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ANALYSIS OF mRNA IN β-THALASSEMIA/Hb E BY REAL TIME PCR DETECTION

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Reverse transcriptase-polymerase chain reaction (RT-PCR) has been widely used to quantitate different types of mRNA. However, the method still requires post-PCR manipulation which is labor- and time-intensive. Therefore, in this study, real time RT-PCR has been conducted to quantitate the α/β globin mRNA in β-thalassemia/Hb E patients as compare to normal control. Since patients with β-thalasssemia/Hb E show remarkable variability in severity, thus, the α/β mRNA was determined. The result is shown in figure 1 supporting that the imbalanced protein synthesis in β-thalassemia reflects both impairment of reduced β-globin chain mRNA and translation.

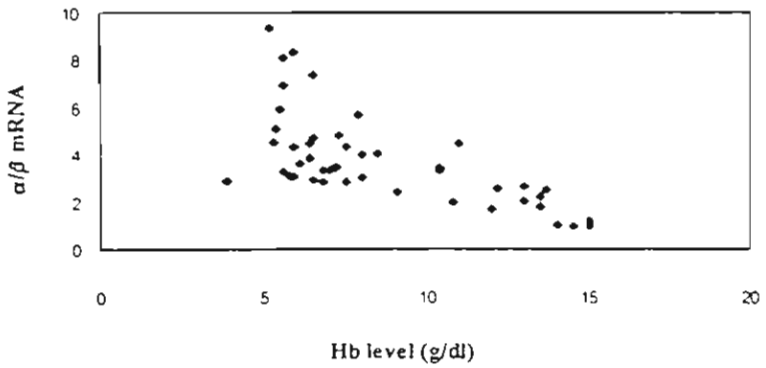

Fig.1 Comparison of Hb levels and α/β mRNA in β–thalassemia/Hb E patients compared to normal controls

GENOTYPE-PHENOTYPE INTERACTION IN β-THALASSEMIA : EXPERIENCE IN THAILAND

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Beta thalassemia is a very heterogeneous disorder due to variations in inactivation mechanism of the β-globin genes. Point mutations and small deletions or insertions in the nucleotide sequence are the main molecular defects responsible for most β-thalassemia. Homozygous β-thalassemia and β-thalassemia/Hb E are major β-thalassemic syndromes in S.E. Asia. In spite of seemingly identical genotypes, severity of β-thalassemic patients can vary greatly. Heterogeneity in the clinical manifestation of β-thalassemic diseases may occur from the nature of β-globin gene mutation, α-thalassemia gene interaction and difference in the amount of Hb F production which is partly associated with a specific β-globin haplotype.

A total of 144 β-thalassemia patients, 116 Thai and 28 Chinese from Nanning, were divided into mild, intermediate and severe cases according to clinical data and hemoglobin concentration. Clinical data included the age onset of presentation, age at first blood transfusion, requirement of blood transfusion, hepatosplenomegaly and growth development. Using these criteria 46 patients who had Hb ≥ 7.5 g/dl had a mild clinical symptom; 8 were $\beta^+\beta^+$-thalassemia or β^+-thalassemia/Hb E and 38 patients were $\beta^0\beta^+$-thalassemia or β^0-thalassemia/Hb E, 17 of whom also had coinheritance of α-thalassemia genes. Of the 43 patients with severe clinical symptom, 26 were $\beta^0\beta^0$-thalassemia, 5 $\beta^0\beta^+$-thalassemia and 12 β^0-thalassemia/HbE. Coinheritance of α-thalassemia was found in 8 cases of $\beta^0\beta^0$-thalassemia but it did not amenorate the severity of the disease (Table 1).

Table 1 Summary of genetic factors that may determine the severity of β-thalassemia disease

Clinical symptom	Homozygous β-thalassemia			β-Thalassemia/Hb E		
Genetic Factors	Mild	Intermedia	Severe	Mild	Intermedia	Severe
Total subjects (No.)	16	11	31	30	44	12
β^+-Thal/β^+-thal (Hb E)	4	0	0	4	0	0
β^0-Thal/β^+-thal (Hb E)	12	4	5	26	44	12
β^0-Thal/β^0-thal	0	7	26	0	0	0
α-Thal 1 trait	3	2	2	0	1	0
α-Thal 2/α-thal 2	1	0	1	0	0	0
α-Thal 2 trait	2	2	3	8	0	0
Hb Constant Spring (CS)	0	1	0	4	2	0
Xmn I, --	11	7	26	2	8	4
Xmn I, ++	0	0	0	1	1	0
Xmn I, -+	4	4	5	25	32	8

Application of Real-time PCR detection for mRNA quantitation in β-Thalassemia/Hb E

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ABSTRACT

β-Thalassemia and hemoglobin (Hb) E patients, with seemingly identical genotype, have a remarkable variability in severity. As most of β-thalassemia mutations normally exhibit recessive inheritance resulting in a reduced or no β-globin chain synthesis. Therefore, many groups tried to quantify the amount of β-globin mRNA whether there is a correlation with the reduced β-globin chain. Premature termination mRNA is also unstable but no significant systematic attempts to look at mRNA stability among different genotypes. In this work, the one-step real time PCR has been performed as it detected the amount of mRNA in real time during log phase of amplicon accumulation. This circumvents many other problems associated with quantitation in the plateau stage of a PCR amplification. The results show the increase in α/β mRNA ratio (2.4-9.4) in β^{0}-thalassemia/Hb E patients with chain termination mutation as compare to β^{+}-thalassemia/Hb E patients (3.1-5.1), normal controls (1.0-1.2) and Hb E traits (1.7-2.6). This could be due to the pathway called nonsense-mediated mRNA decay (NMD) which accelerated the degradation of unstable mRNA that could be toxic to cells and not be translated along their full length in β-thalassemia /Hb E patients. The difference in the α/β mRNA ratio of patients with the same genotypes could be due to the difference in NMD activity in each individual.

INTRODUCTION

Thalassemia and hemoglobinopathies are widespread recessive, inherited diseases. There are more than 1,000 mutations, including frameshifts, deletions,

insertions, etc have been detected in the human globin system, resulting in the synthesis of structurally abnormal proteins or in thalassemic types of disorders. β-Thalassemia, one of the most common single gene disorder, is characterized by abnormal β-globin gene function causing a decrease or absence of β-globin protein (1-3). More than 180 mutations affecting almost every known stage of β-globin gene expression result in a reduction (β^+) or complete absence (β^0) of β-globin chain synthesis from the affected alleles (1, 4). Such mutations may cause defects in transcription, RNA splicing and translation. In β^+-thalassemia, β-chain synthesis remains but at a markedly reduced rate and there is a correspondingly reduced level of β-globin mRNA in reticulocytes (5, 6). In β^0-thalassemia, no β-globin chains are synthesized. There are three main clinical phenotypes of β-thalassemia: thalassemia trait, thalassemia intermedia and thalassemia major (1). Thalassemia traits with one allele are clinically asymptomatic. Thalassemia intermedia is an ill-defined clinical term used to describe patients with phenotypes that are more severe than the asymtomatic thalassemia trait but milder than the transfusion-dependent thalassemia major. Most complications of β-thalassemia, including those related to extramedullary haemopoiesis including orthodontic abnormalities, fractures and spinal cord compression (7-9).

Hemoglobin E (Hb E) is the most common structural Hb variant among Southeast Asian populations. It is a β-globin variant resulting from the mutation at codon 26, G→A (Glu→Lys). The mutation in codon 26 of the β^E-globin gene can activate an adjacent cryptic splice site located at codon 24 to 27 giving rise to an alternative splicing which produces no β-globin chain whereas the normal spliced mRNA which contains the exon 1 mutation at codon 26 can produce β^E-globin (10). Hb E traits have minimal

hematologic manifestations except for microcytosis. Individuals who are homozygous for Hb E usually shown to be clinically normal, however, compound heterozygote for β-thalassemia and Hb E have a remarkable variability in severity and may have a severe β-thalassemia. The most severely affected individuals have an anemia similar to that of homozygous β-thalassemia disease. Despite apparently identical genotypes, severity of the patients can vary greatly, with Hb level ranging from 2.5 to 13.5 g/dl, in the steady state, has been shown in 803 patients with β°-thalassemia/Hb E (11). The difference in severity of β-thalassemia/Hb E has been affected by many confounding genetic factors. Mutations causing β-thalassemia can produce either severe (e.g. mutation in IVS-1 position 5, G→C) or mild clinical symptom (*e.g.* mutation at codon 26, Hb E) (10, 12). Co-inheritance of a gene for hereditary persistance of fetal hemoglobin (HPFH) or other determinants which elevate Hb F expression can alleviate the severity of β-thalassemia (13). In addition, co-inheritance of α-thalassemia or Hb Constant Spring significantly ameliorates the severity of the disease (14). Recently, inheritance of a β-thalassemia chromosome with *Xmn* I cleavage site (+) at position -158 of the γ^G-globin gene was shown to be associated with milder anemia (15). Two copies of these alleles were necessary to produce significant clinical effect. Why should patients with the same genotypes have different phenotypes?

In this report, the one-step quantitative reverse transcriptase polymerase chain reaction (QC RT-PCR) method has been used for quantitaion of β-globin mRNA in both normal control and patients with β-thalassemia with many advantages: (1) as a one-step assay, it requires less labor, and sample handling, which minimizes the risk of sample-cross contamination; (2) no post PCR processing is necessary, quantitative results are

obtained in less time; and (3) the assay spans a large dynamic range, which eliminates the need for extensive sample dilution.

MATERIALS AND METHODS

Subjects

Six normal adults, eight hemogonbin E traits, and twenty β°Thalassemia/Hb E, eleven β^{+}Thalassemia/Hb E who have the normal α-globin genotype were studied.

Isolation of Cellular RNA

Five ml of blood samples were collected in vacutainers with EDTA as anticoagulant and transported in ice to the laboratory. RNA was extracted from the reticulocyte-enriched cells with the method of Chomczynski (16). Hematological data were determined with automated cell counter and other routine procedures. Hb analysis was performed during steady state by high performance liquid chromatographic procedure (15). Clinical assessment was based on severity of anemia, blood transfusion requirement and degree of growth impairment and spleen enlargement.

Oligonucleotides.

Synthetic oligonucleotide primers and the fluorescently labeled oligonucleotide hybridization probes were synthesized by Perkin Elmer Applied Biosystem (Foster City,

CA, USA) (Table 1). The α-globin probe was labeled with TET at 5' end, whereas the β- ← Tab
globin probe was labeled with FAM at 5' end. Both probes were labeled with the quencher fluor TAMRA at the 3' end. Primers and probes were designed using Oligo 4.0 software, following guideline suggested in Model 7700 Sequence Detection Instrument manual.

One-step QC RT-PCR.

All samples were assayed in triplicate in a MicroAmp Optical 96-well reaction plate that were frosted to prevent light from reflecting. Tube caps were similar to MicroAmp Caps but specially designed to prevent light scattering (Perkin Elmer). Reagents for the one-step RT-PCR were from the TaqMan™ Gold RT-PCR Kit (Perkin-Elmer, Norwalk, CT, USA) and the procedure recommended by the manufacturer. RNA from a normal sample was used as standard for quantitation of both α- and β-mRNA. The assay was performed in a 50 μl volume containing a mixture of 1 x TaqMan buffer (500 mM KCl, 0.1 mM EDTA, 100 mM Tris-HCl, pH 8.3, and 600 nM passive reference), 5.5 mM magnesium chloride, 300 μM dATP, dCTP, dGTP, 600 μM dUTP, 200 nM forward and reverse primers, 100 nM fluorescent-labelled probe, 0.025 U/μl AmpliTag Gold DNA polymerase (5 U/μl AmpliTag Gold DNA polymerase in 20 mM Tris-HCl, pH 9.0, 100 mM KCl, 0.1 mM EDTA, 1.0 mM DTT, 50% glycerol, and 0.5% (w/v) Tween 20), 0.25 U/ml Multiscribe reverse transcriptase (100 U/μl of recombinant Moloney Murine Leukemia Virus (MuLV) reverse transcriptase in 20 mM Tris-HCl, pH 7.5, 0.1 mM Na_2EDTA, 1 mM DTT, 0.01% (v/v) NP-40, and 50% (v/v) glycerol) and 0.4

U/ml RNase inhibitor. The optimized RT-PCR reaction was performed at 48°C, 30 min followed by AmpliTaq Gold activation at 95°C, 10 min then PCR amplification with the denaturation temperature at 95°C, 15 sec and annealing/extension step at 60°C, 1 min for 40 cycles. RT-and cDNA amplification were carried out in an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystem). Reactions were programmed on a Power Macintosh G3 linked directly to the ABI PRISM 7700 Sequence Detection System. The probes used contain a fluorescent reporter dye, FAM or TET which is covalently linked to the 5' end of the probe, and the fluorescent quencher dye TAMRA is located near the 3' end. In addition, the probe contains a 3' blocking phosphate group to prevent probe extension during amplification (17). When the probe is intact, the reporter dyes fluorescent emission is suppressed due to the close proximity of the quencher dye. During PCR, if the target sequence is present, the probe anneals specifically between the forward and reverse primers. The 5'→3' nuclease activity of the Taq DNA polymerase cleaves the annealed probe between the reporter and quencher dyes, thereby releasing a measurable increase in fluorescence. The cleaved probe is then displaced, and polymerization of the strand continues (18-20). A detector monitors the increase in fluorescent emission in real time without further post-PCR analysis. Since the Taq DNA polymerase does not digest free probe and fluorescent emission requires that both primer and probe are complementary to the target sequence, nonspecific amplification is not detected (20). This type of sequence detection system has been used for both quantitative PCR (21-23) and RT-PCR (18) for specific gene targets. The amplification plots were obtained and the amount of mRNA was analyzed on the Macintosh computer. RT-PCR amplifications were also examined by agarose gel electrophoresis. After ethidium

bromide staining, bands were visible only at the expected molecular weights for the α- and β- mRNA products.

RESULTS

Amplification plot and PCR Detection in Real Time

A PCR amplification of a 71 and 74 bp of α- and β- globin mRNA using primers as shown in Table 1. During the extension phase of the PCR cycle, the 5'→3' nucleolytic activity of the Taq DNA polymerase cleaves fluorescent hybridization probe releasing the reporter dye, TET or FAM, from the α- or β-globin probe. The expected molecular weights for the α- and β- mRNA products, 71 and 74 bp, respectively were also obtained after the RT-PCR reaction on an agarose gel electrophoresis (Fig. 1). ← Fig. 1

Quantitative analysis of α- and β- globin mRNA

Patients with different types of β-thalassemia/Hb E and normal samples were studied as mentioned in materials and methods. The α/β globin mRNA ratios from 6 normal individuals are between 1.0-1.2, Hb levels are 14.0-15.0 g/dl, whereas 20 patients with β°-thalassemia/Hb E (β^{-4bp}-thalassemia/Hb E, β^{17}-thalassemia/Hb E) are ranging from 2.4-9.4, Hb levels are 5.3-9.1 g/dl. In addition, 11 patients with β⁺-thalassemia/Hb E (β^{28}-thalassemia/Hb E, β^{654}-thalassemia/Hb E, Hb E/Hb E), the α/β globin mRNA ratios are ranging from 3.1-5.1 and Hb levels are 5.4-11.0 g/dl. In 8 Hb E traits, the α/β globin mRNA ratio is ranging from 1.7-2.6, Hb levels are from 12.0-13.5

g/dl. The α/β globin mRNA ratios in different types of β-thalassemia/Hb E patients as compared to normal controls is shown in Fig. 2. The results indicated that the average ← Fig. 2 α/β globin mRNA ratios in β°-thalassemia/Hb E (β^{-4bp}-thalassemia/Hb E, β^{17}-thalassemia/Hb E) were higher than in β^{+}-thalassemia/Hb E (β^{28}-thalassemia/Hb E, β^{654}-thalassemia/Hb E, Hb E/Hb E) which were higher than that of normal controls and Hb E traits. Previous work by Smetanina et al. (24) showed that the α/β globin mRNA ratios of 5.2-10.5 in β-thalassemia intermedia due to combination of promoter mutations and a classical type of β-thalassemia confirming the mild suppression of β-mRNA synthesis. They also showed the α/β globin mRNA ratios in two subjects with a β^{o}-thalassemia heterozygosity of 8.3 and 7.3 were considerably higher than that for a normal control (4.4). In addition, Smetanina *et al* (25) also showed that premature termination of β-globin mRNA translation in codon 39 (C→T) nonsense mutation, results in mRNA degradation products in the cytoplasm with the α/β globin mRNA ratio of approximately 8. They also showed that in IVS-I-110 (G→A) mutation containing base changes in an intervening sequence may produce a stable but non-functional mRNA with the α/β globin mRNA ratio of 6.0-7.0. They suggested that the differences in the quantitative synthesis of β-mRNA, higher relative levels of the β-message are present in the patients with a milder phenotypes.

DISCUSSION

Remarkable progress in molecular biology of the red cell, and methodology has been developed to evaluate the relative quantities of the different mRNAs in the

reticulocytes present in a few milliliter of blood from normal controls and patients with the various hemoglobin disorders. Although PCR has provided a powerful tool, it is imperative that it be used properly for quantitation (21). Many previous studies of quantitative PCR and RT-PCR described quantitation of the PCR product but did not measure the initial target quantity. In addition, these methods still require post-PCR manipulation including gel electrophoresis, Southern blot hybridization etc which is time- and labour-intensive, the sample throughput of some methods is limited.

In our study, the quantitation of α/β globin mRNA ratio in patients with β-thalassemia/Hb E were carried out using a new quantitative RT-PCR technique. The method is based on the use of fluorescent hybridization probes and ABI Prism 7700 Sequence Detection System. The real time PCR method offers many advantages. First of all, it is performed in a closed tube system and requires no post-PCR processing of sample. Therefore, PCR contamination is reduced as the PCR products can be analyzed and disposed off without opening the tubes. Secondly, this method supports the use of a normalization gene for quantitative RT-PCR controls. The C_T value, used for quantification, is performed in real time during log phase of amplicon accumulation. This circumvents many other problems associated with quantification in the plateau stage of a PCR amplification (21). Finally, the sample throughput will be increased as no post-PCR processing time, using 96-well format is highly compatible with automation technology.

High specificity can be obtained by using the real time RT-PCR. The Taq DNA polymerase enzyme requires that both probe and primers are complementary to the target sequence for nucleolytic cleavage and releasing of fluorescence emission. In addition, the use of Amplitaq Gold, which remains in an inactive state until a temperature well

above annealing is reached, minimizes primer dimer formation and amplification of non-specific products.

As compound heterozygotes for β-thalassemia/Hb E have a remarkable variability in severity. The most severely affected individuals have an anemia similar to that of homozygous β-thalassemia disease. However, variations in anemia, growth development, hepatospleenomegaly, and transfusion requirements are also observed. As it has been known that in β-thalassemia, α/β globin chain synthesis ratio affects severity of the disease. Premature termination mRNA is unstable and the cells do not want truncated proteins which are toxic to the cells, but no systematic attempts have been made to look at mRNA stability among different genotypes of β-thalassemia/Hb E. Interestingly, determination by real time PCR detection method of the α/β globin mRNA ratio in normal individuals are in the range between 1.0-1.2, which is different from the previous study of approximately 4.3 (24) determined by two-step RT-PCR followed by detection and quantitation of PCR products on a non-denaturing gel, and densitometric scanning of the autoradiograms. For mRNA ratio determination to be valid using an RT-PCR based method, all measurement must be taken during the exponential phase and the primer sets involved must have a very similar amplification efficiency. To confirm that our protocol fulfilled these requirements, the α/β globin DNA ratio was determined using the technique of real time PCR detection in normal sample and the results show that the ratio is ~1.9 (data not shown) which correlates with the α/β globin gene ratio of 2. As we know that individuals without globin gene abnormalities have 2 α-globin genes for every β-globin gene. Thus the technique is reliable, not an artifact. The ratio in normal individuals are in the range between 1.0-1.2 could be due to a correction of the message

at the transcriptional step, having the β globin gene as the rate limiting step, finally leading to an equal α/β globin chain synthesis.

Mutations that cause deletion or frameshift and premature termination, β°-thalassemia/Hb E (β^{-4bp}-thalassemia/Hb E, β^{17}-thalassemia/Hb E), the α/β globin mRNA ratios in 20 patients show the increase in α/β mRNA ratio (2.4-9.3) as compare to β^{+-} thalassemia/Hb E patients (3.1-5.1), normal controls (1.0-1.2) and Hb E traits (1.7-2.6). The increase in the α/β globin mRNA ratios could be partly due to premature (truncated) termination which leads to unstable mRNA that can not be translated along their full length are rapidly degraded. This helps in reducing the accumulation of rouge proteins that might be deleterious. The pathway that accelerated mRNA decay is referred to as nonsense-mediated mRNA decay (NMD) (26). The difference in the α/β globin mRNA ratios among β°-thalassemia/Hb E patients with same genotypes could be due to the difference in the activities of NMD pathway in each individual and the age of the red blood cells in the circulation. As the proportion of mRNA decreases with erythroid maturation (27). The results correlate with the work by Smetanina *et al* (24) showed that the the α/β globin mRNA ratios in two subjects with a β°-thalassemia heterozygosity of 8.3 and 7.3 were considerably higher than that for a normal control (4.4) confirming the mild suppression of β mRNA synthesis. The work by Smetanina *et al* (25) also indicated that the differences in the quantitative synthesis of β-mRNA, higher relative levels of the β-message are present in the patients with milder phenotypes.

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Table 1. Primer and probes sequences of α- and β-globin gene

Oligonucleotide	Sequence (5'→3')	Location
α-globin gene		
α-globinF primer	5'GAGGCCTGGAGGATGTTC 3'	95-114
α-globinR primer	5'CGTGGCTCAGGTCGAAGTG 3'	165-183
α-globin probe	5'TGTCCTTCCCCACCACCAAGACCTACT3'	115-141
β-globin gene		
β-globinF primer	5'GGGATCTGTCCACTCCTGATG 3'	190-210
β-globinR primer	5'GGCACCGAGCACTTTCTTG 3'	263-281
β-globin probe	5'TGGGCAACCCTAAGGTGAAGGCTCA3'	217-241

Fig. 1. The α-and β- globin cDNA obtained from one-step RT-PCR (Real time PCR detection) of normal α-and β-mRNA using primers and probes as listed in Table 1. Ethidium bromide stained 2.0% agarose gel was visualized. The lanes are represented by lane1: DNA Mass Ladder (Gibco BRL), lane7: β- globin cDNA (74 bp), lane 8: α- globin cDNA (71 bp)

Fig. 2. Comparison of the α/β globin mRNA ratio in subjects with β^{-4bp}/β^{E}, β^{17}/β^{E} β^{654}/β^{E}, β^{28}/β^{E} and β^{E}/β^{E}as compare to normal control (β^{A}/β^{A}) and Hb E traits (β^{A}/β^{E}). The one-step RT-PCR (Real time PCR detection) was carried out as mentioned in methods using α-and β-primers and probes as listed in Table 1. The optimized condition used was 48°C, 30 min. for reverse transcription followed by Amplitaq Gold activation at 95°C, 10 min. then PCR amplification with the denaturation temperature at 95°C, 15 min. and annealing/extension step at 60°C, 1 min for 40 cycles

(Legend)

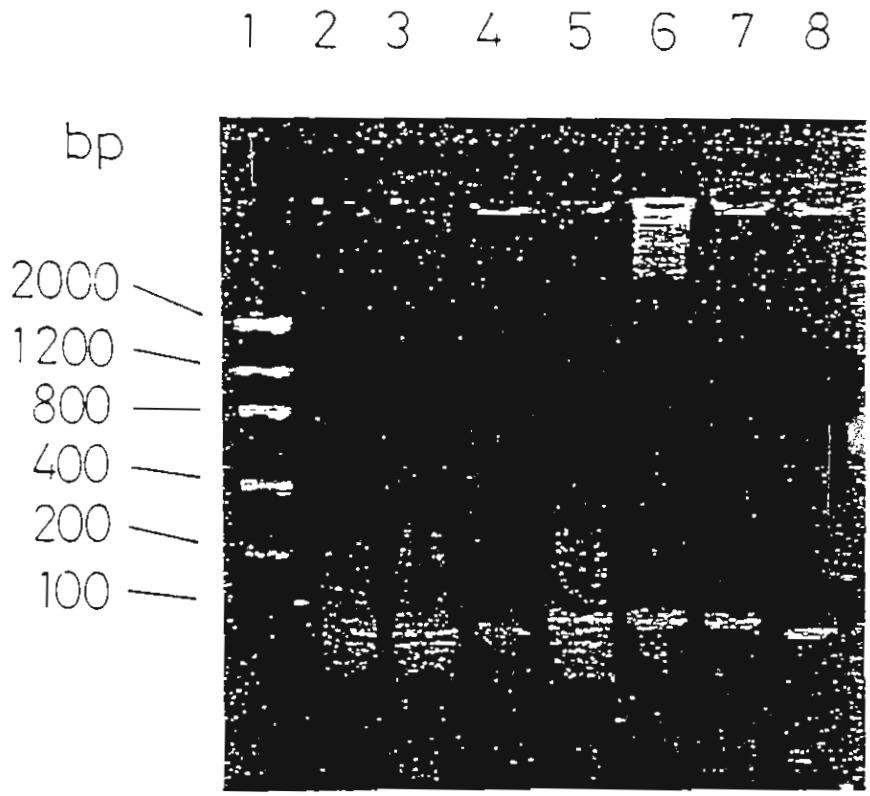

Fig. 1

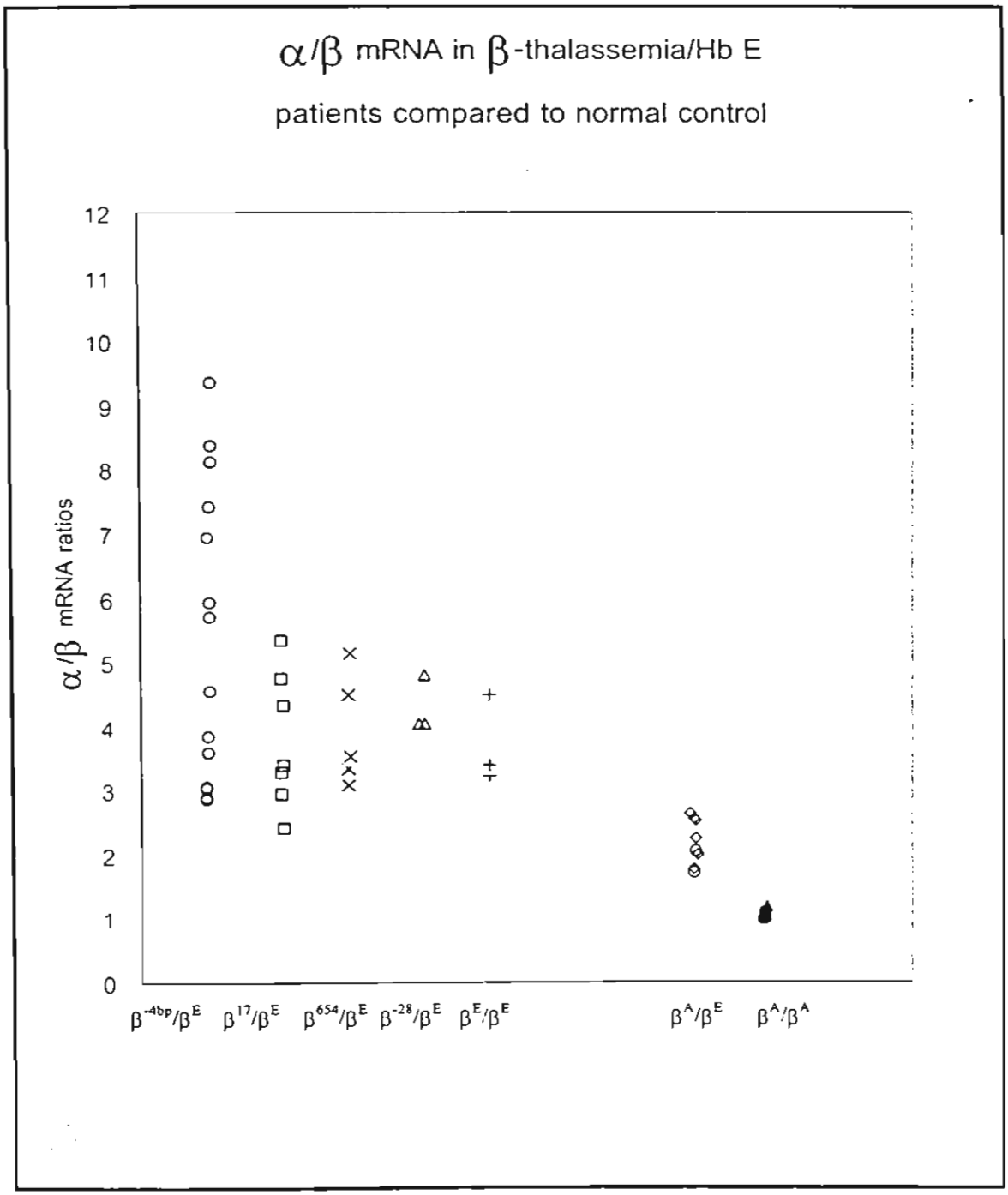
α/β mRNA in β-thalassemia/Hb E
patients compared to normal control
α/β mRNA ratios
12
11
10
9
8
7
6
5
4
3
2
1
0
β-4bp/βE
β17/βE
β654/βE
β-28/βE
βE/βE
βA/βE
βA/βA

Fig. 2