Rapid Screening for Deleted Form of β-thalassemia by Real-Time Quantitative PCR

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Background: Thalassemia is the most common single gene disease in human beings. The prevalence rate of β-thalassemia in Taiwan is approximately 1–3%. Previously methods to reveal and diagnose severe deleted form of α- or β-thalassemia were insufficient and inappropriate for prenatal diagnosis. *Methods:* A real-time quantitative PCR method was set up for rapid screening of the deleted form of β-thalassemia. *Results:* Our results show that $\Delta\Delta Ct$ between deleted form of β-thalassemia and normal individuals were 1.0674 ± 0.0713. On the contrary, mutation form β-thalassemia showed no difference with normal healthy control. The *HBB/CCR5* ratio for deleted form of β-thalassemia patients was 0.48, whether normal individuals and mutation form of β-thalassemia was 1.0. *Conclusion:* This RQ-PCR technique is an alternative rapid screening assay for deleted form of β-thalassemia. In addition, it could also identify undefined type. Our technique by using RQ-PCR to quantify gene copies is a reliable and time-saving method that can screen deleted form of β-thalassemia.
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Key words: real-time quantitative PCR; β-thalassemia

INTRODUCTION

Thalassemia is the most common single gene disease in human beings. The decrease or absence of β-globin production leads to β-thalassemia (1, 2). The β-globin gene cluster is composed of five genes in the arrangement of 5'-ε-γ^G-γ^A-δ-β–3' and is located at minus strand of chromosome 11 (3). The whole length of gene cluster spans around 60 kb. More than 250 mutations in β-globin gene have been reported to cause β-thalassemia, and most of them are point mutations (4–6). Until now, more than 20 β-globin gene mutated or deleted forms have been found in Taiwanese population (7). The prevalence rate of β-thalassemia in Taiwan is 1–3% approximately (7, 8). Four common types of the point mutations, namely HBB:c.-78A>G (promoter −28 A→G), c.52A>T (codon 17 AAG→TAG), c.125_128delTCTT (codon 41–42-TCTT) and c.316-197C>T (IVS-2 nt 654 C→T) mutations, were the main cause of β-thalassemia in more than 80% of patients in Taiwan (9). However, 3–5% cases belong to deleted form of β-thalassemia, including U01317.1:g.37242_116449del79208 (Southeast Asian type of hereditary persistence of hemoglobin F; HPFH-SEA), U01317.1:g.40454_119300del78847 (Chinese $G\gamma^+(A\gamma\delta\beta)^\circ$ type), and Yunnanese type (10–14). To diagnose those deleted forms of β-thalassemia, duplex PCR was used to amplify the junction in clinical laboratories (13–15). According to Globin Gene Server (http://globin.cse.psu.edu/), there are 17 different large-fragment β-globin deleted forms that have been reported but not able to be identified by current duplex PCR in clinical laboratories (16).

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Previous studies showed that cDNA/DNA hybridization techniques were used to discover severe deleted form of α- or β-thalassemia (17–19). However, those techniques are not only inappropriate for prenatal diagnosis but also laborious in a clinical laboratory. Therefore, we try to set up a real-time quantitative PCR (RQ-PCR) method for rapid screening of the deleted form of β-thalassemia.

DESIGN AND METHODS

Patients and Controls

From 2002 to 2007, a total of 112 patients from Kaohsiung Medical University Hospital were enrolled for this study. Out of the 112 patients, eight patients were detected as β-globin heterozygous deletions, 34 patients as mutated β-thalassemia and 70 healthy persons served as normal controls. Genomic DNA was extracted using a NucleoSpin® genomic DNA from blood kit according to the manufacturer's instructions (Macherey-Nagel GmbH & Co., Düren, Germany).

Detection of β-thalassemia by PCR-RFLP and Duplex PCR

To detect point mutated β-thalassemia, PCR-restriction fragment length polymorphism (PCR-RFLP) method was used as described in our previous report (9). To detect HPFH-SEA, a duplex PCR was used with three primers bridging the 3' breakpoint (13). The upstream primer 5'-TGGTATCTGCAGCAGTTGCC-3' (NT 5207166 to 5207147 in NC_000011) and the downstream primer 5'-AGCCTCATGGTAGCAGAATC-3' (NT 5179380 to 5179399 in NC_000011) were used to amplify the breakpoint region. For normal control, another upstream primer 5'-ATTGTTGAGTTGCAAGATCG-3' (NT 5179944 to 5179925 in NC_000011) with the downstream primer around the breakpoint region were used. To detect Yunnanese deletion (Aγδβ)°, a upstream primer 5'-TTCCCCACACTATCTCAATGC-3' (NT 5227764 to 5227744 in NC_000011) and a downstream primer 5'-CAAGGCCAGGGAGAACTGC-3' (NT 5207049 to 5207068 in NC_000011 of GenBank) were used to amplify breakpoint region. Identical upstream primer with downstream primer 5'-AGAGGACAGGTTGCCAAAGC-3' (NT 5227336 to 5227355 in NC_000011) was used to amplify the normal 5' area around the breakpoint region (14). To detect Chinese deletion ($G\gamma^{+}(A\gamma\delta\beta)°$), γA1 5'-GCTGGACACATATAAAATGCTGC-3' (NT 5226740 to 5226718 in NC_000011) and γA3 5'-AGAAATTGCCTCATGTCTCT- 3' (NT 5147639 to 5147658 in NC_000011) were used to amplify the breakpoint region. γA2 5'-TGCAGGTAGTTGTTCCCCTTCA-3' (NT 5226443 to 5226464 in NC_000011) and γA1 were used to amplify normal 5' sequence around the breakpoint region (12). Approximately 0.2~1.0 μg genomic DNA was mixed with 50 ng of each primer and 200 μM of each dNTP in 50 μl reaction buffer containing 10 mM Tris-HCl (pH 8.3) 50 mM KCl, 1.5 mM $MgCl_2$, and 2.5 units of Taq polymerase. PCR amplification was carried out in an ABI 9700 thermal cycler. The mixture was incubated at 95°C for 5 min, followed by thirty-six amplification cycles of 95°C for 1 min denaturation, 52°C for 1 min annealing and 72°C for 2 min extension. The amplified PCR product was further incubated at 72°C for 7 min to ensure the complete extension. The PCR products were analyzed by electrophoresis on 2% agarose gel and visualized in UV light.

PCR Primers for Real-Time Quantitative PCR (RQ-PCR)

Primers for RQ-PCR were designed by a DNASTAR PrimerSelect. The primers for *HBB* gene were *HBB*145F 5'-TGGTATCAAGGTTACAAGACAGGTTT-3' (NT 5204733 to 5204708 in NC_000011) and *HBB*245R 5'-CAGAGAGAGTCAGTGCCTATCAGAA-3' (NT 5204633 to 5204654 in NC_000011). The internal control primers for *CCR5* gene were *CCR5*F 5'-GAACCTTGACGGCATTGC-3' (NT 46391197 to 46391214 in plus strand of NC_000003) and *CCR5*R 5'-CCTCCCTCCTTCCCATCC-3' (NT: 46391394 to 46391377 in plus strand of NC_000003).

Real-Time Quantitative PCR

The RQ-PCR combined with the dissociation curve analysis was used to determine *HBB* gene copy number of patients. *CCR5* gene served as a reference to normalize and enable the comparability of RQ-PCR data. Delta *C*t value (cycle threshold value; Δ*C*t) was calculated by subtracting $Ct_{(HBB)}$ from $Ct_{(CCR5)}$. Delta *C*t was calculated by subtracting Δ*C*t (β-thalassemia group) from Δ*C*t (normal healthy control). RQ-PCRs were performed on a LightCycler 1.0 or LightCycler 480 (Roche Diagnostics; F. Hoffmann-La Roche Ltd., Basel, Switzerland). Each reaction was carried out in 2 μl of 100 ng/μl genomic DNA and 8 μl reaction mixture containing 2.4 mM $MgCl_2$, 1 U of FastStart DNA Master Mix SYBR Green I (containing Taq DNA polymerase, SYBR Green I, and deoxynucleoside triphosphate mix), 0.6 μM of the primers (forward and reverse). PCRs were subjected to 10 min of 95°C hot-start enzyme activation, and 40 cycles of 95°C denaturation for 5 sec, 64°C annealing

for 5 sec, 72°C elongation for 12 sec, and 80°C signal detection for 0 sec. To avoid cross-contamination and sample carryover, pre- and post-PCR sample processing were performed in separate rooms. All fluid transfers were carried out with plugged pipette tips to eliminate aerosols. Diethyl pyrocarbonate (DEPC)-treated water served as the template for negative control amplifications, which were included with each PCR run. Amplified DNA products were detected by melting curve analysis which consisted of 95°C for 5 sec, 65°C for 30 sec, and continuously heated at the rate of 0.1°C/sec until 95°C. For analysis of the melting curves, the Light Cycler instrument's software that automatically converts them into melting peaks was used. The T_m's of the peaks were analyzed using the best-fit analysis software provided by Roche Diagnostics (F. Hoffmann-La Roche Ltd.).

Statistical Analysis

Results were analyzed by SPSS version 11.5 for Windows (SPSS Inc., Chicago, IL). One-way ANOVA and Scheffe post hoc analysis were used to test continuous variables in three different groups. A P value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

All of the β-thalassemia forms were diagnosed by the PCR-RFLP and duplex PCR methods. Figure 1 shows the duplex PCR result of a case of $G\gamma^+(A\gamma\delta\beta)^\circ$ of Chinese type. For RQ-PCR screening, the PCR efficiency of these two primer pairs was of no difference. *HBB*145F and *HBB*245R primer pair was used for detection of target *HBB* gene, whereas *CCR5*F and *CCR5*R primer pair for *CCR5* gene severed as a reference control. In this study, genomic DNA from normal populations were serial diluted and quantified by RQ-PCR using *HBB*145F and *HBB*245R, and *CCR5*F and *CCR5*R primer pairs to test their PCR efficiency. The amplification curve was shown in the Figure 2a. According to the cycle number vs. concentration for each standard, standard curve for target *HBB* gene were constructed (figures were not shown). The average slope of *HBB*145F and *HBB*245R primer pair was 3.738 ± 0.18 and the average PCR efficiency was 1.852 ± 0.02. However, the average slope of *CCR5*F and *CCR5*R primer pair was 3.707 ± 0.15 and the average PCR efficiency was 1.872 ± 0.02. The melting point of PCR product of *HBB* gene was 82.76°C, whereas product of *CCR5* gene was 88.06°C (Fig. 2b). No other non-specific product was found in melting curve analysis and electrophoresis.

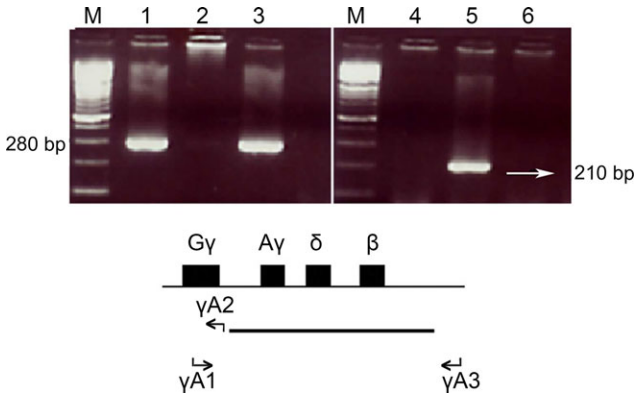

Fig. 1. The PCR-RFLP result of the breakpoint in a case of Chinese $G\gamma^+(A\gamma\delta\beta)^\circ$-thalassemia. The γA1 and γA2 primer pair amplifies the normal upstream sequence around the breakpoint region, the PCR product is 280 bp. The γA1 and γA3 primer pair amplifies the breakpoint region, the product is 210 bp. M represents 100 bp ladder marker. The lower panel shows the relative location of the primers. The diagram of β-gene cluster is not the true scale.

The copy numbers of *HBB* gene in deleted form of β-thalassemia were significantly different from normal individuals ($P < 0.001$). The mean $\Delta\Delta C$t in each run of Q-PCR was 0.0035 ± 0.1765 in normal individuals, 0.0558 ± 0.2209 in mutational form of β-thalassemia, and 1.0709 ± 0.1759 in deleted form of β-thalassemia. One-way ANOVA and Scheffe post hoc were used to test the difference of $\Delta\Delta C$t among the three groups (Fig. 3). The result showed $F = 113.189$ and $P < 0.001$ in one-way ANOVA. The mean difference of $\Delta\Delta C$t was 1.0674 ± 0.0713 between deleted form of β-thalassemia and normal individuals, and 1.0151 ± 0.0751 between deleted form and mutation form of β-thalassemia. The *HBB*/*CCR5* ratio for deleted form of β-thalassemia patients was 0.48, whereas for normal individuals and mutation form of β-thalassemia, it was 1.0.

This RQ-PCR technique is an alternative rapid screening assay for deleted form of β-thalassemia and could be used to find out undefined type. The cDNA/DNA hybridization technique is used to discover severe deletion forms of α and β-thalassemia (18). In this method, the initial step is the isolation of messenger RNA and translation by incubating with wheat germ cell-free system containing [^{14}C]-leucine. Then, cDNA is hybridized to mRNA, β-enriched cDNA, and total DNA to determine whether mismatched hybrids of low stability are formed at the lower temperature. It takes a long time for each step. In 1979, Orkin et al. (16) used restriction endonuclease mapping of cell DNA to investigate the deleted form of β-thalassemia. The diagnostic procedure started from using EcoRI,

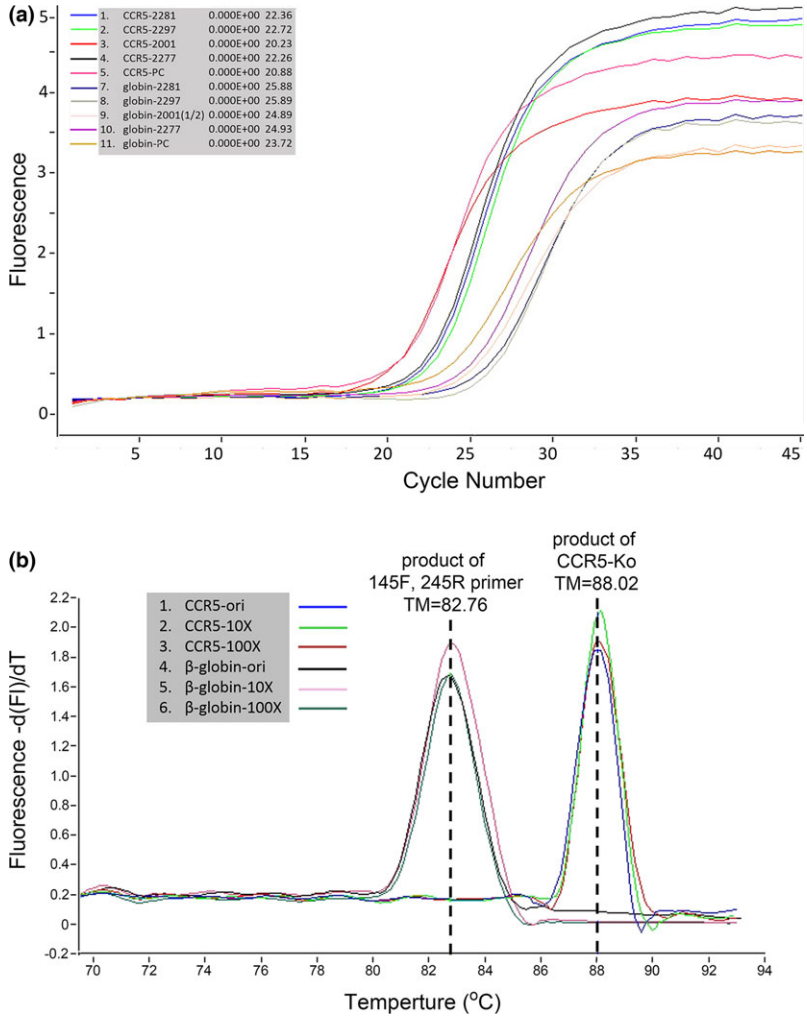

Fig. 2. The amplification and melting curves of *HBB* and *CCR5* gene: (a) The amplification curve of *CCR5* and *HBB* gene, respectively. (b) The melting point of PCR product of *HBB* gene was 82.76°C, whereas product of *CCR5* gene was 88.06°C.

	Mean	SD
Normal	0.0035	0.1765
B-thalassemia		
Mutation form	0.0558	0.2209
Deletion form	1.0709	0.1759
Total	0.0956	0.3320

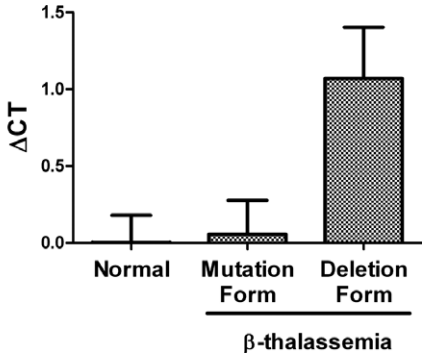

Fig. 3. The difference of ΔΔ*C*t among the three groups: The copy numbers of *HBB* gene in deleted β-thalassemia were significantly different from normal individuals and mutated β-thalassemia ($P < 0.001$).

Pst I, Bgl II, and BamHI to digest cellular DNA samples and followed by electrophoresis in 1% agarose gel. Then, β-globin [^{32}P] cDNA is used to hybridize the sample. The shortage of this technique is that it only can detect small length deletion (no more than 1,000 bps) of β-globin. In 1986, Henthorn et al. (20)

used breakpoint junction PCR/gel electrophoresis method to detect Indian HPFH $^{G}\gamma^{A}\gamma(\delta\beta)^{0}$-thalassemia. In 1990, field-inversion gel electrophoresis was used to detect Belgian $G\gamma^{+}(A\gamma\delta\beta)^{0}$-thalassemia (21). Fluorescent in situ hybridization with β-globin genes as probe to detect the β-globin gene copy is also a reliable method (22). However, this method is time consuming and need relative more viable cells. Currently, duplex PCR with three primers bridging the 3' breakpoint is a widely used method as it can only detect each of well-known deleted forms (12–14). A real-time PCR Light-Cycler technology using fluorescence and melting curve analysis has been developed in 1998 (23). Since then the nucleic acid-based diagnostics were widely used in clinical practice for the detection of microorganisms (24), gene polymorphisms (25), lymphoma (26, 27), and many other diseases (28, 29).

In conclusion, the deleted β-thalassemia, including HPFH-SEA, Chinese $G\gamma^{+}(A\gamma\delta\beta)^{\circ}$ type and Yunnanese type, can be screened through the change in $\Delta\Delta Ct$ and it could also identify undefined type of deleted β-thalassemia. Our technique, RQ-PCR, to quantify gene copies is a reliable and time-saving method that can screen deleted forms of β-thalassemia.

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AUTHORSHIP

L-YK designed the study, participated in data collection, analysis, interpretation, and wrote the draft of the manuscript; T-CL participated in designing the study, and in data collection, analysis, and interpretation, and approved the final version; J-GC and C-SC co-ordinated the research and recruited the patients; and C-WL and L-LS performed the laboratory work.

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