adjustment the amount of DNA samples to 50 ng provided reproducible PCR products that range from 40–50, 50–60, and 55–65 μg/mL for normal, heterozygous, and homozygous SEA α-thalassemic samples, respectively. Therefore, our 10 μL of PCR product corresponds to an amount of DNA that ranges from 0.4–0.6 μg. Moreover, the use of 10-μL PCR products without a DNA measuring step was also convenient for application of this technique to routine practice.

Under the optimized condition, the frequency changes obtained from a representative sample of genetically normal α-globin, SEA α-thalassemia heterozygote, and homozygote were 206 ± 11, 256 ± 5, and 307 ± 3 Hz,

respectively. This finding indicated that the developed DNA-based piezoelectric biosensor could discriminate among 3 types of samples. When the fabricated biosensor was applied to characterize 18 blind samples that consisted of 7 genetically normal α-globin, 8 heterozygous, and 3 homozygous SEA α-thalassemia samples, we found that the frequency change signals were statistically different among all 3 groups ($P < 0.01$). Notably, we found no overlapping among the means $\pm$ 3SD of the frequency shift from 3 groups of samples (Fig 3, *A*). This finding supports the use of DNA-based piezoelectric biosensors coupled with PCR in the diagnosis of SEA α-thalassemia. Nevertheless, our fabricated biosensor device could not be used for detection of other types of α-thalassemias, which includes those that develop from gene mutations. The application of QCM piezoelectric biosensor to diagnose point mutation type α-thalassemia has been reported recently by using the immobilized DNA probe complementary to the site of genetic mutation.[16] Feriotto et al[14,15] have reported the use of surface plasmon resonance–based biospecific interaction analysis and biosensor technology for the multiple detections of point mutations in β-thalassemic samples. Thus, both types of biosensors provide strong potential to be modified for identification of the mutation type α-thalassemia.

The reusability of biosensor is an important factor that should be concerned in terms of cost effectiveness for massive screening in high-risk populations. A complete removal of the bound target DNA from the hybridized complexes without adverse effect on the immobilized probes on the gold surface of the electrode is a possible strategy to enhance biosensor reusability. Our fabricated biosensor can be reused for 3 times when 1-mmol/L HCl was used as washing solution,[16] which drastically reduces the cost per patient for SEA diagnosis.

CONCLUSION

The developed method that couples PCR with a piezoelectric biosensor device provides a sensitive, specific, convenient, and less hazardous technique for the detection of SEA α-thalassemia, which is one of the most common genetic disorders in Thailand, in both carrier and disease forms. The system required only 3 primers and a single oligonucleotide probe. It is a pilot project that used a DNA-based biosensor for the rapid and accurate diagnosis of SEA α-thalassemia. This system can be reused for 3 times and uses AT-cut quartz crystals that are commercially available in electronic markets to reduce the cost for large-scale screening of SEA α-thalassemia carriers.

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