Original Article

Comparisons between RT-PCR, Real-time PCR, and *In Vitro* Globin Chain Synthesis by α/β Ratio Calculation for Diagnosis of α- from β-thalassemia Carriers

Fereshteh Maryami MD[1], Reza Mahdian MD PhD[1], Somayeh Jamali MSc[1], Mohsen Karimi Arzanani PhD[1], Shohreh Khatami PhD[2], Fahimeh Maryami BSc[1], Parastou Bayat BSc[2], Sedigheh Sadeghi MLD[2], Morteza Karimipour MD PhD[1], Sirous Zeinali PhD•[1]

Abstract

Background: Thalassemia, which may be due to point mutations, translocations, and deletions involving the α or βglobin gene, is the most prevalent single gene disorder in Iran.This study aims to calculate the α/β ratio in normal cases, α- and β-thalassemia carriers by RT-PCR, real-time PCR, and *in vitro* globin chain synthesis (GCS) in order to establish the most accurate technique to distinguish between α- and β-thalassemia carriers in suspicious cases.

Methods: The α/β ratios were calculated in all samples by RT-PCR, real-time RT-PCR, and *in vitro* GCS.

Results: Using RT-PCR, the ratios were 1.09 ± 0.07 in normal samples, 1.2 ± 0.17 in β-thalassemia, 1.08 ±0.19 in mild α-thalassemia, and 0.96 ± 0.19 in severe α-thalassemia carriers. In real-time RT-PCR, the ratios were 2.21 ± 1.36 in normal samples, 5.12 ± 1.83 in β-thalassemia, 2.88 ± 0.81 in mild α-thalassemia, and 1.18 ± 0.52 in severe α-thalassemia carriers. With GCS, the ratios were 1.03 ± 0.1 in normal samples, 1.9 ± 0.37 in β-thalassemia, 0.8 ± 0.13 in mild α-thalassemia, and 0.59 ± 0.12 in severe α-thalassemia carriers.

Conclusion: To determine the most accurate technique, we statistically analyzed the α/β ratios obtained from the three standard methods. The ratio obtained by GCS and real-time PCR were helpful in distinguishing between α and β carriers in suspicious patients in whom the mutation detection was limited and the risk for offspring was not clear. The use of this technique is more obvious when time is restricted (i.e. during the pregnancy period).

Keywords: Comparative threshold cycle method, globin chain synthesis, real-time PCR, RT-PCR, thalassemia

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Introduction

Thalassemia syndromes are the most prevalent autosomal recessive single gene disorders in Iran.[1,2] Interactions of different types of hemoglobinopathies can lead to thalassemia syndromes with a variety of phenotypes that range from asymptomatic to severe anemia. The diversity of thalassemia phenotypes depends on the amount of imbalances created between α- and non-α-globin chains.[3,4]

In normal erythropoiesis, there is a subtle balance between the productions of α- and β-globin chains. Ineffective erythropoiesis, decreased erythrocyte lifespan, and clinical manifestations of β-thalassemia are all related to the extent of imbalances in the globin chain. The defective β-globin gene is responsible for the production of excessive α-globin chains, which would be precipitated in red blood cells (RBCs) and would lead to ineffective erythropoiesis.[5,6] The most direct method for evaluating the imbalances between α- and β-globin chain ratios is *in vitro* globin chain synthesis (GCS).[3,4] Recently, Irenge and Chaisue[4,7,8] have developed a quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) that quantifies the expression of the defective β-globin gene, which has revealed that qRT-PCR is an accurate technique to evaluate the transcriptional impact of the β-globin gene.

Chaisue et al.[4] also measured the α/β ratio by the real-time PCR (qRT-PCR) and $2^{-\Delta\Delta CT}$ method and determined the presence of a relationship between the α/β ratio and disease severity. According to their findings, the α/β ratio in α-thalassemia carriers was less than non-carriers, whereas in β-thalassemia carriers, this ratio was more when compared with non-carriers. By following the methods used by Han et al.,[9] it has been shown that the α/β and α/(β+γ)-globin gene mRNA ratios varied significantly among normal adults, normal infants, β-thalassemia minors, and β-thalassemia majors. According to reports, real-time PCR (qRT-PCR) compared with competitive RT-PCR was proven to be faster, less labor-intensive, and did not need molecular carryover.[4,10] Khatami et al.[11] measured the α/β ratio using GCS to differentiate rare types of normal A2 heterozygous β-thalassemia from α-thalassemia. According to their results, this method was capable of differentiating between silent β-thalassemia and α-thalassemia carriers. In the present study we determined the α/β globin and their mRNA ratios using GCS, RT-PCR, and qRT-PCR.

Patients and Methods

Patients

After obtaining informed consent, 19 blood samples were taken

Authors' affiliations: [1]Biotechnology Research Center, Department of Molecular Medicine, Pasteur Institute of Iran, Tehran, Iran. [2]Department of Biochemistry, Pasteur Institute of Iran, Tehran, Iran.

•Corresponding author and reprints: Sirous Zeinali PhD, Iranian Molecular Medicine Network, Biotechnology Research Center, Pasteur Institute of Iran, Pasteur St., Tehran, Iran. CEO, Kawsar Biotech Co. and Kawsar Genomics & Biotech Center, No. 41 Majlesi St., Vali Asr St., Tehran 1595645513, Iran. Fax/Tel: +98 218-893-9142; +98-218-893-9140; E-mail: zeinali@kawsar.ir; sirouszeinali@yahoo.com

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from normal individuals in whom CBC indices were normal and who had no family history of thalassemia. Blood samples were also taken from 12 mild α-thalassemia carriers who had a deletion of one α-globin gene, 6 severe α-thalassemia carriers with the deletion of two α-globin genes, and 16 known β-thalassemia carriers who had been referred to the Prenatal Diagnosis Center at Pasteur Institute of Iran and whose molecular defects had already been determined at this center. A total of 10 mL of blood was taken and collected in tubes that contained EDTA for subsequent analyses. Hematological parameters were determined with hematology analyzers (models K1000 and KX21; Sysmex, Kobe, Japan). Hemoglobin electrophoresis was performed using conventional techniques in an alkaline medium.[11,12] HbA2 was measured by micro column chromatography.[11,12] The iron status was normal in all cases.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from 50 μL of blood sample using the Sigma TRI reagent. RNA concentration was measured using BioRad Smart Spect™ plus spectrophotometer; high quality RNAs were selected for subsequent analysis. cDNA was synthesized from total RNA using Roche M-MLV reverse transcriptase (Roche, Germany) according to the method given by the manufacturer.

Multiplex PCR was performed using specific primers (Table 1). All primers were designed using Gene Runner software (Hastings Software, Inc., version 3.05). Primer sequences were evaluated using Blast (http://www.ncbi.nlm.nih.gov/BLAST/) to check for sequence specificity.

Quantitative Real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

The primers were designed using Primer Express V.3.0 software (Applied Biosystems, Foster City, CA, USA). To avoid any homology to other parts of the human genome, the primer sequences were verified at http://www.ncbi.nlm.nih.gov/BLAST/. The characteristics of the primers are summarized in Table 1. A total of 25 ng of cDNA, 5 pM of each primer, and 12.5 μL of SYBR Green PCR master mix (Applied Biosystems, ABI, Foster City, CA, USA) were used to set up the amplification reactions. Melting curve analysis of the PCR products was performed after each amplification reaction.

In vitro globin chain synthesis (GCS)

The GCS procedure as reported by Khatami et al.[11] consisted of four fundamental steps: 1) incubation of reticulocyte-enriched red cells with (L-4,5H^3) leucine in a leucine free medium; 2) globin chain analysis on hemolysate samples by a high performance liquid chromatography (HPLC) system. A mono-S 5/5 R column was used as in the previously described method[11]; 3) determination of incorporated activity in the area of the α and β peak by using a beta counter instrument; and 4) calculation of the α/β globin chain ratio by dividing the ΣCPM incorporated activity in the area of the α peak to that of the β peak.[11]

Results

Agarose gel images (Figure 1) obtained by the RT-PCR method were analyzed by PhotoCapt software (PhotoCapt version 99.01 for Windows). The α- and β-peak areas were calculated with PhotoCapt software (Figure 2).

In the qRT-PCR technique, the amplification plot of the α-globin, β-globin, and β-actin is demonstrated in Figure 3. The standard curve was drawn, and PCR efficiency was calculated for both test and reference genes (Figure 4). The CT value of α-, β-globin, and β-actin genes were determined in each cDNA sample (Figure 4). In order to normalize the expression of the test genes to that of the reference gene, ΔΔCT values were calculated for each sample based on the formula: $\Delta\Delta CT = (mC_{T\ \alpha\text{-globin}} - mC_{T\ \beta\text{-actin}}) - (mC_{T\ \beta\text{-globin}} - mC_{T\ \beta\text{-actin}})$. Then, the α/β globin mRNA expression ratios were determined using the $2^{-\Delta\Delta CT}$ formula[4] in different groups.

In the GCS technique, the α/β globin chain ratios were calcu-

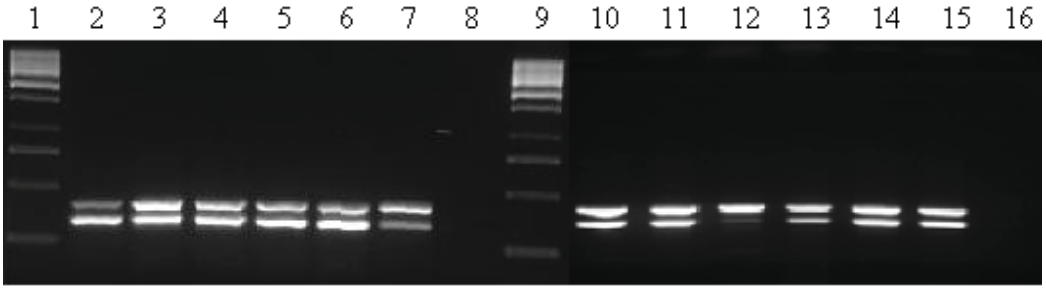


Figure 1. Electrophoresis gel photo. 1 = ladder; 2 = severe α-thalassemia carrier; 3, 4 = normal cases; 5, 6 = mild α- thalassemia carriers; 7 = β-thalassemia carrier; 8 = negative control; 9 = ladder; 10, 11 = normal cases; 12 = major β-thalassemia; 13 = β-thalassemia carrier; 14, 15 = normal cases; 16 =negative control.

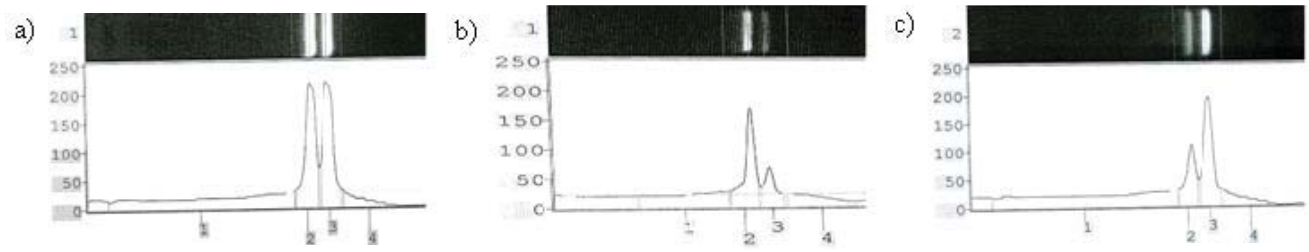


Figure 2. Gel analysis by PhotoCapt software; **a)** normal individual, α area/β area = 1.03; **b)** β-thalassemia carrier, α area/β area = 1.3; **c)** α-thalassemia carrier, α area/β area = 0.58.

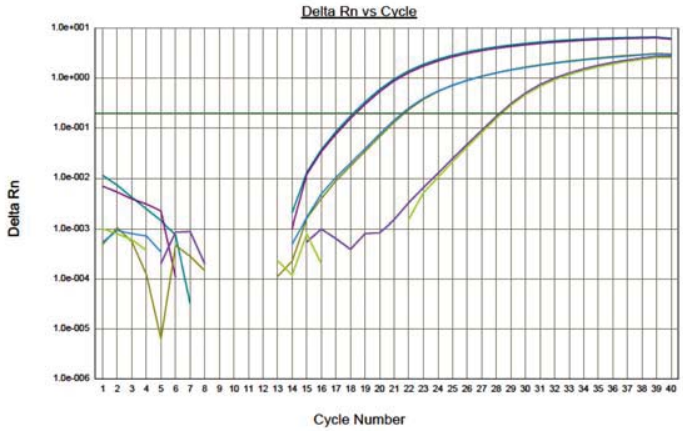
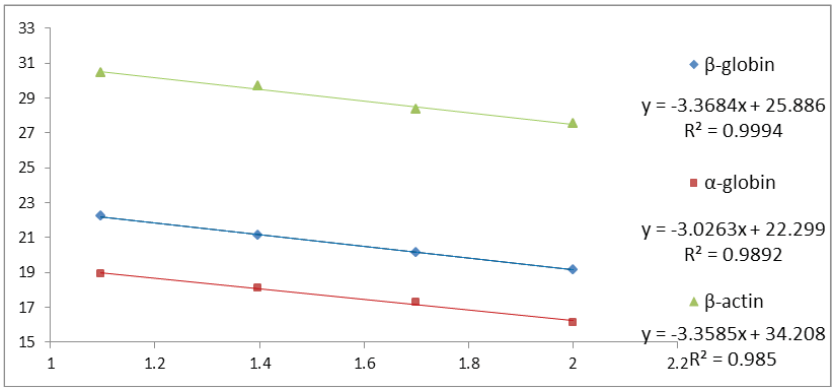


Figure 3. Amplification curves of α-globin, β-globin, and β-actin cDNAs drawn in a real-time PCR experiment.



	α-globin	β-globin	β-actin
Slope	-3.02	-3.36	-3.35
Efficiency (%)	97.8	98.01	98.01

Figure 4. Standard curve and PCR efficiency for α-globin, β-globin, and β-actin cDNAs.

lated by dividing the ΣCPM incorporated activity of α peak area to that of the β peak area. A descriptive analysis was performed on each sample group to determine the α/β ratio reference interval.[11] α/β ratios obtained from the three different methods are shown in Table 2.

Discussion

For countries implementing control of thalassemia or hemoglobinopathies, borderline CBC parameters are almost always a problem. They are more problematic when one partner is a definite carrier of either α- or β-thalassemia and the carrier status of the other partner is not definite. If time is restricted (i.e., during later stages of pregnancy), the situation becomes more critical. Therefore, the use of rapid and accurate techniques is essential for the diagnosis and evaluation of probable risk to the fetus.

Taking the results of this study into account, *in vitro* GCS is one of the most accurate methods of distinguishing between different types of thalassemia carriers and normal cases at the protein level. However, molecular techniques can only determine the mRNA content, which may not correspond well with protein because mRNA may rapidly degrade or may not be translated properly. In this regard, GCS has been shown to be more accurate.

The determination of α/β ratios by a reliable technique may provide useful information for diagnosing atypical cases. This technique could be valuable in differentiating between microcytosis produced by silent β-thalassemia and that caused by α-thalassemia.[11,13] *In vitro* GCS might also be useful in the diagnosis of rare large α- or β-globin gene deletions or, less commonly, point mutation defects. However, *in vitro* GCS involves an incubation of reticulocytes with radioactive amino acids, such as 3H-leucine, which require not only handling the radioisotopes (with their difficulties and possible harm to the users), but also a chromatographic separation step of the two types of globin chains.[3,4] Because of the considerable overlapping of ranges, this cannot be used as the sole diagnostic tool for thalassemia carrier detection.[11] This technique is also time consuming.

As reported by Chaisue et al.,[4] the α/β globin mRNA ratio calculation in thalassemia, using qRT-PCR, has shown that the deviations in α/β globin mRNA ratio from the normal control value are good indicators of thalassemia disease severity. They illustrated that multiplex qRT-PCR was a convenient and accurate method of measuring α/β globin mRNA ratio from α- and β-thalassemia carriers. In α-thalassemia, the α/β globin mRNA ratio correlated with the number of functional α-globin genes present, whereas in β-thalassemia this ratio provided a good indicator of disease severity.

Conclusion

Data analysis revealed that the RT-PCR method is appropriate for discrimination between β-thalassemia carriers and normal

Table 1. Primers used in RT-PCR and qRT- PCR.

Primer name	Technique	Gene	Position on gene	Primer sequence	Product length (bp)
alpha-FS	RT-PCR	α-globin	Exon I	5' CCG ACA AGA CCA ACG TCA AGG 3'	320
alpha-RS	RT-PCR	α-globin	Exon II	5' GGT ATT TGG AGG TCA GCA CGG 3'	
beta-FQ	RT-PCR	β-globin	Exon I	5' CCT GAG GAG AAG TCT GCC GTT AC 3'	280
beta-RS	RT-PCR	β-globin	Exon II	5' CCA GCA CAC AGA CCA GCA CG 3'	
a-real-F	Real-time PCR	α-globin	Exon II	5' CGCACAAGCTTCGGGTGGAC 3'	125
a-real-R	Real-time PCR	α-globin	Exon III	5' CAGGAACTTGTCCAGGGAGGCG 3'	
b-real-F	Real-time PCR	β-globin	Exon I	5' ACGTGGATGAAGTTGGTGGTGAGG 3'	126
b-real-R	Real-time PCR	β-globin	Exon II	5' TCACCTTAGGGTTGCCCATAACAG 3'	
act-real-F	Real-time PCR	β-actin	Exon III	5' CCAAGGCCAACCGCGAGAAG 3'	135
act-real-R	Real-time PCR	β-actin	Exon IV	5' CACCGGAGTCCATCACGATGC 3'	

Table 2. α/β ratios obtained by three different methods in different groups.

Method	Normal cases	β-thal carriers	Mild α-thal carriers	Severe α-thal carriers
In vitro GCS	1.03 ± 0.1 (95%CI: 0.99–1.07)	1.9 ± 0.37 (95%CI: 1.72–2.08)	0.8 ± 0.13 (95%CI: 0.73–0.87)	0.59 ± 0.12 (95%CI: 0.5–0.68)
qRT-PCR	2.21 ± 1.36 (95%CI: 1.68–2.74)	5.12 ± 1.83 (95%CI: 4.23–6.01)	2.88 ± 0.81 (95%CI: 2.43–3.33)	1.18 ± 0.52 (95%CI: 0.77–1.0)
RT-PCR	1.09 ± 0.07 (95%CI: 1.06–1.16)	1.2 ± 0.17 (95%CI: 1.12–1.37)	1.08 ± 0.19 (95%CI: 0.98–1.18)	0.96 ± 0.19 (95%CI: 0.81–1.11)

cases (sensitivity: 80%; specificity: 22%) or severe α-thalassemia carriers (sensitivity: 60%; specificity: 83%). However, it should not be used to distinguish between mild and severe α-thalassemia carriers or normal cases in which the specificity is very low. QRT-PCR can distinguish between normal cases and β-thalassemia carriers (sensitivity: 75%; specificity: 76%) or severe α-thalassemia carriers (sensitivity: 80%; specificity: 61%) but no valuable results can discriminate between mild α-thalassemia carriers and normal cases. Taking the results of this study into account, *in vitro* GCS has been determined to be the most accurate technique and accurately distinguished between normal cases and mild α-thalassemia carriers (sensitivity: 80%; specificity: 73%). In addition, it was able to distinguish normal cases from β- or severe α-thalassemia carriers with a sensitivity and specificity of more than 90%. However, qRT-PCR could be a good substitute for GCS when the use of this method is limited. Furthermore, qRT-PCR can be a helpful method in combination with *in vitro* GCS when the ratios overlap. These two techniques can also confirm each other and be used in combination to diagnose atypical cases of thalassemia carriers.

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