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Low cost biosensor-based molecular differential diagnosis of α-thalassemia (Southeast Asia deletion)

Abstract

Background: Thalassemias are genetic hematologic diseases which the homozygous form of α-thalassemia can cause either death in utero or shortly after birth. It is necessary to accurately identify high-risk heterozygous couples. We developed a quartz crystal microbalance (QCM) to identify the abnormal gene causing the commonly found α-thalassemia1, [Southeast Asia (SEA) deletion]. This work is an improved method of our previous study by reducing both production cost and analysis time.
Methods: A silver electrode on the QCM surface was immobilized with a biotinylated probe. The α-globin gene fragment was amplified and hybridized with the probe. Hybridization was indicated by changes of quartz oscillation. Each drying step was improved by using an air pump for 30 min instead of the overnight air dry. The diagnostic potency of the silver QCM was evaluated using 70 suspected samples with microcytic hypochromic erythrocytes.
Results: The silver QCM could clearly identify samples with abnormal α-globin genes, either homozygous or heterozygous, from normal samples. Thirteen out of 70 blood samples were identified as carrier of α-thalassemia1 (SEA deletion). Results were consistent with the standard agarose gel electrophoresis. Using silver instead of gold QCM could reduce the production expense 10-fold. An air pump drying the QCM surface could reduce the analysis time from 3 days to 4 h.
Conclusions: The silver thalassemic QCM was specific, sensitive, rapid, cheap and field applicable. It could be used as a one-step definite diagnosis of α-thalassemia1 (SEA deletion) with no need for the preliminary screening test.
Keywords: biosensor; DNA; quartz crystal microbalance; SEA deletion; thalassemia.

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Introduction

Thalassemias are hemoglobinopathies resuling from quantitative abnormal hemoglobin synthesis. The most severe form is Hb Bart's hydrops fetalis or homozygous α-thalassemia1 which leads to death in utero or shortly after birth [1]. Identification of high-risk couples is essential to prevent the birth of new cases [2]. Usually, the carriers of abnormal α-gene are identified by the evaluation of erythrocyte parameters, morphology and osmotic fragility [3]. Confirmation needs hemoglobin typing and identification of the abnormal gene(s) [4]. This study developed a piezoelectric biosensor based on quartz crystal microbalance (QCM) to identify the abnormal α-globin gene causing α-thalassemia1 [Southeast Asia (SEA) deletion]. The QCM diagnostic potency and clinical application were also elucidated.

Materials and methods

Amplification of α-globin gene

The genomic DNA was purified by a PUREGENE Blood Core Kit (Qiagen, USA). Primers were synthesized (1st BASE, Singapore) as described [5]. Three primers composed of one common forward (F: CTCTGTGTTCTCAGTATTGGAG) and two reverse primers of normal α-globin gene (RN: GGTTCCCTGAGCCCCGACACG) and α-thalassemia1 gene (RT: ATATATGGGTCTGGAAGTGTATC).

Gene amplification was performed using the PCR master mix solution (*i*-Taq, 2x) (iNtRON Biotechnology, Korea) and were analyzed by agarose gel electrophoresis at 100 volts for 45 min [5].

The specific DNA probe was designed using Primer 3 software [6] and synthesized with biotin tagged at the 5′ end (Biotin-TTTTTTGAAGGAGGGGAGAAGCTGAG) (1st BASE, Singapore).

Preparation of thalassemic QCM

The 12 MHz AT-cut quartz crystals with silver fabricated on both sides (4 mm diameter/0.1257 cm^2) were provided locally (Metal Inter Supply Ordinary Partnership, Thailand). Each measurement was performed on one side of the silver coated quartz surface. The resonance frequency was measured by an in-house oscillation counter as described [5–8].

The QCM silver surface was cleaned with 1:10 diluted Piranha solution containing 3 parts of H_2SO_4 and 1 part of H_2O_2. The baseline resonance frequency (f_0) was recorded. Sequential surface chemical modifications were performed [5, 8] to activate the carboxyl groups on the silver electrode for an avidin attachment. Avidin at various concentrations (0.0, 0.005, 0.01, 0.05, 0.1, 0.15 and 0.2 g/L) were individually immobilized on the modified QCM surface. The resonance frequency after each avidin immobilization (f_1) was recorded. The optimal avidin concentration was determined and used in all experiments.

Various concentrations (0.0, 0.125, 0.25, 0.5, 0.75, 1.0, 1.5 and 2 μmol/L) of the biotinylated probe were immobilized via an avidin-biotin interaction. The resonance frequency (f_2) was measured and the optimal probe concentration was determined. The probe immobilized QCM was kept in a sealed cap at room temperature and used as the silver thalassemic QCM.

Each immobilization was incubated at room temperature for 30 min in a dark moisture chamber. Washing with distilled water and drying overnight in a desiccator were performed [5, 8]. The numbers of immobilized either avidin or biotinylated probes were estimated by a Sauerbrey equation [9].

Evaluation of the thalassemic QCM

This study was approved by the Mahidol Ethical Committee on Institutional Review Broad (MU-IRB 2011/010.0609). Blood samples carrying normal α-globin gene, gene of α-thalassemia1 (SEA deletion) and gene of α-thalassemia1 disease (Hb Bart's hydrops fetalis) were used to the test diagnostic potency of the thalassemic QCM. The amplified target DNA of each blood sample was diluted in hybridization buffer to various concentrations from 0, 25, 50, 75, 100 and 150 mg/L. Then 10 μL of each dilution was denatured at 95°C for 10 min and hybridized with the immobilized probe at room temperature for 30 min. After hybridization, the QCM was rinsed with distilled water and dried overnight in a desiccator. The resonance frequency (f_3) was recorded and the frequency shifts ($\Delta f = f_2 - f_3$) indicated hybridization.

Clinical application of the QCM was evaluated using 70 suspected samples with microcytic hypochromic red blood cells (MCV<80 fL).

Evaluation of the thalassemic QCM storage stability

The thalassemic QCM was kept in a sealed cap at room temperature for 0, 5, 10, 15, 20 and 30 days. During these time intervals, the QCM was tested for its diagnostic potency to identify the abnormal α-globin gene.

Improvement of the QCM preparation

The conventional process of QCM preparation and target DNA hybridization as described previously [5, 8] needed three washing and drying steps. Each drying step was performed by an overnight air-drying in a desiccator leading to the long analysis time (3–4 days). This study improved the drying step by using a MILLIPORE® air-pump (BENSON HORBOR, MI, USA) at 2.5 psi for 30 min. After washing, the QCM was placed in a closed box connected to an air-pump. The diagnostic potency was compared between the overnight air-dried and using an air-pump.

Statistical analysis

All measurements in this study were performed in triplicate. The data were analyzed by a two-tailed Student's t-test or one way ANOVA.

Results

Amplification of α-globin gene

The target α-globin DNA was amplified and analyzed by 1% agarose gel electrophoresis (Figure 1). The amplification of normal α-globin gene generated a single PCR product at 314 bp while that of Hb Bart's hydrops fetalis was also a single band at 660 bp. The amplification of α-thalassemia1 (SEA deletion) showed two bands at both positions.

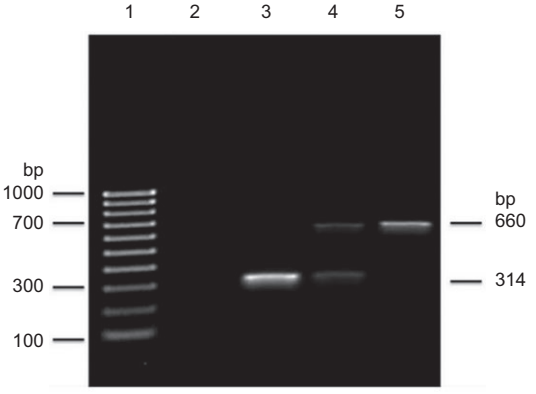

Figure 1 The amplified target DNA of blood samples carrying normal α-globin gene (lane 3), abnormal gene of either α-thalassemia1 carrier (SEA type) (lane 4) or Hb Bart's hydrops fetalis (lane 5), respectively.
The 100 bp DNA markers (lane 1) and the PCR control without template DNA (lane 2) were included. Agrose gel electrophoresis was performed at 100 volt for 45 min.

Preparation of thalassemic QCM

The silver surface of QCM was modified with various concentrations of avidin. Figure 2A shows saturated avidin coating with the maximum binding at 0.1 g/L. This optimal concentration was used in all experiments. The number of avidin at this concentration was estimated by a Sauerbrey equation [9] showing 5.77×10^{11} molecules of avidin immobilized on the QCM silver reaction area (0.126 cm^2).

Then each concentration of the biotinylated probe was immobilized. Figure 2B shows saturated probe coating with the maximum binding at 0.5 μmol/L. This optimal probe concentration was used in all experiments. Estimation using the Sauerbrey equation [9] indicated the number of immobilized probes at $1.28\pm0.19\times10^{12}$ molecules.

The binding ratio between immobilized avidin and probe on the QCM surface was $5.77\pm0.43\times10^{11}$: $1.28\pm0.19\times10^{12}$ or 1:2.2.

Evaluation of the thalassemic QCM

The QCM was preliminarily verified using diagnosed blood samples. The binding of target DNA to the biotinylated probe in all cases was saturated at concentrations >100 mg/L (Figure 3). The frequency shifts of Hb Bart's hydrops fetalis were highest while those of normal globin gene were lowest. The heterozygous of thalassemia1 (SEA deletion) showed frequency shifts between normal and Hb Bart's hydrops fetalis.

There was no statistically significant difference among the three genotypes at DNA concentrations <25 mg/L (p-value >0.05). At DNA concentrations 50 and 75 mg/L, the QCM could differentially identify normal α-globin gene from genes causing α-thalassemia1 but could not identify between heterozygous and homozygous gene abnormalities. The three different genotypes could be differentially diagnosed at 100 mg/L of target DNA (p-value <0.05). This was the optimal DNA concentration and was used in all experiments.

The clinical application of the silver QCM was evaluated using 70 suspected blood samples with microcytic anemia (MCV <80 fL). The genomic DNA was extracted and then α-globin DNA was amplified and identified by the QCM (Table 1) compared to the agarose gel electrophoresis (Figure 4).

Thirteen samples with two amplified DNA bands, one consistent with normal α-globin gene and the other consistent with abnormal gene corresponded to α-thalassemia1 (SEA deletion). These 13 samples were carriers of α-thalassemia1 (SEA deletion). The other 57 samples showed a single band that corresponded to normal α-globin gene. All of these samples were also analyzed by the new thalassemic QCM (Table 1).

It was found that the frequency shifts of the 13 samples corresponded to the α-thalassemia1 (SEA deletion) as diagnosed by gel electrophoresis (Figure 4). They showed frequency shifts at 155+7.11 Hz while the other 57 samples showed statistical different frequency shifts at 92+15.67 Hz (p-value <0.05). The results indicated that diagnostic efficiency of the new thalassemic QCM was completely consistent with that of the standard agarose gel electrophoresis.

Table 1 also showed the difference of red blood parameters between the normal and α-thalassemic carrier. The mean cell volume (MCV) of the 13 thalassemia carriers was significantly lower than that of 57 non-carriers (p-value <0.05). No difference in mean hemoglobin concentration and mean cell hemoglobin concentration between the two groups.

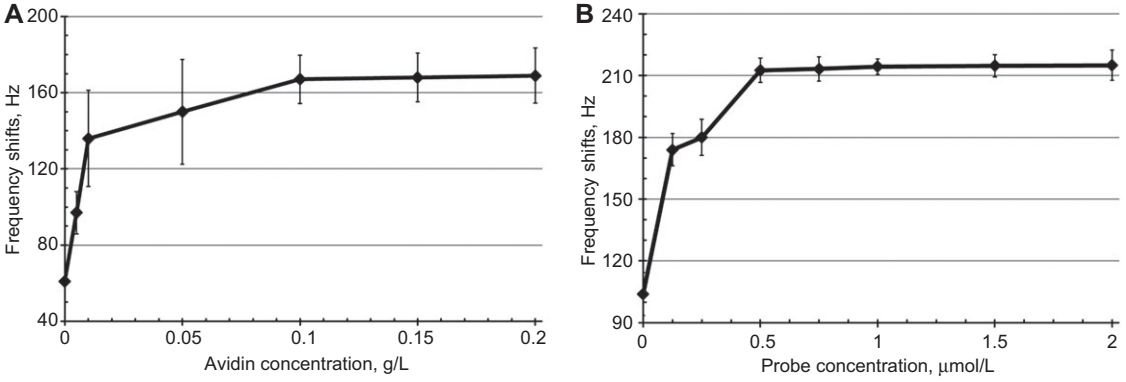

Figure 2 Binding of avidin and biotinylated probe to the QCM silver surface.
The frequency shifts after avidin (A) and bionylated probe (B) were immobilized on the QCM silver surface.

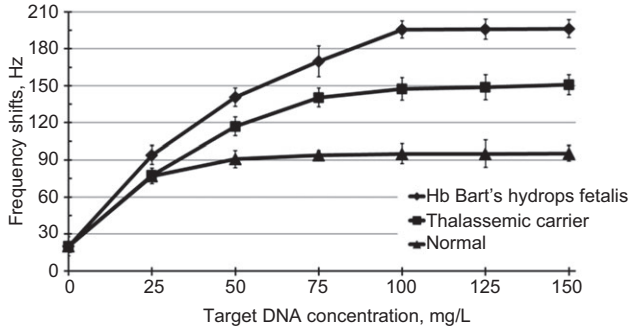

Figure 3 The diagnostic potency of the silver QCM.
Each amplified target DNA of blood from normal, α-thalassemia1 carrier (SEA deletion) and Hb Bart's hydrops fetalis was hybridized with the immobilized biotinylated probe on the QCM surface. The frequency shifts were measured.

Evaluation of the thalassemic QCM storage stability

The silver thalassemic QCM was tested for its storage stability by keeping the freshly prepared sensor in a sealed cap at room temperature for 0, 5, 10, 15, 20 and 30 days. During these time intervals, the diagnostic potency was tested using 100 mg/L of amplified target DNA from blood of normal globin genotype, α-thalassemia1 carrier (SEA deletion) and Hb Bart's hydrops fetalis. Keeping at room temperature up to 30 days did not affect the QCM diagnostic potency when compared to the freshly prepared QCM (p-value <0.05). It could be efficiently used to differentially diagnose normal α-globin genes from the abnormal α-thalassemic genes.

Improvement of the QCM preparation

In the conventional process of QCM preparation, all the drying steps used overnight were air-dried in a desiccator. The total analysis time was 3 days and 3 h. The drying step was improved by using an air pump for 30 min. The frequencies when using an air-pump drying were slightly lower than those of overnight air-dried but there was no statistical difference (p-value >0.05). This improvement could reduce the total analysis time to 4 h with no effect on the QCM diagnostic potency.

By using an air pump, the silver QCM could be manually used to analyze approximately 30–50 samples in 4 h of analysis time. The throughput limitation is the surface drying after each chemical immobilization.

Discussion

Homozygous α-thalassemia1 or Hb Bart's hydrops fetalis is the most severe form of hereditary hemolytic anemia. Couples who are carriers of α-thalassemia1 have 25% chance to produce babies born with this deadly disease [10, 11]. The effective prevention program needs an accurate identification of the carriers and family planning [12]. Many methods have been used to screen the carriers but the definite diagnosis requires confirmation at the gene level [13–16]. The molecular diagnosis composed of polymerase chain reactions (PCR) and post-PCR methods to identify the amplified gene(s) [17, 18]. However, the electrophoresis needs ethidium bromide staining which is carcinogenic. This study developed silver QCM as a post-PCR method which needs no carcinogenic staining and it is a label-free technique.

Biosensor has been used in clinical application [19–21]. It was successfully modified to differentially identify normal globin gene from abnormal gene causing Hb Bart's hydrops fetalis (SEA deletion) [5, 8, 22]. Recently, it was applied in malaria genotyping [8]. However, most of the QCM used was gold fabricated which might be expensive for developing countries. Presently, we used the silver fabricated QCM which was 10 times cheaper than the gold QCM.

It has been suggested that the target DNA fragments detected by QCM sensor should not be longer than 500 bp because of the steric hindrance [8, 22]. This study designed three primers to amplify either the normal α-globin gene or gene causing Hb Bart's hydrops fetalis (SEA deletion). The primers produced a single product at

Blood samples	Frequency shifts, Hz	Hemoglobin, g/L	Mean cell volume, fL	Mean hemoglobin concentration, g/L
α-Thalassemia1 (SEA deletion) (n=13)	155±7.11	110.46±10.10	64.45±2.53	320.05±6.40
Other microcytic anemia (n=57)	92±15.67	120.78±10.68	74.81±4.71	320.69±7.60
p-Value	0.005	0.24	0.022	0.55

Table 1 Clinical application of the silver QCM. Seventy microcytic hypochromic blood samples were diagnosed by the QCM.

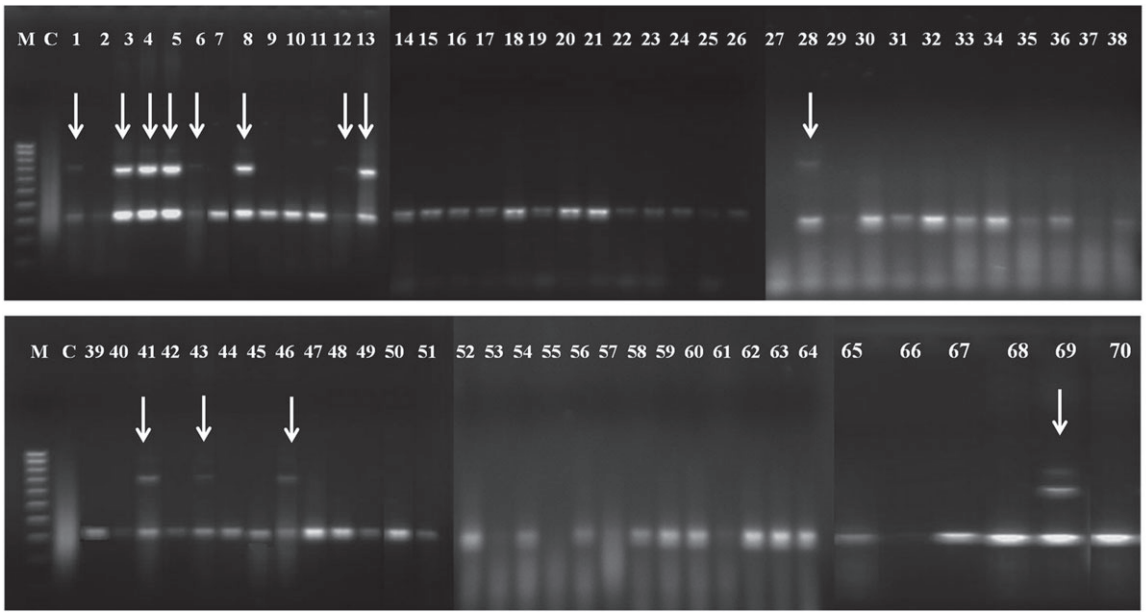

Figure 4 Analysis of the suspected thalassemic samples by agarose gel electrophoresis.
M and C were 100 bp molecular markers and the control without DNA template. No.1–70 was amplified α-globin gene of each sample. The arrows indicated abnormal α-globin DNA corresponded to α-thalassemia1 (SEA type).

either 314 or 660 bp, respectively (Figure 1). The heterozygous α-thalassemia1 (SEA deletion) showed two amplified DNA fragments at both positions. These amplified products were corresponded to the expected size when primers were designed.

QCM has been developed to diagnose the abnormal α-thalassemic gene using a large (2.54 cm in diameter) gold fabricated sensor [22]. To reduce the production cost, the small sensor (4 mm in diameter) was developed [5]. This could reduce the production cost from $100 to $2–$3. In this study, we used the small silver fabricated sensor since it is 10 times cheaper than the gold QCM.

However, since silver has higher chemical reactivity and lower acid resistance than gold [23], the high strength of sulfuric acid used in conventional Piranha solution [5, 8] can cause the dark QCM surface. This dark surface made the QCM not suitable in clinical diagnosis. Therefore, the 10× diluted Piranha solution was used to clean the QCM silver surface in all experiments.

The clean silver QCM surface was sequentially coated with immobilizing chemicals to prepare the carboxyl groups for binding with the amine groups of avidin [5, 8]. Avidin immobilization on the silver QCM surface was saturated with the maximum binding at 0.1 g/L (Figure 2A). At this concentration, there were $5.77\pm0.43\times10^{11}$ molecules of avidin attached on the QCM reaction area (0.126 cm^2). Calculation from the three dimensions of avidin (6.6×5.5×4 nm) [24] indicated the averaged 5.18×10^{11} molecules of avidin could possibly bind as a monolayer on this reaction area. The estimated number of immobilized avidin ($5.77\pm0.43\times10^{11}$ molecules) suggested an almost monolayer binding as suggested in QCM development [25].

The biotinylated probe was attached via a strong avidin-biotin interaction which could tolerate many manipulations during QCM preparation [24]. The probe was saturated immobilized with the maximum binding at concentration 0.5 μmol/L (Figure 2B). The estimated numbers of probe were $1.28\pm0.19\times10^{12}$ molecules. So the binding ratio of immobilized avidin to biotinylated probe was 1:2.2 which was lower than 1:4 of the free floating binding ratio [25]. The 1:2.2 ratios are reasonable since the immobilized avidin would not be freely accessed by the biotinylated probe.

The probe immobilized QCM developed in this study was used to diagnose the abnormal α-globin gene using amplified products as shown in Figure 1. The target DNA fragments were analyzed by hybridization with the immobilized biotinylated probe on the QCM. Figure 3 shows the ability of QCM in differential diagnosis between normal α-globin gene and gene causing α-thalassemia1 (SEA deletion), either carrier or disease forms. The optimal DNA concentration at 1 μg was consistent with the previous study [5].

Moreover, Figure 3 also shows the frequency shift after hybridization of normal α-gene and gene of Hb Bart's hydrops fetalis. The frequency shift was consistent with

the size of amplified DNA fragments, 314 bp of normal and 660 bp of Hb Bart's hydrops fetalis. The frequency shift of the carrier was almost in the middle since it is a heterozygous composed of normal globin gene on one arm of chromosome and thalassemic globin gene on the other.

The clinical application of the silver QCM for discrimination between normal α-globin gene and gene causing α-thalassemia1 (SEA deletion) was further tested using the suspected 70 blood samples with microcytic hypochromic anemia (MCV <80 fL). The target gene was amplified and analyzed by the QCM compared to the agarose gel electrophoresis. Figure 4 demonstrated 13 heterozygous α-thalassemia1 (SEA deletion) showing two amplified DNA bands at 314 and 660 bp. The other 57 samples showed one normal band at 314 bp. This result corresponded to that of silver QCM (Table 1). The frequency shifts of 13 carriers (155±7.11 Hz) were significantly higher than those of 57 samples with normal genotype (92±15.67 Hz) (p-value=0.005). These findings were consistent with the previously developed gold fabricated QCM [5]. The data showed high potency of the silver QCM as a one-step molecular diagnosis of α-thalassemia1 (SEA deletion).

The silver QCM was stable for up to 30 days after keeping at room temperature. The keeping stability indicated ability of the QCM to be transferred and used in the remote area away from the production site.

The QCM drying step was improved by using an air-pumped for 30 min instead of an overnight air-dried [5, 8]. This improvement could reduce the analysis time from 3 days to 4 h with no change in diagnostic potency (p-value >0.05).

Conclusively, this study could develop a new silver fabricated QCM to differential identify α-globin genes between normal and α-thalassemia1 (SEA deletion). This QCM is a promising one step definite diagnosis which is simple, specific, sensitive, cheap, rapid, stable and field applicable.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.
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